UTILIZATION OF WOOD-DECAY FUNGI FOR BIOKRAFT PULPING OF SOFTWOOD

by

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"..... taking into consideration the whole of the southern part of Africa, there can be no doubt of its being a sterile country."

Charles Darwin, Journal of Researches during the voyage of H.M.S. Beagle (1837)

This thesis is dedicated to my family

CONTENTS

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PREFACE

The forest products industry is one of the most important earners of foreign exchange for South Africa (Kruger *et al.*, 1995). Products such as mining timber, construction timber, veneer logs, particle and fibreboard and pulpwood chips make an important contribution to the GDP. However, the major focus of this industry is the production of pulp and paper with an annual pulp capacity of 2,4 million tons (Rockey, 1998).

The pulping and paper-making process is a predominantly chemical process that utilizes biological raw materials (Ferris, 1997). During pulping, wood fibres are either separated by mechanical pulping, or lignin is dissolved by chemical cooking to free cellulose (Sjöström, 1981). Roundwood can only be utilized directly, in one method of mechanical pulping (ground wood pulping), but chipped wood is used for other forms of pulping. Logs are, therefore, debarked and chipped before storage, because chips are more economical to handle than logs (Zabel & Morrell, 1992). Wood chips are then pulped by various methods, depending on the required pulp characteristics. Mechanical pulping yields pulp with a high yield and with low

strength properties that can be used for newsprint (Sjöström, 1981). Chemical pulp, on the other hand, has lower yields, but with better strength properties that are used for a wider range of paper products such as corrugated containers, fine paper and tissue. Many paper grades obligate the removal of residual lignin in pulp by bleaching with oxidizing chemicals. Bleached pulp and, sometimes, unbleached pulp are then formed into paper sheets with different strength and optical properties that are determined by the end use. Unfortunately, all of these processes generate potentially hazardous effluents. This results in considerable criticism against the pulp and paper industry (Bergbauer & Eggert, 1992).

The paper and pulp industry relies on physical and chemical processes for most of the unit operations (Sjöström, 1981). However, biotechnology offers the industry the potential to produce higher quality products at reduced cost and with less environmental impact (Eriksson, 1991; Ferris, 1997; Kirk, 1989). These advantages are the result of more specific, but natural reactions that are catalyzed by microorganisms or their products (Eriksson, 1990).

Only a limited number of biological systems are currently available to the industry (Ferris, 1997), but they have been researched for little more than 20 years (Shimada, 1996). The focus of this research has moved to different unit operations as determined by the needs of the industry. However, the rationale for all developments has been to save energy (Akhtar *et al.*, 1997), improve product quality (Buchert *et al.*, 1994; Popius-Levlin *et al.*, 1997; Viikari *et al.*, 1993), increase production (Lascaris *et al.*, 1997) or reduce the environmental impact of conventional processes (Eriksson,

1991). Most of these processes utilize fungi or fungal products (Eriksson, 1991; Shimada, 1996). The justification for the use of fungi, lies in their ability to rapidly colonize lignocellulosic substrates (Kirk, 1989). Filamentous fungi have the ability to penetrate solid substrates (Messner & Srebotnik, 1994), and wood-inhabiting fungi are also able to produce important enzymes required for degradation of lignocellulose (Eriksson, 1990) and wood extractives (Blanchette *et al.*, 1992b; Farrell *et al.*, 1997; Fischer *et al.*, 1996). The white-rot Basidiomycetes are, for example, the only group of organisms that can degrade lignin on a significant scale (Akhtar *et al.*, 1997).

The application of fungi or fungal products has been investigated for the following operations in the forest products industry:

- Biological control of sap stain in felled lumber by application of *Ophiostoma piliferum* (Behrendt *et al.*, 1995) and *Phlebiopsis gigantea* (Behrendt & Blanchette, 1997).
- Improvement of debarking of logs through the application of *P. gigantea* (Behrendt & Blanchette, 1997).
- Biological control of sap stain of wood chips by applying *O. piliferum* (Farrell *et al.*, 1994; Schmitt *et al.*, 1998; Wall *et al.*, 1995)
- Biological treatment of wood prior to pulping to improve mechanical and chemical pulping processes (Akhtar *et al.*, 1998; Iverson *et al.*, 1997; Jacobs *et al.*, 1998; Jacobs-Young *et al.*, 1998; Wall *et al.*, 1994; Wall *et al.*, 1995; Wall *et al.*, 1996).

- Biological treatment of wood for pitch control (Blanchette *et al.*, 1992b; Farrell *et al.*, 1997; Fischer *et al.*, 1996; Haller & Kile, 1992; Qin & Chen, 1997; Wall *et al.*, 1995).
- Biological pre-treatment of non-wood fibre (Hatakka *et al.*, 1996; Johnsrud *et al.*, 1987; Sabharwal *et al.*, 1996; Wolfaardt *et al.*, 1998).
- Modification of the papermaking properties of fibre by application of enzymes, to strengthen pulp webs and improve drainage on paper machines (Jeffries, 1992; Laleg & Pikulik, 1992; Lascaris *et al.*, 1997; Sarkar, 1997).
- Biologically assisted bleaching of pulp by fungi, cell free cultures and enzymes (Buchert *et al.*, 1994; Jeffries, 1992; Kirkpatrick *et al.*, 1989; Kirkpatrick *et al.*, 1990; Paice *et al.*, 1995; Popius-Levlin *et al.*, 1997; Onysko, 1993; Reid & Paice, 1994; Senior *et al.*, 1997; Viikari *et al.*, 1993).
- De-inking of secondary fibres by using fungal enzymes (Prasad, 1993; Rutledge-Cropsey *et al.*, 1998).
- Treatment of pulp mill effluents by white-rot fungi to reduce phenolics and chlorolignins (Bergbauer *et al.*, 1991; Bryant *et al.*, 1992; Müncnerová & Augustin, 1994).

Applications where the most significant contributions have been made to date, are biopulping, biobleaching and treatment of waste water (Eriksson, 1991). Biopulping is potentially the most important of these processes, because it can influence all downstream operations in the papermaking processes. It was for this reason that Sappi Ltd. (Now Sappi Forest Products) entered into an agreement with the Foodtek and Forestek divisions of the CSIR (Council for Scientific and Industrial

Research), to develop a biopulping process. The biopulping process was developed for the treatment of softwood at the Sappi Kraft Ngodwana mill in Mpumalanga. This mill is the largest and most modern of Sappi's South African mills, but it is also situated in an environmentally sensitive area.

The Ngodwana mill lies in close proximity to the Elands River that is a tributary to the Crocodile River. The Crocodile River forms the southern boundary of the Kruger National Park and water from the river is also used to irrigate tobacco, a crop that is very sensitive to chlorides. For these reasons, the Ngodwana mill does not release effluent into the river system, but is irrigated onto pastureland after treatment. However, the threat of chloride migration in the soil water has compelled Sappi to investigate the use of environmentally benign processes in upstream operations.

Sappi Ngodwana utilizes softwood and hardwood in its chemical pulping operations and softwood for its groundwood fibre line. About 5400 tons of softwood chips are used daily. The chips have an average residence time of three days, but never more than seven days on the chip pile. The chip pile is managed on a first in first out basis by using a traversing stacker and reclaimer. The softwood supply consists of *Pinus patula* (40 %), *P. elliottii* (40 %) and *P. taeda* (20 %). Wood is chipped within three weeks after felling, but 18 to 20 % of the wood is acquired as chips from sawmill waste.

The technical planning of the biopulping project that is reported on in this thesis included the following primary goals:

- **Collection of South African wood-decay fungi from diverse habitats.**

  It was decided to evaluate the potential of local white-rot fungi, because the search for superior fungal strains is seen as one of the main target areas for the development of a successful biopulping process (Reid, 1991). An added incentive was the potential to obtain proprietary ownership of novel strains. The establishment of the culture collection of local fungal strains and characterization of these strains in culture is discussed in Chapter 2.

- **Screening of fungi for application in a biokraft pulping process.**

  Several methods have been used to screen fungi for application in biopulping processes (Bechtold *et al.*, 1993; Blanchette *et al.*, 1992a; Job-Cei *et al.*, 1991; Otjen *et al.*, 1987). However, we chose to conduct mini pulping trials and to relate the performance of fungal strains to the process in which they were to be applied (Chapter 3).

- **Development of techniques for the optimal production of fungal biomass for application as inoculum.**

  Different methods of inoculum production were investigated. The production of inoculum from a pre-inoculum of homogenized mycelium provided the most efficient means of biomass production. The best fungal growth was obtained in a medium containing Corn Steep Liquor (CSL) as a nitrogen source and sucrose (unpublished results). This work was directed by other members of the research team and is, therefore, not included in this thesis.

- **Optimization of physical parameters for solid-substrate fermentation.**

  Many of the requirements for the optimal growth of biopulping fungi such as aeration, temperature control and moisture control have been investigated. Strategies to control these factors on chip piles or in reactors have already been developed by Akhtar *et al.* (1996) and Wall *et al.* (1993). Studies to control these parameters were, therefore, limited during this project. Chapter 4 deals with an investigation to determine the microenvironment of the Ngodwana chip pile. In Chapter 5, we investigated the inhibitory influence of microbial competitors and monoterpenes on biopulping.

- **Evaluation of supplements to improve solid substrate fermentation.**

  Addition of nutritional supplements has proved to enhance competitive ability of fungi and selective delignification (Akhtar *et al.*, 1989; Kirk *et al.*, 1976). Our team also showed that biopulping efficiency can be increased by addition of CSL and sucrose to the wood during inoculation (unpublished results), however the work was directed by other members of the research team and is, therefore, not included in this thesis.

- **Optimization of kraft pulping parameters.**

  The results of pulping of fungal-treated wood under different conditions are discussed in Chapter 6. The effects of alkali charge, liquor to wood ratio and pulping time on pulp quality and yield were evaluated. These results were used to model the kraft biopulping process. The model was used to determine the exact benefits of biopulping for a kraft process and to evaluate the economic feasibility of such a biopulping.

Appendices have been included at the end of the thesis to provide more complete data where these data did not make up an integral part of the different chapters. Some of the data from the appendices have been included in condensed form in chapters.

REFERENCES

AKHTAR, M., BLANCHETTE, R.A. & KIRK, T.K. 1997. Fungal delignification and biomechanical pulping of wood. *Adv. Biochem. Eng. Biotechnol.* 57: 159-195.

AKHTAR, M., KIRK, T.K. & BLANCHETTE, R.A. 1996. Biopulping: An overview of consortia research. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 187–192. Facultas-Universitätsverlag, Vienna, Austria.

AKHTAR, M., LENTZ, M.J., BLANCHETTE, R.A. & KIRK, T.K. 1989. Advances in biotechnology in pulp and paper manufacture: Overview of the 1989 International Conference. *Tappi J.* 72(5): 33-43.

AKHTAR, M., LENTZ, M.J., SWANEY, R.E., SCOTT, G.M., HORN, E. & KIRK, T.K. 1998. Commercialization of biopulping for mechanical pulping. Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada. Vol. A: 55-58.

BECHTOLD, R., GONZÀLES, A.E., ALMENDROS, G., MARTINEZ, M.J. & MARTINEZ, A.T. 1993. Lignin alteration by *Ganoderma australe* and other white-rot fungi after solid-state fermentation of beech wood. *Holzforschung* 47(2): 91-96.

BEHRENDT, C.J., BLANCHETTE, R.A. & FARRELL, R.L. 1995. An integrated approach, using biological and chemical control, to prevent blue stain in pine logs. *Can. J. Bot.* 73: 613-619.

BEHRENDT, C.J. & BLANCHETTE, R.A. 1997. Biological processing of pine logs for pulp and paper production with *Phlebiopsis gigantea. Appl. Environ. Microbiol.*. 63: 1995-2000.

BERGBAUER, M. & EGGERT, C. 1992. Differences in the persistence of various bleachery effluent lignins against attack by white-rot fungi. *Biotechnol. Lett.* 14(9): 869-874.

BERGBAUER, M., EGGERT, C. & KRAEPELIN, G. 1991. Degradation of chlorinated lignin compounds in bleach plant effluent by the white-rot fungus *Trametes versicolor*. *Appl. Microbiol. Biotechnol.* 35: 105-109.

BLANCHETTE, R.A., BURNES, T.A., EERDMANS, M.M. and AKHTAR, M. 1992a. Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. *Holzforschung* 46(2): 109-115.

BLANCHETTE, R.A., FARRELL, R.L., BURNES, T.A., WENDLER, P.A., ZIMMERMAN, W., BRUSH, T.S. & SNYDER, R.A. 1992b. Biological control of pitch in pulp and paper production by *Ophiostoma piliferum*. *Tappi J.* 75: 102-106.

BRYANT, C.W., AVENELL, J.J., BARKELEY, W.A. & THUT, R.N. 1992. The removal of chlorinated organics from conventional pulp and paper waste water treatment systems. *Wat. Sci. Technol.* 26: 417-425.

BUCHERT, J., TENKANEN, M., KANTELINEN, A. & VIIKARI, L. 1994. Applications of xylanases in the pulp and paper industry. *Bioresource Technol.* 50: 65-72.

ERIKSSON, K.-E.L. 1990. Biotechnology in the pulp and paper industry. *Wood Sci. Technol.* 24: 79-101.

ERIKSSON, K.-E.L. 1991. Biotechnology: three approaches to reduce the environmental impact of the pulp and paper industry. *Sci. Progress* 75: 175-189.

FARRELL, R.L. & ALLISON, R.W. 1997. Effect of Cartapip 97® Pretreatment on the kraft pulping of radiata pine. *Tappi Biological Sciences Symposium*, pp. 15-18. Tappi Press, Atlanta, USA.

FARRELL, R.L., BRUSH, T.S., FRITZ, A.R., BLANCHETTE, R.A. & IVERSON, S. 1994. Cartapip®: A biological product for control of pitch and resin acid problems in pulp mills. *Tappi Biological Sciences Symposium*, pp. 85-87. Tappi Press, Atlanta, USA.

FERRIS, J.L. 1997. Challenges in adapting biotechnology to pulp and paper: The academic role. *Tappi Biological Sciences Symposium*, pp. 11-13. Tappi Press, Atlanta, USA.

FISCHER, K., AKHTAR, M., MESSNER, K., BLANCHETTE, R.A. & KIRK, T.K. 1996. Pitch reduction with the white-rot fungus *Ceriporiopsis subvermispora*. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the 6th International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 193–198. Facultas-Universitätsverlag, Vienna, Austria.

HALLER, T.M. & KILE, G. 1992. Cartapip® treatment of wood chips to reduce pitch and improve processing. *Tappi Pulping Conference*, pp. 1243-1252. Tappi Press, Atlanta, USA.

HATAKKA, A., METTÄLÄ, A., HÄRKÖNEN, T. & PAAVILAINEN, L. 1996. Biopulping of gramineous plants by white-rot fungi. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 229–232. Facultas-Universitätsverlag, Vienna, Austria.

IVERSON, S., BEHRENDT, C., WILLIAMS, D., BLANCHETTE, R. & WALL, M.B. 1997. *Phlebiopsis gigantea*, a white rot fungus for biomechanical pulping. *Tappi Biological Sciences Symposium*, pp. 25-28. Tappi Press, Atlanta, USA.

JACOBS, C.J., VENDITTI, R.A. & JOYCE, T.W. 1998. Effect of enzymatic pretreatments on conventional kraft pulping. *Tappi. J.* 81(2): 143-147.

JACOBS-YOUNG, C.J., VENDITTI, R.A. & JOYCE, T.W. 1998. Effect of enzymatic pretreatment on the diffusion of sodium hydroxide in wood. *Tappi. J.* 81(1): 260-266.

JEFFRIES, T.W. 1992. Enzymatic treatment of pulps. In: Emerging technologies for materials and chemicals from biomass. Eds. R.M. Rowell, T.P. Schultz & R. Narayan, pp. 313–329. American Chemical Society, Washington, USA.

JOB-CEI, C., KELLER, J. & JOB, D. 1991. Selective delignification of sulphite pulp paper: Assessment of 40 white-rot fungi. *Material u. Organismen* 26(3): 215-226.

JOHNSRUD, S.C., FERNANDEZ, N., LOPEZ, P., GUTIÉRREZ, I., SAEZ, A. & ERIKSSON, K.-E. 1987. Properties of fungal pretreated high yield bagasse pulps. *Nord. Pulp Pap. Res. J.* Special issue: 47-52.

KIRK, T.K. 1989. Advances in biotechnology in pulp and paper manufacture: Overview of the 1989 International Conference. *Tappi J.* 72(5): 33-43.

KIRK, T.K., CONNORS, W.J. & ZEIKUS, J.G. 1976. Requirement for a growth substrate during lignin decomposition by two wood rotting fungi. *Appl. Environ. Microb.* 32(1): 192-194.

KIRKPATRICK, N., REID, I.D., ZIOMEK, E., HO, C. & PAICE, M.G. 1989. Relationship between fungal biomass production and the brightening of hardwood kraft pulp by *Coriolus versicolor*. *Appl. Environ. Microbiol.* 55: 1147-1152.

KIRKPATRICK, N., REID, I.D., ZIOMEK, E. & PAICE, M.G. 1990. Biological bleaching of hardwood kraft pulp using *Trametes (Coriolus) versicolor* immobilized in polyurethane foam. *Appl. Microbiol. Biotechnol.* 33: 105-108.

KRUGER, F.J., EVERARD, D.A. & MANDERS, P.T. 1995. Forestry and environmentally sustainable development in South Africa. *Commonwealth For. Rev.* 74(1): 71-75.

LALEG, M. & PIKULIK, I.I. 1992. Strengthening of mechanical pulp webs by chitosan. *Nord. Pulp Pap. Res. J.* 4: 174-199.

LASCARIS, E., LONERGAN, G. & FORBES, L. 1997. Drainage improvement using a starch degrading enzyme blend in a recycling paper mill. *Tappi Biological Sciences Symposium*, pp. 271-277. Tappi Press, Atlanta, USA.

MESSNER, K. & SREBOTNIK, E. 1994. Biopulping: An overview of developments in an environmentally safe paper-making technology. *FEMS Microb. Rev.* 13: 351 - 364.

MÜNCNEROVÁ, D. & AUGUSTIN, J. 1994. Fungal metabolism and detoxification of polycyclic aromatic hydrocarbons: A review. *Bioresource Technol.* 48: 97-106.

ONYSKO, K.A. 1993. Biological bleaching of chemical pulps: A review. *Biotech. Adv.* 11: 179-198.

OTJEN, L., BLANCHETTE, R., EFFLAND, M. and LEATHAM, G. 1987. Assessment of 30 white rot basidiomycetes for selective lignin degradation. *Holzforschung* 41(6): 343-349.

PAICE, M.G., BOURBONNAIS, R., REID, I.D., ARCHIBALD, F.S. & JURASEK, L. 1995. Oxidative bleaching enzymes: A review. *J. Pulp Paper Sci.* 21(8): 280-284.

POPIUS-LEVLIN, K., WANG, W., RANUA, M., NIKU-PAAVOLA, M.-L. & VIIKARI, L. 1997. Biobleaching of chemical pulps by laccase/mediator systems. *Tappi Biological Sciences Symposium*, pp. 329-333. Tappi Press, Atlanta, USA.

PRASAD, D.Y. 1993. Enzymatic deinking of laser and xerographic office wastes. *Appita* 46(4): 289-292.

QIN, M.H. & CHEN, J.X. 1997. Biological pitch control of masson pine groundwood pulp by lipases. *Tappi Biological Sciences Symposium*, p. 369. Tappi Press, Atlanta, USA.

REID, I.D. 1991. Biological pulping in paper manufacture. *Tibtech* 9(8): 262-265.

REID, I.D. & PAICE, M.G. 1994. Biological bleaching of kraft pulps by white-rot fungi and their enzymes. *FEMS Microb. Rev.* 13: 369-376.

ROCKEY, N. 1998. Annual Review: South Africa. *Pulp Pap. Int.* 40(7): 84.

RUTLEDGE-CROPSEY, K., KLUNGNESS, J.H. & ABUBAKR, S.M. 1998. Performance of enzymatically deinked recovered paper on machine runnability. *Tappi. J.* 81(2): 148-151.

SABHARWAL, H.S., AKHTAR, M., YU, E., D'AGOSTINO, D., YOUNG, R.A. & BLANCHETTE, R.A. 1996. Development of biological pulping processes for non-woody plants. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 233–236. Facultas-Universitätsverlag, Vienna, Austria.

SARKAR, J.M. 1997. Recycle paper mill trial using enzyme and polymer for upgrading recycled fibre. *Appita* 50(1): 57-60.

SCHMITT, E.K., MIRANDA, M.B. & WILLIAMS, D.P. 1998. Applications of fungal inocula in the pulp and paper industry. Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada. Vol. A: 13-16.

SENIOR, D.J., BERNHARDT, S.A., HAMILTON, J. & LUNDELL, R. 1997. Mill implementation of enzymes in pulp manufacture. *Tappi Biological Sciences Symposium*, pp. 163-168. Tappi Press, Atlanta, USA.

SHIMADA, M. 1996. Biopulping: Recent advances in microbial, enzymatic, and biomimetic lignin degradation research. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the 6th International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 17–23. Facultas-Universitätsverlag, Vienna, Austria.

SJÖSTRÖM, E. 1981. Wood chemistry: Fundamentals and Applications, 2nd ed. Academic Press, San Diego, USA.

VIIKARI, L., TENKANEN, M., BUCHERT, J., RÄTTÖ, M., BAILEY, M., SIIKA-AHO, M. & LINKO, M. 1993. Hemicellulases for industrial applications. In: Hemicellulases for industrial applications. ed. J.N. SADDLER, pp. 131–182. CAB International Press, Wallingford, UK.

WALL, M.B., BRECKER, J., FRITZ, A., IVERSON, S. & NOEL, Y. 1994. Cartapip® treatment of wood chips to improve chemical pulping efficiency. *Tappi Biological Sciences Symposium*, pp. 67-76. Tappi Press, Atlanta, USA.

WALL, M.B., CAMERON, D.C. & LIGHTFOOT, E.N. 1993. Biopulping process design and kinetics. *Biotech. Adv.* 11: 645-662.

WALL, M.B., NOËL, Y., FARRELL, R.L., IVERSON, S. & FRITZ, A. 1995. The use of *Ophiostoma piliferum* treatment to improve chip quality. *Tappi Pulping Conference*, pp. 829-833. Tappi Press, Atlanta, USA.

WALL, M.B., STAFFORD, G., NOEL, Y., FRITZ, A., IVERSON, S. & FARRELL, R.L. 1996. Treatment with *Ophiostoma piliferum* improves chemical pulping efficiency. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the 6th International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 205–210. Facultas-Universitätsverlag, Vienna, Austria.

WOLFAARDT, F., DU PLOOY, A., DUNN, C., GRIMBEEK, E. & WINGFIELD, M. 1998. Biopulping of sugarcane bagasse: Improvement of the Semi-Alkaline Sulphite-Anthraquinone process. Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada. Vol. B: 53-55.

ZABEL, R.A. & MORRELL, J.J. 1992. Wood microbiology: Decay and its prevention. Academic Press, Inc., San Diego, USA.

CHAPTER 1

APPLICATION OF FUNGI AND FUNGAL PRODUCTS IN BIOPULPING PROCESSES: A REVIEW

Coriolus versicolor

ABSTRACT

Biopulping is a solid-substrate fermentation (SSF) process where lignocellulosic materials are treated with fungi prior to pulping to improve pulping. Research has focussed on the utilization of lignin degrading fungi for biopulping. The only commercial process currently available, utilizes *Ophiostoma piliferum*, a sap staining and not a lignin-degrading fungus. Filamentous fungi are well adapted for biopulping because of their ability to penetrate and transfer enzymes into the woody substrate, but because biopulping is a SSF process it requires control of temperature, moisture and aeration. Biopulping has been evaluated for improvement of mechanical and chemical pulping as well as for depitching and the control of degradation of the raw materials. The diversity of these pulping processes requires that different SSF processes should be developed to achieve unique benefits. The aim of this review is to consider the work done on different pulping methods and to elucidate the benefits and economic implications of biopulping.

INTRODUCTION

Biopulping has been defined as the treatment of lignocellulosic materials with lignin-degrading fungi prior to pulping (Akhtar *et al.*, 1997c). This definition stresses the use of lignin degrading fungi. However, Wall *et al.* (1994; 1996) have shown that a biopulping effect can also be achieved with *Ophiostoma piliferum,* a fungus that does not degrade lignin. Improvement of biopulping by *O. piliferum* has been achieved through the reduction of wood extractives to improve penetration of pulping chemicals. Wood chips have also been treated successfully with fungal enzymes to improve the penetration of pulping liquor (Jacobs *et al.*, 1998; Jacobs-Young *et al.*, 1998). The process that utilizes lignin degrading fungi as well as the process based on *O. piliferum* have been developed to a stage where they can be applied on a mill scale (Akhtar *et al.*, 1998; Schmitt *et al.*, 1998). These procedures are aimed at the treatment of wood chips in a solid-substrate fermentation (SSF) process. Filamentous fungi are ideally suited for biopulping because of their ability to penetrate and transfer enzymes into the woody substrate (Messner & Srebotnik, 1994; Mitchell, 1992).

Despite the unique ability of fungi to successfully modify wood and to improve the pulping process, biopulping is hampered by several obstacles that require engineering and management solutions (Akhtar *et al.*, 1997c; Mitchell & Lonsane, 1992; Wall *et al.*, 1993). One of the most important problems is the control of competing microorganisms. Fungi such as *Trichoderma* spp. and *Aspergillus* spp. are important in this respect (Messner & Srebotnik, 1994, Chapter 5), but pre-sterilization of wood chips is regarded as uneconomical (Wall *et al.*, 1993). Freshly cut wood also

contains inhibitory compounds such as monoterpenes, to which white-rot fungi are especially susceptible (Cobb *et al.*, 1968; Pearce *et al.*, 1996; Chapter 5). A certain measure of asepsis of wood chips as well as reduction of the inhibitory compounds in wood can be achieved with a brief steam treatment (Akhtar *et al.*, 1996; Wall *et al.*, 1993). The SSF process occurs outdoors on chip piles during the normal storage, but the fungi that are recommended for utilization, require a relatively controlled environment for growth. Special measures to control temperature, moisture and aeration must, therefore, be taken (Akhtar *et al.*, 1997c; Wall *et al.*, 1993). Mitchell & Lonsane (1992) have discussed the engineering problems that have to be considered in the development of SFF processes in detail and they will not be repeated here.

The special requirements of SSF apply to all of the biopulping methods. However, the specific processes of biomechanical pulping, biochemical pulping and pitch control all have unique requirements to obtain different benefits. Different types of fungi are used to achieve the specific aims with each method (Akhtar *et al.*, 1998; Farrell *et al.*, 1997; Fischer *et al.*, 1996; Iverson *et al.*, 1997; Qin & Chen, 1997; Wall *et al.*, 1993; Wall *et al.*, 1996) and process parameters need to be changed accordingly.

BIOMECHANICAL PULPING

Mechanical processes are responsible for 25 % of the worldwide production of pulp (Akhtar *et al.*, 1997c). Mechanical pulps are characterized by their high yield, that is obtained at the cost of high energy inputs (Sjöström, 1981). These pulps also have a reduced strength compared to chemical pulp. The aim of biomechanical pulping is, therefore, to reduce the energy consumption during pulping and to improve pulp strength (Akhtar *et al.*, 1998). Development of such a biopulping procedure has mainly been focussed on thermomechanical pulping where wood chips (which are easier to treat than roundwood) are used. The benefit of biomechanical pulp is that it has similar properties to that of chemithermomechanical pulp (Reid, 1991). It could, therefore, compete with this type of pulp for a share of the world market.

The biopulping consortia of industry and universities at the Forest Products Laboratory of the USDA Forest Service developed a method of biomechanical pulping over a period of eight years (Akhtar *et al.*, 1996). *Ceriporiopsis subvermispora* (Coriolaceae) was identified as the most efficient fungus for biopulping of soft and hardwood, with the ability to grow on both softwood and hardwood. A United States patent was issued for this method (Blanchette *et al.*, 1991). Application of *C. subvermispora* on *Pinus taeda* resulted in a 42 % saving in energy, 32 kN/g improvement of burst index and 67 mN m^2/g improvement of tear index (Akhtar *et al.*, 1996). A reduction of pulp brightness was experienced during initial trials, but this problem was solved by bleaching with alkaline hydrogen

peroxide or sodium hydrosulphite. Brightness stability was lower than refiner mechanical pulp, but higher than chemithermomechanical pulp.

Several parameters for efficient SSF with *C. subvermispora* were also optimized (Akhtar *et al.*, 1996). Mycelial suspensions of the fungus as well as pre-colonized chips were effective biopulping inocula and aeration was required, but at a low flow rate. One obstacle was the inability of *C. subvermispora* to colonize unsterilized wood chips (Wall *et al.*, 1993), notwithstanding earlier statements that this fungus could be applied to unsterilized chips (Blanchette *et al.*, 1991). *Phanerochaete chrysosporium* (Meruliaceae), on the other hand, was able to colonize unsterilized chips at its optimal growth temperature of 39 °C (Akhtar *et al.*, 1996). *Phanerochaete chrysosporium* was, however, less efficient than *C. subvermispora* to improve pulping. Steaming wood chips at atmospheric pressure resulted in a sufficient degree of asepsis, to allow *C. subvermispora* to colonize chips. Economic evaluation of a biomechanical pulping process showed that treatment of chips in a packed bed reactor yielded a return on investment of 21 % before tax. However, when a chip pile based system was used, the return on investment was a least 106 %. These calculations were based on an industrial chip pile that had been modified to regulate moisture and temperature. The packed bed bioreactor consisted of a bed of chips that had been designed to control process conditions, but required larger capital investment (Akhtar *et al.*, 1996).

A major variable cost factor in the economic evaluation of biopulping is the cost of inoculum (Akhtar *et al.*, 1997a; Wolfaardt *et al.*, 1998). Initial studies showed

that a very high inoculum dosage of 3 kg/ton wood (dry weight) was required for efficient biopulping. It was then discovered that corn steep liquor (CSL) could be added as an inexpensive nutrient source to improve colonization (Akhtar *et al.*, 1997b). By adding CSL, the inoculum requirement was reduced to 0,25 g/ton wood (Akhtar *et al.*, 1996).

Analysis of the waste water from the first pass of treated aspen chips through the refiner indicated that biopulping had reduced the environmental impact (Akhtar *et al.*, 1996). The toxicity was substantially reduced but chemical oxygen demand (COD) values were higher. The increase in COD values was ascribed to the release of products resulting from lignin degradation by the fungi (Akhtar *et al.*, 1996).

In more recent developments, *Phlebiopsis gigantea* (Meruliaceae) has been identified as a fungus that can potentially be applied for biopulping (Behrendt & Blanchette, 1997; Iverson *et al.*, 1997). This fungus is able to grow on a variety of hard and softwoods and, because it is a primary colonizer of fresh wood, it can compete effectively with contaminating microbes. The fungus can grow well at temperatures as high as 37 °C. *Phlebiopsis gigantea* offers protection against blue stain and is also able to reduce the extractives content of wood (up to 69 %) (Iverson *et al.*, 1997). Studies have shown that this fungus can be applied to logs directly after felling, thereby allowing biopulping to start in the forest (Behrendt & Blanchette, 1997).

Treatment of *Pinus taeda* and *P. resinosa* wood with *P. gigantea* resulted in reduced energy consumption during refining (up to 27 % on *P. resinosa*). Paper properties were improved for burst strength (17 %), tear strength (20 %) and tensile strength (13 %), but pulp brightness was reduced (Behrendt & Blanchette, 1997). This technique to treat logs instead of chips could lead to significant savings, because chip sterilization and chip pile aeration is not required (Behrendt & Blanchette, 1997).

BIOCHEMICAL PULPING

The application of biopulping in chemical pulping has not been researched to the same extent as biomechanical pulping (Reid, 1991). However, the effect of fungal treatment on wood has been investigated for the two most important chemical pulping methods namely kraft and sulphite pulping (Sjöström, 1981) as well as organosolv pulping (Aleksandrova *et al.*, 1995).

Biosulphite pulping with white-rot fungi

The use of sulphite pulping has declined in recent years. It is a process that takes place in an acidic pulping liquor that contains a high percentage of free sulphur dioxide (Kouris, 1996). The sulphite base may be calcium, sodium, magnesium or ammonia. Two research groups, one based in Austria (Messner *et al.*, 1992) and the other in the U.S.A. (Scott *et al.* 1995), have focussed on the biological treatment of wood prior to sulphite pulping. Scott et al. (1995) has evaluated the effect of fungal treatment on *P. taeda* chips for different sulphite processes. Chips were treated for

two weeks with two strains of *C. subvermispora* and pulped in a semi alkaline sodium based sulphite as well as an acidic calcium sulphite process. The first process resulted in pulp with a kappa number that was reduced by 27 % compared to the untreated control or, alternatively, a 30 min shorter pulping time to reach the same kappa number. A significant reduction of yield (3,5 %) was also observed. Similar results were obtained with both fungal strains, but strain CZ-3 resulted in the greatest improvement when the calcium based method was used. The kappa number of calcium sulphite pulp was reduced by 49 %, while the yield remained similar to untreated wood. An alternative benefit was pulping time that was reduced by 30min. The improvement by the fungal pretreatment was ascribed to degradation of lignin or its modification for easier removal (Scott *et al.* 1995). In these trials no change in chemical consumption was observed. However, the authors commented that *C. subvermispora* was selected for application in biomechanical processes and that it might not be the most suitable organism for biosulphite pulping (Scott *et al.* 1995).

The potential of fungal pretreatment of wood chips for magnesium based sulphite pulping has been demonstrated in a study by Messner *et al.* (1992) in collaboration with Leykam-Mürtztaler, an Austrian pulp and paper company. It was found that several fungi were able to reduce the kappa number of birch pulp and also increase brightness of pulp, but with a loss of strength. It was also observed that the beneficial effect for biochemical pulping was obtained by a different mechanism from biomechanical pulping (Messner *et al.*, 1992). The effect of fungal treatment on mechanical pulping was ascribed to the reduction of the binding capacity of fibres. In

chemical pulping, the beneficial effect is caused by an increase in lignin solubility (Messner *et al.*, 1992).

Biokraft pulping with white-rot fungi

Kraft pulping is an alkaline process with cooking liquor that contains sodium hydroxide and sodium sulphide (Kouris, 1996). Kraft pulping accounts for 80 % of the world chemical pulp production (Sjöström, 1981). Biokraft pulping has, however, been restricted to studies using blue-stain fungi (Wall *et al.*, 1994), studies utilizing white-rot fungi on hardwood (Chen & Schmidt, 1995; Oriaran *et al.*, 1990; 1991), and the work presented in this thesis (Chapter 6). Valuable information does, however, exist on the kraft pulping properties of softwood that has been decayed by white-rot fungi under natural conditions (Hunt, 1978b; 1978c). These studies focussed on the effect of wood from decadent stands on kraft pulping parameters. The degradation of wood occurred under uncontrolled conditions and results can, therefore, only be applied to biopulping to a limited extent. The most obvious benefit of fungal pre-treatment, is the reduction of lignin content (Oriaran *et al.*, 1990; 1991) or alternatively reduction of the pulping time (Oriaran *et al.*, 1991; Scott *et al.* 1995). These improvements also seem to be associated with reduction in pulp yield (Oriaran *et al.*, 1990; Hunt, 1978b) and chemical consumption (Hunt, 1978a; 1978c).

In one study, aspen chips were compressed into bales after application of *Phanerochaete chrysosporium* inoculum and covered with foil (Chen & Schmidt, 1995). Sufficient fungal growth was obtained with this method without the addition of nutrients, sterilization of chips or stringent control of incubation conditions.

However, the cost of strapping and foil wrapping was not specified. Some loss of wood mass occurred during incubation, but changes in pulp yield was not determined. The strength properties of pulp from treated wood were improved and the rate at which water drained from the pulp (freeness) was reduced (Chen & Schmidt, 1995). However, brightness of the pulp was also reduced.

Our own studies on the kraft pulping of softwood treated with *Stereum hirsutum* showed a substantial reduction of the lignin content of pulp (Chapter 6). Pine chips were treated for three weeks and pulping conditions varied to determine the optimal pulping conditions for fungal treated wood. Under optimal conditions, a 30 % reduction in kappa number was observed. Pulping time could also be reduced to obtain pulp with the same kappa number as the control which could be translated to increased pulp production. However, due to non-selective delignification that occurred, the pulp yield was reduced. Most of this reduction in yield occurred as loss of wood mass before pulping, but some of the reduction also occurred during pulping. The degree of polymerization was not negatively influenced by fungal action, but was found to be a factor of kappa number. The most important disadvantage of this process was an increased alkali consumption (Chapter 6). It is, however, possible to reduce the use of chemicals during the bleaching stages when pulp with a lower kappa number is used (Macleod, 1993).

Organosolv pulping

One of the more recent developments to reduce the environmental impact of the pulping industry has been the application of organosolv pulping. Organic solvents

such as ethanol are utilized to eliminate toxic sulphur-containing wastes (Aleksandrova *et al.*, 1995). Fungal treatment of small samples (15 g) of aspen wood can be combined with aqueous-ethanol pulping to reduce kappa number (Table 1). Aleksandrova *et al.*, (1995) described an increase in yield that was obtained with this method, because an apparent increase in cellulose yield occurred during pulping. However, wood mass was reduced by all three of the fungal strains used and when yield was calculated on a mass balance basis, cellulose yield was reduced (Table 1). Selective delignification was, nonetheless, still improved during biopulping and yield increased by two percentage points when wood treated with *Trametes villosus* was pulped to the same kappa number as untreated wood (Aleksandrova *et al.*, 1995). This biopulping process has, to our knowledge, not been scaled up.

Table 1. Effect of different fungal treatments on the wood mass, cellulose yield and kappa number of treated aspen chips (adapted from Aleksandrova *et al.*, 1995).

Biopulping fungus		**Wood mass (g)**	**Cellulose yield (g)[a]**	**Kappa no.**
Phanerochaete chrysosporium	0 days	15,0	7,9	19,8
	15 days	13,7	7,0	12,9
	Change (%)	**-8,5**	**-11,8**	**-34,8**
Phanerochaete sanguinea	0 days	15,0	7,9	19,3
	15 days	14,7	7,7	14,9
	Change (%)	**-1,7**	**-1,9**	**-22,8**
Trametes villosus	0 days	15,0	7,9	19,7
	15 days	14,3	7,6	15,1
	Change (%)	**-4,8**	**-4,6**	**-23,4**

a Based on the mass balance that includes loss in wood mass.

BIOPULPING WITH CARTAPIP®

Ophiostoma piliferum is a primary colonizer of softwood and is also involved in sap staining (Blanchette *et al.*, 1992). A melanin deficient strain of this fungus (Zimmerman *et al.*, 1995) has been sold commercially since 1990 (Farrell *et al.*, 1993) and is currently the only fungal product that is available for commercial biopulping (Schmitt *et al.*, 1998). The inoculum consists of lyophilized mycelium and conidia and is sold under the trade name Cartapip®97 (Schmitt *et al.*, 1998). Cartapip® is suspended in fresh water and sprayed onto wood chips before stacking (Farrell *et al.*, 1994). The fungus is able to colonize freshly cut wood and utilize sugars and extractives in the wood, but is unable to degrade cellulose or lignin (Farrell *et al.*, 1994). The fungus colonizes wood via ray parenchyma cells and resin canals and disrupts pit membranes (Blanchette *et al.*, 1992). It is, therefore, applied to control staining and decay of wood chips, reduce pitch in mechanical pulp and for biochemical pulping (Schmitt *et al.*, 1998). An incubation time of seven to fourteen days is required to achieve any benefit (Schmitt *et al.*, 1998).

Biomechanical pulping

Ophiostoma piliferum (Cartapip®) was the first fungus to be applied commercially in a biopulping process (Farrell *et al.*, 1993). Cartapip® was develop in collaboration with Bear Island Paper Company (BIPCo), a thermomechanical pulp mill (Haller & Kile, 1992). The mill has applied Cartapip® since 1991 and obtains the same benefits that were previously obtained by aging of chips (Haller & Kile,

1992). However, with Cartapip® no loss of brightness due to chip staining occurred (Schmitt *et al.*, 1998).

At pulp mills, the storage of wood chips is preferred to the storage of logs, because chips are more economical to handle (Hulme, 1979). However, during prolonged storage, contaminating fungi cause a darkening of chips that leads to decreased brightness of thermomechanical pulp (Farrell *et al.*, 1993). One of the characteristics of *O. piliferum* is, that it is a primary colonizer that can compete strongly with other fungi on freshly chipped wood (Farrell *et al.*, 1993). The contaminating fungi include sap-staining as well as decay fungi (Lindgren & Eslyn, 1961). By reducing staining and decay, application of Cartapip® can reduce the bleaching requirement of pulp and improve pulp yield. The fungus grows in tracheids, ray parenchyma cells and resin ducts while metabolizing extractives. The disruption of parenchyma cells weakens the binding of tracheids, thereby allowing easier separation of wood fibres (Blanchette *et al.*, 1992). The benefit of loosened fibres is that energy is saved during mechanical pulping.

At BIPCo, approximately 1200 tons of freshly cut, unsterilized southern yellow pine (50 % *P. taeda* and 50 % *P. virginiana*) chips are treated per day with Cartapip® at a screw conveyor (Haller & Kile, 1992). Incubation occurs on an unmodified chip pile for 14 days (Schmitt *et al.*, 1998), but the chip pile is managed by turning it, to prevent overheating. Biopulping resulted in improved brightness (0,9 %), tensile strength (5,4 %), tear strength (3,4 %) (Haller & Kile, 1992) and burst strength (3,3 %) (Schmitt *et al.*, 1998). Pilot trials with *P. taeda* have shown that it is

also possible to reduce the energy requirement for mechanical pulping with application of Cartapip ® (Kohler *et al.*, 1995). For the same energy input, pulp with a greater tensile strength was obtained. Fibre length increased and fines were reduced in lab scale trials during the same study.

Depitching

A large variety of compounds, found in wood, are soluble in neutral organic solvents or water. These compounds are collectively called extractives or pitch (Sjöström, 1981). Wood extractives are important causes of production and quality problems in pulp as well as paper mills (Hassler, 1988). Pulp produced from wood with high pitch content has reduced strength (Farrell *et al.*, 1993) and optical properties (Hillis, 1962). The presence of extractives in pulp could also lead to breakage of sheets on paper machines (Farrell *et al.*, 1993).

One of the methods available to control pitch, is the application of Cartapip® to stored wood chips before pulping (Farrell *et al.*, 1993). The fungus reduces the amount of pitch in the wood by metabolizing it (Blanchette *et al.*, 1992). Treatment of southern yellow pine chips at BIPCo resulted in reduction of triglycerides, resin acids as well as an unidentified extractives fraction (Schmitt *et al.*, 1998). The diminished extractives content (-37,5 %) reduced the requirement for alum (-31,7 %) to control pitch while the control of chip discolouration resulted in a reduction in the use of bleaching chemicals (-36,9 %) (Haller & Kile, 1992). Application of Cartapip® reduces the time that chips have to be seasoned to reduce extractives (Wall *et al.*, 1994). The wood inventory can, therefore, also be reduced to save money. The

effect of Cartapip® on the extractives content of wood from other soft- and hardwood species has also been determined (Table 2). The extractives content of wood from all these species was reduced by at least 11 % compared to fresh chips. This improvement was not as high when compared to aged chips, but the improvement was achieved in a shorter time (Table 2).

Table 2. Effect of treatment with Cartapip® on extractives content on wood from different species (adapted from Schmitt *et al.*, 1998).

	Reduction in extractives content (%)	
Species	Compared to fresh chips	Compared to aged chips
Southern Yellow Pine	40	22
Jack Pine (*P. banksiana*)	31	22
Radiata pine (*P. radiata*)	11	0
Red Pine (*P. resinosa*)	33	23
Hemlock	11	0
Aspen (*Populus* sp.)	40	20
Maple (*Acer* sp.)	26	1
Cottonwood	40	14
Birch (*Betula* sp.)	32	11

Biochemical pulping

The presence of wood extractives impairs the penetration of pulping chemicals into chips, thereby increasing chemical consumption and pulping time (Gardner & Hillis, 1962). Cartapip® can increase the porosity of wood, which allows faster penetration of pulping chemicals, by the consumption of pitch and opening of pit membranes (Wall *et al.*, 1994). Smaller amounts of chemicals and shorter pulping times are, therefore, required.

In one study by Wall *et al.* (1994), 10 tons of white fir (*Abies concolor*) chips were treated with Cartapip® and pulped by means of a sulphite process. The K-number of pulp was reduced by 3,2 %. Pulp yield and viscosity increased by 4,2 % and 32 % respectively. These results illustrate that extractives are more importance in sulphite pulping, because fatty acids are not saponified during pulping as in kraft pulping (Wall *et al.*, 1994).

Mill-scale trials for biosulphite pulping of aspen wood have shown that K-number of unbleached pulp was reduced by 7 % while the amount of rejects was reduced by 12 % and the viscosity increased by 19 % (Schmitt *et al.*, 1998). An increase of 1 % in brightness of unbleached pulp has contributed to a 7 % to 10 % decrease in consumption of bleaching chemicals (Schmitt *et al.*, 1998). One of the downstream benefits was the reduction in use of sizing agents that could translate to a saving of US$ 6 per ton of pulp (Schmitt *et al.*, 1998).

Biokraft pulping trials with Cartapip® on hardwood have only been completed on laboratory scale (Wall *et al.*, 1994). Up to 20 % reduction in the active alkali requirement was obtained under these conditions or, alternatively, pulp was produced with a 29 % lower kappa number under the same pulping conditions (Schmitt *et al.*, 1998). Viscosity improved when fungal treated samples were pulped to the same kappa number as control samples (Wall *et al.*, 1994). Pulp yield remained unchanged, because *O. piliferum* is unable to degrade cellulose (Schmitt *et al.*, 1998).

Small samples (500 g) of fresh softwood chips have been treated with Cartapip® for kraft pulping (Wall *et al.*, 1994). Samples that contained 70 % *P. banksiana* and 30 % *Picea abies* were treated for two weeks to produce pulp with a reduced (12 %) Kappa number. Less active chlorine (9 %) was required in the D/C stage during bleaching of the pulp in an O-D/C-Eo-H-D bleaching sequence (Wall *et al.*, 1994). The pulp also responded better to refining.

BIOPULPING OF NON-WOOD FIBRE

About 7 % of the current world pulp production is from non-wood fibres (Webb, 1992), but non-wood fibre sources are becoming more important in the supply of plant fibre for pulp and paper products (Bolton, 1996). At present 330 mills worldwide produce pulp from non-wood fibre (Croon, 1995), with two mills in South Africa that utilize bagasse as raw material. Biopulping of bagasse is, therefore, of special significance to the South African pulping industry.

One of the hurdles in the pulping of bagasse is the seasonal availability of the raw material (Atchison, 1987). This necessitates special storage practices, such as wet bulk storage, to preserve the fibre (Salabar & Maza, 1971). However, the long periods of storage (sometimes more than one year) also offer an opportunity to pre-treat bagasse to improve the pulping properties. Biopulping of bagasse could have a number of advantages. Large quantities of water is used for the preservation of fibre in wet bulk storage of bagasse (Atchison, 1987). Biopulping fungi would also preserve fibre and could be applied on bagasse with a lower moisture content. In a

semi-arid country such as South Africa the saving of water can be a significant advantage. The lignin content of fibre and, consequently, in pulp could be reduced (Hatakka *et al.*, 1996). Fibre discolouration and pith content could be reduced, resulting in improved wet depithing (Atchison, 1987). The reduced lignin and pith content could result in reduced chemical consumption during pulping and in improved pulp quality (Wolfaardt *et al.*, 1998).

Biopulping of bagasse is one of the relatively unexplored fields in biotechnology for the pulping industry, although many considerations that make such a procedure theoretically feasible. For example, bagasse is stored for long periods (Hurter, 1991), allowing enough time to treat raw material with fungi. Capital and other resources that is currently used for the preservation of bagasse (Hurter, 1991) could be diverted to the biopulping processes. Additionally, the colonization of bagasse by fungi is favoured by the residual sugars and the exposed surface area (Ramaswamy *et al.*, 1989).

The small number of publications that deal with fungal pre-treatment of bagasse (Delgado *et al.*, 1992, Johnsrud *et al.*, 1987) does not refer to the soda pulping method that is widely used. One technique combines fungal pre-treatment with the so-called Cuba-9 process (Johnsrud *et al.*, 1987), which produces newsprint with a modified cold soda process prior to mechanical refining of bagasse. Other publications deal with the fungal treatment of kenaf and jute prior to steam explosion and refining (Sabharwal *et al.*, 1996), gramineous plants prior to soda pulping (Hatakka *et al.*, 1996) and biopulping of wheat straw (Rostanci & Yalinkilic, 1991).

The white-rotting fungi are the organisms most frequently used for biopulping of wood (Messner & Srebotnik, 1994) and also non-wood fibre (Delgado *et al.*, 1992; Hatakka *et al.*, 1996; Johnsrud *et al.*, 1987; Rostanci & Yalinkilic, 1991; Sabharwal *et al.*, 1996). These fungi are known to degrade all the wood components, but lignin is degraded with varying degrees of selectivity (Rostanci & Yalinkilic, 1991). The selectivity with which lignin is degraded depends on specific strains as well as the specific treatment conditions (Rostanci & Yalinkilic, 1991; Ríos & Eyzaguirre, 1992). Cellulase deficient isolates of *Phanerochaete chrysosporium* have been used to enhance selectivity (Johnsrud *et al.*, 1987), but wild type isolates of *P. chrysosporium* and a hybrid strain of *Pleurotus ostreatus* have also been used (Delgado *et al.*, 1992).

Different fungal species have been used to treat other non-wood fibres. An isolate of *C. subvermispora* has been used for biopulping of kenaf and jute (Sabharwal *et al.*, 1996) and *Pleurotus ostreatus* for wheat straw (Rostanci & Yalinkilic, 1991). *Phlebia radiata*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Panus tigrinus*, *Phlebia tremellosa* and *C. subvermispora* were used to treat reed canary grass and tall fescue prior to soda pulping (Hatakka *et al.*, 1996). *Lenzites betulina* (Wolfaardt *et al.*, 1998), *P. ostreatus* and *P. chrysosporium* (Delgado *et al.*, 1992; Johnsrud *et al.*, 1987) have been used for biopulping of sugarcane bagasse.

The most successful method for the production of inoculum of white-rot fungi for biopulping is to use a pre-inoculum of homogenized mycelium (Wolfaardt *et al.*, 1998). A pre-inoculum was also used by Johnsrud *et al.* (1987) and inoculum was

then produced in a fermenter. By using this method, it was possible to harvest inoculum after eight days. Although special media have been used (Johnsrud *et al.*, 1987), molasses can provide a convenient nutrient source, as it can be obtained from nearby sugar mills and is inexpensive.

In small-scale experiments dried, depithed bagasse (60 % to 70 % fibre) was soaked in water, after which moisture was adjusted to various levels between 50 % and 80 % (Johnsrud *et al.*, 1987). The moist bagasse was then sterilized by autoclaving, inoculated and then incubated at 28 °C or 39 °C for 10 to 20 days. Screening experiments with bagasse were done with as little as 2 g of bagasse in 100 ml flasks, but bench scale treatments were done in 1 L flasks or 10 L cylinders (Johnsrud *et al.*, 1987).

In these bench scale experiments, reactors were flushed with oxygen and relative humidity controlled. Polyethylene bags filled with 1 kg sterilized bagasse at 70 % to 75 % moisture have been used in SSF for the production of biopulp and for the production of enriched animal feed (Delgado *et al.*, 1992). Degradation was enhanced by oxygenation compared to aeration, especially when done intermittently (Johnsrud *et al.*, 1987). Optimal conditions were approximately 65 % moisture and 95 % relative humidity (Johnsrud *et al.*, 1987). Moisture of the substrate is one of the key factors influencing SSF. It is important that water is available only as thin films on the bagasse surface to increase the surface-to-volume ratio for oxygen and carbon dioxide transfer (Mudgett & Paradis, 1985; Wall *et al.*, 1993). On the other hand, low moisture increases the risk of fire (Hurter, 1991) and limits fungal growth (Wall *et al.*,

1993). Temperature is one of the most important physical parameters and also the most difficult to control (Prior *et al.*, 1992). Maintaining temperature as close as possible to the optimal biopulping temperature for the selected fungus decreases treatment time and may give the fungus the competitive edge over contaminants (Wall *et al.*, 1993).

Incubation time plays an important role in the degradation of lignocellulosic materials. After biopulping of wheat straw, lignin content, 1 % caustic solubility, cellulose and holocellulose were determined (Rostanci & Yalinkilic, 1991). This analysis showed that most of the lignin was degraded in the latter part of the treatment time. After 20 days of incubation of bagasse under optimal conditions, 2,5 % weight loss and 8,7 % lignin loss were achieved and no discolouration was apparent (Johnsrud *et al.*, 1987). Delgado *et al.* (1992) showed 9,6 % lignin decrease after 11 days of treatment with a hybrid *P. ostreatus* and 14,7 % decrease after 35 days. The hybrid *P. ostreatus* was able to modify bagasse more selectively than a cellulase deficient strain of *P. chrysosporium*.

Some researchers hypothesize that reduction of lignin can lead to savings in consumption of pulping chemicals (Rostanci & Yalinkilic, 1991; Duarte *et al.*, 1996). Optimization of parameters for the kraft pulping of fungal treated wood has shown this not to be true (Chapter 6). The consumption of chemicals by fungal mycelium has been investigated by Johnsrud *et al.* (1987), who showed that the increase in NaOH consumption was due to the dissolution of mycelium. This point is debatable as it was previously thought to be caused by the lower-molecular weight products of

decay (Hunt & Hatton, 1979). The effect of fungal treatment on pith content is unknown and might even reduce chemical consumption by degrading pith that generally causes increased chemical consumption (Giertz *et al.*, 1979; Wolfaardt *et al.*, 1998).

Fungal treatment used in combination with the Cuba-9 process increased the beatability of bagasse pulp and produced hand sheets with strength properties similar to those obtained from reference pulp (Johnsrud *et al.*, 1987). On kenaf and jute, the fungal treatment improved strength properties (Sabharwal *et al.*, 1996). Soda pulping of fungal treated reed canary grass produced fine paper (furnish composition of 40 % grass, 40 % softwood and 20 % talc) with better printing and strength properties (Hatakka *et al.*, 1996).

CONCLUSIONS

The potential of biopulping has been evaluated for most pulping methods and raw materials that are used for the production of paper pulp. It is clear that different fungi are suited to each process and that these processes must be adapted to achieve the full potential of an environmentally friendly technology. It has been demonstrated that biopulping offers some flexibility that will suit the individual requirements of mills. Pulping can, for instance, be adapted to produce chemical pulp with lower lignin content or the pulping time can be reduced to increase production (Wall *et al.*, 1994; Chapter 6).

Certain negative effects such as increased chemical consumption (Chapter 6) and reduction in yield have, unfortunately, also been associated with certain biopulping processes. However, these problems could in most cases be ascribed to the utilization of fungi that were not suitable for the specific type of pulping or SSF that was not conducted under optimal conditions. Utilization of fungi that are more selective in the degradation can, for example, improve the pulp yields. It was found that incubation time plays an important role in selective delignification of wood (Rostanci & Yalinkilic, 1991) and that with long treatment times degradation became less selective. Treatment time will, therefore, play a significant role in the success of an industrial biopulping process.

The most important factors to be considered in the design of a biopulping process have been investigated and described by Wall *et al.* (1993). These factors include the choice of organism, degree of asepsis, size and type of inoculum, control of physical conditions such as temperature and aeration, as well as the addition of nutrients (Kirk *et al.*, 1976). Thus the most important design factors for biopulping are similar to those to be considered for all SSF procedures. The economical viability of biopulping is determined by two contributing factors. The most important capital investment, is that required for the modification of the chip pile to allow SSF (Wall *et al.*, 1993) and the most important variable cost item, is that of the inoculum (Akhtar *et al.*, 1997a; Wolfaardt *et al.*, 1998).

Environmental benefits of biopulping have been demonstrated (Akhtar *et al.*, 1996), but unfortunately, environmental benefits are often difficult to quantify.

Application of biopulping is, therefore, only considered on the basis of economic benefits and the modelling of pulping processes is required for the prediction of the economic benefits.

REFERENCES

AKHTAR, M., BLANCHETTE, R.A. & KIRK, T.K. 1997a. Fungal delignification and biomechanical pulping of wood. *Adv. Biochem. Eng. Biotechnol.* 57: 159-195.

AKHTAR, M., KIRK, T.K. & BLANCHETTE, R.A. 1996. Biopulping: An overview of consortia research. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 187–192. Facultas-Universitätsverlag, Vienna, Austria.

AKHTAR, M., LENTZ, M.J., BLANCHETTE, R.A. & KIRK, T.K. 1997b. Corn steep liquor lowers the amount of inoculum for biopulping. *Tappi J.* 80(6): 161-164.

AKHTAR, M., LENTZ, M.J., SWANEY, R.E., SCOTT, G.M., HORN, E. & KIRK, T.K. 1998. Commercialization of biopulping for mechanical pulping. Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada. Vol. A: 55-58.

AKHTAR, M., LENTZ, M.J., SWANEY, R.E., SHIPLEY, D.F., SCOTT, G.M., HORN, E.G. & KIRK, T.K. 1997c. Meeting biological and engineering challenges during scale-up of biopulping. *Tappi Biological Sciences Symposium*, pp. 35-39. Tappi Press, Atlanta, USA.

ALEKSANDROVA, G., MEDVEDEVA, S., KANITSKAYA, L., BABKIN, V. & ZAKAZOV, A. 1995. An ecologically sound pulping process involving microbiological delignification. Proceedings of the 8th International Symposium on Wood and Pulping Chemistry Vol. 2: 273-278.

ATCHISON, J.E. 1987. Bagasse. In: Pulp and Paper Manufacture (3), Secondary fibers and non-wood pulping. Ed. M.J. Kocurek, pp. 22–70. Tappi Press, Atlanta, USA.

BEHRENDT, C.J. & BLANCHETTE, R.A. 1997. Biological processing of pine logs for pulp and paper production with *Phlebiopsis gigantea*. *Appl. Environ. Microbiol.* 63: 1995-1999.

BLANCHETTE, R.A., FARRELL, R.L., BURNES, T.A., WENDLER, P.A., ZIMMERMAN, W., BRUSH, T.S. & SNYDER, R.A. 1992. Biological control of pitch in pulp and paper production by *Ophiostoma piliferum*. *Tappi J.* 75: 102-106.

BLANCHETTE, R.A., LEATHAM, G.F., ATTRIDGE, M., AKHTAR, M. & MYERS, G.C. 1991. Biomechanical pulping with *C. subvermispora*. *U.S. Pat.* 5,055,159.

BOLTON, J. 1996. Plant derived composites and the future importance of non-wood or annually harvested crops. *Commonwealth For. Rev.* 75(2): 164-168.

CHEN, Y. & SCHMIDT, E.L. 1995. Improving aspen kraft pulp by a novel, low-technology fungal pretreatment. *Wood Fiber Sci.* 27(2): 198-204.

COBB, F.W., KRSTIC, M., ZAVARIN, E. & BARBER, H.W. 1968. Inhibitory effects of volatile oleoresin components on *Fomes annosus* and four *Ceratocystis* species. *Phytopathology.* 58: 1327-1335.

CROON, I. 1995. Straw grass fibres. *Papermaker* December: 33-34.

DELGADO, G., FIDALGO, M.L., MANSUR, M., MARTÍNEZ, E.O. ORTEGA, G., MARTÍNEZ, A.T. & GONZÁLEZ, A.E. 1992. Lignin alteration during the solid-state fermentation of sugarcane wastes with *Pleorotus ostreatus* and *Phanerochaete chrysosporium*. In: Biotechnology in the pulp and paper industry. Proceedings of the 5th International Conference on Biotechnology in the Pulp and Paper Industry. Eds. M. Kuwahara & M. Shimada, pp. 209–214. Uni Publishers Co., Tokyo, Japan.

DUARTE, J., MENDES SOUSA, A., COSTA-FERREIRA, M., MORGADO, J., VASCONCELOS, L. & PEREIRA, H. 1996. Studies on the bio-kraft pulping of eucalyptus wood. In. Proceedings of the 6th International Conference on Biotechnology in the Pulp and Paper Industry. p. 88.

FARRELL, R.L. & ALLISON, R.W. 1997. Effect of Cartapip 97® pretreatment on the kraft pulping of radiata pine. *Tappi Biological Sciences Symposium*, pp. 15-18. Tappi Press, Atlanta, USA.

FARRELL, R.L., BLANCHETTE, R.A., BRUSH, T.S., HADAR, H., IVERSON, S., KRISA, K., WENDLER, P.A. & ZIMMERMAN, W. 1993. Cartapip™: A biopulping product for control of pitch and resin acid problems in pulp mills. *J. Biotechnol.* 30: 115-122.

FARRELL, R.L., BRUSH, T.S., FRITZ, A.R., BLANCHETTE, R.A. & IVERSON, S. 1994. Cartapip®: A biological product for control of pitch and resin acid problems in pulp mills. *Tappi Biological Sciences Symposium*, pp. 85-87. Tappi Press, Atlanta, USA.

FISCHER, K., AKHTAR, M., MESSNER, K., BLANCHETTE, R.A. & KIRK, T.K. 1996. Pitch reduction with the white-rot fungus *Ceriporiopsis subvermispora*. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the 6th International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 193–198. Facultas-Universitätsverlag, Vienna, Austria.

GARDNER, J.A.F. & HILLIS, W.E. 1962. The influence of extractives on the pulping of wood. In: Wood extractives and their significance to the pulp and paper industries, Ed. W.E. HILLIS, pp. 367-403. Academic Press, New York, USA.

GIERTZ, H.W. & VARMA, R.S. 1979. Studies on the pulping of bagasse and influence of pith on paper properties. In: Non-wood plant fiber pulping, Progress Report 10. pp. 53–69. Tappi Press, Atlanta, USA.

HALLER, T.M. & KILE, G. 1992. Cartapip® treatment of wood chips to reduce pitch and improve processing. *Tappi Pulping Conference*, pp. 1243-1252. Tappi Press, Atlanta, USA.

HASSLER, T. 1988. Pitch deposition in papermaking and the function of pitch control agents. *Tappi J.* 71: 195-201.

HATAKKA, A., METTÄLÄ, A., HÄRKÖNEN, T. & PAAVILAINEN, L. 1996. Biopulping of gramineous plants by white-rot fungi. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 229–232. Facultas-Universitätsverlag, Vienna, Austria.

HILLIS, W.E. 1962. The influence of extractives on the color of groundwood in newsprint. In: Wood extractives and their significance to the pulp and paper industries, Ed. W.E. HILLIS, pp. 405-419. Academic Press, New York, USA.

HULME, M.A. 1979. Outside chip storage – Deterioration during OCS. In: Chip quality monograph, Ed. J.V HATTON, pp. 213-244. Tappi Press, Atlanta, USA.

HUNT, K. 1978a. Pulping western hemlock decayed by white-rot fungi. *Pulp Pap. Can.* 79(6): 75-80.

HUNT, K. 1978b. Kraft pulping of decayed wood: western red cedar and alpine fir. *Pulp Pap. Can.* 79(7): 30-35.

HUNT, K. 1978c. How rot-decayed wood affects chipping and kraft pulping. *Pulp Pap. Can.* 79(8): 65-68

HUNT, K. & HATTON, J.V. 1979. Predicting the kraft pulping behavior of decayed pulpwood. *Pulp Pap. Can.* 80(3): 55-57.

HURTER, A.M. 1991. Bagasse storage and deterioration. *Tappi Pulping Conference*, pp. 203-217. Tappi Press, Atlanta, USA.

IVERSON, S., BEHRENDT, C., WILLIAMS, D., BLANCHETTE, R. & WALL, M.B. 1997. *Phlebiopsis gigantea*, a white rot fungus for biomechanical pulping. *Tappi Biological Sciences Symposium*, pp. 25-28. Tappi Press, Atlanta, USA.

JACOBS, C.J., VENDITTI, R.A. & JOYCE, T.W. 1998. Effect of enzymatic pretreatments on conventional kraft pulping. *Tappi. J.* 81(2): 143-147.

JACOBS-YOUNG, C.J., VENDITTI, R.A. & JOYCE, T.W. 1998. Effect of enzymatic pretreatment on the diffusion of sodium hydroxide in wood. *Tappi. J.* 81(1): 260-266.

JOHNSRUD, S.C., FERNANDEZ, N., LOPEZ, P., GUTIÉRREZ, I., SAEZ, A. & ERIKSSON, K.-E. 1987. Properties of fungal pretreated high yield bagasse pulps. *Nord. Pulp Pap. Res. J.* special issue: 47-52.

KIRK, T.K., CONNORS, W.J. & ZEIKUS, J.G. 1976. Requirement for a growth substrate during lignin decomposition by two wood-rotting fungi. *Appl. Environ. Microbiol.* 32(1): 192-194.

KOHLER, L.F., MALCOLM, E.W., FARRELL, R.L., DINUS, R.J., RUDIE, A.W. & BRUSH, T.S. 1995. Improving the softwood mechanical pulp properties with *Ophiostoma piliferum*. *Tappi Pulping Conference*, pp. 303-308. Tappi Press, Atlanta, USA.

KOURIS, M. 1996. Dictionary of paper, 5th ed. Tappi Press, Atlanta, USA.

LINDGREN, R.M.. & ESLYN, W.E. 1961. Biological deterioration of pulpwood and pulp chips during storage. *Tappi J.* 44: 419-429.

MACLEOD, J.M. 1993. Extended delignification: a status report. *Appita* 46(6): 445-451.

MESSNER, K., MASEK, S., SREBOTNIK, E. & TECHT, G. 1992. Fungal pretreatment of wood chips for chemical pulping. In: Biotechnology in the pulp and paper industry: Proceedings of the 5th International Conference on Biotechnology in the Pulp and Paper Industry. Eds. M. Kuwahara & M. Shimada, pp. 9–13. Uni Publishers Co., Tokyo, Japan.

MESSNER, K. & SREBOTNIK, E. 1994. Biopulping: An overview of developments in an environmentally safe paper-making technology. *FEMS Microb. Rev.* 13: 351-364.

MITCHELL, D.A. 1992. Microbial basis of process. In: Solid substrate cultivation, Eds. H.W. Doelle, D.A. Mitchell & C.E. Rolz, pp. 17-28. Elsevier Science Publishers, Barking, UK.

MITCHELL, D.A. & LONSANE, B.K. 1992. Definition, charateristics and potential. In: Solid substrate cultivation, Eds. H.W. Doelle, D.A. Mitchell & C.E. Rolz, pp. 1-16. Elsevier Science Publishers, Barking, UK.

MUDGETT, R.E. & PARADIS, A.J. 1985. Solid-state fermentation of natural birch lignin by *Phanerochaete chrysosporium*. *Enzyme Microb. Technol.* 7: 150-154.

ORIARAN, T.P., LABOSKY, P. & BLANKENHORN, P.R. 1990. Kraft pulp and papermaking properties of *Phanerochaete chrysosporium*-degraded aspen. *Tappi J.* 73(7): 147-152.

ORIARAN, T.P., LABOSKY, P. & BLANKENHORN, P.R. 1991. Kraft pulp and papermaking properties of *Phanerochaete chrysosporium*-degraded red oak. *Wood Fiber Sci.* 23(3): 316-327.

PEARCE, R.B. 1996. Tansley Review No. 87. Antimicrobial defences in the wood of living trees. *New Phytol.* 132: 203-233.

PRIOR, B.A., DU PREEZ, J.C. & REIN, P.W. 1992. Environmental parameters. In: Solid substrate cultivation, Eds. H.W. Doelle, D.A. Mitchell & C.E. Rolz, pp. 65-85. Elsevier Science Publishers, Barking, UK.

QIN, M.H. & CHEN, J.X. 1997. Biological pitch control of masson pine groundwood pulp by lipases. *Tappi Biological Sciences Symposium*, p. 369. Tappi Press, Atlanta, USA.

RAMASWAMY, V., RAMANATHAN, T. & VENKATARAMAN, T.S. 1989. Role of fungi in the biodegradation of wet pile stored bagasse under tropical conditions. *Tappi Pulping Conference*, pp. 803-806. Tappi Press, Atlanta, USA.

REID, I.D. 1991. Biological pulping in paper manufacture. *Tibtech* 9(8): 262-265.

RÍOS, S. & EYZAGUIRRE, J. 1992. Conditions for selective degradation of lignin by the fungus *Ganoderma australis*. *Appl. Microbiol. Biotechnol.* 37: 667-669.

ROSTANCI, S. & YALINKILIC, M.K. 1991. Preliminary studies on biodegradation of wheat straw (*Triticum aestivum* L.) by oyster mushroom (*Pleorotus ostreatus* Jacq.) aimed at producing biopulp. In: Non-wood plant fiber pulping, Progress Report 19. pp. 83–88. Tappi Press, Atlanta, USA.

SABHARWAL, H.S., AKHTAR, M., YU, E., D'AGOSTINO, D., YOUNG, R.A. & BLANCHETTE, R.A. 1996. Development of biological pulping processes for non-woody plants. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 233–236. Facultas-Universitätsverlag, Vienna, Austria.

SALABAR, J. & MAZA, F. 1971. Ritter biological treatment process for bagasse bulk storage. *Non-wood Plant Fiber Pulping Prog. Rep.* 2: 89-136.

SCHMITT, E.K., MIRANDA, M.B. & WILLIAMS, D.P. 1998. Applications of fungal inocula in the pulp and paper industry. Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada. Vol. A: 13-16.

SCOTT, G.M., LENTZ, M. & AKHTAR, M. 1995. Fungal pretreatment of wood chips for sulfite pulping. *Tappi Pulping Conference*, pp. 355-361. Tappi Press, Atlanta, USA.

SJÖSTRÖM, E. 1981. Wood chemistry: Fundamentals and Applications, 2nd ed. Academic Press, San Diego, USA.

WALL, M.B., BRECKER, J., FRITZ, A., IVERSON, S. & NOËL, Y. 1994. Cartapip® treatment of wood chips to improve chemical pulping efficiency. *Tappi Biological Sciences Symposium*, pp. 67-76. Tappi Press, Atlanta, USA.

WALL, M.B., CAMERON, D.C. & LIGHTFOOT, E.N. 1993. Biopulping process design and kinetics. *Biotech. Adv.* 11: 645-662.

WALL, M.B., STAFFORD, G., NOEL, Y., FRITZ, A., IVERSON, S. & FARRELL, R.L. 1996. Treatment with *Ophiostoma piliferum* improves chemical pulping efficiency. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the 6th International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 205–210. Facultas-Universitätsverlag, Vienna, Austria.

WEBB, L. 1992. Paper and the environment – Part 3: Pulping and bleaching. *Paper Focus* 66(6): 20-22.

WOLFAARDT, F., DU PLOOY, A., DUNN, C., GRIMBEEK, E. & WINGFIELD, M. 1998. Biopulping of sugarcane bagasse: Improvement of the Semi-Alkaline Sulphite-Anthraquinone process. Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada. Vol. B: 53-55.

ZIMMERMAN, W., BLANCHETTE, R.A., BURNES, T.A. & FARRELL, R.L. 1995. Melanin and perithecial development in *Ophiostoma piliferum*. *Mycologia* 87: 857-863.

CHAPTER 2

A SURVEY OF SOUTH AFRICAN WOOD-INHABITING BASIDIOMYCETES AND CHARACTERIZATION OF CULTURED STRAINS

Lenzites elegans

ABSTRACT

Wood-inhabiting fungi, especially white-rot fungi, are important for possible application in industrial processes involving the biological degradation of lignin or lignified compounds. Much is known about the diversity and taxonomy of South African wood-inhabiting fungi, but little is known about their potential to degrade lignocellulosic materials. The aim of this study was to collect different species of Basidiomycetes from a variety of substrates, habitats and localities. The aim was, furthermore, to identify the collected fungi, grow and maintain them in culture and investigate their ability to degrade lignin. Two hundred and seventy eight strains of Basidiomycetes with potential to degrade wood were collected from various habitats. This collection included 43 species from 13 families. Two species were collected that have not previously been recorded from South Africa. Different groups of fungal strains were characterized on the basis of the enzymes that they produce and their oxidase reactions. The different strains of fungi could be useful in various biotechnological applications and the information that was obtained on their physiology could assist in the future selection of specific fungi for such applications.

INTRODUCTION

The so-called white-rot fungi gained research attention when their efficiency in degradation of lignin and phenolic compounds was realized (Crowder *et al.*, 1978; Evans, 1987; Joyce, 1992). They have since been considered for application in a variety of processes such as biochemical pulping (Akhtar *et al.*, 1997; Messner *et al.*, 1993; Wolfaardt *et al.*, 1996), biomechanical pulping (Akhtar *et al.*, 1992; Blanchette *et al.*, 1992), biobleaching (Kirkpatrick *et al.*, 1990; Onysko, 1993; Reid & Paice, 1994), treatment of pulp and bleach-plant effluents (Galeno & Agosin, 1990; Bergbauer *et al.*, 1991) and bioremediation of soils contaminated with xenobiotics (Spiker *et al.*, 1992; Field *et al.*, 1993). These various applications appear to have specific requirements (Job-Cei *et al.*, 1991) and different species or strains of wood-decay fungi will most likely be needed to meet unique demands. It has become apparent that application of fungi in biotechnological processes would not be possible without a clear knowledge of the physiology of these fungi.

One of the requirements for fungi that are to be used in biopulping, is that they degrade lignin selectively (Blanchette *et al.*, 1988). These fungi must be able to degrade lignin without substantially degrading cellulose. Although selective white-rot fungi have been recognized as potential agents for biotechnological processes, they are often difficult to identify as white-rot fungi under field conditions (Cookson, 1995). A white type of decay might, for instance, appear brown during the incipient stages (Davidson *et al.*, 1938). It must be borne in mind that the term "white-rot" does not have any taxonomic significance. It is, therefore, more important that strains that

produce specific enzymes to effectively degrade lignified compounds should be recognized. All white-rot fungi produce lignin peroxidases that are responsible for ring opening and cleavage of bonds (Feichter, 1993). It also appears that laccase is one of the important enzymes responsible for the degradation of lignin. This enzyme can oxidize phenols and break C-C and C-O bonds (Feichter, 1993).

One of the most important factors in the development of a biopulping process could be the choice of a suitable fungal strain (Wall *et al.*, 1993). The search for superior fungal strains is also seen as one of the main target areas for the development of a successful biopulping process (Reid, 1991). Despite the emphasis on the utilization of biodiversity, screening of wood-decay fungi has been restricted mainly to previously characterized strains and especially to those from culture collections (Job-Cei *et al.*, 1991; De Jong *et al.*, 1992). This has led to the use of a limited number of strains from only a small number of species for biopulping (Reid, 1991). Comparison of different fungal strains has shown marked differences in the physiological pathways by which they degrade lignin (De Jong *et al.*, 1992). It is, therefore, difficult to predict the suitability of strains from our collection for biotechnological applications on the basis of earlier studies.

Excellent publications on South African wood-inhabiting Basidiomycetes exist (Reid, 1975). These publications are, however, mostly concerned with distribution, morphology and taxonomy and include little information of relevance to applied research (Doidge, 1950; Talbot, 1951; 1958; Van der Byl, 1924; 1928; Van der

Westhuizen, 1971; Van der Westhuizen & Eicker, 1994). These fungi are also not available as cultures and exist only as dried herbarium specimens. It is, therefore, with some difficulty that the use of South African strains of decay fungi in biotechnology can be investigated. In this study wood-inhabiting Basidiomycetes were collected from different geographical regions and habitats and the cultured strains characterized.

White-rot fungi can generally be distinguished from brown-rot fungi by the extracellular oxidase enzymes that they produce (Nobles, 1958). Bavendamm (1928) was the first to use small amounts of gallic and tannic acid in media to detect these differences. Davidson (1938) identified 96 % of the fungi showing positive Bavendamm reactions on these media as white-rotting fungi. Nobles (1948) took this technique one step further with a gum guaiac test to determine the presence of phenolases, but did not differentiate between different enzymes. Differences in the intensity of diffusion zones and growth rate of fungi on these media have since been used universally to differentiate between brown and white-rot fungi (Käärik, 1965; Worrall *et al.*, 1997). Later, different phenolic compounds and organic amines were used by Käärik (1965) to detect oxidative enzyme production. These methods also formed the basis for the identification of species of wood-inhabiting fungi in culture as described by Stalpers (1978). In the present study the oxidase tests (Bavendamm, 1928) and drop tests for different enzymes (Stalpers, 1978) were used to characterize strains from our collection and to group the strains according to their characteristics in culture. Strains with tolerance to phenolic compounds that could be valuable in biotechnological applications were also identified.

MATERIALS AND METHODS

Collection of cultures

From January 1991 to March 1993, macrocarpic wood-inhabiting fungi were collected from different areas in South Africa (Figure 1). The Western Cape Province (winter rainfall area) experienced high rainfall during the two collection periods, but the summer rainfall area, and especially KwaZulu-Natal, was very dry. Collections were made from indigenous forest, commercial pine and eucalyptus plantations as well as stands of exotic *Quercus* and *Acacia*. A few collections were also made from decaying ornamental and fruit trees. The basidiocarps were mostly collected from dead wood found in indigenous forests and commercial plantations and the fungi identified. The systematic system of Hawksworth *et al.* (1995) was used for the classification of specimens. Cultures were made from the sporocarps or colonized wood by aseptically transferring small pieces of tissue to 1,5 % malt extract agar (MEA) plates that were incubated at room temperature. Purified cultures, obtained from these plates, were maintained on MEA slants at 4 °C. A collection of more than 278 South African wood-inhabiting Basidiomycetes is maintained in the culture collection of the CSIR, Pretoria, South Africa.

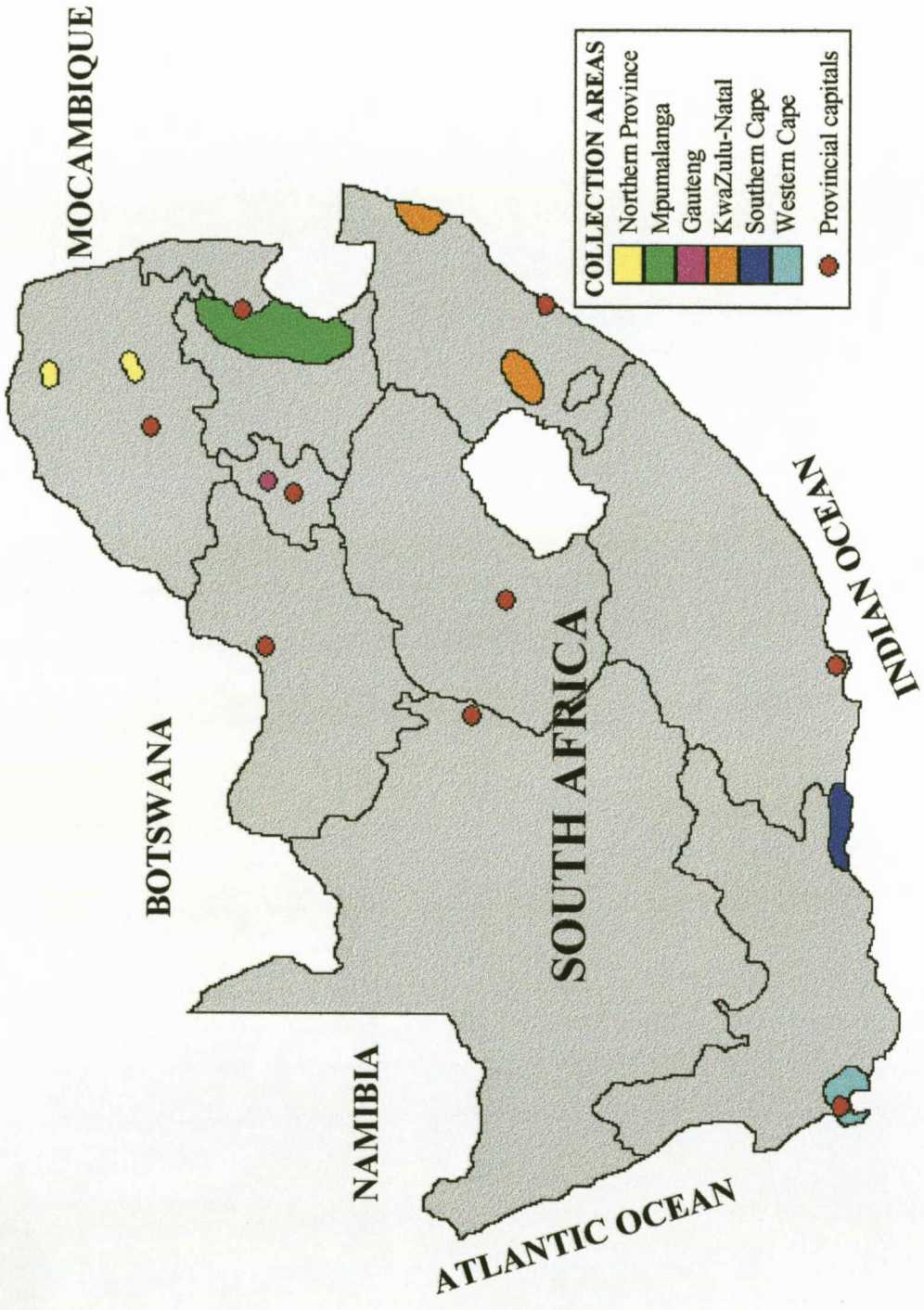


Figure 1. Collection areas of wood-inhabiting fungi in South Africa.

Enzymatic characteristics

The oxidase reactions of strains were determined on duplicate plates of 1,5% malt extract agar containing 0,5 % gallic acid (GAM) or tannic acid (TAM). Plugs (5 mm diameter) were removed aseptically with a cork borer from one-week old cultures on malt extract agar (MEA) and inverted on GAM and TAM. Slow growing strains were only used when sufficient mycelium was produced to yield four inoculum plugs. Plates were incubated at room temperature (22 °C) and reactions and growth determined after seven days. Instead of the six reaction types recorded by Davidson *et al.* (1938), we only distinguished between three reaction types, viz: No darkening of medium was regarded as a negative reaction (rated as 0), discolouration of the medium restricted to the area covered by mycelium was regarded as a positive reaction (rated as 1) and extension of discoloured zone beyond the mycelium was regarded as a strong positive reaction (rated as 2).

Drop tests were performed in duplicate on one-week old cultures on 2 % MEA containing 10 % sucrose, neutralized with 2N KOH. Tests for cytochrome oxidase, laccase, peroxidase and tyrosinase were done by placing a drop of the following test solutions on the colony perimeter (Stalpers, 1978): Tetramethyl-*p*-phenylenediamine dihydrogen chloride (20 mg) in solution with 10 ml ascorbic acid (15 ppm in water) was used to test for the presence of cytochrome oxidase. The positive test for laccase was the development of a purplish colour on the colony when 0,1M α-naphtol in 96 % ethanol (aq) was added. Equal parts of freshly prepared 0,4 % hydrogen peroxide and 1 % pyrogallol in water turned yellowish brown in the presence of peroxidase. The

presence of tyrosinase was indicated by an orange-brown colour when 0,1M *p*-cresol in 96 % ethanol (aq) was added to the mycelium. All reactions were read after 3 h, 24 h and 72 h. Tests on cytochrome oxidase were read after 20 to 30 minutes and compared with a blank control, because atmospheric oxidation also takes place and could interfere with the results after a longer period. Results of these tests were compared with results reported previously (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948, 1958; Stalpers, 1978; Van der Westhuizen, 1971).

The different strains of fungi were grouped according to the production of oxidative enzymes, strength of oxidase reactions and growth rate on GAM and TAM. The growth rate for some groups was described as a range of possible measurements.

RESULTS AND DISCUSSION

Collection of cultures

In total, 278 strains of wood-inhabiting Basidiomycetes were collected over a period of 27 months (Table 1; Appendix A). This collection represented 43 species from 13 families of Homobasidiomycetes and included two species that have, to our knowledge, not been recorded from South Africa, i.e. *Gloeophyllum abietinum* and *Trametes nivosa*. The fungi were collected from different areas and a variety of substrates (Figure 1; Table 1).

Table 1. Number of strains collected in different areas and from different hosts or substrates.

Collection area[a]	Host / Substrate						
	Eucalyptus spp.[b]	Exotic *Acacia* spp.[c]	*Quercus* spp.	Ornamental & Fruit trees	Indigenous Hardwood	Softwood	Miscellaneous Substrates
Western Cape	10	13	41	11	5	43	2
Southern Cape	-	-	-	-	4	9	1
KwaZulu-Natal	9	2	1	-	5	22	-
Mpumalanga	15	5	13	-	3	6	2
Northern Province	5	-	2	-	14	25	3
Gauteng	-	6	-	1	-	-	-
TOTAL	39	26	57	12	31	105	8

a Refers to collection areas shown in Figure 1.
b Exotic species occurring in commercial plantations.
c Exotic species in commercial plantations and invaders of natural vegetation.

The variety of habitats that was visited during collection and the differences in the weather experienced at the time were reflected by the species of fungi that were collected (Appendix A). Of the more abundant species that were collected, only *Phellinus gilvus* and *Pycnoporus sanguineus* were found in all the collection areas. *Skeletocutis* sp. was found only in the Western Cape, but species that were usually abundant such as *Coriolus hirsutus, C. versicolor, Gloeophyllum* spp. and *Stereum hirsutum* were not found in KwaZulu-Natal where it was hot and dry. Under these dry conditions, the predominant species were *P. sanguineus* and *Trametes nivosa.*

The largest variety of fungal species was found on *Quercus* spp. and the fewest on *Pinus* spp. (Appendix A). The fungal diversity on *Pinus* spp. was restricted and

mostly yielded *Gloeophyllum* spp. that cause brown-rot. The large number of strains collected from softwood is a reflection on the effort that was made to collect as many cultures as possible from softwood for potential application in biopulping of pine wood (Table 1). However, the only white-rot fungal species that were collected on more than one occasion from softwood were *P. sanguineus, S. hirsutum* and *T. nivosa.*

Enzymatic characteristics

Characteristics that included the production of different enzymes, oxidase reactions as well as growth rates on GAM and TAM were used to distinguish twelve groups of fungi (Table 2). The strength of the oxidase reactions was the most useful characteristic to differentiate between groups. The culture collection can, therefore, be divided into strong white-rot fungi (Group Ia, Ib, Ic, II and IV), weak white-rot fungi (Group V, VI and VII) and brown-rot fungi (Group VIII, IX, X and XI). Group III represents a group of brown and white-rot fungi that did not test positive for oxidase enzymes, but produced a positive Bavendamm reaction on GAM and TAM.

Each of the fungal strains was assigned to a group on the basis of the enzymes that they produced and oxidase reactions (Table 3; Appendix B). Several species were represented by strains that were placed in more than one group, e.g., *Pycnoporus sanguineus*, *Stereum hirsutum* and *Trametes nivosa.* The variation within species should not be ascribed to a flaw in the assay, but is possibly due to genetic variation within species. Käärik (1965) has demonstrated that large variation exists within

species by comparing monosporic strains from the same sporocarp of *Stereum sanguinolentum*.

Table 2. Criteria used for the grouping of wood-decay fungi and the number of strains occurring in each group.

Group	Enzymes[a]				Gallic acid medium		Tannic acid medium		Number of strains[c]
	Cytochrome Oxidase	Laccase	Peroxidase	Tyrosinase	Oxidase reaction[b]	Growth rate (mm/week)	Oxidase reaction[b]	Growth rate (mm/week)	
Ia	±	+	+	±	2	0,0 - 6,5	2	0,0 - 11,0	173
Ib	±	+	–	±	2	0,0 - 6,5	2	0,0 - 6,5	3
Ic	±	–	+	±	2	0,0 - 6,5	2	0,0 - 6,5	5
II	±	±	+	±	2	0,0 - 18,5	1 / 2	11 - 33,0	13
III	–	–	–	–	2	0,0 - 4,5	0 / 1 / 2	0,0 - 7,5	5
IV	±	+	+	±	2	0,0 - 2,5	0 / 1	0,0 - 2,0	8
V	±	±	±	±	1	1,5 -3,0	0	3,0 - 3,5	3
VI	–	±	+	±	0 / 1	0,0 -6,0	0 / 1	0,0 - 3,0	4
VII	+	+	+	±	0 / 1	0,0 - 8,0	0 / 1	0,0 - 5,0	23
VIII	±	–	–	±	0	8,0 - 24,5	0	13 - 29,5	7
IX	±	±	–	±	0	1,0 - 11	0	1,0 - 11,5	10
X	+	±	+	+	1 / 0	18,5 - 30,0	0	0,0	2
XI	±	–	–	–	1 / 0	0,0 - 8,5	1 / 0	0,0	22

a Presence of enzymes determined with drop tests (Stalpers, 1978): (+) enzyme present, (±) enzyme present for some strains and (-) enzyme absent.

b Strength of oxidase reactions determined as follows: (0) no darkening of media, (1) darkened media not extending beyond mycelium, (2) darkened media extend beyond mycelium.

c Number of strains that were identified to species level.

Our results were mainly in agreement with previous reports. Results of laccase tests were compared with previously published results, because it is the assay most frequently used by other researchers. The results of other enzyme tests were, therefore, not included in the comparison. Results of tyrosinase tests were not considered, as our results were ambiguous for some strains as experienced by Käärik (1965) and Stalpers (1978). According to Davidson *et al.* (1938), significant differences occur between growth rates of strains grown on different sources of gallic and tannic acid. Strength of the oxidase reactions could, therefore, be compared but not the growth rates of fungi. Comparison of our results with those from previous studies was troublesome because the strength of oxidase reactions was reported according to different scales. Comparisons with previous studies are, therefore, based on our interpretation of published results (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948, 1958; Stalpers, 1978; Van der Westhuizen, 1971).

Table 3. Identity of fungal strains and grouping according to oxidase reactions and enzyme production.

Orders, Families and Species[a]	Group[b]	Number of strains
AGARICALES		
Strophariaceae		
Hypholoma fasciculare (Huds.:Fr.) Kummer	Ia	2
Tricholomataceae		
Cyptotrama asprata (Berk.) Redhead & Ginns	VII	1
BOLETALES		
Coniophoraceae		
Coniophora olivacea (Fr.) Karst.	VI	1

Orders, Families and Species[a]	Group[b]	Number of strains
GANODERMATALES		
Ganodermataceae		
Ganoderma applanatum (Pers.: Wallr.) Pat.	Ia	2
	Ic	1
G. curtisii (Berk.) Murr.	Ia	1
G. lucidum (Leyss.: Fr.) Karst.	IV	1
	VII	1
HERICIALES		
Gloeocystidiellaceae		
Laxitextum bicolor (Pers.: Fr.) Lentz	X	1
HYMENOCHAETALES		
Hymenochaetaceae		
Phellinus gilvus (Schw.) Pat.	Ia	7
PORIALES		
Coriolaceae		
Antrodia variiformis (Peck) Donk	VII	1
Bjerkandera adusta (Willd.: Fr.) Karst.	Ia	2
	VII	7
Coriolopsis polyzona (Pers.) Ryvarden	Ia	5
C. strumosa (Fr.) Ryvarden	Ia	1
	VII	1
Coriolus hirsutus (Wulf.: Fr.) Quél.	Ia	9
	VII	1
C. pubescens (Schum.: Fr.) Quél.	Ia	1
C. versicolor (Wulf.: Fr.) Quél.	Ia	27
C. zonata (Nees: Fr.) Quél.	Ia	1
Daedalea quercina L.: Fr.	VIII	1
Fomitopsis lilacino-gilva (Berk.) Wright & Deschamps	VIII	1
	IX	1
Gloeophyllum abietinum (Bull.: Fr.) Karst.	XI	1
G. sepiarium (Wulf.: Fr.) Karst.	III	1
	IV	1
	XI	14
G. trabeum (Pers.: Fr.) Murr.	III	1
	XI	5
Hexagona rigida Berk.	IV	1
Lenzites betulina (L.: Fr.) Fr.	Ia	4
	II	1
L. elegans (Spreng.: Fr.) Pat.	Ic	1
	IV	1
Nigroporus vinosus (Berk.) Murr.	Ia	1
Phaeolus schweinitzii (Fr.) Pat.	Ic	1

Orders, Families and Species[a]	Group[b]	Number of strains
Pycnoporus coccineus (Fr.) Bond & Sing.	Ia	3
P. sanguineus (L.: Fr.) Murr.	Ia	49
	III	1
	IV	2
Trametes cingulata Berk.	Ia	7
T. glabrescens (Berk.) Fr.	Ia	2
T. nivosa (Berk.) Murr.	V	3
	VI	1
	VII	2
	VIII	5
	IX	6
Lentinaceae		
Lentinus stupeus Klotzsch	Ia	5
L. villosus Klotzsch	Ia	2
SCHYZOPHYLLALES		
Schizophyllaceae		
Schizophyllum commune Fr.	IX	2
STEREALES		
Corticiaceae		
Pulcherricium caeruleum (Fr.) Parm.	Ia	1
Hyphodermataceae		
Schizopora paradoxa (Schrad.: Fr.) Donk	III	1
Meruliaceae		
Chondrostereum purpureum (Fr.) Pouz.	Ia	1
	VII	1
Stereaceae		
Stereum fulvum (Lév.) Sacc.	VI	1
	VII	1
S. hirsutum (Wild.: Fr.) S.F. Gray	Ia	9
	Ib	1
	II	10
S. illudens Berk.	Ia	2
	Ic	1
S. ostrea (Blume & Nees: Fr.) Fr.	Ia	3
	Ib	1
	VII	1
S. rimosum Berk.	Ia	1
S. sanguinolentum (Alb. & Schw.: Fr.) Fr.	Ib	1

a Classification of genera in orders and families follows the system of the Dictionary of the Fungi (Hawksworth *et al.*, 1995).

b Groups are based on the criteria defined in Table 2.

Unfortunately, only a small number of species in our collection have also been studied by other workers. Many similarities and a few discrepancies were noted between our results and those previously published, and these warrant further explanation. Some of the tested species were also included in one or more of the descriptions by Davidson *et al.* (1938), Käärik (1965), Nobles (1948, 1958), Stalpers (1978) and Van der Westhuizen (1971). The following comparisons only note the characteristics that have been described previously:

Bjerkandera adusta

(Group Ia). The two strains that were examined produced diffusion zones on GAM that extended beyond the mycelium, interpreted as a strong reaction. Previous reports indicate negative or weak reactions (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948; Van der Westhuizen, 1971). The positive laccase test suggests that strong reactions should be expected.

(Group VII). Seven strains produced laccase and oxidase reactions similar to the descriptions of Davidson *et al.* (1938), Käärik (1965), Nobles (1948, 1958) and Van der Westhuizen (1971).

Chondrostereum purpureum

(Group Ia). One strain produced laccase and oxidase reactions as described in literature (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1958; Stalpers, 1978).

(Group VII). One of the tested strains showed a weak reaction on GAM and TAM. Previous results (Davidson *et al.*, 1938; Käärik, 1965) describe strong reactions, which is a result that is supported by the production of laccase.

Coniophora olivacea

(Group VI). Only one strain was tested and it showed a weak diffusion zone on TAM, compared to a reported (Käärik, 1965) negative reaction.

Coriolopsis polyzona

(Group Ia). All five of the tested strains produced laccase as described by Stalpers (1978).

Coriolus hirsutus

(Group Ia). The nine strains in this group produced laccase and strong oxidase reactions. These results have previously been described by Käärik (1965), Nobles (1958) and Stalpers (1978) and by Davidson *et al.* (1938), Käärik (1965) and Nobles (1948) respectively.

(Group VII). One strain showed a weak reaction on GAM. This is contrary to the characteristics of nine other strains that produced strong reactions, which is similar to all previous records (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948).

Coriolus versicolor

(Group Ia). Results of oxidase reactions and laccase production of 27 strains tested agree with those published (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948, 1958; Stalpers, 1978; Van der Westhuizen, 1971).

Coriolus pubescens

(Group Ia). The tested strain produced laccase, and strong oxidase reactions on GAM and TAM as described by Stalpers (1978) and Nobles (1948) respectively.

Coriolus zonata

(Group Ia). Laccase was produced, which is consistent with results published by Käärik (1965) and Stalpers (1978). Positive oxidase reactions confirm those published previously (Davidson *et al.*, 1938; Nobles, 1948).

Daedalea quercina

(Group VIII). The test for laccase was negative as described by Käärik (1965), Stalpers (1978) and Van der Westhuizen (1971). Negative oxidase reactions are consistent with previous studies (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948).

Fomitopsis lilacino-gilva

(Group VIII and Group IX). The test for laccase showed negative results for the strains in both groups, as described by Stalpers (1978).

Ganoderma applanatum

(Group Ia and Group Ic). Strains in both groups tested positive for laccase and showed strong oxidase reactions. These results confirm those of Nobles (1948; 1958) and Stalpers (1978).

Ganoderma curtisii

(Group Ia). The tested strain produced oxidase reactions as described by Davidson *et al.* (1938).

Ganoderma lucidum

(Group IV). The strong oxidase reaction on GAM is consistent with the results of Davidson *et al.* (1938). The single strain that was tested produced no reaction on TAM, although Davidson *et al.* (1938) described a weak positive reaction on TAM. All our tests for oxidative enzymes were positive, which contradicts results of the Bavendamm reaction.

(Group VII). The one strain that was tested, showed a weak reaction on GAM which is in contrast to the strong positive reaction reported by Davidson *et al.* (1938). However, all enzyme tests for this strain were positive, which supports the results of Davidson *et al.* (1938).

Gloeophyllum sepiarium

(Group III). One strain produced weak oxidase reactions despite the fact that Davidson *et al.* (1938) suggested that brown-rot fungi should not produce any reaction.

(Group IV). One of the strains tested showed positive reactions to all enzyme tests as well as displaying diffusion zones on GAM and TAM. These reactions could be ascribed to non-enzymatic processes, such as oxidation by H_2O_2 (Ritschkoff & Viikari, 1991).

(Group XI). Fourteen strains displayed negative oxidase reactions and tests for laccase, which is consistent with previous reports (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948, 1958; Stalpers, 1978; Van der Westhuizen, 1971).

Gloeophyllum trabeum

(Group III). One strain showed a strong reaction on GAM, presumably also because of H_2O_2 production.

(Group XI). Five strains displayed negative oxidase reactions and as well as negative tests for laccase, which is consistent with previous reports (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948; Stalpers, 1978; Van der Westhuizen, 1971).

Hypholoma fasciculare

(Group Ia). The two strains tested showed strong Bavendamm reactions and tested positive for laccase as described by Käärik (1965).

Lentinus villosus

(Group Ia). Two strains tested positive for laccase as described by Stalpers (1978).

Lenzites betulina

(Group Ia and II). Strong oxidase reactions and positive tests for laccase were consistent with previous reports (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948, 1958; Stalpers, 1978; Van der Westhuizen, 1971).

Nigroporus vinosus

(Group Ia). The one strain in this study tested positive for laccase as described by Stalpers (1978).

Phaeolus schweinitzii

(Group Ic). The strain tested showed strong reactions on GAM and TAM. Only negative reactions or weak reactions have been described previously (Käärik, 1965). All tests for oxidative enzymes, except for laccase, were positive. According to Nobles (1958), the Bavendamm reaction can indicate that either laccase or tyrosinase is present.

Phellinus gilvus

(Group Ia). Seven strains tested positive for laccase as reported by Stalpers (1978) and showed strong oxidase reactions similar to those reported previously (Davidson *et al.*, 1938; Nobles, 1948).

Pulcerricium caeruleum

(Group Ia). One strain was collected and tested positive for laccase as described by Stalpers (1978).

Pycnoporus coccineus

(Group Ia). Three strains tested positive for laccase as described by Stalpers (1978).

Pycnoporus sanguineus

(Group Ia, Group III and Group IV). All of the 52 tested strains were able to produce laccase as described by Stalpers (1978).

Schizophyllum commune

(Group IX). Neither of the two strains tested showed a reaction on TAM. Other reporters observed weak positive reactions (Davidson *et al.*, 1938; Nobles, 1948).

Schizopora paradoxa

(Group III). Contradictory results were obtained with the one strain that was tested. No enzyme tests were positive, but strong diffusion zones were produced. Stalpers (1978) reported the presence of laccase, while Van der Westhuizen (1971) observed no reaction on TAM and a weak reaction on GAM. The species is described as a white-rot fungus by Van der Westhuizen (1971). Therefore, the Bavendamm test must be seen as most reliable for this strain.

Stereum hirsutum

(Group Ia, Group Ib and Group II). The positive test for laccase and strong reactions on GAM and TAM for all 20 strains are consistent with the results of Käärik and (1965) and Stalpers (1978).

Stereum ostrea

(Group Ia, Group Ib and Group VII). The five strains from these groups all produced laccase as observed by Stalpers (1978).

Stereum sanguinolentum

(Group Ia). The single tested strain produced laccase and strong oxidase reactions, which are consistent with previously published results (Käärik, 1965; Nobles, 1948; 1958; Stalpers, 1978).

Trametes cingulata

(Group Ia). Seven strains were tested and were laccase positive with strong oxidase reactions such as those described by Stalpers (1978) and Van der Westhuizen (1971).

CONCLUSIONS

A culture collection was established by sampling from a variety of substrates, habitats and a diversity of climatic and topographic regions. As a result of this study, two species were recorded for the first time from South Africa. This survey also provides valuable additional information on the occurrence and distribution of wood-inhabiting Basidiomycetes.

Current methods to identify species of wood-decay fungi in culture do not include the utilization of Bavendamm's test (Stalpers, 1978). The laccase test with guaiacol was introduced by Nobles (1958) to replace the cumbersome tests on GAM and TAM. More recently, tests with polymeric dyes were used to determine the type of decay caused by wood-inhabiting fungi (Cookson, 1995). The Bavendamm and drop tests are, however, still useful in characterizing cultures and as an instrument in the screening programme for biotechnological applications. It has become important to obtain as much information as possible in order to select fungi for biotechnological application. The growth of fungi on GAM and TAM can, for instance, provide important information on the tolerance of fungi to phenolic compounds (Davidson *et al.*, 1938), which would be valuable in the selection of fungi for degradation of phenolic pollutants (Collett, 1992).

The variation in results of the present study and some disagreements with previously published studies (Davidson *et al.*, 1938; Käärik, 1965; Nobles, 1948, 1958;

Stalpers, 1978; Van der Westhuizen, 1971) indicates that considerable variation exists between strains of the same species. The extent of genetic variation was also demonstrated by Käärik (1965). It is, therefore, clear that as many strains as possible should be included in screening trials.

Progress in the field of biotechnology has brought new perspectives to the criteria previously used to characterize and group wood-decay fungi. These tests can indicate the potential of decay fungi to be used in industrial processes, while previous studies utilized these assays for identification purposes only. This study represents a contribution towards our understanding of the ability of South African wood-inhabiting fungi to produce lignin degrading enzymes and cause wood decay.

REFERENCES

AKHTAR, M., ATTRIDGE, M.C., MYERS, G.C., KIRK, T.K. & BLANCHETTE, R.A. 1992. Biomechanical pulping of loblolly pine with different strains of the white-rot fungus *Ceriporiopsis subvermispora*. *Tappi J.* 75(2): 105-109.

AKHTAR, M., BLANCHETTE, R.A. & KIRK, T.K. 1997. Fungal delignification and biomechanical pulping of wood. *Adv. Biochem. Eng. Biotechnol.* 57: 159-195.

BAVENDAMM, W. 1928. Über das vorkommen und den nachweis von oxydasen bei holzzerstörenden pilzen. *Z. Pflanzenkr. Pflanzenschutz* 38: 257-276.

BERGBAUER, M., EGGERT, C. & KRAEPELIN, G. 1991. Degradation of chlorinated lignin compounds in a bleach plant effluent by the white-rot fungus *Trametes versicolor*. *Appl. Microbiol. Biotechnol.* 35: 105-109.

BLANCHETTE, R.A., BURNES, T.A., EERDMANS, M.M. & AKHTAR, M. 1992. Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. *Holzforschung* 46: 109-115.

BLANCHETTE, R.A., BURNES, T.A., LEATHHAM, G.F. & EFFLAND, M.J. 1988. Selection of white-rot fungi for biopulping. *Biomass* 15: 93-101.

COOKSON, L.J. 1995. Reliability of Poly B-411, a polymeric anthraquinone-based dye, in determining the rot type caused by wood-inhabiting fungi. *Appl. Environ. Microbiol.* 61: 801-803.

CROWDER, A.L., EUDY, W.W. & SETLIFF, E.C. 1978. Industrial aspects of lignin biodegradation. In: Developments in Industrial Microbiology, Ed. L.A Underkofler, pp. 63-68. Society for Industrial Microbiology, Gaithersburg, USA.

COLLETT, O. 1992. Aromatic compounds as growth substrates for isolates of the brown-rot fungus *Lentinus lepideus* (Fr. ex Fr.) Fr. *Mater. u. Organ.* 27: 67-77.

DAVIDSON, R.W., CAMPBELL, W.A. & BLAISDELL, D.J. 1938. Differentiation of wood-decaying fungi by their reactions on gallic or tannic acid medium. *J. Agric. Res.* 57: 683-695.

DE JONG, E., DE VRIES, F.P., FIELD, J.A., VAN DER ZWAN, R.P. & DE BONT, J.A.M. 1992. Isolation and screening of basidiomycetes with high peroxidative activity. *Mycol. Res.* 96: 1098-1104.

DOIDGE, E.M. 1950. The South African fungi and lichens. *Bothalia* 5: 1-1094.

EVANS, C.S. 1987. Lignin degradation. *Process Biochem.* Aug.: 102-105.

FEICHTER, A. 1993. Function and synthesis of enzymes involved in lignin degradation. *J. Biotechnol.* 30: 49-55.

FIELD, J.A., DE JONG, E., FEIJOO-COSTA, G. & DE BONT, J.A.M. 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. *Tibtech* 11: 44-49.

GALENO, G.D. & AGOSIN, E.T. 1990. Screening of white-rot fungi for efficient decolourization of bleach pulp effluents. *Biotechnol. Lett.* 12: 869-872.

HAWKSWORTH, D.L., KIRK, P.M., SUTTON, B.C. & PEGLER, D.N. 1995. Ainsworth & Bisby's Dictionary of the Fungi, 8th ed. CAB International, Wallingford, UK.

JOB-CEI, C., KELLER, J. & JOB, D. 1991. Selective delignification of sulphite pulp paper: Assessment of 40 white-rot fungi. *Mater. u. Organ.* 26: 215-226.

JOYCE, T.W. 1992. Applications of biotechnology in the manufacture of paper. Preprints: Papfor 92 Conference. St. Petersburg, Russia: 269-277.

KÄÄRIK, A. 1965. The identification of mycelia of wood-decay fungi by their oxidation reactions with phenolic compounds. *Stud. For. Suec.* 31: 1-80.

KIRKPATRICK, N., REID, I.D., ZIOMEK, E. & PAICE, M.G. 1990. Biological bleaching of hardwood kraft pulp using *Trametes (Coriolus) versicolor* immobilized in polyurethane foam. *Appl. Microbiol. Biotechnol.* 33: 105-108.

MESSNER, K., SCIEFERMEIER, M., SREBOTNIK, E. & TECHT, G. 1993. Bio-sulfite pulping: Current state of research. In: Lignin Biodegradation and Transformation: Biotechnological applications, Eds. J.C. DUARTE, M.C. FERREIRA & P. ANDER, pp. 197-200. Forbitec Editions, Lisbon, Portugal.

NOBLES, M.K. 1948. Studies in forest pathology. VI. Identification of wood-rotting fungi. *Can. J. Res.* 26: 281-431.

NOBLES, M.K. 1958. A rapid test for extracellular oxidase in cultures of wood-inhabiting hymenomycetes. *Can. J. Bot.* 36: 91-99.

ONYSKO, K.A. 1993. Biological bleaching of chemical pulps: A review. *Biotechnol. Adv.* 11: 179-198.

REID, D.A. 1975. Type studies of the larger Basidiomycetes described from southern Africa. Contributions from the Bolus Herbarium 7. University of Cape Town, Cape Town, South Africa.

REID, I.D. 1991. Biological pulping in paper manufacture. *Tibtech* 9(8): 262-265.

REID, I.D. & PAICE, M.G. 1994. Biological bleaching of kraft pulps by white-rot fungi and their enzymes. *FEMS Microbiol. Rev.* 13: 369-376.

RITSCHKOFF, A-C. & VIIKARI, L. 1991. The production of extracellular hydrogen peroxide by brown-rot fungi. *Mater. u. Organ.* 26: 157-167.

SPIKER, J.K., CRAWFORD, D.L. & CRAWFORD, R.L. 1992. Influence of 2,4,6-Trinitrotoluene (TNT) concentration on the degradation of TNT in explosive contaminated soils by the white-rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58: 3199-3202.

STALPERS, J.A. 1978. Identification of wood-inhabiting fungi in pure culture. Studies in Mycology 16. CBS, Baarn, Netherland.

TALBOT, P.H.B. 1951. Studies of some South African resupinate Hymenomycetes II. *Bothalia* 6: 1-116.

TALBOT, P.H.B. 1958. Studies of some South African resupinate Hymenomycetes II. *Bothalia* 7: 131-187.

VAN DER BYL, P.A. 1924. Descriptions of additional South African Polyporeae. *S. Afr. J. Sci.* 21: 308-313.

VAN DER BYL, P.A. 1928. Aantekeninge oor enige Suid-Afrikaanse swamme. *S. Afr. J. Sci.* 25: 181-184.

VAN DER WESTHUIZEN, G.C.A. 1971. Cultural characters and carpophore construction of some poroid Hymenomycetes. *Bothalia* 10: 137-658.

VAN DER WESTHUIZEN, G.C.A. & EICKER, A. 1994. *Mushrooms of Southern Africa*, Struik Publishers, Cape Town, South Africa.

WALL, M.B., CAMERON, D.C. & LIGHTFOOT, E.N. 1993. Biopulping process design and kinetics. *Biotech. Adv.* 11: 645-662.

WORRALL, J.J., ANAGNOST, S.E. & ZABEL, R.A. 1997. Comparison of wood decay among diverse lignicolous fungi. *Mycologia* 89(2): 199-219.

WOLFAARDT, J.F., BOSMAN, J.L., JACOBS, A., MALE, J.R. & RABIE, C.J. 1996. Bio-kraft pulping of softwood. In: Biotechnology in the Pulp and Paper Industry: Recent advances in applied and fundamental research, Eds. E. Srebotnik & K. Messner, pp. 211-216. Facultas Unversitätsverlag, Vienna, Austria.

CHAPTER 3

ASSESSMENT OF WOOD-INHABITING BASIDIOMYCETES FOR BIOKRAFT PULPING OF SOFTWOOD CHIPS

Stereum ostrea

ABSTRACT

Wood-inhabiting Basidiomycetes have been screened for various industrial applications in the pulp and paper industry and it is evident that different fungi need to be used to suit the specific requirements of each application. Special screening techniques have, therefore, been developed to select fungi for biopulping that selectively degrade lignin and are able to grow under specific environmental conditions. This study assesses the suitability of a collection of 278 strains of South African wood-decay fungi for the pre-treatment of softwood chips prior to kraft pulping. The influence of these fungi on kappa number, yield and strength properties of pulp was evaluated. A number of these strains were more efficient in reducing kappa number than the frequently used strains of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*. Seven of the tested strains, including six strains of *Stereum hirsutum* and a strain of an unidentified specie were able to reduce the kappa number significantly without a significant influence on the pulp yield. Treatment of wood with two strains of *S. hirsutum,* one strain of *Peniophora* sp. and a strain of an unidentified sp. resulted in paper with improved strength properties.

INTRODUCTION

White-rot fungi are the most efficient degraders of lignin (Kirk *et al.*, 1980) and they are, therefore, probably also the most suitable organisms to be utilized in an industrial process that requires delignification (Messner & Srebotnik, 1994). White-rot fungi are not only capable of producing lignin degrading enzymes, but are also able to penetrate the substrate to transport enzymes into material such as wood chips (Messner & Srebotnik, 1994). A variety of possible applications for these fungi exist, including bio-chemical pulping processes. The potential benefits of bio-chemical pulping include decreased lignin content of pulp, reduction of pulping time, reduced consumption of bleaching chemicals (Messner & Srebotnik, 1994; Wall *et al.*, 1996) and an increase in the strength properties of pulp (Oriaran *et al.*, 1990, 1991). The biological treatment of wood, prior to chemical pulping, has not been investigated to the same extent as biomechanical pulping (Messner & Srebotnik, 1994) and biokraft pulping is almost unexplored (Chen & Schmidt, 1995). Only a small number of publications deal with kraft pulping of degraded wood (Oriaran *et al.*, 1990, 1991; Wall *et al.*, 1996), although kraft pulping accounts for more then 80 % of the world's annual pulp production (Sjöström, 1981). To our knowledge, no results on the evaluation of fungi for biokraft pulping of softwood have been published.

It has been suggested that different biotechnological applications have specific requirements (Job-Cei *et al.*, 1991) and that it is difficult to predict the effect of biological treatment on pulping (Messner & Srebotnik, 1994). Our approach was, therefore, to evaluate the potential of a South African collection of wood-inhabiting

Basidiomycetes as biopulping organisms. By testing the ability of strains to produce lignin degrading enzymes (Stalpers, 1978) and by means of tests for oxidase reactions (Davidson *et al.*, 1938), fungi with the ability to degrade lignin were selected for further screening (Wolfaardt *et al.*, 1993).

Many different screening procedures have been developed to select organisms with appropriate characteristics. These methods include differential staining and microscopy (Otjen *et al.*, 1987), scanning and transmission electron microscopy (Job-Cei *et al.*, 1991), determination of weight loss (Job-Cei *et al.*, 1991, Blanchette *et al.*, 1992) and analysis of degradation products (Otjen *et al.*, 1987, Bechtold *et al.*, 1993). Unfortunately, none of these methods can be used to accurately predict the effect of fungal treatment on chemical pulping; possibly because an improvement in pulping could simply be ascribed to an improvement in cooking liquor penetration (Oriaran *et al.*, 1991).

The purpose of this study was to evaluate a number of South African strains of lignin-degrading fungi for potential application as a pre-treatment of softwood chips prior to kraft pulping. We chose to do mini pulping trials and to rank strains according to their improvement of the resultant pulp. A number of criteria routinely used in the pulping industry were applied to determine the effect of fungal treatment. These parameters included kappa number, pulp yield and paper strength.

MATERIALS AND METHODS

Fungi and inoculum

Two hundred and seventy eight strains of wood-inhabiting Basidiomycetes representing 44 genera were collected in South Africa (Chapter 2). The different strains were deposited in the culture collection of the Division of Food Science and Technology at the Council for Scientific and Industrial Research (CSIR). All of these strains as well as strains of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* were used in screening trials. Inoculum was produced by growing fungi on 1,5 % malt extract agar (MEA) plates. Plugs overgrown with mycelium were used to start pre-inoculum in 200 ml liquid medium containing 3 % (v/v) corn steep liquor (CSL) and 1 % (w/v) sucrose. The pre-inoculum was incubated in stationary culture for seven days at 28 °C before being homogenized with an Ultra Turrax (Janke Kunkel). The homogenized culture (20 ml) was inoculated into 200 ml CSL and sucrose medium in 500 ml baffled flasks. Cultures were incubated at 28 °C on a rotary shaker at 100 rpm for seven days, after which they were again homogenized to produce inoculum for wood chips.

Wood and solid-substrate fermentation

Softwood chips (40 % *Pinus patula*, 40 % *P. elliottii* and 20 % *P. taeda*) from Sappi's Ngodwana mill were dried for five days at 50 °C to a moisture content of 3 % and stored at room temperature. The dried wood was placed in 2 L bioreactors (100 g/ reactor) and supplemented with 160 ml of the CSL and sucrose medium to provide additional carbon and nitrogen sources (Kirk *et al.*, 1976). The wood was

autoclaved for 15 min (121 °C, 100 kPa) and inoculated with 20 ml homogenized inoculum, which brought the moisture content to 60 %. The control treatment consisted of 20ml CSL and sucrose medium that was added to the wood in stead of inoculum. The treated wood was incubated for three or eight weeks at 28 °C and a relative humidity of 85 % to 95 %. This experimental procedure allowed control of solid-substrate fermentation conditions such as temperature and moisture, wood sterilization, nutrient supplementation and a treatment period of three weeks. These factors would, however, have important economic implications to be considered during scale-up.

Pulping conditions

Batches of up to 20 chip samples (50 g dry weight/ sample) were cooked in stainless steel mesh bags in a mini (20 L) digester. In the present study, the effect of biopulping was observed only when the active alkali charges and liquid to wood ratios that are used in mills were modified. This could possibly be ascribed to the heterogeneous cooking batch, or to a greater consumption of cooking chemicals by decayed wood (Hunt, 1978; Hunt & Hatton, 1979). The active alkali charge and liquid to wood ratio were, therefore, increased to 32 % and 10,0:1 respectively. The digester was charged with kraft cooking liquor (32 % active alkali, 25 % sulphidity) at a liquid to wood ratio of 10:1. The pulping conditions were: ambient to 170 °C in 90 min. and 90 min. at 170 °C.

Screening procedure

All the fungal strains in the collection (280 including strains of *P. chrysosporium* and *C. subvermispora*) were subjected to initial screening on wood chips. Practical considerations limited the second screening step to 20 % of the total number of strains. Initial treatments were done in duplicate, and the best 20 % of strains selected on their ability to reduce the kappa number of pulp. These strains were all able to reduce kappa number by 13 % after three weeks or by 23 % after eight weeks in one of the duplicate treatments (Appendix C). These levels of reduction were selected as In a second screening step, 36 selected strains were evaluated in three different experiments, because of restricted space in the digester (Table 1). A control sample was included in each experiment as reference and each treatment replicated three times. A randomized block design was used for each experiment. Replications of treatments were included in separate cooking batches that were analyzed as different blocks.

Table 1. Trial design for the pulping of treated wood to evaluate different fungal strains.

			Replications (Blocks)		
		Samples	**1**	**2**	**3**
Experiments	**A**	14 fungal treatments + Control A	Cook 1	Cook 2	Cook 3
	B	7 fungal treatments + Control B	Cook 4	Cook 5	Cook 6
	C	15 fungal treatments + Control C	Cook 7	Cook 8	Cook 9

Pulp evaluation

To determine yield, pulp was dried at 55 °C until the weight remained constant. Pulp yields were calculated to reflect losses caused by fungal treatment as well as losses incurred during pulping. Kappa numbers were determined according to Tappi Test Method, T236. The reduction in kappa numbers and pulp yields were calculated as a percentage of the controls that were included in each experiment. Pulp from all three replications was bulked to obtain enough material for strength tests. Handsheets were made from the pulp and bursting strength (Tappi Test Method, T403) and tearing strength (Tappi Test Method, T220) determined. Strength ratings were calculated by expressing the average of bursting and tearing strengths of the treated sample as a percentage of the average of bursting and tearing strengths of the control. The data from each experiment were statistically analyzed in a two-way analysis of variance. The multiple comparisons of treatment means were done with Tukey's method (Winer, 1971) at the 5 % level.

RESULTS AND DISCUSSION

The initial screening of 280 strains resulted in the selection of 36 strains that were evaluated in a second screening step (Table 2). The reference strains, *P. chrysosporium* and *C. subvermispora* were not selected, because they failed to reduce kappa number by the required 13 %. A number of the strains that were able to cause a notable reduction in kappa number in the first screening step, were not as effective in the second cycle of experiments. This could possibly be ascribed to some strains that

were selected as result of incubation for eight weeks during the initial screening step (Appendix C). The long incubation could have allowed better wood colonization in some samples. Considerable variation between cooking batches were also observed in the initial screening, possibly because these batches were composed of a heterogeneous combination of treatments. The variability in results obtained in the initial screening was eliminated to a large extent in the second screening step. The randomized block experimental design compensated for differences between cooking batches, and the composition of batches was less heterogeneous. In some cases no significant differences were found between the experimental blocks that represented different cooks.

Twenty-two of the strains tested in the second screening, resulted in a significant modification of lignin, as reflected by reduction in kappa number of the pulp (Table 2). *Stereum hirsutum* was very efficient in reducing kappa numbers, with all nine tested strains causing significant ($p \leq 0,05$) reductions. *Pycnoporus sanguineus* was less efficient, with only two out of eight tested strains causing significant reductions. Two out of five strains of *Coriolus hirsutus* tested, caused significant reductions. The most efficient strains were *P. sanguineus* (WR 398), *Pycnoporus* sp. (WR 270) and *S. hirsutum* (WR 310). These strains were able to reduce the kappa number by 18,1 %, 18,5 % and 18,5 % respectively, compared to the control sample, but also caused significant losses in pulp yield (Table 2). The most promising results were obtained with *S. hirsutum* (WR 3), which reduced the kappa number by 15,2 % without causing a significant reduction of pulp yield.

Table 2. Influence of treatment of wood chips with selected fungal strains on the lignin content and yield of kraft pulp.

	Treatment	Strain[a]	Kappa number[b]	Δ Kappa (%)[c]	Pulp yield (g/100 g wood)[b]	Δ Yield (%)[c]
Experiment A	Control A		31,3		42,2	
	Coriolus hirsutus	WR 83	28,6	-8,6	39,6*	-6,2
	Pycnoporus coccineus	WR 132	29,0	-7,5	42,1	-0,2
	Pycnoporus sanguineus	WR 93	28,8	-8,1	39,9*	-5,5
		WR 114	27,5*	-12,2	37,8*	-10,3
		WR 130	29,2	-6,7	38,8*	-8,1
		WR 131	28,7	-8,3	38,3*	-9,2
		WR 170	29,0	-7,4	39,7*	-5,9
	Stereum hirsutum	WR 3	26,5*	-15,2	40,8	-3,3
		WR 9	27,0*	-13,7	40,3	-4,5
		WR 25	27,6*	-11,7	40,9	-3,0
		WR 91	28,0*	-10,7	40,4	-4,3
		WR 95	26,5*	-15,3	40,2*	-4,7
		WR 156	27,7*	-11,5	40,7	-3,6
	Trametes glabrescens	WR 120	29,8	-4,8	40,2*	-4,7
Experiment B	Control B		31,3		41,9	
	Coriolus hirsutus	WR 61	29,7	-5,0	38,8*	-7,4
		WR 141	29,2	-6,8	40,8	-2,6
	Lentinus stupeus	WR 24	29,5	-5,6	38,7*	-7,6
	Pycnoporus sanguineus	WR 124	28,8	-7,9	39,0*	-6,9
		WR 146	29,4	-6,0	38,2*	-8,8
	Stereum hirsutum	WR 22	27,8*	-11,3	41,0	-2,8
	Stereum ostrea	WR 19	29,6	-5,3	39,0*	-6,9
Experiment C	Control C		32,1		41,8	
	Coriolopsis polyzona	WR 308	29,6	-7,8	40,6	-2,9
	Coriolus hirsutus	WR 407	27,7*	-13,9	36,1*	-13,6
		WR 255	27,7*	-13,8	36,7*	-12,2
	Ganoderma curtisii	WR 349	28,3*	-11,9	36,6*	-12,4
	Gymnopilus sp.	WR 351	27,8*	-10,6	35,5*	-15,1
	Lentinus villosus	WR 339	28,1*	-12,5	37,2*	-11,0
	Lenzites betulina	WR 402	27,7*	-13,9	37,2*	-11,0
	Peniophora sp.	WR 286	26,7*	-17,0	36,7*	-12,2
	Pycnoporus sanguineus	WR 398	26,3*	-18,1	35,8*	-14,4
	Pycnoporus sp.	WR 270	26,2*	-18,5	34,9*	-16,5
	Stereum hirsutum	WR 297	26,8*	-16,7	36,6*	-12,4
		WR 310	26,2*	-18,5	37,6*	-10,1
	Trametes cingulata	WR 340	27,1*	-15,6	33,9*	-18,9
		WR 345	28,5*	-11,2	35,9*	-14,1
	Unidentified sp.	WR 251	28,0*	-12,8	40,0	-4,3

a Culture numbers of strains maintained in the collection of wood-inhabiting fungi at the CSIR.
b Average of three replications.
c Change calculated as the percentage reduction compared to the control treatment of the experiment.
* Significantly different from the control treatment at the 5 % level by Tukey's test.

Of the 22 strains that were able to cause a significant reduction in kappa number, only seven did not cause a significant loss in the yield (Table 2). These strains were six strains of *S. hirsutum* (WR 3, WR 9, WR22, WR 25, WR 91 and WR 156) and a strain of an unidentified specie (WR 251). The identity of strain WR 251 is in doubt. It was originally identified as *Laetiporus sulphureus* (Fr.) Murr. *miniatus* (Jungh.) Imaz. on the basis of the dried fruit body, but these results indicate that it could be a white-rot fungus with some selective lignin-degrading potential. We, therefore, regard this strain as an unidentified specie. The pulp yields (Table 2) appear to be low, compared with pulp yields obtained on a commercial scale. These yields should, however, be compared with those of the control samples that were also low and were possibly related to specific pulping conditions.

Handsheets were made from biopulp produced by the seven fungal strains that did not reduce the yield significantly, as well as four other promising strains and of the control treatments. The strength of biopulp from the unidentified specie (WR 251), *Peniophora* sp. (WR 286) and two strains of *S. hirsutum* (WR 156 and WR 310) was better than that of pulp from untreated wood (Table 3). The strongest pulp was produced from treatment with *S. hirsutum* (WR 310) that was 5 % stronger than the control. This improvement in strength can be ascribed to the large improvement in the bursting index and to a small increase in the tearing index. In general, the bursting strength of handsheets from biopulp was higher and the tearing strength lower than those of the controls.

Table 3. Influence of treatment of wood chips with eleven selected fungal strains on bursting and tearing indices and the strength rating of kraft pulp.

	Treatment	Strain[a]	Bursting index (MN/kg)	Tearing index (Nm^2/kg)	Strength rating[b] (%)
Experiment A	Control A		6,4	15,4	100
	Pycnoporus sanguineus	WR 114	6,2	14,3	94
	Stereum hirsutum	WR 3	6,2	13,8	92
		WR 9	6,5	14,6	96
		WR 25	6,7	14,4	97
		WR 91	6,5	14,4	96
		WR 95	6,3	15,5	100
		WR 156	6,7	15,6	102
Exp. B	Control B		6,4	15,4	100
	Stereum hirsutum	WR 22	6,5	14,5	96
Exp. C	Control C		6,4	12,5	100
	Peniophora sp.	WR 286	6,9	13,1	104
	Stereum hirsutum	WR 310	6,9	12,7	105
	Unidentified sp.	WR 251	6,8	12,4	101

a Culture numbers of strains maintained in the collection of wood-inhabiting fungi at the CSIR.

b Average of bursting and tearing strengths expressed as a percentage of the burst and tearing strengths of the controls.

CONCLUSIONS

The collection of wood-inhabiting Basidiomycetes provided us with new strains of previously tested species as well as some species that have not previously been tested for use in biopulping. The strategy, to collect new strains with the aim of selecting superior ligninolytic strains, was followed by De Jong *et al.* (1992). This strategy proved to be successful in the present study because a number of strains gave better results than the reference strains. One strain each of the widely used biopulping species, *P. chrysosporium* (ATCC 32629) and *C. subvermispora* (CZ-3) (Akhtar *et al.*, 1996), were included in the initial screening trials. However, these strains were

not able to reduce the kappa number by the required 13 % within three weeks (Appendix C).

A number of strains from certain species (e.g., *Pycnoporus sanguineus* and *Stereum hirsutum*) were collected and included in the screening trials. These strains were included, because of the abundance of the species in nature, but also because of the diversity that exists within species (Otjen *et al.*, 1987; Chapter 2). Two strains, *S. hirsutum* (WR 156) and unidentified specie (WR 251), were identified as potential biopulping fungi that will now be tested in scale-up and optimization experiments.

The mini pulping of the large number of samples was time consuming and labour intensive; however, it allowed us to screen different strains for a specific application (kraft pulping) and on a specific substrate (pine wood). The treatment of the specific substrate is important, since different rates of delignification have been described for different substrates (Otjen *et al.*, 1987; Homolka *et al.*, 1994). Pulping conditions similar to those used at pulp mills have been used for the pulping of fungal treated hardwood (Oriaran *et al.* 1991; Wall *et al.* 1996). However, the present study required special pulping conditions to accommodate the large number of samples generated by the screening trials. A change from these experimental pulping conditions to mill conditions will, therefore, be necessary and it will be necessary to optimize conditions for the large scale pulping of fungal treated wood and to do an economic evaluation of the process.

The results of this study showed that most of the 38 strains tested, caused a reduction in pulp yield of treated wood, possibly due to degradation of cellulose as non-selective delignification took place. Fungi capable of "selective delignification" are, therefore, regarded as the most suitable organisms for biopulping (Otjen *et al.*, 1987). Several fungi are able to degrade lignin with some degree of selectivity (Blanchette *et al.*, 1988), however absolute selectivity as reported for *Ganoderma australe* (Bechtold *et al.*, 1993) appears to be unattainable (Erikson & Kirk, 1986). Selectivity of delignification has, for example, been shown to change, depending on the substrate and environmental conditions (Otjen *et al.*, 1987). Pulp yields are often based on the oven dry weight of treated chips that are placed into the digester (Oriaran *et al.*, 1990) and it is then possible to observe an increase in yield due to an increase in holocellulose to lignin ratio in the digester (Oriaran *et al.*, 1991). However, mass balance calculations based on the weight of wood before fungal treatment showed a yield loss (Oriaran *et al.*,1990).

The increased pulp strength observed in this study, is consistent with the results of kraft pulping of fungal treated hardwoods (Oriaran *et al.*, 1990, 1991). The change in strength properties, demonstrates that non-selective delignification was caused by fungal treatment. The increase in bursting strength and decrease in tearing strength indicate an improvement in fibre flexibility and a reduction in fibre length respectively (Chen & Schmidt, 1995).

The results presented in this paper demonstrate the importance of screening to select superior fungal strains for use in biopulping. Under the specific pulping conditions of the screening trials, 38 strains of white-rot fungi were tested that were more suitable than the reference strains of *P. chrysosporium* and *C. subvermispora*. The importance of mini pulping experiments to select the strains with the greatest benefit in a biokraft pulping process was also evident.

REFERENCES

AKHTAR, M., KIRK, T.K. & BLANCHETTE, R.A. 1996. Biopulping: An overview of consortia research. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds E. Srebotnik & K. Messner, pp. 187–192. Facultas-Universitätsverlag, Vienna, Austria.

BECHTOLD, R., GONZÀLES, A.E., ALMENDROS, G., MARTINEZ, M.J. & MARTINEZ, A.T. 1993. Lignin alteration by *Ganoderma australe* and other white-rot fungi after solid-state fermentation of beech wood. *Holzforschung* 47(2): 91-96.

BLANCHETTE, R.A., BURNES, T.A., EERDMANS, M.M. & AKHTAR, M. 1992. Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. *Holzforschung* 46(2): 109-115.

BLANCHETTE, R.A., BURNES, T.A., LEATHAM, G.F. & EFFLAND, M.J. 1988. Selection of white-rot fungi for biopulping. *Biomass* 15: 93-101.

CHEN, Y. & SCHMIDT, E.L. 1995. Improving aspen kraft pulp by a novel, low-technology fungal pretreatment. *Wood Fiber Sci.* 27(2): 198-204.

DAVIDSON, R.W., CAMPBELL, W.A. & BLAISDELL, D.J. 1938. Differentiation of wood-decaying fungi by their reactions on gallic or tannic acid medium. *J. Agric. Res.* 57: 683-695.

DE JONG, E., DE VRIES, F.P., FIELD, J.A., VAN DER ZWAN, R.P. & DE BONT, J.A.M. 1992. Isolation and screening of basidiomycetes with high peroxidase activity. *Mycol. Res.* 96(12): 1098-1104.

ERIKSSON, K.-E. & KIRK, T.K. 1986. Biopulping, biobleaching and treatment of kraft bleaching effluents with white-rot fungi. In: Comprehensive biotechnology in industry, agriculture and medicine. Ed. M. Moo-Young, pp. 271-294. Pergamon Press, Oxford, UK.

HUNT, K. 1978. How rot-decayed wood affects chipping and kraft pulping. *Pulp Pap. Can.* 79(8): 65-68.

HUNT, K. & HATTON, J.V. 1979. Predicting the kraft pulping behavior of decayed pulpwood. *Pulp Pap. Can.* 80(3): 55-57.

HOMOLKA, L., NERUD, F., KOFRONOVA, O., NOVOTNÀ, E. & MACHUROVÀ, V. 1994. Degradation of wood by the basidiomycete *Coriolopsis occidentalis*. *Folia Microbiol.* 39(1): 37-43.

JOB-CEI, C., KELLER, J. & JOB, D. 1991. Selective delignification of sulphite pulp paper: Assessment of 40 white-rot fungi. *Mater. u. Organ.* 26(3): 215-226.

KIRK, T.K., CONNORS, W.J. & ZEIKUS, J.G. 1976. Requirement for a growth substrate during lignin decomposition by two wood rotting fungi. *Appl. Environ. Microb.* 32(1): 192-194.

KIRK, T.K., HIGUCHI, T. & CHANG, H. 1980. Lignin biodegradation: Summary and perspectives. In: Lignin biodegradation: Microbiology, chemistry, and potential applications Vol 2. Eds. T.K. Kirk, T. Higuchi & H. Chang, pp. 235-243. CRC Press Inc, Boca Raton, USA.

MESSNER, K. & SREBOTNIK, E. 1994. Biopulping: An overview of developments in an environmentally safe paper-making technology. *FEMS Microb. Rev.* 13: 351-364.

ORIARAN, T.P., LABOSKY, P. & BLANKENHORN, P.R. 1990. Kraft pulp and paper making properties of *Phanerochaete chrysosporium*-degraded aspen. *Tappi J.* 73(7): 147-152.

ORIARAN, T.P., LABOSKY, P. & BLANKENHORN, P.R. 1991. Kraft pulp and paper making properties of *Phanerochaete chrysosporium*-degraded red oak. *Wood Fiber Sci.* 23(3): 316-327.

OTJEN, L., BLANCHETTE, R., EFFLAND, M. & LEATHAM, G. 1987. Assessment of 30 white-rot basidiomycetes for selective lignin degradation. *Holzforschung* 41 (6): 343-349.

SJÖSTRÖM, E. 1981. Wood chemistry: Fundamentals and Applications, 2nd ed. Academic Press, San Diego, USA.

STALPERS, J.A. 1978. Identification of wood-inhabiting fungi in pure culture. Studies in Mycology 16. CBS, Baarn, Netherland.

WALL, M.B., STAFFORD, G., NOEL, Y., FRITZ, A., IVERSON, S. & FARRELL, R.L. 1996. Treatment with *Ophiostoma piliferum* improves chemical pulping efficiency. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds E. Srebotnik & K. Messner, pp. 205–210. Facultas-Universitätsverlag, Vienna, Austria.

WINER, B.J. 1971. Statistical principles and experimental design, 2nd ed. Mc Graw-Hill Book Co., USA.

WOLFAARDT, J.F., BOSHOFF, I.E., BOSMAN, J.L., RABIE, C.J. & VAN DER WESTHUIZEN, G.C.A. 1993. Lignin degrading potential of South African wood-decay fungi. In: FEMS Symposium: Lignin Biodegradation and Transformation. Book of Proceedings. Eds. J.C. Duarte, M.C. Ferreira & P. Ander, pp. 67-68. Forbitec Editions, Lisbon, Portugal.

CHAPTER 4

EVALUATION OF THE MICROCLIMATE, AND ENUMERATION OF FUNGI IN A STORED SOFTWOOD CHIP PILE

Outside chip storage at Sappi Ngodwana

ABSTRACT

Storage of wood chips is preferred to storage of round wood. The deterioration of chips, however, causes considerable problems in chip piles. Factors contributing to deterioration have, therefore, been studied to manage chip storage. The requirements of biopulping processes have renewed interest in the microclimate and microbial populations in these piles. The aim of this study was to investigate conditions such as temperature, moisture, CO_2 and microbial populations that develop over a three-week period in a softwood chip pile of 3000 tons, and to assess the suitability of the chip pile for colonization of a biopulping fungus. The results show that zones formed within the pile where temperature, CO_2 concentration, moisture and microbial contamination varied. The high temperatures that developed in some areas in the chip pile would make a large volume (29 %) of the chip pile unsuitable for colonization by mesophilic white-rot fungi. The moisture content in 24 % of the chip pile reached 55%, but is not expected to have a large impact on biopulping. The areas of high temperature and high moisture were also overlapping. Special management practices would, however, be required to produce a suitable environment in the chip pile for colonization by biopulping fungi. High levels of CO_2 (12,7 %) accumulated for a short period in some areas, but results have shown that biopulping could still be effective at these levels.

INTRODUCTION

Storage of wood chips is preferred to storage of logs, because they are more economical to handle, degradation is reduced and smaller storage areas are required (Zabel & Morrell, 1992). Pulping properties such as colour and fibre yield of chips can, however, deteriorate rapidly when the chip piles are not managed carefully (Bergman & Nilsson, 1979; Hulme, 1979; Vanderhoff, 1992). Chip deterioration is mostly caused by sap stain and decay fungi. In order to assist with the management of outside chip storage (OCS), several studies have evaluated factors that contribute to chip deterioration (Bergman & Nilsson, 1979; Fuller, 1985; Hatton & Hunt, 1972; Tarocinski, 1976). These investigations emphasized the importance of microclimate and microbial populations in chip pile management (Tarocinski, 1976) as well as the interactions between these factors.

The changes in chip piles can be described in terms of respiration of wood cells, chemical oxidation and microbe activity (Hulme, 1979). Initially, respiration of living parenchyma cells in green wood causes an increase in temperature and carbon dioxide concentration (Springer & Hajny, 1970). Air is moved by convection in the centre of the pile as it is heated, cooler air is drawn in through the sides and humidified as it passes over moist chips. The movement of moisture then redistributes heat by absorbing it during evaporation and emitting it during condensation (Hulme, 1979). An increase in temperature to 40 °C leads to auto oxidation of carbohydrates that becomes increasingly important as temperatures continue to rise (Springer *et al.*, 1971). The heat lost to the surroundings eventually

equals the heat generated so that stability is reached (Springer *et al.*, 1971). At this point heat generated by microbes would maintain the temperature. Although microorganisms are already present at the time of chipping, their contribution to heat generation only becomes important when sufficient microbial biomass has been produced (Springer & Hajny, 1970; Springer *et al.*, 1971).

Biopulping has been defined as the treatment of wood chips with fungi to improve pulping processes (Akhtar *et al.*, 1998). This fungal treatment can be described as a solid-state fermentation process during which asepsis or control of contaminating microbial populations, aeration and temperature is of utmost importance. Factors such as carbon dioxide concentration, moisture content and temperature influence the degradation of wood (Fuller, 1985; Nishimoto *et al.*, 1975) and it can, therefore, be expected that these factors will have a considerable impact on the activity of biopulping fungi in chip piles. Recent advances in biopulping research have renewed interest in the chemical and physical changes occurring in chip piles (Akhtar *et al.*, 1998; Eriksson, 1998; Wall *et al.*, 1993). Suppression of biopulping fungi by populations of naturally occurring microorganisms can also play an important role in proposed biopulping processes (Akhtar *et al.*, 1998; Messner & Srebotnik, 1994). It is, therefore, important to study all these conditions on the specific chip pile where such an operation is envisaged.

During a project to evaluate the potential of a biopulping process for a kraft mill, a comprehensive study was done on physical and microbiological parameters in

the OCS. This paper presents the results of this study on an un-inoculated softwood chip pile at Sappi's Ngodwana mill in Mpumalanga, South Africa.

MATERIALS AND METHODS

Physical conditions

The study of chip pile conditions commenced on May 3rd 1994 (early winter) in a section of a chip pile that was stacked by a traversing stacker. The traversing direction was east to west, and the temperature was monitored in the centre and the southern (shaded) half of the pile. The chip pile consisted of mixed softwood [*Pinus patula* (40 %), *P. elliottii* (40 %) and *P. taeda* (20 %)] that was chipped (18 mm) within three weeks after felling. Trees were less than 15 years old. The chip pile contained c.a. 3000 tons of wood (oven dry) and was 12 m high and 28 m wide at the base.

Nine sampling points were used in the chip pile and ambient conditions were monitored from one point outside the chip pile (Figure 1). Temperature was measured with constantan-copper thermocouples. Carbon dioxide was monitored with a Lira 202 analyzer (MSA Instruments, Pittsburgh, USA), by pumping air from the chip pile through 4 mm copper tubing. Temperature and CO_2 were monitored at two-hour intervals for 24 hours after stacking of the pile commenced. Monitoring for periods of 24 hours was repeated one, two and three weeks after stacking. A factorial experiment with a completely randomized design was used to determine the influence of storage time on temperature and CO_2 at nine different positions in the chip pile.

Three factors were considered: age of chip pile (one, two and three weeks), time of day (day or night) and position in the chip pile. The observations made from 08:00 to 18:00 were used as six replications for day observations and observations made from 20:00 to 06:00 as replications for night observations. One-way analysis of variance was done and means were compared using Tukey's test (Winer, 1971).

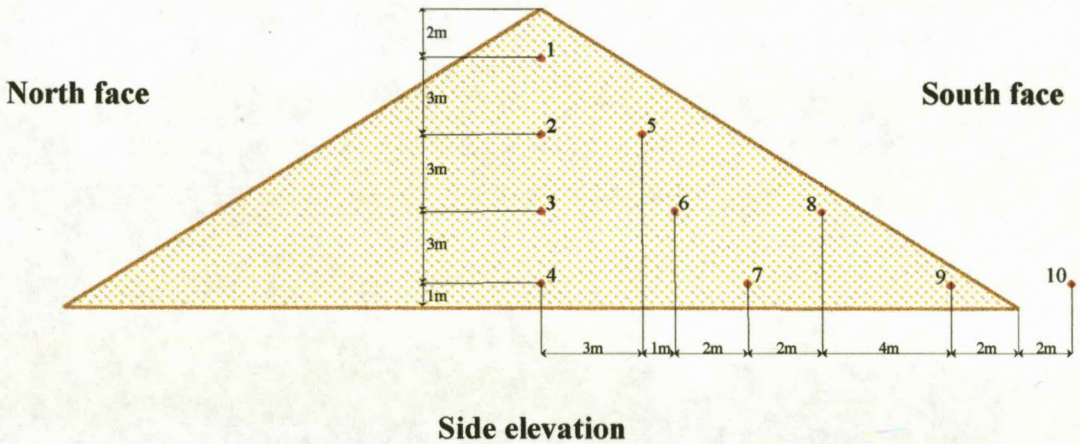


Figure 1. Chip pile dimensions (in metres) and monitoring positions within the pile.

Five chip samples (100 g oven dry) were collected at two-hourly intervals during the stacking of the chips to determine the initial moisture content of wood. Two replicate samples were also collected during reclamation of the chip pile from the area close to each of the nine temperature and CO_2 probes for determination of moisture content. Samples were also collected from the surface of the chip pile close to the top, where free water was visible after three weeks. Chips were dried at 50 °C

for 72 h and weighed to determine moisture content on a dry weight basis. One-way analysis of variance was done and means compared using Tukey's test.

Microbial populations

Five chip samples (c.a. 50 g) were collected in duplicate at two-hourly intervals during the stacking of the chips, to enumerate the fungal populations on fresh wood. Pieces of wood (approximately 9 x 2 x 1 mm) were removed from 100 chips in each sample and were placed onto 1,5 % potato dextrose agar (Difco) (PDA) and incubated at 25 °C. Samples were collected during reclamation of the chip pile from the area close to the temperature and CO_2 probes. Three of these chip samples (from position 5, 6 and 7) were discarded due to exceptionally large mite infestations in those areas. Duplicate chip samples were also collected from the north face, south face and top of the chip pile to enumerate the fungi occurring on the surface of the OCS. Two hundred pieces of wood per sample were picked at random and plated onto PDA (five pieces per plate). Half of each sample (100 pieces) was incubated at 25 °C to allow development of mesophilic and thermotolerant fungi, and the other half incubated at 54 °C to allow growth of thermophilic fungi. The total number of each fungal species occurring on the wood was counted and in some cases a piece of wood yielded more than one species. Identification was based on macroscopic, microscopic and culture morphology. Non-sporulating cultures were purified and exposed to near-ultra violet radiation at 25 °C to allow sporulation.

RESULTS AND DISCUSSION

Physical conditions

Analysis of variance showed that the two-factor interactions between temperature and the age of the chip pile, time of day and monitoring position were significant ($p \leq 0,01$). Mean day-time and night-time temperatures within the OCS changed significantly over three weeks, although the changes were small (Table 1, Appendix D). Three distinct temperature zones, which could be observed by using nine sampling points, already developed after one week within the chip pile (Figure 2). Significant differences were found between temperatures of these zones, although significant differences in temperature were also observed within zones (Table 2). Significant, but small differences (≤ 1,3 °C) were observed between the day and night temperatures at the outer positions in the pile, but except for position 2, day and night temperatures in the core positions did not differ significantly (Table 3).

Table 1. Influence of chip age on temperatures in outside chip storage (OCS).

	Day temperatures (°C)		Night temperatures (°C)	
Age	**OCS[a]**	**Ambient[b]**	**OCS[a]**	**Ambient[b]**
1 Week	38.9 a	15.4	38.5 a	8.4
2 Weeks	39.6 b	21.3	39.4 b	9.6
3 Weeks	36.8 c	13.4	34.7 c	1.3

a Means of six measurements at nine positions. Means in the same column followed by the same letter do not differ significantly ($p \leq 0,01$; Tukey's test).

b Means of six measurements at one position outside chip pile.

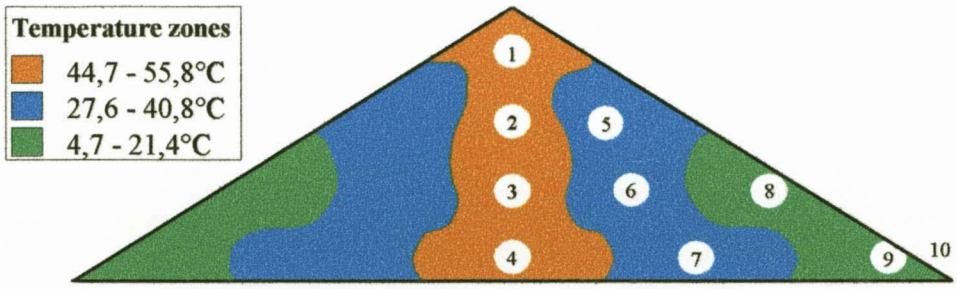


Figure 2. Extent of temperature zones that had developed in the chip pile after storage for three weeks. Numbers 1 to 10 indicate sampling positions.

Table 2. Mean temperature (°C) at different positions in a chip pile at weekly intervals.

Temperature Zone	Monitoring position	Age of chip pile		
		1 Week	2 Weeks	3 Weeks
Hot	1	51,6a	55,8a	51,2bc
	2	52,1a	53,9a	50,3c
	3	51,6a	55,6a	53,8a
	4	44,7b	54,9a	52,5ab
Warm	5	40,0c	36,6b	30,9d
	6	40,8c	36,0b	30,9d
	7	32,7d	30,7c	27,6e
Cool	8	20,9e	21,4d	15,2f
	9	13,6f	11,1f	4,7h
	Ambient	11,9f	16,4e	7,3g

a, b, c, d, e, f, g, h. Means in the same column followed by the same letter do not differ significantly ($p \leq 0,01$; Tukey's test)

Table 3. Mean temperature at different positions during day-time and night –time observations.

	Monitoring position	Time of measurement	
		Day temperatures (°C)	Night temperatures (°C)
Inner positions	1	52,9a	53,0a
	2	52,5a	51,7b
	3	53,6a	53,3a
	4	50,5a	50,9a
Outer positions	5	36,5a	35,2b
	6	36,5a	35,2b
	7	30,4a	30,2a
	8	19,4a	18,9b
	9	10,3a	9,3b
	Ambient	16,4a	6,4b

a, b. Means in the same row followed by the same letter do not differ significantly ($p \leq 0,01$; Tukey's test)

The highest CO_2 measurement of 12,7 % was made after one week at position 4 (Appendix E). It is possible that the CO_2 levels were higher during the first week after stacking, but conditions in the chip pile were not observed on a daily basis. Analysis of variance showed that the three-factor interaction between the age of the chip pile, time of day and monitoring position had a significant ($p \leq 0,01$) influence on CO_2 concentration. The weekly CO_2 levels decreased significantly ($p \leq 0,01$) in the centre, bottom (positions 3 and 4) of the pile (Table 4). In adjacent positions, this reduction only became significant after three weeks, possibly because the initial levels of CO_2 were lower. The largest reductions in CO_2 concentrations were observed in the core of the chip pile. Significant differences between day and night-time CO_2 concentrations were observed only in the centre, bottom of the chip pile (positions 3 and 4 after one week and position 4 after three weeks) (Table 5).

Table 4. Weekly CO_2 concentrations (%) at different positions, monitored in the day and night over three weeks of chip pile storage.

Monitoring position	Day			Night		
	1 Week	2 Weeks	3 Weeks	1 Week	2 Weeks	3 Weeks
1	1,6a	1,4ab	0,9b	1,3a	1,2ab	0,7b
2	2,3a	1,9a	1,2b	1,8a	1,7a	1,1b
3	5,9a	2,6b	1,4c	3,5a	2,4b	1,6c
4	9,6a	3,1b	2,3c	4, 8a	3,0b	1,6c
5	0,8a	0,8a	0, 5a	0,8a	0,7ab	0,2b
6	1,1a	0,8a	0,5a	0,9a	0,6ab	0,2b
7	0,9a	0,5a	0,3a	0,7a	0,4ab	0,1b
8	0,3a	0,2a	0,2a	0,4a	0,2a	0,0a
9	0,2a	0,1a	0,1a	0,2a	0,1a	0,0a
Ambient	0,1a	0,1a	0,1a	0,2a	0,1a	0,0a

a, b, c. Means of six replications. Means for day-time observations in the same row, and means for night-time observations in the same row that is followed by the same letter do not differ significantly ($p \leq 0,01$; Tukey's test)

Table 5. CO_2 concentrations (%) at different positions in the day and night, monitored weekly over three weeks of chip pile storage.

Monitoring position	1 Week		2 Weeks		3 Weeks	
	Day	Night	Day	Night	Day	Night
1	1,6a	1,3a	1,4a	1,2a	0,9a	0, 7a
2	2,3a	1,6a	1,9a	1,7a	1,2a	1,1a
3	5,8a	3,5b	2,6a	2,4a	1,5a	1,6a
4	9,6a	4,8b	3,1a	3,0a	2,3a	1,6b
5	0, 8a	0,8a	0,8a	0,7a	0,5a	0,2a
6	1,1a	0,9a	0,8a	0,6a	0,5a	0,2a
7	0,9a	0,7a	0,5a	0,4a	0,3a	0,1a
8	0,3a	0,4a	0,2a	0,2a	0,2a	0,0a
9	0,2a	0,2a	0,1a	0,1a	0,1a	0,0a
Ambient	0,1a	0,2a	0,1a	0,1a	0,1a	0,0a

a, b. Means of six replications. Observations for the same chip pile age and in the same row that is followed by the same letter do not differ significantly ($p \leq 0,01$; Tukey's test)

The significant reduction of CO_2 concentrations during the night in the lower centre of the pile could possibly be ascribed to increased aeration of the chip pile. The possibility that the increased influx of air was temperature driven, was

investigated by plotting ambient temperature and CO_2 concentrations at these positions against time (Figure 3). The reduction of CO_2 concentrations with reduction in ambient temperature points to a system where aeration of the chip pile is driven by a temperature gradient between the ambient and the inside of the chip pile. Temperatures in the central positions of the chip pile did not differ significantly, therefore, the gradient changed because of fluctuations in ambient temperature. The relationship between CO_2 concentrations, at those positions where significant differences were observed between ambient temperature during the day and night was, therefore, tested. Analyses showed that correlation coefficients were 81 %, 77 % and 76 % for positions 4 and 3 after one week and position 4 after three weeks respectively.

In the three-week old chip pile, the moisture content at only two positions differed significantly ($p \leq 0,01$) from the moisture content of chips that were collected during stacking (Figure 4). The moisture content of the sample collected from the top, where free water was visible on the chip surface, was 9 % higher than the initial moisture content of 56 %. The driest chips were collected from the centre, bottom (position 4) of the OCS, where the moisture content was reduced by 28 %. The drying of chips in this area can be ascribed to moisture migration within the pile. The most important area to which moisture was transferred, was the top of the chip pile creating the wet lens as described by Hulme (1979).

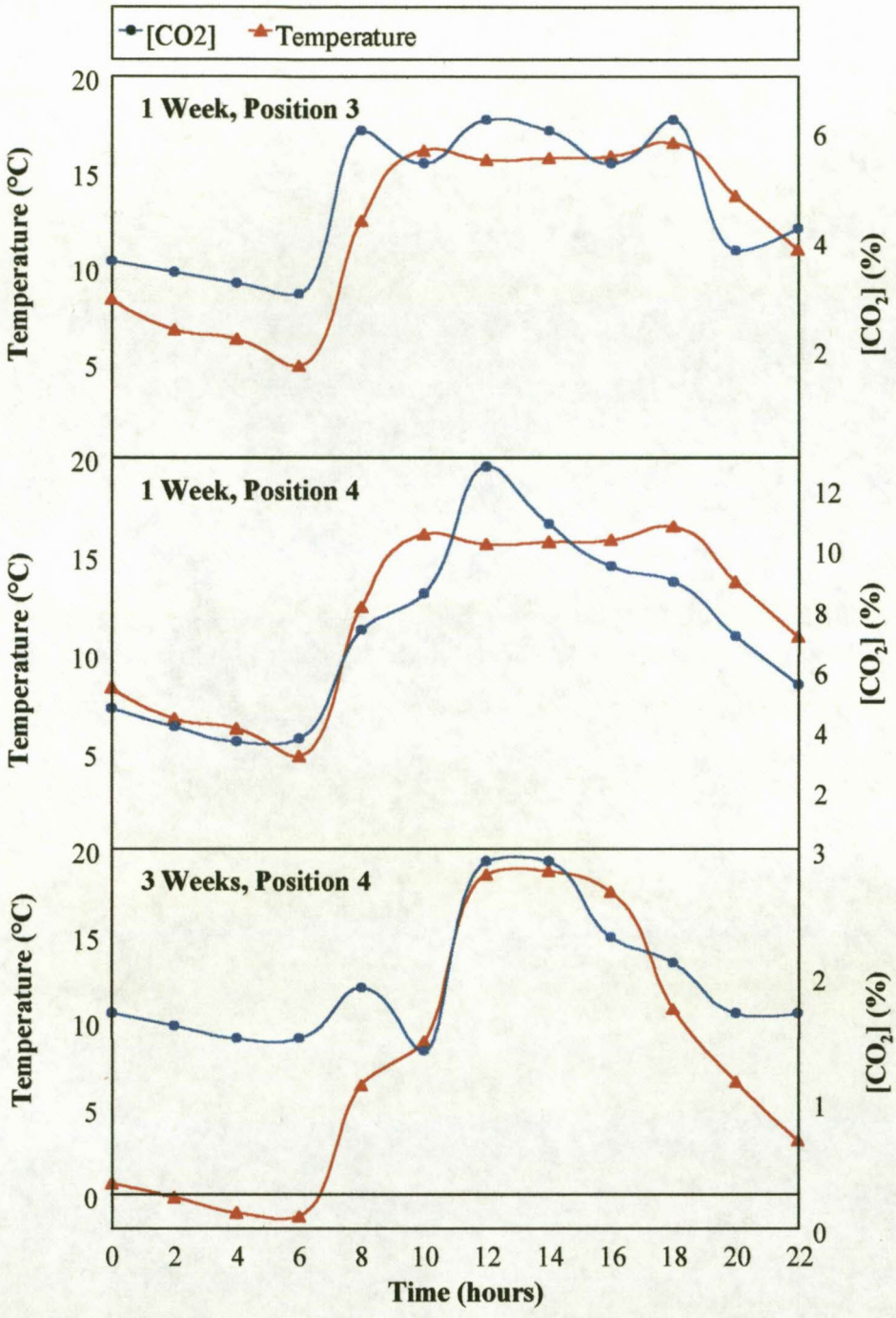


Figure 3. Change in ambient temperature and CO_2 concentration at positions where significant differences between day and night time temperatures were observed. Positions where these observations were made are shown in Figure 1.

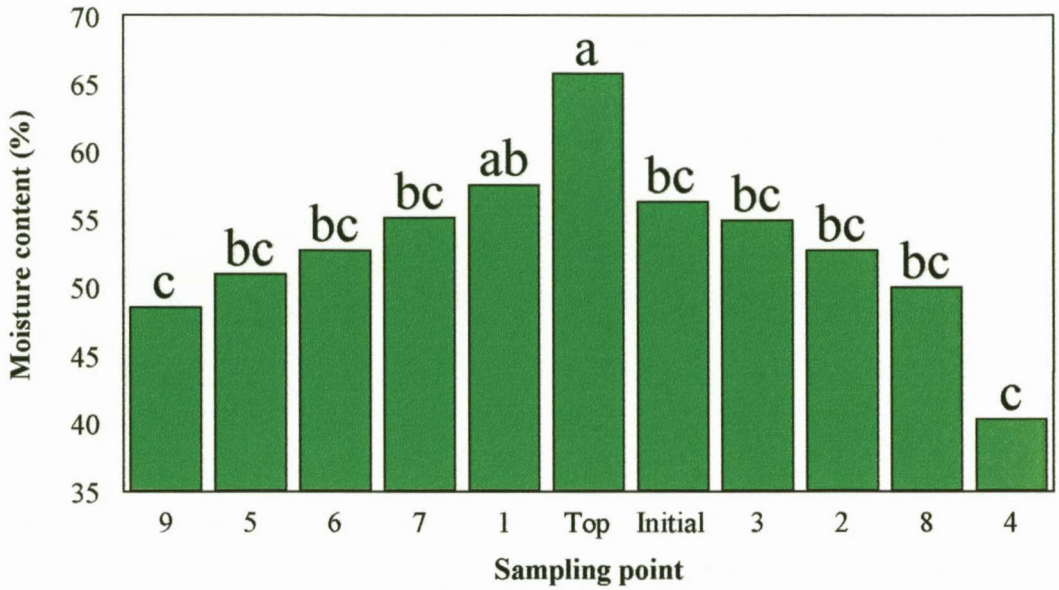


Figure 4. Average moisture content of chip samples collected after three weeks at different positions, compared to samples collected during stacking.
(a, b, c, d: Columns with the same letter do not differ significantly, $p \leq 0,01$; Tukey's test)

Microbial populations

The distribution of fungal species and frequency of isolation in the chip pile reflected the temperature of the positions from where they were collected (Table 6). As expected, most of the thermophiles were isolated from the hot zone (44,7 to 55,8 °C). In this zone *Paecilomyces* sp., *Thermoascus* sp. and *Thermomyces* sp. predominated, but a thermotolerant *Trichoderma* sp. and *Gliocladium* sp. were also isolated frequently. In the cooler zones (4,7 to 21,4°C), the occurrence of *Paecilomyces* sp. and *Thermomyces* sp. was notably reduced, while *Aspergillus fumigatus* became one of the dominant species. Chips on the surface of the OCS were mostly contaminated by the same species that were present deeper in the pile. This observation could possibly be ascribed to the contamination that occurs during the

reclaiming of adjacent chip piles. Large percentages of chips from the surface were, however, also colonized by *Mucor* sp., *Dictyostelium* sp. and *Sphaerographium* sp. Although the *Dictyostelium* sp. was present in some of the hot areas of the chip pile, it was not restricted to the wetter areas that would suit development of bacteria for this Myxomycete to feed on (Alexopoulos & Mims, 1979). The *Sphaeropsis* sp. that was present on a large percentage of chips during stacking was almost completely displaced from the chip pile, but the *Trichoderma* sp. maintained its dominance on chips during the storage period. It is, however, not possible to determine the amount of fungal biomass present by comparing the frequency of isolations. The thermophilic species were not observed on chips during stacking, but occurred in large numbers in the hot zones after three weeks. It is possible that inoculum was released during the reclaiming of adjacent chip piles and that spores were transferred to the centre of the pile by air movement. However, propagules might also have been present on chips during stacking, but have been undetected at the incubation temperature of 25 °C. Sap-staining fungi (*Ophiostoma* sp., *Graphium* sp. etc.) were only detected on a small number of the chips from the cool zones and the surface. If any wood-decay Basidiomycetes were present, the techniques used were inadequate to isolate these fungi.

Table 6. Isolation frequency of different fungal genera (%) from different zones in a pile of softwood chips in outside storage.

	Hot zones[a]	Cool zones[b]	OCS Surface[c]	Total[d]	Stacking[e]
Thermophilic fungi					
Humicola sp.	9			4	
Paecilomyces sp.	100	45	55	73	1
Thermoascus sp.	41	37	10	29	
Thermomyces sp.	22	1	1	10	
Thermotolerant fungi					
Aspergillus fumigatus	9	40	2	14	
Gliocladium sp.	41	40	74	52	50
Mucor sp.	8	2	46	19	25
Rhizopus sp.			4	1	7
Trichoderma sp.	97	100	94	97	99
Mesophilic fungi					
Alternaria sp.					1
Dictyostelium sp.	2	1	18	1	
Fusarium sp.	1		1	1	
Graphium sp.			3	1	
Leptographium sp.		2		1	
Ophiostoma sp.		5	2	2	
Penicillium sp.					1
Phoma sp.	1	7	4	3	2
Sphaerographium sp.	3	1	15	6	
Sphaeropsis sp.			1	1	57

a Mean percentage of four samples of 100 pieces of wood each (positions 1, 2, 3 and 4).
b Mean percentage of two samples of 100 pieces of wood each (positions 8 and 9).
c Mean percentage of three samples of 100 pieces of wood each (North face, South face and top).
d Mean percentage of nine samples of 100 pieces of wood each.
e Mean percentage of six duplicate samples of 100 pieces of wood each, collected during stacking.

CONCLUSIONS

White-rot fungi have been the subject of biopulping studies, because of their ability to degrade lignin (Messner & Srebotnik, 1994). Most of these fungi are, however, mesophilic (Anonymous, 1994) and would probably not be effective at

temperatures higher than 40 °C. At least 29 % of the chip pile would, therefore, be unsuitable for colonization by most white-rot fungi, because of the high temperatures in the hot zone. The changes in temperature observed in the experimental chip pile occurred in accordance with previously described mechanisms. Initially, temperatures increased in the whole pile due to respiration of parenchyma cells that are still viable in the first week (Springer *et al.*, 1971). However, during the second week, heat transfer due to the migration of moisture became more important (Hulme, 1979), especially at the centre of the pile. The different temperature zones that developed within the chip pile in this study agree with the chimney-model that was described for large chip piles by Hulme (1979).

This study showed that the concentration of CO_2 reached a maximum of 12,7 % within the first week after OCS stacking. This period of higher CO_2 production also agrees with the time in which parenchyma cells are viable (Feist *et al.*, 1971). Freshly harvested young wood, with a relatively large sapwood content was chipped in the present study. Viable parenchyma would, therefore, play a more important role in temperature and CO_2 generation (Springer *et al.*, 1971). Conditions observed during the present study were, therefore, mainly determined by respiration of parenchyma, because more viable parenchymal tissue was present. A biopulping trial with selected white-rot fungi (Appendix F) showed that it was possible to improve pulping at CO_2 concentrations of 15 %. This level of CO_2 is much higher than the level of 5% that was suggested by Nishimoto *et al.* (1975) as a level that would retard wood degradation. Akhtar *et al.* (1998) and Wall *et al.* (1993) proposed

processes to improve aeration of chip storage for biopulping. Our results indicate that the most important effect of aeration would be to improve temperature control.

The moisture content of the bulk of the Ngodwana chip pile remained fairly constant between 48 % and 58 %. In 24 % of the chip pile moisture contents increased to more than 55 %. However, temperatures were also very high in these areas, and high moisture content does not add to the volume of the chip pile that is unsuitable for colonization by biopulping fungi. Moisture conditions were, therefore, favourable for fungal colonization in largest part of the OCS. A three-fold increase in moisture in some areas of a *Picea glauca* chip pile has been recorded while it was dried to 50 % of the initial moisture content in other areas (Hatton & Hunt, 1972). However, smaller changes (similar to the results of the present study) were observed on stored softwood *Pinus contorta* chips (Hatton & Hunt, 1972). The moisture content and distribution will be influenced by measures to control temperature and aeration (Akhtar *et al.*, 1998; Wall *et al.*, 1993), as well as by water added during inoculation of chips with biopulping fungi. Moisture conditions at the surface and the top of the studied chip pile, also present a problem to a biopulping process. Chips on the slopes and bottom of the pile were dried out while moisture condensed on chips at the top of the pile to reach a moisture content of 65 % (Figure 4) in the wet lens described by Hulme (1979). In the drier zones (on the surface of the pile), water for metabolic activity would be restricted and fungal colonization would be limited, although existing inoculum would probably survive (Morrell *et al.*, 1996). It has also been shown that moisture migration plays a very important role in heat transfer

(Hulme, 1979). It is thus very important to consider the effect of moisture in order to maintain a suitable environment for biopulping fungi.

Based on the weight of wood, more fungal propagules are present on chipped wood than on logs, because it provides a larger surface area (Miller & Rothrock, 1968). On average, each chip in the present study was contaminated with more than two fungal propagules, half of which was *Trichoderma* sp. The number of isolates from chips increased after storage for three weeks in the OCS, while fungi were also isolated from the most inhospitable areas in the chip pile. Fungi were isolated from areas where temperatures rose to 57 °C, areas that were saturated with water as well as areas where the moisture content was as low as 40%. The fungi most frequently isolated belong to genera, that have previously been described as common in chip piles (Hulme, 1979; Zabel & Morrell, 1992). However, *Thermomyces* sp. and *Dictyostelium* sp., that were isolated frequently from the warm areas and the surface of the OCS respectively, were not listed (Hulme, 1979; Zabel & Morrell, 1992). *Chaetomium* spp. and Basidiomycetes are wood-decay fungi that are usually present on chips in consequential numbers (Hulme, 1979), but we did not find these fungi. Few sap staining and ophiostomatoid fungi (*sensu* Upadhyay, 1993) were isolated compared to numbers recorded in other studies (Hulme, 1979; Zabel & Morrell, 1992).

The number of yeasts, bacteria and Actinomycetes were not determined in the present study, because they cause little decay (Hulme, 1997). These organisms do, however, play an important role in the inhibition of other organisms (Chapter 5),

especially in the initial stages after OCS stacking, when low molecular weight nutrients are available (Hulme, 1997).

The reduction in CO_2 levels in the second week suggests that microorganisms did not have a significant impact on physical conditions in the OCS within the first three weeks of stacking. However, increased CO_2 concentrations, caused by microorganisms, should have been expected after two weeks (Fuller, 1985; Zabel & Morrell, 1992). The frequency of occurrence and diversity of fungi does, however, suggest that they could play a role in competing with biopulping fungi. The negative role of *A. fumigatus* and *Trichoderma* sp. in competition with biopulping fungi has already been highlighted (Messner & Srebotnik, 1994) and control of these contaminants will require special management practices. Pasteurization and higher inoculum dosages are two of the methods that could be employed to reduce the adverse effects of contaminants (Messner & Srebotnik, 1994; Wall *et al.*, 1993).

This study on physical and microbiological parameters in a South African chip pile has shown that changes occur in a softwood chip pile within three weeks after stacking. Zones where temperature and carbon dioxide varied, developed after one week. Unfortunately, some of these conditions do not favour the colonization of OCS by biopulping fungi. Special OCS management practices and capital investments would, therefore, be required to maintain the OCS under optimal conditions for biopulping. It is also important to bear in mind that the present study was conducted on an un-inoculated chip pile. All of the factors discussed above could be changed by the addition of a fungal inoculum. Water added with inoculum could have a large

influence on heat transfer, while activity of the inoculum could lead to quicker buildup of CO_2 in the chip pile. Fungal growth on the surface of chips could also impede aeration and lead to increased temperatures and CO_2 concentrations.

REFERENCES

ANONYMOUS. 1994. List of cultures, 33rd ed. Centraal Bureau voor Schimmelcultures., Baarn, Netherlands.

AKHTAR, M., LENTZ, M.J., HORN, E., SCOTT, G.M., SWANEY, R.E. & KIRK, T.K. 1998. Commercialization of biopulping for mechanical pulping. Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada. Vol. A: 55-58.

ALEXOPOULOS, C.J. & MIMS, C.W. 1979. Introductory Mycology. John Wiley & Sons, Inc., New York, USA.

BERGMAN, Ö. & NILSSON, T. 1979. An experiment on outdoor storage of whole-tree chips. *Swed. Univ. Agric. Sci.* R 109: 1-21.

ERIKSSON, K.-E.L. 1998. Past successes and future possibilities for biotechnology in the pulp and paper industry. Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada. Vol. A: 7-10.

FEIST, W.C., SPRINGER, E.L. & HAJNY, G.J. 1971. Viability of parenchyma cells in stored green wood. *Tappi. J.* 54(8): 1295-1297.

FULLER, W.S. 1985. Chip pile storage – a review of practices to avoid deterioration and economic losses. *Tappi J.* 68(8): 48-52.

HATTON, J.V. & HUNT, K. 1972. Effect of prolonged outside chip storage on yield and quality of kraft pulps from *Picea glauca* and *Pinus contorta* chips. *Tappi. J.* 55(1): 122-126.

HULME, M.A. 1979. Outside chip storage. In: Chip quality monograph, Ed. J.V. Hatton, pp. 213-244. Joint Textbook Committee of the Paper Industry, Atlanta, USA.

MESSNER, K. & SREBOTNIK, E. 1994. Biopulping: An overview of developments in an environmentally safe paper-making technology. *FEMS Microb. Rev.* 13: 351-364.

MILLER, R.L. & ROTHROCK, C.W. 1968. Why outside chip storage is increasing. *Pulp. and Pap.* June 1968: 30-32.

MORRELL, J.J., FREITAG, C.M., SMITH, S.M., CORDEN, M.E. & GRAHAM, R.D. 1996. Basidiomycete colonization in Douglas-fir poles after 3 or 6 months of air-seasoning. *For. Prod. J.* 46(5): 56-63.

NISHIMOTO, K., AKINO, S., TSUTSUI, I., YODOMURA, K. & TSUDA, M. 1975. Prevention of wood deterioration during outside chip storage. 3. Prevention of wood deterioration by oxygen cut. *Japan Tappi.* 28(11): 534-544. [Japanese; Eng Summ.]

SPRINGER, E.L. & HAJNY, G.J. 1970. Spontaneous heating in piled wood chips. I. Initial mechanism. *Tappi. J.* 53(1): 85-86.

SPRINGER, E.L., HAJNY, G.J. & FEIST, W.C. 1971. Spontaneous heating in piled wood chips. II. Effect of temperature. *Tappi. J.* 54(4): 589-591.

TAROCINSKI, E. 1976. Evaluation criteria of changes occurring in wood chips stored in piles. *Zesz. Probl. Postepow Nauk Roln.* 178: 105-112. [Polish; Eng Summ.]

UPADHYAY, H.P. 1993. Classification of Ophiostomatoid fungi. In: *Ceratocystis* and *Ophiostoma*: Taxonomy, ecology and pathogenicity, Eds. M.J. Wingfield, K.A. Seifert and J.F. Webber, pp. 7-13. APS Press, St. Paul, USA.

VANDERHOFF, J.E. 1992. Handling chips. *Papermaker.* October 1992: 22-24.

WALL, M.B., CAMERON, D.C. & LIGHTFOOT, E.N. 1993. Biopulping process design and kinetics. *Biotech. Adv.* 11: 645-662.

WINER, B.J. 1971. Statistical principles and experimental design, 2nd ed. Mc Graw-Hill Book Co., USA.

ZABEL, R.A. & MORRELL, J.J. 1992. Wood microbiology: Decay and its prevention. Academic Press, Inc., San Diego, USA.

CHAPTER 5

COLONIZATION OF FRESHLY CHIPPED SOFTWOOD BY WHITE-ROT FUNGI.

Arial view of Ngodwana chip piles

ABSTRACT

Biopulping is a solid-substrate fermentation process in which wood chips are treated with fungi to improve the pulping process. Problems are, however, experienced with poor colonization of freshly chipped softwood. The insufficient growth of biopulping fungi can be ascribed to competition with contaminating fungi or to inhibition by wood components. In this study, the relative importance of different inhibitory factors present in fresh wood as well as contaminating microbes was determined and the effect of different wood pre-treatments evaluated.

It was found that the inhibition of biopulping fungi by monoterpenes was greater than that of any group of contaminating microbes. Steaming mitigated the inhibition by fungi as well as by extractives. Steaming for ten minutes under atmospheric pressure would be the most economical method to improve colonization by biopulping fungi. However, biopulping fungi with a good competitive ability and tolerance to monoterpenes, in addition to their potential to improve pulping, should preferably be selected.

INTRODUCTION

Biopulping has been defined as the treatment of wood chips with lignin-degrading fungi prior to pulping (Akhtar *et al*, 1996). The biopulping process is applied to wood chips where free water is limited. Therefore, it is a solid-substrate fermentation process that requires measures to ensure asepsis or at least measures to reduce contaminant growth (Wall *et al.*, 1993). Messner & Srebotnik (1994) have discussed the effect of fungal contamination in a review on biopulping. The most cost-effective way of reducing contaminant loads appears to be a brief exposure of chips to steam under atmospheric pressure (Akhtar *et al.*, 1998). Aseptic treatment is not a prerequisite for biopulping as it is for some fermented products, but is required for sufficient and reproducible growth (Wall *et al.*, 1993).

Biopulping fungi are not only inhibited by competing microorganisms, but they also interact with the wood-chip substrate. The growth of biopulping fungi can, therefore, be inhibited by the metabolic products of wood cells. Biopulping fungi are sometimes applied to wood chips immediately after chipping (Wall *et al.*, 1993) when the freshly cut wood cells are not dead and are still respiring (Springer & Hajny, 1970). Some of the chemical compounds that protect living trees against microbial attack could, therefore, still be present in these wood cells and in exudates. These inhibitory compounds include tannins, phenols and oleoresins (Pearce, 1996). The ability of lignin degrading fungi to produce enzymes that catalyze the oxidation of phenols and tannins is well known (Barz & Weltring, 1985; Davidson *et al.*, 1938; Käärik, 1965). However, compounds in the oleoresin fraction could play an

important role in the inhibition of fungi (Cobb *et al.*, 1968; De Groot, 1972; Pearce, 1996; Rudman, 1965), but their effects on Basidiomycetes are not well documented. Cobb *et al.* (1968) have shown that components of the oleoresin fraction are inhibitory to pathogenic fungi, but oleoresin can also act as a mechanical barrier to infection (Pearce, 1996). Oleoresin is found in all living parenchyma cells (Mirov, 1961; Pearce, 1996) and, therefore, also in freshly chipped wood. The volatile fraction can be removed by steam distillation and is referred to as turpentine (Mirov, 1961) that is primarily composed of volatile monoterpenoids and sesquiterpenoids (Sjöström, 1981). The most abundant components in the terpene fraction of *Pinus patula* Schl. & Cham. are α-pinene (5 %) and β-phellandrene (70 % to 80 %) (Mirov, 1961). The fungistatic effect of both these compounds has been demonstrated on *Heterobasidion annosum* and four species of *Ceratocystis* and *Ophiostoma* (Cobb *et al.*, 1968). However, few studies have been conducted on the inhibition of Basidiomycetes that could potentially be used for biopulping. In one such experiment, De Groot (1972) compared the effect of terpenes on two white-rot species (*Phlebiopsis gigantea* and *Schizophyllum commune*) with the effect it had on a brown-rot fungus (*Gloeophyllum sepiarium*), and also on *Ceratocystis minor* and *Trichoderma viride.* It was found that the pioneer white-rot fungus, *P. gigantea*, was inhibited less than the other species tested.

Identification of the most important inhibitory factors is necessary in order to either select biopulping fungi that are tolerant to inhibition or, alternatively, to develop suitable control measures to alleviate inhibition. The aim of this study was, therefore, to determine the relative importance of monoterpenes and microbial

contaminants in the suppression of successful colonization of *P. patula* chips by biopulping fungi.

MATERIALS AND METHODS

Wood preparation

Pinus patula wood constitutes 40 % of the softwood for kraft pulping at the Sappi Ngodwana mill, Mpumalanga, South Africa. *Pinus patula* chips (18 mm long) were, therefore, collected at the mill immediately after chipping. The wood chips were moved to our laboratories within four hours and samples (220 g) subjected to one of six treatments (referred to as heat treatments). These treatments included: (1) Autoclaving for 15 min at 121 °C; (2) Steaming for 20 min at 80 °C to 85 °C and atmospheric pressure; (3) Steaming for 10 min at 80 °C to 85 °C and atmospheric pressure; (4) Drying at room temperature for two days; (5) Drying at room temperature for four days; (6) An untreated control. Steaming of samples were done in a retort and drying in containers that were covered to allow evaporation, but not contamination. Samples that were subjected to each of these treatments were used for biopulping experiments, for the enumeration of microbes and for the determination of monoterpene contents.

Enumeration of microbes

The microbes that occur on samples that were subjected to heat treatments were enumerated by plating out pieces of wood. One hundred pieces of wood, each from a different chip, were plated onto 1,5 % malt extract agar (five per plate). Plates

were incubated at 22 °C for five days and colonies identified or transferred and purified for later identification. The numbers of microbes were expressed as the total number of colonies that developed from 100 pieces of wood.

Biopulping

The highest moisture content (60 %) of wood chips that were subjected to the heat treatments was measured in the samples that were steamed for 20 min. The moisture content of the other samples was, therefore, adjusted to 60 % (wet basis). Biopulping treatment of the wood was done in 2 L bioreactors as described previously (Chapter 3). A factorial experiment with a randomized block design was used. The experiment consisted of six heat treatments and three biopulping treatments; each combination replicated three times. The biopulping treatments were inoculated with strains of *Pycnoporus sanguineus* (WR 124) and *Stereum hirsutum* (WR 95) and only growth medium [3 % corn steep liquor (v/v), 1 % sucrose (w/v)] as a control. The wood samples, treated with different inocula, were incubated at 28 °C and 85 % RH. The treated wood was harvested after three weeks and the predominant fungi present on the wood in each of the bioreactors recorded. The treated wood was dried to a moisture content of 3 % and pulped.

Kraft pulping of samples (50 g dry weight) was done in stainless steel mesh bags in a mini digester (20 L). Three different cooks were done, with each cook containing one replication of all treatments. The digester was charged with 32 % active alkali (25 % sulphidity) at a liquid to wood ratio of 10:1. The pulping times were: 90 min to 171 °C and 90 min at 171 °C.

The kappa number of each sample was determined to ascertain the efficiency of biopulping (Tappi Test Methods, T236). A two way analysis of variance was conducted and Tukey's method for multiple comparison used to identify significant differences between treatment means at the 95 % confidence level (Winer, 1971).

Extraction and analyses of monoterpenes

One chip sample (220 g) from each of the six heat treatments was frozen immediately at –4 °C, after preparation. These samples were thawed prior to use and ca. 1 g of the chips cut into pieces (18 x 2 x 1 mm) for the extraction of monoterpenes. The wood was placed in 4 ml vials and extraction solution added. The extraction solution consisted of 200 µl ethyl acetate with 10,5 g/l ρ-cymene as an internal standard that was made up to 1 ml with n-pentane. The samples were shaken for 10min on a vortex mixer and extracted for 4 h at room temperature. The samples were shaken again for 10 min and filtered through absorbent cotton wool.

The samples were analyzed on a Hewlett Packard 5890 Series II gas chromatograph with a microprocessor that utilized a multistage programme. The gas chromatograph was equipped with a flame ionization detector and a 30 m x 0,23 mm column. The initial temperature was 80 °C for 3 min. It was then raised by 3 °C/min to 170 °C and after 1 min by 30 °C/min to 290 °C. The inlet and detector temperatures were 185 °C and 300 °C respectively. The different monoterpene peaks (α-pinene and β-phellandrene) were identified by comparison with the retention times

of the standards. The mass spectra were also compared with those of known standards with the aid of a Hewlett Packard 5972 Mass Selective Detector.

Influence of α-pinene on fungi

Twenty-nine strains of wood-inhabiting fungi were tested for their ability to grow in an atmosphere that was saturated with α-pinene (Cobb *et al.*, 1968). These fungi represent five groups from different habitats, affiliated to either the Basidiomycetes or Ascomycetes (Table 1). Some mitosporic fungi were grouped with the Ascomycetes, where a link had been established.

The experimental procedures were similar to those used by Cobb *et al.* (1968). Inoculum discs (5 mm diameter) were transferred from the margins of cultures growing on 3 % potato-dextrose agar (PDA) to Petri dishes (6,5 cm diameter) with PDA. Each strain was replicated three times. Discs of filter paper (55 mm diameter) were saturated with 40 µl of 100 % pure α-pinene (Fluka Chemical Corp. New York) and stuck to the lids of Petri dishes. The cultures were stacked in 2 L jars with a vial containing 200 µl of α-pinene to saturate the environment in the jars. Water was placed on the filter paper and in the jars for the control treatments. The control treatment was done in triplicate.

Cultures were incubated in the dark at 23 °C until the colonies covered 90 % of the plate surface on either the control or α-pinene treatment. Growth in the α-pinene treatment was recorded as a percentage relative to the mean colony diameter of the three control plates. The effect of α-pinene on the relative growth rates of

different groups of fungi was tested for significance using the T-method for *a priori* contrasts at 95 % confidence (Zar, 1984).

Table 1. Taxonomic affiliation and habitat of fungal strains that were exposed to an environment saturated with α-pinene.

Taxonomic affiliation and Habitat	**Species**	**Strain**	**Source[a]**
Ascomycetes	*Botryosphaeria dothidea*	CHS52	1
Live hardwoods	*Ceratocystis virescens*	CMW3276	1
	Cylindrocladium candelabrum	CMW3911	1
	Ophiostoma piliferum	CMW2524	1
	Ophiostoma quercus	CMW2519	1
	" "	CMW2542	1
Ascomycetes	*Ceratocystis minuta*	CMW1477	1
Live softwoods	*Ophiostoma ips*	CMW84	1
	Ophiostoma minus	CMW1993	1
	Ophiostoma piceae	CMW1367	1
	Ophiostoma piliferum	CMW2506	1
	Sphaeropsis sapinea	CWS30	2
Basidiomycetes	*Amylostereum aureolatum*	A11	1
Live softwoods	" "	M7W	1
	" "	SN13.3B	1
	Armillaria heimii	CMW2717	1
	" "	CMW2742	1
Basidiomycetes	" "	CMW3164	1
Live hardwoods	*Armillaria mellea*	CMW3179	1
	" "	CMW3974	1
	Chondrostereum purpureum	WR66	3
	Cylindrobasidium laeve	SCC282	4
	Unidentified sp.	WR251	3
Basidiomycetes	*Ceriporiopsis subvermispora*	CZ-3	5
Dead wood	*Pycnoporus sanguineus*	WR330	3
	" "	WR124	3
	Phanerochaete chrysosporium	ATCC32629	6
	Stereum hirsutum	WR3	3
	" "	WR95	3

a Culture collections of: 1. Forestry and Agricultural Biotechnology Institute, University of Pretoria. 2. Department of Plant Pathology, University of the Orange Free State. 3. CSIR, Foodtek, Pretoria. 4. Plant Protection Research Institute, Stellenbosch. 5. USDA Forest Products Laboratory, Madison, Wisconsin. 6. ATCC, Rockville, Maryland.

RESULTS AND DISCUSSION

Enumeration of microbes

The enumeration of microbes after heat treatment of chips, showed that autoclaving was the most successful method of decontamination (Table 2), followed by steaming for either 10 or 20 min. It is not clear why the 20 min steam treatment resulted in the total colonization of chips by *Sporothrix* sp. It is possible that the contamination occurred during or after steaming, because *Sporothrix* was not isolated from wood that was subjected to any other treatment. Only 11 % of chips that were treated with steam for 10 min were contaminated with microbes (Table 2). A short steam treatment can, therefore, provide a successful and, presumably, a more economical alternative to autoclaving. Treatment of wood chips by air drying did not reduce the contamination load as reflected by the total number of isolations (Table 2).

Table 2. Isolation frequency of microorganisms from *Pinus patula* chips that were subjected to different heat treatments.

Microbial contaminant	Heat treatment					
	Autoclaving	Steam (20 min)	Steam (10 min)	Air dried (48 h)	Air dried (96 h)	Untreated Control
Unidentified bacteria	1		5	18	40	22
Unidentified yeasts			1	44	50	53
Aspergillus sp.				1		
Cladosporium spp.			1	2	3	
Fusarium spp.					3	
Mucor spp.				4	20	7
Penicillium spp.			3	76	75	63
Rhizopus spp.				2	36	20
Sporothrix spp.		100				
Trichoderma spp.			1	25	19	37
Total isolations	1	100	11	172	246	202

Biopulping

Trichoderma and *Aspergillus* spp. were the most common contaminants in biopulping reactors (Table 3). These fungi were, however, not the most common on wood after heat treatment (Table 2). The success of these fungi in reactors could possibly be ascribed to the suitability of the incubation conditions as well as their ability to rapidly colonize lignocellulosic substrates and also to sporulate abundantly. The presence of these fungi is of particular concern in biopulping applications, because of their abundance in chip piles (Chapter 4). *Stereum hirsutum* could only proliferate on chips that had been treated with steam, while *P. sanguineus* was dominant in all the reactors (Table 3). This is an indication of the superior ability of *P. sanguineus* to colonize freshly chipped softwood compared to that of *Stereum hirsutum*.

Table 3. Dominant fungal growth observed in bioreactors with wood chips that had been subjected to different heat treatments and inoculated with biopulping fungi.

	Biopulping inoculum		
Treatment	**Control**	***S. hirsutum***	***P. sanguineus***
Autoclaving	No growth	*S. hirsutum*	*P. sanguineus*
Steam (20min)	*Trichoderma sp.*	" "	" "
Steam (10min)	*Aspergillus sp.*	" "	" "
Air dried (48h)	" "	*Aspergillus sp.*	" "
Air dried (96h)	" "	" "	" "
Control	*Trichoderma sp.*	*Trichoderma sp.*	" "

The superior ability of *P. sanguineus* to colonize freshly chipped wood (control) was also reflected by the reduction in the lignin content (kappa number) of the wood (Figure 1). When *P. sanguineus* was applied to treated chips, a significant

($p \leq 0,05$) reduction in the kappa number of pulp was observed except in the air dried chips. *Stereum hirsutum* caused a reduction in lignin content only on wood that received a steam treatment prior to inoculation. These results indicate that the vigour of biopulping fungi can be strongly influenced by the presence of contaminating microorganisms. Treatments that were the most effective in sanitation of wood also allowed the most efficient colonization and lignin degradation by the biopulping fungi.

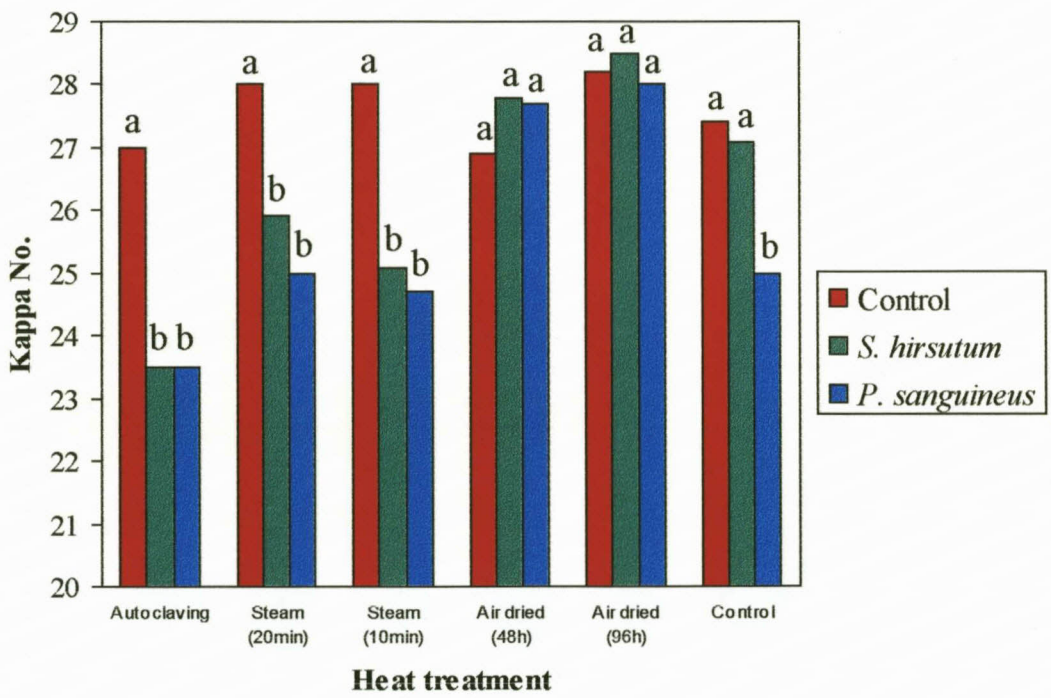


Figure 1. Influence of different treatments on the efficacy of biopulping fungi. (a, b: Columns for the same heat treatment, and with the same letter do not differ significantly, $p \leq 0,05$, Tukey's test)

Extraction and analysis of monoterpenes

The terpene content of untreated chip samples were similar to those reported by Mirov (1961) (5 % α-pinene and 70 to 80 % β-phellandrene). Analyses after steaming or drying showed that significant changes occurred in the concentration of monoterpenes (Figures 2 & 3). The most important turpentine fractions were α-pinene (15,7 µg/mg in control sample) and β-phellandrene (133,7 µg/mg). Only autoclaving caused a significant ($p \leq 0,05$) reduction in α-pinene concentration (Figure 2). However, significant reduction in β-phellandrene concentrations occurred with all treatments except the short steam treatment of ten minutes (Figure 3).

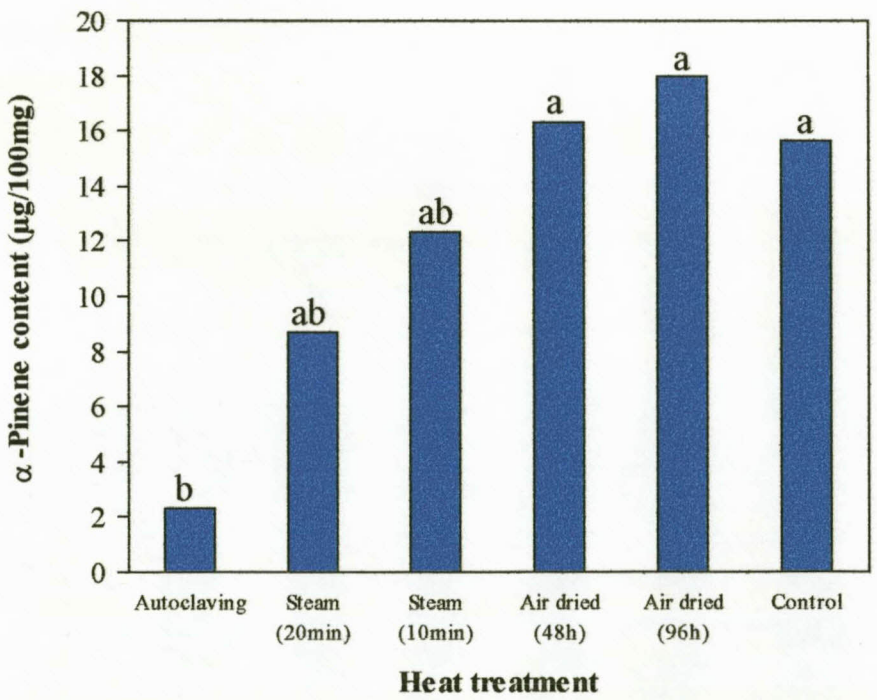


Figure 2. Influence of different treatments on the α-pinene content of wood. (a, b: Columns with the same letter do not differ significantly, $p \leq 0,05$, Tukey's test)

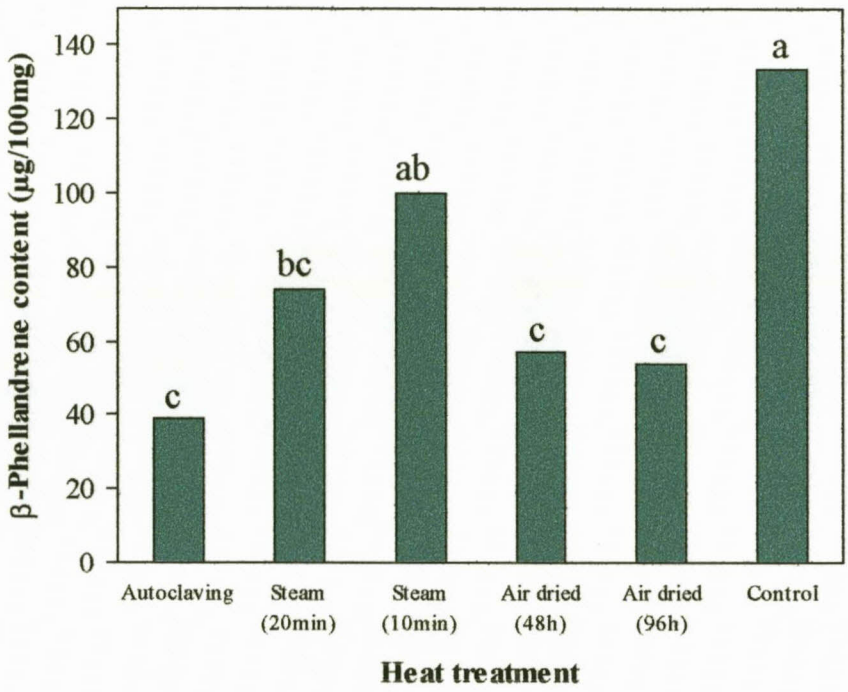


Figure 3. Influence of different heat treatments on the β-phellandrene content of wood. (a, b, c: Columns with the same letter do not differ significantly, $p \leq 0,05$, Tukey's test)

The heat treatments influenced the contaminating microbial populations as well as the concentrations of extractives that could compete or inhibit the action of biopulping fungi (Table 2; Figures 2 & 3). Analysis of data to determine the relationship between these inhibitory factors and the efficiency of biopulping (as reflected by kappa number) showed a strong correlation for all inhibitory factors except β-phellandrene (Table 4). Levels of inhibitory factors were not correlated with the lignin content of control treatments. The influence that these factors had on the kappa numbers of treated wood should, therefore, be ascribed to its influence on the biopulping fungi. The influence of these inhibitory factors on different biopulping strains also differed (Table 4). High correlation coefficients (>0,8) were observed for

the relationship between the quantities of inhibitory factors and biopulping efficiency of *S. hirsutum* (high kappa numbers were correlated with high levels of the inhibitory factors). Lower, but still consequential values (>0,7) were observed for *P. sanguineus*. An increase in the concentration of α-pinene and numbers of contaminating bacteria, yeasts and fungi has, therefore, lead to inefficient lignin degradation during biopulping. Both *S. hirsutum* and *P. sanguineus* were more susceptible to increased α-pinene concentration than to other inhibitory factors (Table 4). However, α-pinene concentration did not only have a direct influence on the growth of biopulping fungi, but also on the numbers of contaminating microbes.

Table 4. Correlation coefficients reflecting the relationship between increased quantities of inhibitory factors in wood and increased kappa numbers obtained for pulp.

	Biopulping treatment		
Inhibitory factor	**Control**	***S. hirsutum***	***P. sanguineus***
α-Pinene	0,15	0,89	0,80
β-Phellandrene	0,13	0,13	0,21
Bacteria	0,13	0,81	0,76
Filamentous fungi	0,13	0,88	0,74
Yeasts	-0,09	0,83	0,70

High correlation coefficients were observed for the relationship between the concentration of α-pinene and the occurrence of yeasts, filamentous fungi and bacteria (Table 5). The influence of β-phellandrene on biopulping (Table 4) and on contaminating microbes (Table 5) appears to be insignificant, although its concentration in *P. patula* wood is higher than that of α-pinene (Mirov, 1961). Correlation between the numbers of yeasts, fungi and bacteria also indicated a strong interaction between these factors (Table 5). Inefficient colonization is the result of

high concentrations of monoterpenes (especially α-pinene) as well as competition with contaminants.

Table 5. Correlation between the levels of different inhibitory factors.

Inhibitory factor	Yeasts	Filamentous fungi	Bacteria	β-Phellandrene
α-Pinene	0,83	0,75	0,82	0,36
β-Phellandrene	0,24	0,14	0,05	
Bacteria	0,89	0,77		
Filamentous fungi	0,81			

Influence of α-pinene on fungi

Basidiomycetes that were collected from dead wood, were the fungi most susceptible to inhibition by α-pinene (Figure 4, Appendix G). Ascomycetes that were isolated from softwood were the most tolerant group to α-pinene (Figure 4). The mean growth rate of these Ascomycetes was greater than the growth rate on the control medium. It, therefore, appears that α-pinene stimulated the growth of these fungi.

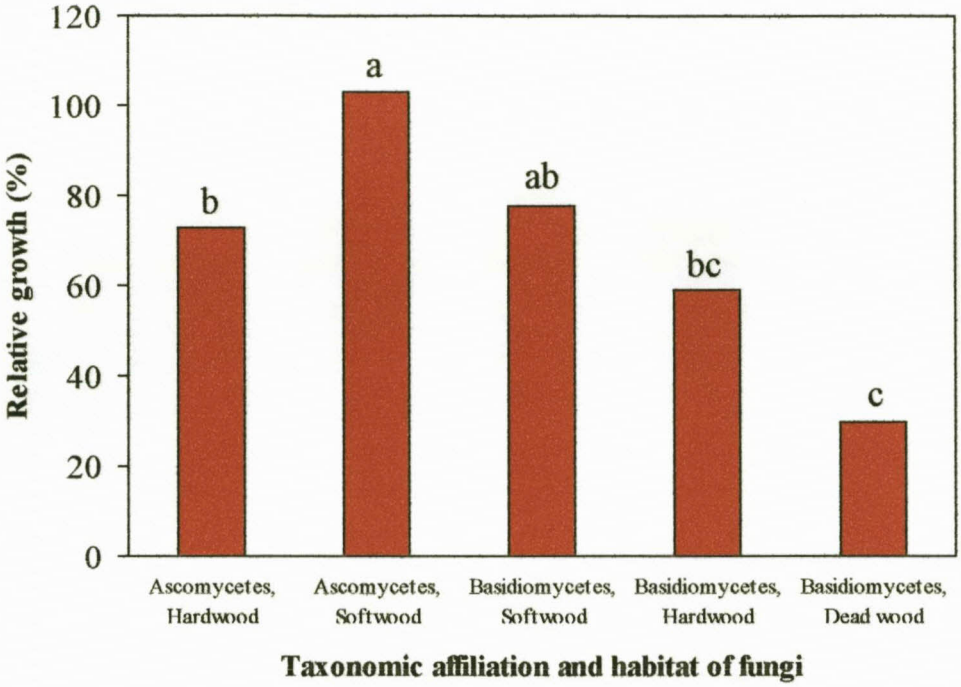


Figure 4. Ability of different groups of wood-inhabiting fungi to grow in an environment saturated with α-pinene. (a, b, c: Columns with the same letter do not differ significantly, $p \leq 0,05$, *a priori* contrast test)

CONCLUSIONS

In the past, poor colonization of fresh wood by biopulping fungi was only ascribed to competition with contaminating microbes (Akhtar *et al.*, 1998; Messner & Srebotnik, 1994; Wall *et al.*, 1993). Volatile oleoresin fractions were, on the other hand, seen as compounds that play a doubtful role in the resistance mechanism of live trees against fungal attack (Cobb *et al.*, 1968; De Groot, 1972; Pearce, 1996; Rudman, 1965). The present study has demonstrated that α-pinene play an important role in

the inhibition of biopulping fungi and that no single factor is responsible for the inadequate colonization of wood chips.

Colonization of softwood chips and degradation of lignin by biopulping fungi improved when the chips were treated with steam before inoculation. The best results were obtained when chips were autoclaved. Unfortunately, this method can not be applied on an industrial scale, because of the high energy costs that will be required for steam generation (Wall *et al.*, 1993). Steam treatments under atmospheric pressure for as little as ten minutes also improved the biopulping effect significantly. It has been demonstrated under laboratory conditions (Wall *et al.*, 1993) and with mill trials (Akhtar *et al.*, 1998) that steaming under atmospheric pressure could be a commercially viable alternative to autoclaving. The cost effectiveness of steam treatments should be evaluated during the scale-up of biopulping processes.

Steaming is not the only possible solution to problems that are experienced when freshly cut chips are inoculated. Results of this study showed that saprophytic Basidiomycetes, which are frequently used for biopulping (Messner & Srebotnik, 1994; Wall *et al.*, 1993; Chapter 3), are the group of fungi that are the most susceptible to inhibition by α-pinene. Ascomycetes were more tolerant to inhibition by α-pinene than saprophytic Basidiomycetes. Cobb *et al.* (1968) also demonstrated that four *Ophiostoma* and *Ceratocystis* species were more tolerant to inhibition by terpenes than the Basidiomycete *Heterobasidion annosum*. This might indicate that ascomycetous fungi are more suitable for application as biopulping agents on

softwood than saprophytic Basidiomycetes. They are, however, not known as efficient degraders of lignin (Crawford & Crawford, 1980).

Inoculation of untreated chips with *P. sanguineus* was more successful than inoculation with *S. hirsutum*, demonstrating that some fungi within the groups described in this study, are better able to compete with contaminants or tolerate wood extractives better than others. De Groot (1972) demonstrated that the biopulping fungus *Phlebiopsis gigantea* was more tolerant to monoterpenes than fungi from other taxonomic groups. *Phlebiopsis gigantea* was described as a pioneer fungus and should, therefore, be more tolerant to inhibitory compounds. It was also noted that species such as *Phanerochaete chrysosporium* were better able to colonize fresh chips than *Ceriporiopsis subvermispora* (Wall *et al.*, 1993). Costly treatment processes for chips could, therefore, be avoided by the correct choice of a biopulping organism. Fungi that are tolerant to wood extractives could be selected on the basis of in vitro experiments (Cobb *et al.*, 1968).

The competitive ability of biopulping fungi could be enhanced by developing the correct formulation of inoculum. Inoculum consisting of fragmented mycelium instead of spores can for instance improve the competitive ability of biopulping fungi by reducing the lag phase (Wall *et al.*, 1993).

REFERENCES

AKHTAR, M., KIRK, T.K. & BLANCHETTE, R.A. 1996. Biopulping: An overview of consortia research. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 187 – 192. Facultas-Universitätsverlag, Vienna, Austria.

AKHTAR, M., LENTZ, M.J., SWANEY, R.E., SCOTT, G.M., HORN, E. & KIRK, T.K. 1998. Commercialization of biopulping for mechanical pulping. Proceedings of the 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, Canada. Vol. A: 55-58.

BARZ, W. & WELTRING, K.-M. 1985. Biodegradation of aromatic extractives of wood. In: Biosynthesis and biodegradation of wood components, Ed. T. Higuchi, pp. 607-664. Academic Press, Orlando, USA.

COBB, F.W., KRSTIC, M., ZAVARIN, E., & BARBER, H.W. 1968. Inhibitory effects of volatile oleoresin components on *Fomes annosus* and four *Ceratocystis* species. *Phytopathol.* 58: 1327-1335.

CRAWFORD, D.L. & CRAWFORD, R.L. 1980. Microbial degradation of lignin. *Enzyme Microb. Technol.* 2: 11-22.

DAVIDSON, R.W., CAMPBELL, W.A. & BLAISDELL, D.J. 1938. Differentiation of wood-decaying fungi by their reactions on gallic or tannic acid medium. *J. Agric. Res.* 57: 681-695.

DE GROOT, R.C. 1972. Growth of wood-inhabiting fungi in saturated atmospheres of monoterpenoids. *Mycologia* 64: 863-870.

KÄÄRIK A. 1965. The identification of mycelia of wood-decay fungi by their oxidation reactions with phenolic compounds. *Stud. For. Suec.* 31: 1-80.

MESSNER, K. & SREBOTNIK, E. 1994. Biopulping: An overview of developments in an environmentally safe paper-making technology. *FEMS Microb. Rev.* 13: 351 - 364.

MIROV, N.T. 1961. Composition of gum turpentines of pines. *U.S. Dep. Agr. Tech. Bull.* 1239: 158p.

PEARCE, R.B. 1996. Tansley Review No. 87. Antimicrobial defences in the wood of living trees. *New Phytol.* 132: 203-233.

RUDMAN, P. 1965. The causes of natural durability in timber. XVIII. Further notes on the fungitoxicity of wood extractives. *Holzforschung.* 19: 57-58.

SJÖSTRÖM, E. 1981. Wood chemistry: Fundamentals and Applications, 2nd ed. Academic Press, San Diego, USA.

SPRINGER, E.L. & HAJNY, G.J. 1970. Spontaneous heating in piled wood chips. I. Initial mechanism. *Tappi. J.* 53(1): 85-86.

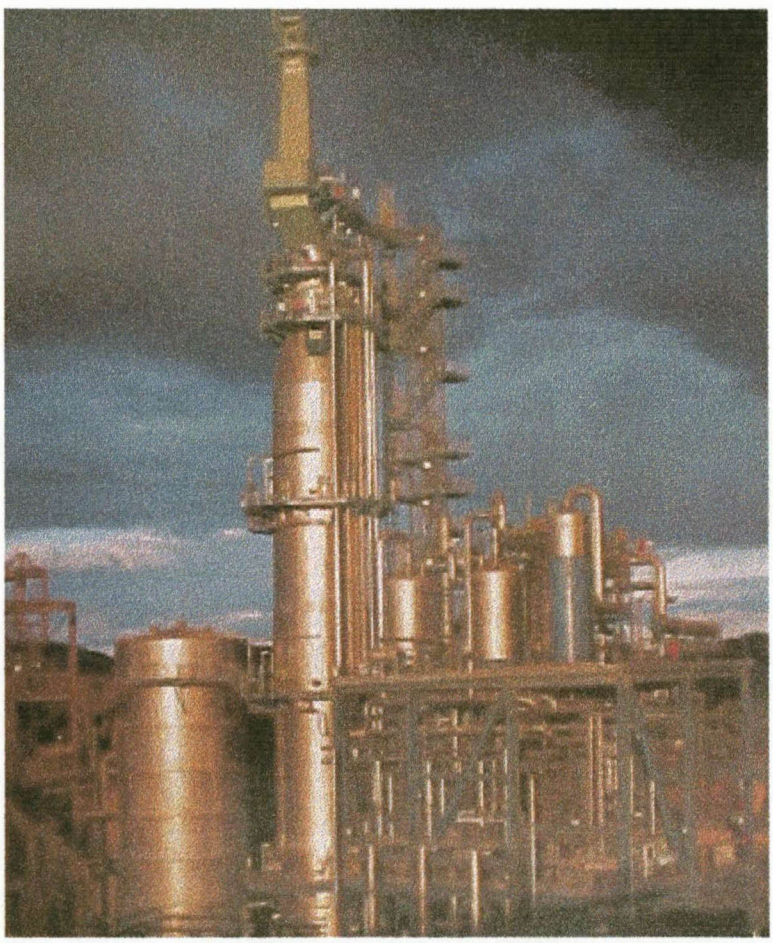
WALL, M.B., CAMERON, D.C. & LIGHTFOOT, E.N. 1993. Biopulping process design and kinetics. *Biotech. Adv.* 11: 645-662.

WINER, B.J. 1971. Statistical principles and experimental design, 2nd ed. Mc Graw-Hill Book Co., USA.

ZAR, J.H. 1984. Biostatistical analysis, 2nd ed. Prentice-Hall Inc., Englewood Cliffs, USA.

CHAPTER 6

KRAFT PULPING OF PINE WOOD, PRE-TREATED WITH A STRAIN OF *STEREUM HIRSUTUM*



Continuous kraft digester at Usutu

ABSTRACT

The successful application of biopulping in most pulping processes has not been exploited to the same extent as for kraft pulping. The influence of biopulping of pine wood on kraft pulping parameters was, therefore, investigated. *Pinus patula* wood chips were pre-treated with a selected strain of *Stereum hirsutum*. Wood was pulped on a small scale and the pulping conditions were varied. Lignin content, yield and viscosity of the pulp were evaluated and the alkali consumption determined. The relationships between these parameters were used to model a biokraft pulping process. Fungal pre-treatment reduced kappa number and yield, but not the degree of polymerization of cellulose. Alkali consumption increased when fungal pre-treated wood was pulped. This study showed that biopulping can reduce the kappa number of pulp or reduce the pulping time, but pulp yield is also reduced and chemical consumption increased. The implementation of biopulping on industrial scale would consequently be determined by the specific requirements of a mill that enables it to exploit specific economic benefits.

INTRODUCTION

Biopulping has been defined in the narrow sense as the pre-treatment of wood with lignin degrading fungi before the mechanical or chemical production of pulp (Messner *et al.*, 1992). Wood chips are usually pre-treated with fungi in a solid-substrate fermentation process (Reid, 1989). Extensive screening has resulted in the identification of a number of white-rot fungi that are, to some extent, selective degraders of lignin (Blanchette *et al.*, 1992b; Job-Cei *et al,*. 1991; Messner & Srebotnik, 1994; Otjen *et al.*, 1987). The result of degradation by these fungi is wood in which the lignin has been utilized or modified. Consequently the efficiency of chemical pulping (Scott *et al.*, 1995; Wall *et al.*, 1996) or fibre separation during mechanical pulping is improved (Blanchette *et al.*, 1992a).

The successful application of biological treatment prior to mechanical pulping is the result of eight years of research by two international consortia based at the Forest Products Laboratory, Madison, Wisconsin (Akhtar *et al.*, 1996). This research led to the issuing of a patent (Blanchette *et al.*, 1991) and to mill scale trials (Akhtar *et al.*, 1996). These investigations showed that pre-treatment of loblolly pine chips with *Ceriporiopsis subvermispora* resulted in energy savings of 38 %. The pulp produced during this process had improved strength properties, with an increase in burst and tear indices (Akhtar *et al.*, 1996).

The pre-treatment of wood with white-rot fungi prior to chemical pulping has not been researched to the same extent as biomechanical pulping (Reid, 1991).

However, Messner *et al.* (1992) and Scott *et al.* (1995) demonstrated the potential of biopulping in a sulphite processes. Fungal pre-treatment of birch chips produced pulp with a reduced kappa number and improved brightness, but with decreased strength properties (Messner *et al.*, 1992). Pre-treatment of loblolly pine chips with *Ceriporiopsis subvermispora* resulted in pulp with a reduced kappa number or pulping time (Scott *et al.*, 1995).

Kraft pulping accounts for 80 % of the chemical pulp production in the world (Sjöström, 1981). Biokraft pulping has, however, been restricted to studies using blue stain fungi (Wall *et al.*, 1994; 1996), or those utilizing white-rot fungi on hardwood (Oriaran *et al.*, 1990; 1991). Valuable information does, however, exist on the kraft pulping properties of softwood decayed by white-rot fungi (Hunt, 1978b; 1978c). These studies have focussed on the utilization of wood from decadent stands for kraft pulping. The degradation of this wood occurred under uncontrolled conditions and results can, therefore, only be applied to biopulping to a limited extent.

From the studies on the kraft pulping of decayed wood, it is clear that several pulping parameters and pulp properties need thorough investigation to allow economic evaluation of a biokraft pulping process. The most obvious benefit of fungal pre-treatment, is the reduction of lignin content (Oriaran *et al.*, 1990; 1991) or, alternatively, the pulping time (Oriaran *et al.*, 1991; Scott *et al.*, 1995). These improvements also appear to be associated with negative changes in pulp yield (Oriaran *et al.*, 1990; Hunt, 1978b) and chemical consumption (Hunt, 1978a; 1978c).

The aim of this study was, therefore, to determine the influence of fungal pre-treatment in a controlled environment on biokraft pulping parameters and pulp properties. In this study, *Pinus patula* chips were pre-treated with *Stereum hirsutum* (WR 95). This white-rot fungus was selected in earlier studies, where it showed potential for application in biokraft pulping (Chapter 3).

MATERIALS AND METHODS

Fungal pre-treatment

Pinus patula chips were collected at Sappi's Ngodwana kraft mill, dried to 3 % moisture content and stored until used. Thirty kilograms (dry weight) of chips were prepared in 25 L polypropylene bioreactors (3 kg/reactor) by adjusting moisture content to 60 % with a 3 % (v/v) corn steep liquor and 1 % sucrose solution. Corn steep liquor, containing 50 % solids, was obtained from African Products (P.O. Box 554, Germiston 1400, South Africa). The wood was autoclaved on two consecutive days for one hour.

Inoculum of *Stereum hirsutum* (WR 95) was produced as described for screening trials (Chapter 3). Fragmented mycelium was used to inoculate chips at a dosage of 3g dry weight per kilogram wood. Cultures were incubated for nine weeks and aerated with humidified air (31 °C, 72 % relative humidity, 500 ml/min) that was pre-sterilized using an air incinerator (New Brunswick, U.S.A.). An equivalent mass of uninoculated chips with sterile growth medium was used as a control. Chips were

harvested and fungal activity was halted by drying at 55 °C to a moisture content of 3 %. Weight loss of the wood that occurred during fungal pre-treatment was determined to use as a correction factor in the calculation of final pulp yield.

Pulping

Pre-treated wood was bulked and divided into 800 g samples and pulped in a 15 L rotating digester at 170 °C. In order to obtain the same pulping conditions, a control sample (100 g) in a canister was included in the digester with the fungal pre-treated sample. The different active alkali charges, liquor to wood ratios and pulping times that were used for different treatments are shown in Table 1. The series of pulping experiments was started by pulping wood under conditions similar to those used at the Sappi Ngodwana mill. These conditions were: 22 % active alkali, 5,4 : 1 liquor to wood ratio and 85 min at the pulping temperature (Table 1). At first, only the liquor to wood ratio was changed to 4,5 : 1 and 7,5 : 1. When it was found that alkali consumption increased for the fungal pre-treated wood, the alkali charge was also increased and tested with different liquor to wood ratios. A reduced pulping time of 65 min and 75 min at 170 °C was used for the last pulping treatments (Table 1).

Table 1. Different pulping treatments to determine the optimal conditions for pulping of wood pre-treated with *Stereum hirsutum*.

Pulping Treatment	Active Alkali Charge (%)	Liquor to Wood Ratio	Alkali Activity	Pulping Time (min)[a]
1	22	4,5	48,9	85
2	22	5,4	40,7	85
3	22	7,5	29,3	85
4	25	8,0	31,3	85
5	26	4,5	57,8	85
6	26	5,4	48,1	85
7	26	7,5	34,7	85
8	27	7,5	36,0	85
9	27	9,0	30,0	85
10	30	7,5	40,0	85
11	32	5,4	59,3	85
12	32	10,0	32,0	85
13	22	5,4	40,7	75
14	22	5,4	40,7	65

a Time at temperature (170°C) and 85 minutes to temperature

Pulp evaluation

The amount of delignification during pulping was determined as the reduction of kappa number using Tappi Test Method (T236). Pulp viscosity was determined with a capillary viscometer using Tappi Test Method (T230). Pulp yields were determined by weighing the pulp (oven dry) and applying a correction factor to account for weight loss during fungal pre-treatment. Alkali consumption was determined by analysis of black liquor according to Tappi Test Method (T625).

RESULTS AND DISCUSSION

Lignin content

The fungal pre-treatment of wood, pulped under all of the test conditions, resulted in reduction of lignin content, as reflected by kappa number (Table 2). The most successful pulping was done in Treatment 9, with fungal pre-treated wood that was pulped with 27 % active alkali at a liquor to wood ratio of 9 : 1 (Table 1). Under these conditions, pulp with a 30,6 % lower kappa number was obtained compared to untreated wood pulped under similar conditions (Table 2).

Table 2. Influence of conditions of different pulping treatments on pulp quality and yield of wood, pre-treated with *Stereum hirsutum* compared to untreated wood.

Pulping Treatment	Kappa Number	Δ Kappa No. (%)[a]	Pulp Yield (g/100 g)[b]	Δ Yield (%)[a]	Viscosity (cm^3/g)	Δ Viscosity (%)[a]
1	21,3	-12,3	41,5	-3,7	782	-12,6
2	23,1	-17,5	41,9	-4,8	873	-12,8
3	36,0	-15,3	42,7	-2,5	922	-17,9
4	25,4	-24,6	42,3	-3,2	891	-15,9
5	19,5	-3,0	40,5	-1,7	770	-2,0
6	16,3	-15,5	39,2	-7,5	697	-18,2
7	23,7	-22,3	40,3	-7,1	882	-13,3
8	22,3	-28,3	41,3	-6,3	853	-16,3
9	25,4	-30,6	42,3	-4,3	912	-15,5
10	18,9	-26,2	40,6	-3,1	806	-27,5
11	12,2	-23,3	38,8	-2,5	558	-15,3
12	21,1	-24,6	41,3	n.d.	857	-14,1
13	24,4	-23,3	42,7	-3,6	899	-12,3
14	30,2	-20,1	43,3	-3,8	978	-11,4

a Change compared to untreated wood pulped under similar conditions

b Based on the oven dry weight of wood before treatment

n.d. Not determined

As result of the varied conditions used for different pulping treatments, pulp with a range of kappa numbers was obtained. The pulping data could, therefore, be used to derive the following equation to calculate the effect of pulping conditions on kappa number:

$$K = 71{,}8276 - 6{,}1F - 0{,}299T - 1{,}531C + 3{,}219L \quad (R^2 = 88\ \%) \qquad (1)$$

Where K is the kappa number, F is the pre-treatment (0 = untreated and 1 = fungal pre-treatment), T is the pulping time (min) at 170 °C, C is the active alkali charge (%) and L is the liquor to wood ratio.

Equation 1 shows that fungal pre-treatment reduced the kappa number by 6,1 units in the experimental range. In comparison, kraft pulping of wood decayed by white-rot fungi, produced variable results. Western hemlock, decayed by *Echinodontium tinctorium* and *Phellinus pini*, produced pulp with a 2,5 % and a 2,4 % respective increase in permanganate number (≈ kappa number/0,66) compared to sound wood (Hunt, 1978a). Another report showed a 3 % decrease in permanganate number of pulp from decayed softwood (Hunt, 1978c). These contradictory results make the mechanism of delignification in decayed wood more difficult to interpret.

The reduction in lignin content of pulp can be ascribed to the degradation or modification of the lignin polymer (Scott *et al.*, 1995). This results in improved penetration of cooking liquor and leads to more efficient pulping (Sjöström, 1981).

Modifications to cell walls and utilization of extractives can, therefore, also increase delignification during pulping (Wall *et al.*, 1994).

Pulp yield and degree of polymerization

The ability of fungi to selectively degrade lignin has been an important criterion in the selection of fungi for use in biopulping processes (Blanchette *et al.*, 1992b; Job-Cei *et al*,. 1991). The kappa number of pulp from fungal pre-treated wood is, therefore, often expressed in terms of yield and viscosity (Oriaran *et al.*, 1990; 1991; Wall *et al.*, 1996). Absolute selectivity does not appear to be possible in biopulping processes (Eriksson & Kirk, 1986) and in this study 1,1 % weight loss of wood occurred during fungal pre-treatment for nine weeks. This weight loss was included in the final yield calculation to maintain the mass balance (Table 2). Benefits in lignin reduction should be weighed-up against losses in yield. During this study, yield loss and reduction of viscosity accompanied reduction in kappa number (Table 2).

Equations to describe linear relationships between pulp yield and kappa number have been proposed for kraft pulping of fungal pre-treated aspen and red oak (Oriaran *et al.*, 1990; 1991). However, from the results (Table 2) the following non-linear equations were the most effective for the estimation of yield at a specific kappa number (Figure 1):

$$\mathbf{Yc} = 37 + 8[1-e^{-0,1(K-12)}] \qquad (R^2 = 86\ \%) \qquad (2)$$

$$\mathbf{Yf} = 37 + 7[1-e^{-0,1(K-12)}] \qquad (R^2 = 86\ \%) \qquad (3)$$

Where Yc (g/100 g wood) is the pulp yield of untreated wood and Yf the yield of biopulped wood.

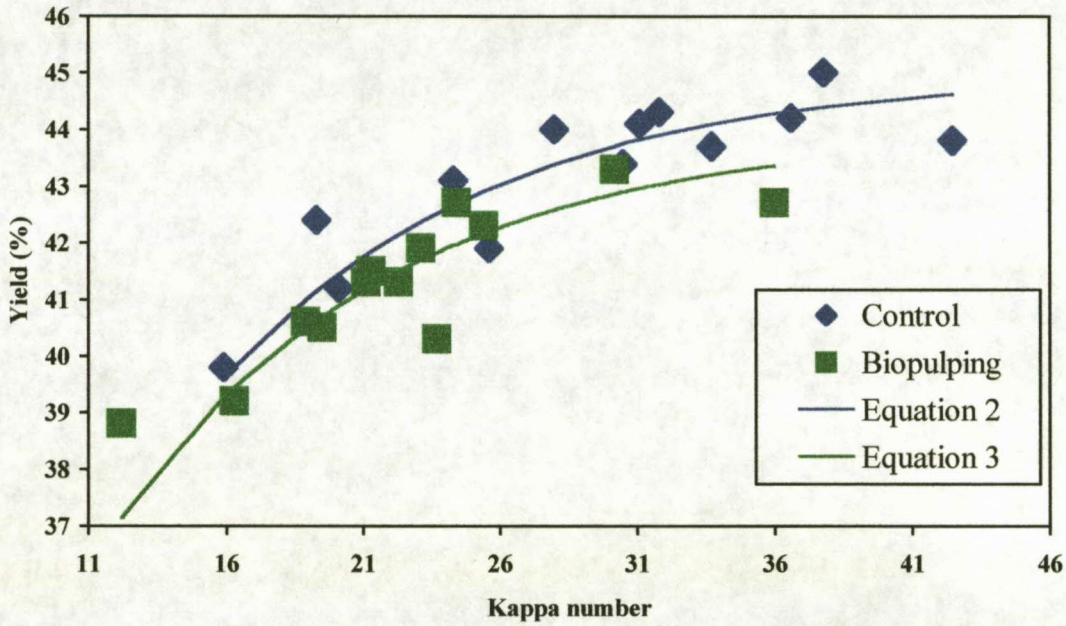


Figure 1. Relationship between pulp yield and kappa number for fungal pre-treated wood and untreated wood, pulped under different conditions.

The influence of kappa number on cellulose viscosity in pulp from untreated and pre-treated wood (Figure 2) is described by:

$$V = 1330(1 - e^{-0,046K}) \qquad (R^2 = 86\ \%) \qquad (4)$$

Where V is viscosity (cm^3/g).

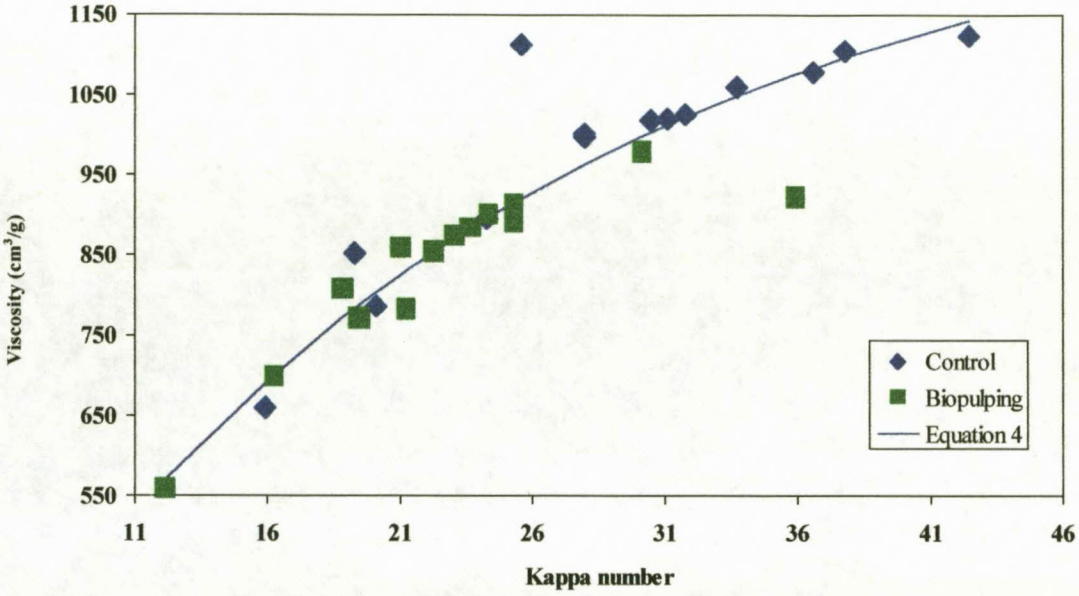


Figure 2. Relationship between cellulose viscosity and kappa number for fungal pre-treated wood and untreated wood, pulped under different conditions.

Equations 2 and 3 can be used to show that the pulp yield of fungal pre-treated wood, compared to untreated wood is reduced by 0,84 g/100 g and 0,56 g/100 g wood when pulped to a kappa number of 30 and 20 respectively. These data show that biopulping reduces yield by less than 1 % in the experimental range for a given kappa number. The loss of yield also becomes smaller as wood is pulped to a lower kappa number. Viscosity, however, is a function only of kappa number and is not influenced by the fungal pre-treatment (equation 4, Figure 2). The loss of pulp yield observed during this study could be further reduced by using a shorter pre-treatment period, because it has been shown that selectivity of lignin degradation decreases with increased incubation time (Messner *et al.*, 1992).

Alkali consumption

An increase in consumption of cooking chemicals is a major concern when decayed wood is used for pulping (Hunt, 1978a; 1978b; 1978c). Results of this study showed that residual alkali can be calculated by using the equations (Figure 3):

$$\mathbf{Rc} = 12 + 20 \times e^{-0,11(\mathbf{K}-15)} \qquad (R^2 = 92\ \%) \qquad (5)$$

$$\mathbf{Rf} = 10 + 17 \times e^{-0,15(\mathbf{K}-12)} \qquad (R^2 = 92\ \%) \qquad (6)$$

Where Rc is the residual alkali of the black liquor from the control treatment and Rf is the residual alkali in black liquor from the fungal pre-treatment.

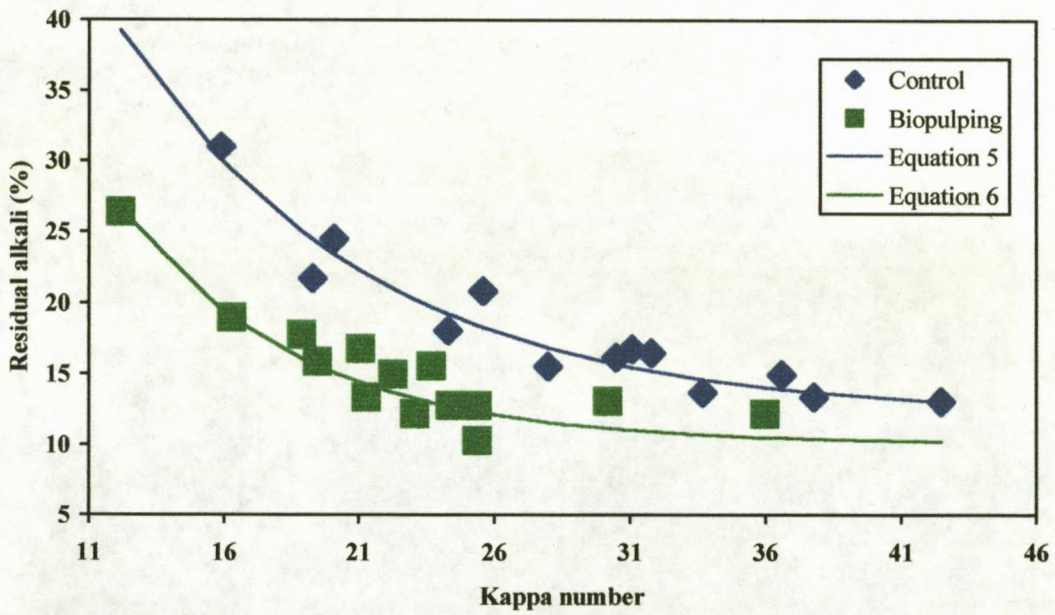


Figure 3. Relationship between residual alkali in the black liquor of fungal pre-treated wood and untreated wood, pulped under different conditions and kappa number.

Alkali consumption is then calculated using:

$$\mathbf{U = 100(A - R) / A} \qquad (7)$$

Where A is the activity of the cooking liquor and R is the residual alkali in the black liquor.

This model (equation 7) can be used to show that alkali consumption increase from 61,1 % to 72,7 %, when fungal pre-treated samples are pulped to a kappa number of 30 using conditions similar to those of a pulp mill (Table 3). These data are comparable with those reported by Hunt (1978b) for the pulping of decadent western red cedar. The increase in alkali consumption has been ascribed to the increase in products of the fungal degradation, such as low molecular weight polysaccharides (Hunt & Hatton, 1979). The reduction in yield is, therefore, correlated with the increased alkali consumption (Hunt & Hatton, 1979). The increased organic solids in the black liquor contribute additional material to the recovery boiler (Hunt, 1978b) that, frequently, has a limited capacity. However, expansion of the recovery capacity to increase the pulping capacity, would increase the fixed cost of pulp (Christie, 1979).

Table 3. Expected influence of fungal pre-treatment of wood chips on kraft pulping parameters.

Parameter Parameters	Example No.		
	1	2	3
Pre-treatment	Control	*Stereum hirsutum*	*Stereum hirsutum*
Alkali charge (%)	22	22	22
Liquor to wood ratio	5,4 : 1	5,4 : 1	5,4 : 1
Kappa number	30	30	24[a]
Time at 170°C (min.)	85,0	79,4[a]	85,0
Yield (g/100g wood)	43,7[b]	42,8[c]	41,9[c]
Viscosity (cm^3/g)	995[d]	995[d]	889[d]
Alkali consumption (%)	61,1[e]	72,7[f]	68,6[f]

a Values calculated using model equation 1.
b Value calculated using model equation 2.
c Values calculated using model equation 3.
d Values calculated using model equation 4.
e Value calculated using model equations 5 and 7.
f Values calculated using model equations 6 and 7.

Pulping time

Variation of pulping times has shown that pre-treated wood can be pulped to a similar kappa number as untreated wood, in a shorter time (Table 3). The estimated reduction in pulping time of 5,6 min was calculated using the model equations (Table 3). Reduction of pulping time has also been reported for biokraft pulping of aspen (Oriaran *et al.*, 1990) and for biosulphite pulping, where the pulping time was reduced from 5,75 h to 5,25 h (Scott *et al.*, 1995).

The reduction of pulping time can be ascribed to modification of wood, which in turn allows quicker and more uniform impregnation by the cooking liquor (Wall

et al., 1994). This is of specific importance in the kraft process, where the initial phase of delignification is controlled by diffusion (Sjöström, 1981).

CONCLUSIONS

The fungal pre-treatment of softwood can cause a substantial reduction of the lignin content of pulp. Under optimal pulping conditions, fungal pre-treatment reduced kappa number by more than 30 %. An alternative benefit is a shorter pulping time. Pre-treated wood could be pulped to the same kappa number (kappa 30) as untreated wood in a shorter time at pulping temperature (6,6 % less than untreated wood). This saving in pulping time could be translated to increased output.

Fungal pre-treatment of wood reduced the pulp yield, due to the non-selective delignification by *S. hirsutum* (WR 95). This yield reduction does, however, become less important when pulping is done to low kappa number. Selectivity of delignification is also influenced by many factors (Otjen *et al.*, 1987) and because solid substrate fermentation is used, these factors are often difficult to control. A reduction of pulping time from nine weeks to three weeks could, for instance, reduce the amount of cellulose that is degraded. The model (equation 4) shows that cellulose viscosity of pulp is a factor of kappa number. The degree of polymerization was, therefore, not negatively influenced by fungal action.

Alkali consumption was increased during kraft pulping of fungal pre-treated wood. It is, however, possible to reduce the use of bleaching chemicals due to the reduced lignin content of the pulp (Macleod, 1993).

We do not expect that different results to those presented in this study, will be obtained by using other fungal strains for biokraft pulping. Our results have corroborated results of previous studies obtained with decayed wood (Hunt, 1978a; 1978b; 1978c; Hunt & Hatton, 1979). The benefits of biopulping, such as reduced kappa number and pulping time might be enhanced by the use of other fungal strains. Increased chemical consumption would, however, remain an important obstacle to a commercially viable process.

REFERENCES

AKHTAR, M., KIRK, T.K. & BLANCHETTE, R.A. 1996. Biopulping: An overview of consortia research. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the Sixth International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 187–192. Facultas-Universitätsverlag, Vienna, Austria.

BLANCHETTE, R.A., LEATHAM, G.F., ATTRIDGE, M., AKHTAR, M. & MYERS, G.C. 1991. Biomechanical pulping with *C. subvermispora*. *U.S. Pat.* 5,055,159.

BLANCHETTE, R.A., AKHTAR, M. & ATTRIDGE, M.C. 1992a. Using Simons stain to evaluate fiber characteristics of biomechanical pulps. *Tappi J.* 75(11): 121–124.

BLANCHETTE, R.A., BURNES, T.A., EERDMANS, M.M. & AKHTAR, M. 1992b. Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. *Holzforschung* 46(2): 109-115.

CHRISTIE, R.D. 1979. The use of decayed wood in pulping process. In: Chip Quality Monograph. Ed. J.V. Hatton, pp. 111-128. Tappi Press, Atlanta, USA.

ERIKSSON, K. -E. & KIRK, T.K. 1986. Biopulping, biobleaching and treatment of kraft bleaching effluents with white-rot fungi. In: Comprehensive biotechnology in industry, agriculture and medicine. Ed. M. Moo-Young, pp. 271-294. Pergamon press, Oxford, UK.

HUNT, K. 1978a. Pulping western hemlock decayed by white-rot fungi. *Pulp Pap. Can.* 79(6): 75-80.

HUNT, K. 1978b. Kraft pulping of decayed wood: western red cedar and alpine fir. *Pulp Pap. Can.* 79(7): 30-35.

HUNT, K. 1978c. How rot-decayed wood affects chipping and kraft pulping. *Pulp Pap. Can.* 79(8): 65-68.

HUNT, K. & HATTON, J.V. 1979. Predicting the kraft pulping behavior of decayed pulpwood. *Pulp Pap. Can.* 80(3): 55-57.

JOB-CEI, C., KELLER, J. & JOB, D. 1991. Selective delignification of sulphite pulp paper: Assessment of 40 white-rot fungi. *Mater. u. Organ.* 26(3): 215-226.

MACLEOD, J.M. 1993. Extended delignification: a status report. *Appita* 46(6): 445-451.

MESSNER, K., MASEK, S., SREBOTNIK, E. & TECHT, G. 1992. Fungal pretreatment of wood chips for chemical pulping. In: Biotechnology in the pulp and paper industry. Proceedings of the 5th International Conference on Biotechnology in the Pulp and Paper Industry. Eds. M. Kuwahara and M. Shimada, pp. 9–13. Uni Publishers, Tokyo , Japan.

MESSNER, K. & SREBOTNIK, E. 1994. Biopulping: An overview of developments in an environmentally safe paper-making technology. *FEMS Microb. Rev.* 13: 351-364.

ORIARAN, T.P., LABOSKY, P. & BLANKENHORN, P.R. 1990. Kraft pulp and papermaking properties of *Phanerochaete chrysosporium*-degraded aspen. *Tappi J.* 73(7): 147-152.

ORIARAN, T.P., LABOSKY, P. & BLANKENHORN, P.R. 1991. Kraft pulp and papermaking properties of *Phanerochaete chrysosporium*-degraded red oak. *Wood Fiber Sci.* 23(3): 316-327.

OTJEN, L., BLANCHETTE, R., EFFLAND, M. & LEATHAM, G. 1987. Assessment of 30 white rot Basidiomycetes for selective lignin degradation. *Holzforschung* 41(6): 343-349.

REID, I.D. 1989. Optimization of solid-state fermentation for selective delignification of aspen wood with *Phlebia tremellosa*. *Enzyme Microb. Technol.* 11: 804–809.

REID, I.D. 1991. Biological pulping in paper manufacture. *Tibtech* 9(8): 262-265.

SCOTT, G.M., AKHTAR, M. & LENTZ, M. 1995. Fungal pretreatment of wood chips for sulfite pulping. *Tappi Pulping Conference*, p. 355-361. Tappi Press, Atlanta, USA.

SJÖSTRÖM, E. 1981. Wood chemistry: Fundamentals and Applications, 2nd ed. Academic Press, San Diego, USA.

WALL, M.B., BRECKER, J., FRITZ, A., IVERSON, S. & NOËL, Y. 1994. Cartapip® treatment of wood chips to improve chemical pulping efficiency. *Tappi Biological Sciences Symposium*, pp. 67-76. Tappi Press, Atlanta, USA.

WALL, M.B., STAFFORD, G., NOEL, Y., FRITZ, A., IVERSON, S. & FARRELL, R.L. 1996. Treatment with *Ophiostoma piliferum* improves chemical pulping efficiency. In: Biotechnology in the pulp and paper industry: Recent advances in applied and fundamental research. Proceedings of the 6th International Conference on Biotechnology in the Pulp and Paper Industry. Eds. E. Srebotnik & K. Messner, pp. 205–210. Facultas-Universitätsverlag, Vienna, Austria.

SUMMARY

KEY WORDS: Basidiomycetes, Biodiversity, Biopulping, Biotechnology, Fungi, Inhibition, Kraft, Pulping, White-rot, Wood.

The forest products industry is one of the most important earners of foreign exchange for South Africa. The major focus of the industry is the production of pulp with an annual capacity of 2,4 million tons. Wood from plantations of exotic trees is the most important source of fibre, but other fibre sources are also used.

Biotechnology can play a significant role in the industry to produce high value products at lower cost and could reduce the environmental impact of conventional processes. Biopulping is potentially the most important of these biotechnological processes, because it can influence all downstream operations. The aim of this study was, therefore, to develop a biopulping process for the treatment of softwood at the Sappi Ngodwana kraft mill in Mpumalanga.

Initially, 278 strains of wood-decay fungi were collected from various natural habitats. This collection represents a diversity of fungal families and included species that have not previously been recorded from South Africa. The first step in selecting suitable fungal strains for biopulping was to characterize different groups on the basis

of the enzymes that they produce and their oxidase reactions. The suitability of these strains for the pre-treatment of softwood chips prior to kraft pulping was subsequently assessed by evaluating their influence on kappa number, yield and strength properties of pulp. Seven of the strains tested were able to reduce the kappa number of pulp significantly, without having a significant influence on the pulp yield. These strains were more efficient than strains of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* that have been patented for other biopulping applications. Treatment of wood with strains of *Peniophora* sp., an unidentified specie as well as two strains of *Stereum hirsutum* resulted in pulp with improved strength properties.

The envisaged biopulping process aimed at treating wood chips in outside chip storage with a biopulping fungus. The aim of one study was to investigate conditions such as temperature, moisture, CO_2 and microbial populations that develop in a chip pile, and to determine the suitability of the chip pile for colonization by biopulping fungi. High temperatures and high moisture levels were observed in some areas of the chip pile, which suggested that part of the pile was unsuitable for colonization by mesophilic fungi. It will, therefore, be necessary to manage the chip pile to maintain a suitable environment for biopulping.

Problems were experienced with poor colonization of freshly chipped softwood by biopulping fungi. The effect of contaminating microbes and inhibitory compounds present in wood was, therefore, studied. It was found that the inhibition of biopulping fungi by α-pinene and by contaminating microbes were both very important. The inhibition by microbes, as well as by extractives, was mitigated by a

short steam treatment of wood chips. Steaming for ten minutes under atmospheric pressure could be an economical method to improve colonization by biopulping fungi. Alternatively biopulping fungi with good competitive ability and tolerance to monoterpenes could be selected.

Pinus patula wood chips were pre-treated with a selected strain of *Stereum hirsutum* to determine the optimal conditions for the kraft pulping of pre-treated softwood and to do an economic evaluation of the process. Chips were pulped on a small scale under various pulping conditions. Lignin content, yield, and viscosity of the pulp as well as alkali consumption were evaluated. The results were used to develop models for biokraft pulping. This study showed that biopulping can reduce the kappa number of pulp or reduce the pulping time. Pulp yield losses were relatively small when pulps with low kappa number were produced. Increased alkali consumption was, however, an important factor in the economic evaluation.

OPSOMMING

SLEUTELWOORDE: Basidiomisete, Biodiversiteit, Biotegnologie, Bioverpulping, Fungi, Hout, Inhibisie, Kraft, Verpulping, Witvrot,.

Die bosbou-industrie is een van die belangrikste bronne van buitelandse valuta vir Suid-Afrika. Die industrie fokus hoofsaaklik op die produksie van pulp met 'n jaarlikse produksiekapasiteit van 2,4 miljoen ton. Die belangrikste roumateriaal vir die industrie is hout uit plantasies van uitheemse bome, maar ander bronne word ook benut.

Biotegnologie kan 'n belangrike rol in die industrie speel om hoë-waarde produkte te vervaardig teen 'n laer koste en om die omgewingsimpak van konvensionele prosesse verminder. Bioverpulping is potensieël die belangrikste metode, omdat alle verdere vervaardigingsprosesse daardeur beïnvloed word. Die doel van hierdie studie was dus om 'n bioverpulpingsproses te ontwikkel vir die behandeling van sagtehout by die Sappi Ngodwana kraft-meule in Mpumalanga.

Aanvanklik is 278 isolate van witvrot fungi versamel uit verskillende natuurlike habitatte. Hierdie versameling het 'n verskeidenheid fungus families verteenwoordig en het ook spesies ingesluit wat nie voorheen in Suid-Afrika beskryf

is nie. Die eerste stap om geskikte isolate vir bioverpulping te kies, was om verskillende groepe te karakteriseer in terme van hulle produksie van ensieme en oksidase-reaksies. Die geskiktheid van hierdie isolate vir die behandeling van houtskerfies voor kraft-verpulping is vervolgens beslis deur hulle invloed op kappagetal, opbrengs en sterkte van pulp te bepaal. Sewe van die isolate wat getoets is, het die kappagetal van pulp betekenisvol verlaag sonder om pulpopbrengs betekenisvol te verlaag. Hierdie isolate was meer doeltreffend as isolate van *Phanerochaete chrysosporium* en *Ceriporiopsis subvermispora,* wat vir ander toepassings van bioverpulping gepatenteer is. Behandeling van die hout met 'n isolaat van *Peniophora* sp., 'n isolaat wat nie geidentifiseer is nie en twee isolate van *Stereum hirsutum,* het pulp met verbeterde sterkte-eienskappe gelewer.

Die voorgestelde bioverpulpingsproses het ten doel gehad om houtskerfies met die bioverpulpingsfungus te behandel waar dit in die buitelug gestoor word. Die doel van een studie was om toestande soos temperatuur, houtvog, CO_2 en populasies van mikrobe te ondersoek, sodat die geskiktheid van die hope vir kolonisasie deur bioverpulpingsfungi bepaal kon word. Hoë temperature en houtvog wat in sommige areas van die houthope waargeneem is, het laat blyk dat gedeeltes van die hope ongeskik sal wees vir kolonisasie deur mesofiele fungi. Dit sou dus nodig wees om bestuurspraktyke toe te pas om 'n geskikte omgewing vir bioverpulping te handhaaf.

Probleme is ondervind met swak kolonisasie van varsgekapte sagtehoutskerfies deur bioverpulpingsfungi. Die invloed van kontaminerende mikrobes en inhiberende verbindings in hout is daarom ondersoek. Dit is gevind dat

inhibisie van bioverpulpingsfungi deur α-pineen en deur kontaminerende mikrobes beide baie belangrik is. Die inhibisie deur mikrobe sowel as vlugtige bestanddele is verlig deur die houtskerfies vir 'n kort periode met stoom te behandel. Stoom vir tien minute by atmosferiese druk kan 'n ekonomiese oplossing wees om kolonisasie van bioverpulpingsfungi te verbeter. Alternatiewelik kan isolate vir bioverpulping met goeie kompeterende vermoë en verdraagsaamheid vir monoterpene geselekteer word.

Pinus patula-skerfies is met 'n geselekteerde isolaat van *Stereum hirsutum* behandel om die optimale toestande vir die kraft-verpulping van fungusbehandelde hout te bepaal en 'n ekonomiese evaluasie van die bioverpulpingsproses te doen. Skerfies is op 'n klein skaal onder verskillende toestande verpulp en die lignien inhoud, opbrengs en viskositeit van die pulp asook alkali-verbruik bepaal. Die data is gebruik om modelle te ontwikkel vir biokraft-verpulping. Die studie het getoon dat die kappagetal of verpulpingstyd deur bioverpulping verminder kan word. Verlies aan pulpopbrengs was relatief laag waar pulp met lae kappagetalle geproduseer is. Die verhoogde alkali-verbruik was egter 'n belangrike faktor by die ekonomiese evaluasie.

APPENDICES

APPENDIX A: ORIGIN OF FUNGAL STRAINS IN CULTURE COLLECTION.

Substrates or hosts, and locality from where different strains of wood-inhabiting fungi were collected.

Strain no.[a]	Species	Substrate / host	Area	Locality
WR 1	*Coriolopsis polyzona*	Acacia wood	KwaZulu-Natal	Howick
WR 2	*Coriolus hirsutus*	"	"	"
WR 3	*Stereum hirsutum*	Oak wood	"	Underberg
WR 4	*Pycnoporus sanguineus*	Pine wood	"	Dukuduku SF.
WR 5	*Lenzites elegans*	Indigenous Hw.	"	"
WR 6	*Coriolopsis polyzona*	Acacia wood	Mpumalanga	Kaapsehoop
WR 7	*Stereum ostrea*	Eucalyptus wood	"	"
WR 8	*Pycnoporus sanguineus*	Acacia wood	"	"
WR 9	*Stereum hirsutum*	Eucalyptus wood	"	"
WR 10	*Lenzites betulina*	"	"	"
WR 11	*Lentinus stupeus*	"	"	"
WR 12	*Pycnoporus sanguineus*	Oak wood	"	Sabie
WR 13	*Laxitextum bicolor*	"	"	"
WR 14	*Pycnoporus sanguineus*	"	"	"
WR 17	*Lenzites elegans*	"	"	"
WR 18	*Coriolopsis polyzona*	"	"	"
WR 19	*Stereum ostrea*	"	"	"
WR 21	*Pycnoporus sanguineus*	"	"	"
WR 22	*Stereum hirsutum*	"	"	"
WR 23	*Lenzites betulina*	"	"	"
WR 24	*Lentinus stupeus*	"	"	"
WR 25	*Stereum hirsutum*	"	"	"
WR 27	*Stereum ostrea*	Wood (unknown)	Northern Province	Haenertsburg
WR 28	*Stereum ostrea*	"	Southern Cape	Saasveld
WR 30	*Stereum rimosum*	"	Mpumalanga	Sudwala
WR 31	*Trametes cingulata*	Tipuana wood	Gauteng	Pretoria
WR 32	*Stereum hirsutum*	Acacia wood	Mpumalanga	Chrissiemeer
WR 33	*Ganoderma lucidum*	Acacia tree	"	"
WR 34	*Lenzites betulina*	Eucalyptus wood	"	Warburton
WR 36	*Gyrodontium* sp.	"	"	"
WR 37	*Coriolus versicolor*	"	"	"
WR 38	*Stereum illudens*	"	"	Piet Retief
WR 39	*Trametes cingulata*	Acacia wood	"	"
WR 40	*Lentinus villosus*	Eucalyptus wood	"	Iswepe
WR 44	*Coriolus versicolor*	Acacia wood	Western Cape	Stellenbosch
WR 45	*Stereum sanguinolentum*	Pine wood	"	"
WR 46	*Antrodia variiformis*	"	"	"
WR 47	*Schizophyllum commune*	"	"	"
WR 50	*Gloeophyllum trabeum*	"	"	Jonkershoek SF.
WR 51	Unidentified sp.	"	"	"
WR 52	*Gloeophyllum trabeum*	"	"	"
WR 58	*Pycnoporus coccineus*	"	"	"
WR 60	Unidentified sp.	Oak wood	"	"
WR 61	*Coriolus versicolor*	"	"	"
WR 62	*Coriolus pubescens*	"	"	"

Strain no.	Species	Substrate / host	Area	Locality
WR 63	*Daedalea quercina*	Oak wood	Western Cape	Jonkershoek SF.
WR 64	*Stereum hirsutum*	"	"	"
WR 65	*Fomitopsis lilacino-gilva*	"	"	"
WR 66	*Chondrostereum purpureum*	Fruit tree	"	Grabouw
WR 67	*Gloeophyllum sepiarium*	Pine wood	"	"
WR 68	*Gloeophyllum trabeum*	"	"	"
WR 70	*Bjerkandera adusta*	Acacia wood	"	"
WR 71	*Bjerkandera adusta*	"	"	"
WR 73	*Coriolus zonatus*	"	"	"
WR 74	*Bjerkandera adusta*	Fruit tree wood	"	Elgin
WR 75	*Coriolus versicolor*	"	"	"
WR 76	*Coriolus versicolor*	"	"	"
WR 77	*Coriolus versicolor*	Acacia wood	"	"
WR 78	*Chondrostereum purpureum*	Fruit tree wood	"	"
WR 79	*Coriolus versicolor*	"	"	"
WR 80	*Coriolus versicolor*	"	"	"
WR 81	*Coriolus versicolor*	"	"	"
WR 82	*Coriolus versicolor*	"	"	"
WR 83	*Coriolus versicolor*	Oak wood	"	"
WR 84	*Phellinus* sp.	"	"	"
WR 85	*Hypholoma fasciculare*	"	"	"
WR 86	*Coriolus versicolor*	Eucalyptus wood	"	Tokai SF.
WR 87	*Stereum hirsutum*	"	"	"
WR 88	*Grifola* sp.	Indigenous Hw.	"	"
WR 89	*Pycnoporus sanguineus*	Pine wood	"	"
WR 90	*Stereum hirsutum*	Indigenous Hw.	"	"
WR 91	*Stereum hirsutum*	Eucalyptus wood	"	"
WR 92	*Coriolus* sp.	"	"	"
WR 93	*Pycnoporus sanguineus*	Callistemon wood	"	"
WR 94	*Coriolus hirsutus*	"	"	"
WR 95	*Stereum hirsutum*	Eucalyptus wood	"	"
WR 96	*Stereum illudens*	Indigenous Hw.	"	"
WR 97	*Pycnoporus sanguineus*	Pine wood	"	"
WR 98	Unidentified sp.	Plant litter	"	"
WR 99	*Pycnoporus sanguineus*	Pine wood	"	"
WR 100	*Coriolus hirsutus*	Oak wood	"	Cecilia SF.
WR 101	*Bjerkandera adusta*	"	"	"
WR 102	*Pycnoporus coccineus*	"	"	"
WR 103	*Pycnoporus sanguineus*	"	"	"
WR 104	*Phellinus gilvus*	"	"	"
WR 105	*Coriolus hirsutus*	"	"	"
WR 112	*Pycnoporus sanguineus*	"	Southern Cape	Coldstream
WR 113	*Pycnoporus sanguineus*	Indigenous SW.	"	Tsitsikamma SF.
WR 114	*Pycnoporus sanguineus*	Indigenous Hw.	"	"
WR 118	*Lenzites betulina*	"	"	Harkerville
WR 119	*Trametes glabrescens*	"	"	"
WR 120	*Trametes glabrescens*	"	"	"
WR 123	*Pycnoporus sanguineus*	Pine wood	"	Karatara
WR 124	*Pycnoporus sanguineus*	"	"	"
WR 125	*Pycnoporus sanguineus*	"	"	Riverview farm

Strain no.	Species	Substrate / host	Area	Locality
WR 126	*Pycnoporus sanguineus*	Pine wood	Southern Cape	Sedgefield
WR 130	*Pycnoporus sanguineus*	"	"	Hoekwil
WR 131	*Pycnoporus sanguineus*	"	"	Barrington
WR 132	*Pycnoporus coccineus*	"	"	Karatara
WR 133	*Gloeophyllum sepiarium*	"	Western Cape	Grabouw
WR 135	*Pycnoporus sanguineus*	Indigenous Hw.	"	"
WR 136	*Stereum hirsutum*	Eucalyptus wood	"	"
WR 137	*Coriolus versicolor*	"	"	"
WR 138	*Stereum hirsutum*	"	"	"
WR 139	*Hyphodontia* sp.	Pine wood	"	"
WR 140	*Pycnoporus sanguineus*	Indigenous Hw.	"	"
WR 141	*Coriolus versicolor*	Pine wood	"	"
WR 142	*Gloeophyllum sepiarium*	"	"	"
WR 144	*Gloeophyllum sepiarium*	"	"	"
WR 145	*Pycnoporus sanguineus*	Acacia wood	"	"
WR 146	*Pycnoporus sanguineus*	Braniaceae wood	"	"
WR 149	*Gloeophyllum sepiarium*	Pine wood	"	"
WR 150	*Ganoderma applanatum*	"	"	"
WR 151	*Gloeophyllum sepiarium*	"	"	"
WR 152	*Skeletocutis* sp.	"	"	Jonkershoek SF.
WR 153	*Pycnoporus sanguineus*	"	"	"
WR 154	*Skeletocutis* sp.	"	"	"
WR 155	*Gloeophyllum abietinum*	"	"	"
WR 156	*Stereum hirsutum*	"	"	"
WR 157	*Skeletocutis* sp.	Oak wood	"	"
WR 159	*Bjerkandera adusta*	"	"	"
WR 164	*Coriolus versicolor*	"	"	"
WR 165	*Skeletocutis* sp.	Pine wood	"	"
WR 166	*Fomitopsis lilacino-gilva*	Oak wood	"	"
WR 167	*Coriolus versicolor*	"	"	"
WR 168	*Poria* sp.	"	"	"
WR 169	*Coriolus versicolor*	"	"	"
WR 170	*Pycnoporus sanguineus*	Pine wood	"	"
WR 171	*Gloeophyllum trabeum*	"	"	"
WR 172	*Gloeophyllum trabeum*	"	"	"
WR 173	*Pycnoporus sanguineus*	Oak wood	"	"
WR 175	*Phellinus gilvus*	"	"	"
WR 176	*Gloeophyllum sepiarium*	Pine wood	"	Lebanon SF.
WR 177	*Coriolus versicolor*	Oak wood	"	"
WR 178	*Phellinus* sp.	"	"	"
WR 179	*Coriolus versicolor*	"	"	"
WR 180	*Phellinus gilvus*	"	"	"
WR 181	*Bjerkandera adusta*	Acacia wood	"	"
WR 182	*Ganoderma applanatum*	Oak wood	"	"
WR 183	*Skeletocutis* sp.	Pine wood	"	"
WR 184	*Coriolus versicolor*	Eucalyptus wood	"	"
WR 185	*Coriolus versicolor*	Oak wood	"	"
WR 186	*Phellinus gilvus*	"	"	"
WR 188	*Coriolus hirsutus*	Acacia wood	"	"
WR 189	*Skeletocutis* sp.	Pine wood	"	"

Strain no.	Species	Substrate / host	Area	Locality
WR 190	*Coriolus versicolor*	Acacia wood	Western Cape	Lebanon SF.
WR 191	*Pycnoporus sanguineus*	Oak wood	"	"
WR 192	*Pycnoporus sanguineus*	"	"	"
WR 193	*Phellinus gilvus*	"	"	Highlands SF.
WR 194	*Coriolus versicolor*	"	"	"
WR 195	*Bjerkandera adusta*	"	"	"
WR 196	*Coriolus hirsutus*	"	"	"
WR 197	*Stereum hirsutum*	"	"	"
WR 199	*Peniophora* sp.	Pine wood	"	Steenbras SF.
WR 200	*Stereum hirsutum*	Eucalyptus wood	"	"
WR 201	*Hyphodontia* sp.	Pine wood	"	Nuweberg SF.
WR 202	*Hyphodontia* sp.	"	"	"
WR 203	*Antrodia* sp.	"	"	"
WR 204	*Gloeophyllum sepiarium*	"	"	"
WR 205	*Schizophyllum commune*	"	"	"
WR 206	*Skeletocutis* sp.	"	"	"
WR 207	*Peniophora* sp.	"	"	"
WR 208	Unidentified sp.	"	"	Haweqwas SF.
WR 210	*Gloeophyllum sepiarium*	"	"	"
WR 211	*Phellinus gilvus*	Acacia wood	"	"
WR 212	*Pycnopor sanguineus*	Oak wood	"	"
WR 213	*Pycnoporus sanguineus*	"	"	"
WR 214	*Pycnoporus sanguineus*	Pine wood	"	"
WR 216	*Stereum hirsutum*	Acacia wood	"	"
WR 217	*Bjerkandera adusta*	"	"	"
WR 218	*Bjerkandera adusta*	"	"	"
WR 220	*Coriolus versicolor*	Oak wood	"	"
WR 251	Unidentified sp.	Hardwood tree	Northern Province	Entabeni SF.
WR 252	*Cyptotrama asprata*	Dead softwood	"	"
WR 253	*Ganoderma applanatum*	Indigenous Hw.	"	"
WR 255	*Coriolus versicolor*	"	"	"
WR 256	*Stereum* sp.	"	"	"
WR 257	*Schizopora paradoxa*	Hardwood tree	"	"
WR 259	*Gloeophyllum sepiarium*	Pine wood	"	"
WR 260	*Phellinus gilvus*	"	"	"
WR 261	*Stereum ostrea*	Indigenous Hw.	"	"
WR 262	*Crepidotus* sp.	"	"	"
WR 268	*Lentinus* sp.	Eucalyptus wood	"	Hangklip SF.
WR 270	*Pycnoporus* sp.	"	"	"
WR 273	*Pulcherricium caeruleum*	Oak wood	"	"
WR 274	*Coriolus* sp.	"	"	"
WR 277	*Stereum fulvum*	Indigenous Hw.	"	"
WR 278	*Nigroporus vinosus*	Eucalyptus wood	"	Entabeni SF.
WR 279	*Hypholoma fasciculare*	"	"	"
WR 280	*Podoscypha* sp.	Compost	"	"
WR 281	*Stereum* sp.	Indigenous Hw.	"	"
WR 283	*Coriolus versicolor*	"	"	"
WR 285	*Phellinus* sp.	"	"	"
WR 286	*Peniophora* sp.	Shrub wood	"	"
WR 288	*Pycnoporus sanguineus*	Indigenous Hw.	"	"

Strain no.	Species	Substrate / host	Area	Locality
WR 292	*Gloeophyllum sepiarium*	Pine wood	Northern Province	Entabeni SF.
WR 295	*Gloeophyllum sepiarium*	"	"	"
WR 296	*Pycnoporus sanguineus*	"	"	"
WR 297	*Stereum hirsutum*	"	"	"
WR 299	*Gloeophyllum sepiarium*	"	"	"
WR 300	*Pycnoporus sanguineus*	"	"	"
WR 302	*Pycnoporus sanguineus*	"	"	"
WR 303	*Trametes nivosa*	"	"	"
WR 304	*Pycnoporus sanguineus*	"	"	"
WR 307	*Trametes nivosa*	"	"	"
WR 308	*Coriolopsis polyzona*	"	"	"
WR 309	*Trametes nivosa*	"	"	"
WR 310	*Stereum hirsutum*	Indigenous Hw.	"	"
WR 311	*Trametes nivosa*	Pine wood	"	"
WR 312	*Coriolus* sp.	"	"	"
WR 313	*Stereum hirsutum*	"	"	"
WR 315	*Trametes nivosa*	"	"	"
WR 316	*Trametes nivosa*	"	"	"
WR 318	*Gloeophyllum trabeum*	"	"	"
WR 319	*Pycnoporus sanguineus*	"	"	"
WR 323	*Coriolus versicolor*	Indigenous Hw.	"	"
WR 324	*Stereum illudens*	Eucalyptus wood	"	"
WR 326	*Coriolopsis polyzona*	Pine wood	"	"
WR 327	*Trametes nivosa*	"	"	"
WR 329	*Trametes nivosa*	"	"	"
WR 330	*Pycnoporus sanguineus*	Eucalyptus wood	KwaZulu-Natal	Langepan SF.
WR 331	*Schizopora* sp.	"	"	"
WR 332	*Trametes cingulata*	"	"	"
WR 334	*Pycnoporus sanguineus*	"	"	"
WR 337	*Trametes nivosa*	"	"	"
WR 338	*Pycnoporus sanguineus*	"	"	"
WR 339	*Lentinus villosus*	"	"	"
WR 340	*Trametes cingulata*	"	"	"
WR 341	*Trametes cingulata*	"	"	"
WR 342	*Trametes nivosa*	Pine wood	"	"
WR 343	*Pycnoporus sanguineus*	"	"	"
WR 344	*Trametes nivosa*	"	"	Dukuduku SF.
WR 345	*Trametes cingulata*	Indigenous Hw.	"	"
WR 346	*Pycnoporus sanguineus*	Pine wood	"	"
WR 347	*Pycnoporus sanguineus*	"	"	"
WR 348	*Pycnoporus sanguineus*	"	"	Eastern shores SF.
WR 349	*Ganoderma curtisii*	"	"	"
WR 350	*Pycnoporus sanguineus*	"	"	"
WR 351	*Gymnopilus* sp.	"	"	"
WR 352	*Trametes nivosa*	"	"	"
WR 353	*Pycnoporus sanguineus*	"	"	Nyalazi SF.
WR 355	*Trametes nivosa*	"	"	"
WR 356	*Trametes nivosa*	"	"	"
WR 357	*Pycnoporus sanguineus*	"	"	"
WR 358	*Poria* sp.	"	"	"

Strain no.	Species	Substrate / host	Area	Locality
WR 360	*Hexagona rigida*	Indigenous Hw.	KwaZulu-Natal	Nyalazi SF.
WR 361	*Coriolopsis strumosa*	"	"	"
WR 362	*Phellinus* sp.	Hardwood tree	"	"
WR 364	*Trametes nivosa*	Pine wood	"	Kwambonambi SF.
WR 365	*Pycnoporus sanguineus*	"	"	"
WR 366	*Trametes nivosa*	"	"	"
WR 367	*Trametes nivosa*	"	"	"
WR 368	*Pycnoporus sanguineus*	"	"	"
WR 369	*Phellinus* sp.	"	"	"
WR 372	*Ganoderna lucidum*	Acacia wood	Gauteng	Pretoria
WR 373	*Coriolopsis strumosa*	"	"	"
WR 375	*Trametes cingulata*	"	"	"
WR 376	*Stereum fulvum*	"	"	"
WR 377	Unidentified sp.	"	"	"
WR 379	*Coriolus hirsutus*	"	"	"
WR 381	*Gloeophyllum sepiarium*	Pine wood	Mpumalanga	Nelshoogte SF.
WR 382	*Pycnoporus sanguineus*	"	"	"
WR 385	*Lentinus stupeus*	Oak wood	"	"
WR 386	*Stereum hirsutum*	"	"	"
WR 390	*Gloeophyllum sepiarium*	Pine wood	"	"
WR 392	*Pycnoporus sanguineus*	"	"	"
WR 393	*Phaeolus schweinitzii*	"	"	"
WR 395	*Phellinus* sp.	Eucalyptus wood	"	Witklip SF.
WR 397	*Coniophora olivacea*	Plant litter	"	"
WR 398	*Pycnoporus sanguineus*	Indigenous Hw.	"	"
WR 400	*Hexagona* sp.	Eucalyptus wood	"	"
WR 401	*Coriolus hirsutus*	"	"	"
WR 402	*Lenzites betulina*	"	"	"
WR 404	*Lentinus stupeus*	"	"	"
WR 406	*Gloeophyllum sepiarium*	Pine wood	"	"
WR 407	*Coriolus hirsutus*	Indigenous Hw.	"	"
WR 408	*Coriolus hirsutus*	"	"	"
WR 409	*Lentinus stupeus*	Eucalyptus wood	"	Swartfontein SF.

a Refers to the reference numbers of strains that are maintained in the culture collection of the CSIR.

wood Refers to dead wood as opposed to "tree" that refers to living trees.

Hw Hardwood.

Sw Softwood.

SF. State Forest.

APPENDIX B: PHYSIOLOGICAL CHARACTERISTICS OF CULTURES

Results of physiological tests on cultures and grouping of different strains of fungi according to the reactions of cultures.

Species[a]	Group	Strain no.	Culture reactions							
			Enzymes[b]				Gallic acid medium		Tannic acid medium	
			Cytochrome oxidase	Laccase	Peroxidase	Tyrosinase	Oxidase reaction[c]	Growth rate (mm/week)[d]	Oxidase reaction[c]	Growth rate (mm/week)[d]
Antrodia variiformis	VII	WR 46	+		+	+	0	1,5	1	0,0
Bjerkandera adusta	Ia	WR 101	+	+	+	+	2	0,0	2	0,0
		WR 181	+	+	+	+	2	0,0	2	9,5
	VII	WR 70	+	+	+	+	1	2,0	1	2,0
		WR 71	+	+	+	+	0	8,0	0	0,0
		WR 74	+	+	+	+	0	2,5	0	0,0
		WR 159	+	+	+	+	1	2,5	0	0,0
		WR 195	+	+	+	+	0	1,0	0	0,0
		WR 217	+	+	+		0	2,0	1	3,0
		WR 218	+	+	+	+	0	4,0	0	1,5
Chondrostereum purpureum	Ia	WR 66	+	+	+	+	2	0,0	2	0,0
	VII	WR 78	+	+	+	+	1	0,0	1	2,5
Coniophora olivacea	VI	WR 397			+		0	6,0	1	0,0
Coriolopsis polyzona	Ia	WR 1	+	+	+	+	2	0,0	2	0,0
		WR 6	+	+	+	+	2	0,0	2	0,0
		WR 18	+	+	+	+	2	0,0	2	1,0
		WR 308	+	+	+	+	2	0,0	2	1,0
		WR 326		+	+	+	2	0,0	2	0,0
Coriolopsis strumosa	Ia	WR 361	+	+	+		2	0,0	2	0,0
	VII	WR 373	+	+	+	+	1	0,0	0	0,0
Coriolus hirsutus	Ia	WR 2	+	+	+		2	0,0	2	0,0
		WR 94	+	+	+	+	2	0,0	2	0,0
		WR 105	+	+	+	+	2	0,0	2	0,0
		WR 188	+	+	+	+	2	0,0	2	4,5
		WR 196	+	+	+	+	2	0,0	2	11,5
		WR 379		+	+		2	0,0	2	3,5
		WR 401	+	+	+		2	0,0	2	0,0
		WR 407	+	+	+		2	0,0	2	0,0
		WR 408	+	+	+		2	0,0	2	2,5
	VII	WR 100	+	+	+	+	1	0,0	1	0,0
Coriolus pubescens	Ia	WR 62	+	+	+	+	2	0,0	2	0,0

Species[a]	Group	Strain no.	Culture reactions							
			Enzymes[b]				Gallic acid medium		Tannic acid medium	
			Cytochrome oxidase	Laccase	Peroxidase	Tyrosinase	Oxidase reaction[c]	Growth rate (mm/week)[d]	Oxidase reaction[c]	Growth rate (mm/week)[d]
Coriolus versicolor	Ia	WR 37	+	+	+	+	2	0,0	2	0,0
		WR 44	+	+	+	+	2	0,0	2	1,0
		WR 61	+	+	+	+	2	0,0	2	0,0
		WR 75	+	+	+		2	0,0	2	7,5
		WR 76	+	+	+	+	2	0,0	2	1,0
		WR 77	+	+	+	+	2	0,0	2	6,5
		WR 79	+	+	+	+	2	0,0	2	1,0
		WR 80	+	+	+	+	2	0,0	2	9,0
		WR 81	+	+	+	+	2	0,0	2	1,5
		WR 82		+	+	+	2	0,0	2	0,0
		WR 83	+	+	+	+	2	0,0	2	4,0
		WR 86	+	+	+	+	2	0,0	2	2,0
		WR 137	+	+	+	+	2	0,0	2	7,0
		WR 141	+	+	+	+	2	0,0	2	3,0
		WR 164	+	+	+	+	2	0,0	2	4,0
		WR 167	+	+	+	+	2	0,0	2	6,5
		WR 169	+	+	+	+	2	0,0	2	9,0
		WR 177	+	+	+	+	2	0,0	2	9,5
		WR 179	+	+	+	+	2	0,0	2	9,0
		WR 184	+	+	+	+	2	0,0	2	5,0
		WR 185	+	+	+	+	2	0,0	2	9,5
		WR 190	+	+	+		2	0,0	2	10,5
		WR 194	+	+	+	+	2	0,0	2	9,0
		WR 220	+	+	+	+	2	0,0	2	6,0
		WR 255	+	+	+	+	2	0,0	2	9,0
		WR 283	+	+	+	+	2	0,0	2	9,5
		WR 323	+	+	+	+	2	0,0	2	8,5
Coriolus zonata	Ia	WR 73	+	+	+	+	2	0,0	2	4,5
Cyptotrama asprata	VII	WR 252	+	+	+		1	0,0	1	0,0
Daedalea quercina	VIII	WR 63					0	18,0	0	23,5
Fomitopsis lilacino-gilva	VIII	WR 65	+				0	13,0	0	16,5
	IX	WR 166					0	8,5	0	6,5
Ganoderma applanatum	Ia	WR 150	+	+	+	+	2	0,0	2	10,5
		WR 182	+	+	+	+	2	0,0	2	2,5
	Ic	WR 253		+			2	1,5	2	2,5
Ganoderma curtisii	Ia	WR 349	+	+	+	+	2	0,0	2	0,0
Ganoderma lucidum	IV	WR 33	+	+	+		2	2,5	0	0,0
	VII	WR 372	+	+	+	+	1	0,0	1	0,0
Gloeophyllum abietinum	XI	WR 155					0	3,5	0	0,0

Species[a]	Group	Strain no.	Culture reactions							
			Enzymes[b]				Gallic acid medium		Tannic acid medium	
			Cytochrome oxidase	Laccase	Peroxidase	Tyrosinase	Oxidase reaction[c]	Growth rate (mm/week)[d]	Oxidase reaction[c]	Growth rate (mm/week)[d]
Gloeophyllum sepiarium	III	WR 151					1	3,0	1	0,0
	IV	WR 259	+	+	+	+	2	0,0	1	0,0
	XI	WR 67	+				0	2,5	0	0,0
		WR 133	+				1	2,5	0	0,0
		WR 142	+				1	2,5	0	0,0
		WR 144	+				0	2,5	0	0,0
		WR 149					0	2,0	0	0,0
		WR 176					0	2,0	0	0,0
		WR 204					0	2,5	0	0,0
		WR 210					0	2,5	0	0,0
		WR 292	+				0	2,5	0	0,0
		WR 295					0	1,5	0	0,0
		WR 299					0	1,0	1	0,0
		WR 381					0	2,0	0	0,0
		WR 390					0	1,5	0	0,0
		WR 406					0	1,0	0	0,0
Gloeophyllum trabeum	III	WR 172	+	+	+	+	2	0,0	2	2,5
	XI	WR 50	+				0	2,0	0	0,0
		WR 52	+				0	3,5	0	0,0
		WR 68	+				0	4,5	0	0,0
		WR 171					0	8,5	0	0,0
		WR 318					0	1,5	0	0,0
Hexagona rigida	IV	WR 360	+	+	+	+	2	0,0	1	0,0
Hypholoma fasciculare	Ia	WR 85	+	+	+	+	2	0,0	2	3,0
		WR 279	+	+	+		2	0,0	2	0,0
Laxitextum bicolor	X	WR 13	+		+	+	0	18,5	0	0,0
Lentinus stupeus	Ia	WR 11	+	+	+	+	2	0,0	2	7,5
		WR 24	+	+	+		2	0,0	2	3,5
		WR 385	+	+	+		2	0,0	2	2,0
		WR 404	+	+	+		2	0,0	2	0,0
		WR 409	+	+	+		2	0,0	2	2,0
Lentinus villosus	Ia	WR 40	+	+	+	+	2	0,0	2	6,0
		WR 339	+	+	+	+	2	0,0	2	2,0
Lenzites betulina	Ia	WR 10	+	+	+	+	2	0,0	2	4,5
		WR 23	+	+	+	+	2	0,0	2	3,0
		WR 118	+	+	+	+	2	0,0	2	8,5
		WR402	+	+	+		2	0,0	2	2,5
	II	WR 34	+	+	+	+	2	0,0	2	9,5

Species[a]	Group	Strain no.	Culture reactions							
			Enzymes[b]				Gallic acid medium		Tannic acid medium	
			Cytochrome oxidase	Laccase	Peroxidase	Tyrosinase	Oxidase reaction[c]	Growth rate (mm/week)[d]	Oxidase reaction[c]	Growth rate (mm/week)[d]
Lenzites elegans	Ic	WR 5	+		+	+	2	0,0	2	0,0
	IV	WR 17		+	+		2	0,0	0	0,0
Nigroporus vinosus	Ia	WR 278	+	+	+	+	2	0,0	2	0,0
Phaeolus schweinitzii	Ic	WR 393	+		+	+	2	3,0	2	1,0
Phellinus gilvus	Ia	WR 104	+	+	+	+	2	0,0	2	0,0
		WR 180	+	+	+	+	2	0,0	2	10,5
		WR 186	+	+	+		2	0,0	2	4,0
		WR 211	+	+	+	+	2	0,0	2	2,0
		WR 175	+	+	+	+	2	0,0	2	10,5
		WR 193	+	+	+	+	2	0,0	2	7,5
		WR 260	+	+	+	+	2	0,0	2	0,0
Pulcherricium caeruleum	Ia	WR 273	+	+	+	+	2	1,5	2	9,0
Pycnoporus coccineus	Ia	WR 58		+	+	+	2	0,0	2	7,0
		WR 102	+	+	+	+	2	0,0	2	6,0
		WR 132	+	+	+	+	2	0,0	2	3,5
Pycnoporus sanguineus	Ia	WR 8	+	+	+		2	2,0	2	0,0
		WR 12	+	+	+	+	2	0,0	2	5,5
		WR 14	+	+	+	+	2	0,0	2	2,0
		WR 21	+	+	+		2	0,0	2	0,0
		WR 89	+	+	+	+	2	4,0	2	3,5
		WR 93	+	+	+		2	0,0	2	1,5
		WR 97	+	+	+	+	2	0,0	2	4,0
		WR 103	+	+	+	+	2	1,0	2	2,0
		WR 112	+	+	+	+	2	0,0	2	3,5
		WR 113	+	+	+	+	2	0,0	2	6,5
		WR 114	+	+	+	+	2	0,0	2	1,0
		WR 123	+	+	+	+	2	0,0	2	2,5
		WR 124	+	+	+		2	1,0	2	1,5
		WR 125	+	+	+	+	2	1,0	2	1,0
		WR 126	+	+	+	+	2	1,0	2	1,0
		WR 130	+	+	+	+	2	0,0	2	3,5
		WR 131	+	+	+	+	2	0,0	2	5,5
		WR 135	+	+	+	+	2	0,0	2	9,0
		WR 140	+	+	+	+	2	0,0	2	3,5
		WR 145	+	+	+		2	0,0	2	5,0
		WR 146	+	+	+	+	2	0,0	2	2,5
		WR 153	+	+	+	+	2	0,0	2	2,0
		WR 170	+	+	+		2	0,0	2	4,5
		WR 173	+	+	+	+	2	0,0	2	5,5
		WR 191	+	+	+	+	2	0,0	2	7,0

Species[a]	Group	Strain no.	Culture reactions							
			Enzymes[b]				Gallic acid medium		Tannic acid medium	
			Cytochrome oxidase	Laccase	Peroxidase	Tyrosinase	Oxidase reaction[c]	Growth rate (mm/week)[d]	Oxidase reaction[c]	Growth rate (mm/week)[d]
Pycnoporus sanguineus	Ia	WR 192	+	+	+		2	0,0	2	9,0
		WR 212	+	+	+	+	2	0,0	2	9,5
		WR 213	+	+	+	+	2	0,0	2	4,5
		WR 214	+	+	+	+	2	0,0	2	9,0
		WR 288	+	+	+		2	0,0	2	0,5
		WR 296		+	+	+	2	0,0	2	3,0
		WR 300	+	+	+	+	2	0,0	2	0,0
		WR 302	+	+	+	+	2	0,0	2	5,5
		WR 304	+	+	+	+	2	0,0	2	1,0
		WR 319	+	+	+	+	2	0,0	2	4,0
		WR 330	+	+	+	+	2	0,0	2	2,0
		WR 334	+	+	+	+	2	0,0	2	2,5
		WR 338	+	+	+	+	2	0,0	2	2,5
		WR 343	+	+	+	+	2	0,0	2	4,0
		WR 346	+	+	+	+	2	0,0	2	0,0
		WR 347	+	+	+	+	2	0,0	2	0,0
		WR 348	+	+	+	+	2	0,0	2	0,0
		WR 350	+	+	+	+	2	0,0	2	3,0
		WR 353		+	+		2	0,0	2	2,5
		WR 357	+	+	+		2	0,0	2	1,0
		WR 368	+	+	+	+	2	0,0	2	1,0
		WR 382	+	+	+	+	2	0,0	2	1,0
		WR 392		+	+		2	0,0	2	1,0
		WR 398	+	+	+		2	0,0	2	1,5
	III	WR 365	+	+	+	+	2	0,0	2	1,0
	IV	WR 4	+	+	+	+	2	0,0	1	2,0
		WR 99	+	+	+	+	2	0,0	1	0,0
Schizophyllum commune	IX	WR 47					0	2,5	0	1,0
		WR 205	+	+			0	6,5	0	2,5
Schizopora paradoxa	III	WR 257					2	1,0	2	7,0
Stereum fulvum	VI	WR 277		+	+		1	0,0	0	0,0
	VII	WR 376	+	+	+		1	0,0	0	0,0

Species[a]	Group	Strain no.	Culture reactions							
			Enzymes[b]				Gallic acid medium		Tannic acid medium	
			Cytochrome oxidase	Laccase	Peroxidase	Tyrosinase	Oxidase reaction[c]	Growth rate (mm/week)[d]	Oxidase reaction[c]	Growth rate (mm/week)[d]
Stereum hirsutum	Ia	WR 9	+	+	+	+	2	5,5	2	8,0
		WR 22	+	+	+	+	2	6,5	2	10,5
		WR 25	+	+	+	+	2	7,0	2	9,0
		WR 90		+	+		2	3,0	2	7,5
		WR 136	+	+	+	+	2	4,0	2	7,0
		WR 200	+	+	+	+	2	0,0	2	10,5
		WR 216	+	+	+	+	2	0,0	2	10,5
		WR 313	+	+	+	+	2	1,0	2	6,5
		WR 386	+	+	+		2	3,0	2	8,0
	Ib	WR 32	+	+		+	2	6,0	2	8,0
	II	WR 3	+	+	+	+	2	10,5	2	14,0
		WR 64	+	+	+	+	2	9,5	1	17,5
		WR 87	+	+	+	+	2	9,0	1	15,5
		WR 91	+	+	+	+	2	14,5	1	17,5
		WR 95		+	+		2	10,0	1	11,0
		WR 138	+	+	+		2	3,5	2	13,5
		WR 156	+	+	+	+	2	14,0	1	15,5
		WR 197	+	+	+	+	2	8,0	2	14,0
		WR 297	+	+	+		2	11,5	2	15,0
		WR 310	+	+	+	+	2	7,5	2	13,0
Stereum illudens	Ia	WR 38	+	+	+	+	2	0,0	2	6,0
		WR 324	+	+	+		2	1,0	2	4,5
	Ic	WR 96			+		2	1,5	2	1,5
Stereum ostrea	Ia	WR 19		+	+	+	2	6,0	2	10,0
		WR 27		+	+	+	2	0,0	2	9,0
		WR 261		+	+	+	2	0,5	2	0,5
	Ib	WR 7		+		+	2	1,5	2	9,0
	VII	WR 28	+	+	+	+	1	1,0	1	1,5
Stereum rimosum	Ia	WR 30	+	+	+	+	2	3,0	2	8,5
Stereum sanguinolentum	Ib	WR 45	+	+			2	0,0	2	3,0
Trametes cingulata	Ia	WR 31	+	+	+	+	2	0,0	2	0,0
		WR 39	+	+	+	+	2	0,0	2	0,0
		WR 332	+	+	+	+	2	0,0	2	0,0
		WR 340	+	+	+		2	0,0	2	0,0
		WR 341	+	+	+		2	0,0	2	0,0
		WR 345	+	+	+	+	2	0,0	2	0,0
		WR 375	+	+	+		2	0,0	2	3,0
Trametes glabrescens	Ia	WR 119	+	+	+	+	2	0,0	2	0,0
		WR 120	+	+	+	+	2	0,0	2	1,0

Species[a]	Group	Strain no.	Culture reactions							
			Enzymes[b]				Gallic acid medium		Tannic acid medium	
			Cytochrome oxidase	Laccase	Peroxidase	Tyrosinase	Oxidase reaction[c]	Growth rate (mm/week)[d]	Oxidase reaction[c]	Growth rate (mm/week)[d]
Trametes nivosa	V	WR 337		+	+	+	1	3,0	0	3,5
		WR 342	+	+	+	+	1	3,0	0	3,5
		WR 367					1	1,5	0	3,0
	VI	WR 311		+	+		0	4,0	1	3,0
	VII	WR 355	+	+	+	+	0	6,5	0	5,0
		WR 366	+	+	+	+	0	7,5	0	5,0
	VIII	WR 307					0	10,0	0	11,5
		WR 309	+				0	12,5	0	15,5
		WR 315					0	9,5	0	13,0
		WR 316					0	11,5	0	16,5
		WR 329					0	8,0	0	13,5
	IX	WR 303	+				0	6,5	0	6,5
	XI	WR 327					0	8,5	0	9,0
		WR 344	+	+			0	4,5	0	4,0
		WR 352				+	0	4,5	0	3,0
		WR 356					0	2,0	0	2,5
		WR 364					0	1,5	0	4,5

a A total of 42 strains were not identified to species level and were, therefore, excluded from this table.

b Qualitative tests + = present.

c 0 = negative reaction; 1 = weak positive reaction; 2 = strong positive reaction.

d Growth rate was determined by measuring the distance from the inoculum plug to the colony margin in four directions after incubation for one week.

APPENDIX C: RESULTS OF THE FIRST SCREENING STEP TO SELECT STRAINS FOR KRAFT BIOPULPING.

Change in kappa number (%) caused by treatment with different strains of fungi compared to control treatments after three or eight weeks.

Strain no.	Species	Treatment time	
		Three weeks	Eight weeks
WR 1	*Coriolopsis polyzona*		-18,2
WR 2	*Coriolus hirsutus*		-10,1
WR 3	*Stereum hirsutum*		-26,1
WR 4	*Pycnoporus sanguineus*		-10,7
WR 5	*Lenzites elegans*		-6,5
WR 6	*Coriolopsis polyzona*		-3,6
WR 7	*Stereum ostrea*		-6,8
WR 8	*Pycnoporus sanguineus*		-9,8
WR 9	*Stereum hirsutum*		-29,1
WR 10	*Lenzites betulina*		-12,0
WR 11	*Lentinus stupeus*		-12,2
WR 12	*Pycnoporus sanguineus*		-0,9
WR 13	*Laxitextum bicolor*		n.d.
WR 14	*Pycnoporus sanguineus*		-9,5
WR 17	*Lenzites elegans*	-12,4	
WR 18	*Coriolopsis polyzona*		-3,9
WR 19	*Stereum ostrea*	-16,7	-19,3
WR 21	*Pycnoporus sanguineus*		-1,9
WR 22	*Stereum hirsutum*	-17,0	-22,0
WR 23	*Lenzites betulina*		+2,8
WR 24	*Lentinus stupeus*		-24,0
WR 25	*Stereum hirsutum*	-16,4	
WR 27	*Stereum ostrea*		-9,1
WR 28	*Stereum ostrea*	-4,6	
WR 30	*Stereum rimosum*		-10,1
WR 31	*Trametes cingulata*		n.d.
WR 32	*Stereum hirsutum*		-15,1
WR 33	*Ganoderma lucidum*		+2,5
WR 34	*Lenzites betulina*		-15,0
WR 36	*Gyrodontium* sp.		n.d.
WR 37	*Coriolus versicolor*		-8,2
WR 38	*Stereum illudens*	-8,4	
WR 39	*Trametes cingulata*		-2,2
WR 40	*Lentinus villosus*		-8,5
WR 44	*Coriolus versicolor*		-5,3
WR 45	*Stereum sanguinolentum*	-0,0	

Strain no.	Species	Treatment time	
		Three weeks	Eight weeks
WR 46	*Antrodia variiformis*		n.d.
WR 47	*Schizophyllum commune*		+11,0
WR 50	*Gloeophyllum trabeum*		-41,8
WR 51	Unidentified sp.		-20,4
WR 52	*Gloeophyllum trabeum*		-32,1
WR 58	*Pycnoporus coccineus*		-21,0
WR 60	Unidentified sp.		n.d.
WR 61	*Coriolus versicolor*	-14,1	
WR 62	*Coriolus pubescens*		+4,8
WR 63	*Daedalea quercina*		+4,5
WR 64	*Stereum hirsutum*		-20,2
WR 65	*Fomitopsis lilacino-gilva*		-10,1
WR 66	*Chondrostereum purpureum*		+17,9
WR 67	*Gloeophyllum sepiarium*		-41,1
WR 68	*Gloeophyllum trabeum*		-24,0
WR 70	*Bjerkandera adusta*		-12,7
WR 71	*Bjerkandera adusta*		-13,0
WR 73	*Coriolus zonatus*		-12,2
WR 74	*Bjerkandera adusta*		+1,2
WR 75	*Coriolus versicolor*		-4,2
WR 76	*Coriolus versicolor*		-6,8
WR 77	*Coriolus versicolor*		n.d.
WR 78	*Chondrostereum purpureum*		-11,0
WR 79	*Coriolus versicolor*	-12,8	
WR 80	*Coriolus versicolor*		-6,8
WR 81	*Coriolus versicolor*		-6,5
WR 82	*Coriolus versicolor*		-2,1
WR 83	*Coriolus versicolor*		-24,9
WR 84	*Phellinus* sp.		-12,4
WR 85	*Hypholoma fasciculare*		n.d.
WR 86	*Coriolus versicolor*		-17,1
WR 87	*Stereum hirsutum*	-7,2	
WR 88	*Grifola* sp.		n.d.
WR 89	*Pycnoporus sanguineus*	-1,5	
WR 90	*Stereum hirsutum*	-12,6	-21,4
WR 91	*Stereum hirsutum*		-32,0
WR 92	*Coriolus* sp.	-7,5	
WR 93	*Pycnoporus sanguineus*		-26,0
WR 94	*Coriolus hirsutus*		-17,0
WR 95	*Stereum hirsutum*	-14,1	
WR 96	*Stereum illudens*	-11,8	

Strain no.	Species	Treatment time	
		Three weeks	Eight weeks
WR 97	*Pycnoporus sanguineus*		-19,0
WR 98	Unidentified sp.		n.d.
WR 99	*Pycnoporus sanguineus*		-19,0
WR 100	*Coriolus hirsutus*	-11,1	
WR 101	*Bjerkandera adusta*		-10,0
WR 102	*Pycnoporus coccineus*		-22,0
WR 103	*Pycnoporus sanguineus*		-21,0
WR 104	*Phellinus gilvus*		-21,0
WR 105	*Coriolus hirsutus*		-18,0
WR 112	*Pycnoporus sanguineus*		-19,0
WR 113	*Pycnoporus sanguineus*		-15,0
WR 114	*Pycnoporus sanguineus*		-24,0
WR 118	*Lenzites betulina*		-17,0
WR 119	*Trametes glabrescens*		-7,0
WR 120	*Trametes glabrescens*		-23,0
WR 123	*Pycnoporus sanguineus*	-12,4	
WR 124	*Pycnoporus sanguineus*		-31,0
WR 125	*Pycnoporus sanguineus*		-20,0
WR 126	*Pycnoporus sanguineus*		-20,0
WR 130	*Pycnoporus sanguineus*		-25,0
WR 131	*Pycnoporus sanguineus*		-30,0
WR 132	*Pycnoporus coccineus*		-34,0
WR 133	*Gloeophyllum sepiarium*	-35,1	
WR 135	*Pycnoporus sanguineus*	-4,5	
WR 136	*Stereum hirsutum*	-6,5	
WR 137	*Coriolus versicolor*	-6,8	
WR 138	*Stereum hirsutum*	-8,9	
WR 139	*Hyphodontia* sp.	-7,8	
WR 140	*Pycnoporus sanguineus*	-9,8	
WR 141	*Coriolus versicolor*	-13,0	
WR 142	*Gloeophyllum sepiarium*	-25,2	
WR 144	*Gloeophyllum sepiarium*	-40,0	
WR 145	*Pycnoporus sanguineus*	-1,6	
WR 146	*Pycnoporus sanguineus*	-15,4	
WR 149	*Gloeophyllum sepiarium*	-30,2	
WR 150	*Ganoderma applanatum*		n.d.
WR 151	*Gloeophyllum sepiarium*	-41,6	
WR 152	*Skeletocutis* sp.	-6,5	
WR 153	*Pycnoporus sanguineus*	-9,5	
WR 154	*Skeletocutis* sp.		n.d.
WR 155	*Gloeophyllum abietinum*	-40,2	

Strain no.	Species	Treatment time	
		Three weeks	Eight weeks
WR 156	*Stereum hirsutum*	-18,5	
WR 157	*Skeletocutis* sp.	-7,9	
WR 159	*Bjerkandera adusta*	-9,4	
WR 164	*Coriolus versicolor*	-10,4	
WR 165	*Skeletocutis* sp.	-2,3	
WR 166	*Fomitopsis lilacino-gilva*	-8,2	
WR 167	*Coriolus versicolor*	-10,5	
WR 168	*Poria* sp.		n.d.
WR 169	*Coriolus versicolor*	-5,9	
WR 170	*Pycnoporus sanguineus*	-14,9	
WR 171	*Gloeophyllum trabeum*	-38,6	
WR 172	*Gloeophyllum trabeum*	-45,9	
WR 173	*Pycnoporus sanguineus*	-3,6	
WR 175	*Phellinus gilvus*		n.d.
WR 176	*Gloeophyllum sepiarium*	-30,0	
WR 177	*Coriolus versicolor*	0,0	
WR 178	*Phellinus* sp.	-2,2	
WR 179	*Coriolus versicolor*	-0,3	
WR 180	*Phellinus gilvus*	-0,0	
WR 181	*Bjerkandera adusta*	-3,1	
WR 182	*Ganoderma applanatum*	-12,3	
WR 183	*Skeletocutis* sp.		n.d.
WR 184	*Coriolus versicolor*	-12,6	
WR 185	*Coriolus versicolor*	-4,2	
WR 186	*Phellinus gilvus*	-3,9	
WR 188	*Coriolus hirsutus*	-7,8	
WR 189	*Skeletocutis* sp.	-1,8	
WR 190	*Coriolus versicolor*	-2,9	
WR 191	*Pycnoporus sanguineus*	-3,2	
WR 192	*Pycnoporus sanguineus*	-3,9	
WR 193	*Phellinus gilvus*		n.d.
WR 194	*Coriolus versicolor*	-1,3	
WR 195	*Bjerkandera adusta*	-3,9	
WR 196	*Coriolus hirsutus*	-3,9	
WR 197	*Stereum hirsutum*	-3,9	
WR 199	*Peniophora* sp.	-5,5	
WR 200	*Stereum hirsutum*	-6,2	
WR 201	*Hyphodontia* sp.	-5,4	
WR 202	*Hyphodontia* sp.	-6,5	
WR 203	*Antrodia* sp.		n.d.
WR 204	*Gloeophyllum sepiarium*	-32,5	

Strain no.	Species	Treatment time	
		Three weeks	Eight weeks
WR 205	*Schizophyllum commune*	-2,3	
WR 206	*Skeletocutis* sp.	-9,1	
WR 207	*Peniophora* sp.	-7,4	
WR 208	Unidentified sp.		n.d.
WR 210	*Gloeophyllum sepiarium*	-41,8	
WR 211	*Phellinus gilvus*		n.d.
WR 212	*Pycnopor sanguineus*	-3,2	
WR 213	*Pycnoporus sanguineus*	-12,3	
WR 214	*Pycnoporus sanguineus*	-5,8	
WR 216	*Stereum hirsutum*	-5,8	
WR 217	*Bjerkandera adusta*	-2,6	
WR 218	*Bjerkandera adusta*	-6,8	
WR 220	*Coriolus versicolor*	-0,6	
WR 251	Unidentified sp.	-16,8	
WR 252	*Cyptotrama asprata*		n.d.
WR 253	*Ganoderma applanatum*	-6,8	
WR 255	*Coriolus versicolor*	-17,9	
WR 256	*Stereum* sp.	-10,7	
WR 257	*Schizopora paradoxa*	-2,5	
WR 259	*Gloeophyllum sepiarium*		n.d.
WR 260	*Phellinus gilvus*	-1,0	
WR 261	*Stereum ostrea*	-10,7	
WR 262	*Crepidotus* sp.	0,0	
WR 268	*Lentinus* sp.	-10,1	
WR 270	*Pycnoporus* sp.	-14,9	
WR 273	*Pulcherricium caeruleum*	-7,4	
WR 274	*Coriolus* sp.	-8,6	
WR 277	*Stereum fulvum*	-12,2	
WR 278	*Nigroporus vinosus*	-2,5	
WR 279	*Hypholoma fasciculare*	-7,5	
WR 280	*Podoscypha* sp.	-7,5	
WR 281	*Stereum* sp.	-5,2	
WR 283	*Coriolus versicolor*	-4,9	
WR 285	*Phellinus* sp.	-16,0	
WR 286	*Peniophora* sp.	-15,2	
WR 288	*Pycnoporus sanguineus*	-10,4	
WR 292	*Gloeophyllum sepiarium*	-35,8	
WR 295	*Gloeophyllum sepiarium*	-25,7	
WR 296	*Pycnoporus sanguineus*	-10,1	
WR 297	*Stereum hirsutum*	-14,6	
WR 299	*Gloeophyllum sepiarium*	-26,9	

Strain no.	Species	Treatment time	
		Three weeks	Eight weeks
WR 300	*Pycnoporus sanguineus*	-12,3	
WR 302	*Pycnoporus sanguineus*	-11,4	
WR 303	*Trametes nivosa*	-22,3	
WR 304	*Pycnoporus sanguineus*	-15,8	
WR 307	*Trametes nivosa*	-8,8	
WR 308	*Coriolopsis polyzona*	-15,4	
WR 309	*Trametes nivosa*	-7,9	
WR 310	*Stereum hirsutum*	-14,6	
WR 311	*Trametes nivosa*	-9,5	
WR 312	*Coriolus* sp.		n.d.
WR 313	*Stereum hirsutum*	-11,1	
WR 315	*Trametes nivosa*	-1,4	
WR 316	*Trametes nivosa*	-8,2	
WR 318	*Gloeophyllum trabeum*	-36,4	
WR 319	*Pycnoporus sanguineus*	-15,9	
WR 323	*Coriolus versicolor*	-3,8	
WR 324	*Stereum illudens*	-14,6	
WR 326	*Coriolopsis polyzona*	-12,3	
WR 327	*Trametes nivosa*	-5,8	
WR 329	*Trametes nivosa*	-0,3	
WR 330	*Pycnoporus sanguineus*	-8,7	
WR 331	*Schizopora* sp.	-11,7	
WR 332	*Trametes cingulata*	-2,1	
WR 334	*Pycnoporus sanguineus*	-11,7	
WR 337	*Trametes nivosa*	-3,3	
WR 338	*Pycnoporus sanguineus*	-11,1	
WR 339	*Lentinus villosus*	-14,3	
WR 340	*Trametes cingulata*	-15,0	
WR 341	*Trametes cingulata*	-12,4	
WR 342	*Trametes nivosa*	-8,1	
WR 343	*Pycnoporus sanguineus*	-4,8	
WR 344	*Trametes nivosa*	-3,9	
WR 345	*Trametes cingulata*	-13,7	
WR 346	*Pycnoporus sanguineus*	-21,1	
WR 347	*Pycnoporus sanguineus*	-11,0	
WR 348	*Pycnoporus sanguineus*	-6,6	
WR 349	*Ganoderma curtisii*	-15,3	
WR 350	*Pycnoporus sanguineus*	-4,8	
WR 351	*Gymnopilus* sp.	-15,6	
WR 352	*Trametes nivosa*	-4,9	
WR 353	*Pycnoporus sanguineus*	-9,3	

Strain no.	Species	Treatment time	
		Three weeks	Eight weeks
WR 355	*Trametes nivosa*	-0,6	
WR 356	*Trametes nivosa*	-3,9	
WR 357	*Pycnoporus sanguineus*	-6,3	
WR 358	*Poria* sp.	-9,0	
WR 360	*Hexagona rigida*	-6,3	
WR 361	*Coriolopsis strumosa*	-7,5	
WR 362	*Phellinus* sp.		n.d.
WR 364	*Trametes nivosa*	-3,6	
WR 365	*Pycnoporus sanguineus*	-10,8	
WR 366	*Trametes nivosa*	-2,7	
WR 367	*Trametes nivosa*	-2,2	
WR 368	*Pycnoporus sanguineus*	-9,3	
WR 369	*Phellinus* sp.		n.d.
WR 372	*Ganoderna lucidum*		n.d.
WR 373	*Coriolopsis strumosa*		n.d.
WR 375	*Trametes cingulata*	-2,3	
WR 376	*Stereum fulvum*	-7,8	
WR 377	Unidentified sp.	-5,8	
WR 379	*Coriolus hirsutus*	-8,7	
WR 381	*Gloeophyllum sepiarium*	-39,8	
WR 382	*Pycnoporus sanguineus*	-6,5	
WR 385	*Lentinus stupeus*	-4,6	
WR 386	*Stereum hirsutum*	-17,4	
WR 390	*Gloeophyllum sepiarium*	-39,5	
WR 392	*Pycnoporus sanguineus*	-4,3	
WR 393	*Phaeolus schweinitzii*	-19,9	
WR 395	*Phellinus* sp.	-13,0	
WR 397	*Coniophora olivacea*		n.d.
WR 398	*Pycnoporus sanguineus*	-14,4	
WR 400	*Hexagona* sp.	-10,2	
WR 401	*Coriolus hirsutus*	-5,2	
WR 402	*Lenzites betulina*	-21,7	
WR 404	*Lentinus stupeus*	-4,6	
WR 406	*Gloeophyllum sepiarium*	-40,5	
WR 407	*Coriolus hirsutus*	-13,0	
WR 408	*Coriolus hirsutus*	-0,3	
WR 409	*Lentinus stupeus*	-5,0	
ATCC32629	*Phanerochaete chrysosporium*	-4,8	
CZ-3	*Ceriporiopsis subvermispora*	-12,3	

n.d. Not determined, because of insufficient growth.

APPENDIX D: TEMPERATURES OBSERVED IN A COMMERCIAL CHIP PILE

Temperature (°C) at different positions, times and at different ages of the experimental chip pile.

Age of pile	Position	Time												
		08:00	10:00	12:00	14:00	16:00	18:00	20:00	22:00	00:00	02:00	04:00	06:00	08:00
1 Day	1				21,1	21,2	21,4	21,4	21,4	21,4	21,6	21,7	21,9	22,1
	2			19,8	20,0	20,2	20,6	20,7	20,8	21,0	21,2	21,6	21,9	22,1
	3			19,4	19,6	19,8	20,1	20,3	20,2	20,4	20,5	20,7	21,3	21,4
	4		19,5	19,5	19,5	19,5	20,5	21,3	21,9	22,7	23,5	24,5	25,4	26,3
	5				20,2	20,4	20,7	20,7	20,7	20,9	21,0	21,2	21,5	21,7
	6			21,3	21,4	21,5	21,6	21,6	21,6	21,6	21,6	21,6	21,6	21,6
	7			19,6	19,8	20,0	20,5	20,5	20,5	20,7	20,9	21,2	21,6	21,9
	8					20,3	20,6	20,6	20,7	20,9	21,1	21,3	21,6	21,8
	9						20,0	20,0	19,9	19,9	19,9	19,9	20,0	19,6
	10						19,7	19,0	16,7	15,9	15,4	15,0	14,9	15,5
1 Week	1	51,7	51,6	52,0	51,9	52,1	52,0	51,6	51,6	51,7	51,9	51,9	52,0	
	2	52,1	52,6	52,5	52,4	52,5	52,5	51,9	51,9	51,8	51,9	51,8	51,8	
	3	51,4	51,6	51,6	51,6	51,6	51,6	51,5	51,6	51,6	51,6	51,6	51,6	
	4	42,7	43,5	43,6	43,9	44,4	44,7	44,6	45,1	45,4	45,8	46,2	46,7	
	5	41,2	40,6	40,7	40,4	40,4	40,4	39,8	39,6	39,6	39,5	39,2	39,1	
	6	42,0	42,0	42,0	41,6	41,5	41,2	40,7	40,4	40,0	39,9	39,5	39,2	
	7	32,7	32,9	32,7	32,6	32,5	32,5	32,3	32,4	32,5	32,8	32,9	33,1	
	8	20,3	20,6	20,9	20,7	20,7	20,9	20,7	20,9	21,0	21,2	21,3	21,4	
	9	14,5	14,9	16,1	14,9	13,4	13,4	13,2	13,3	13,2	12,9	12,3	11,3	
	10	12,4	16,1	15,6	15,7	15,8	16,5	13,7	10,9	8,3	6,7	6,2	4,8	
2 Weeks	1	55,2	54,8	57,1	56,0	56,4	56,3	56,0	55,7	55,6	55,5	55,4	55,2	
	2	54,0	52,9	55,9	54,0	53,8	53,9	53,8	53,7	53,8	53,7	53,8	53,8	
	3	55,3	54,1	56,1	54,9	54,7	54,9	54,8	54,7	54,9	54,9	55,0	55,2	
	4	55,1	54,0	55,6	54,8	54,8	54,9	54,8	54,7	55,0	55,1	55,2	55,3	
	5	35,6	36,3	38,9	37,3	37,1	37,1	36,7	36,3	36,3	36,1	35,8	35,6	
	6	35,2	35,4	38,3	36,5	36,3	36,1	35,9	35,7	35,7	35,5	35,4	35,4	
	7	30,2	29,1	31,8	30,6	30,7	30,9	31,0	31,0	31,1	30,8	30,7	30,5	
	8	19,9	21,7	23,1	22,2	22,1	21,9	21,6	21,3	21,2	21,0	20,5	20,4	
	9	11,3	10,3	11,5	10,8	10,2	10,4	10,8	11,2	11,5	11,6	11,7	11,4	
	10	10,4	23,3	29,2	24,3	22,4	17,9	14,6	11,3	9,6	8,0	7,3	6,8	
3 Weeks	1	48,9	49,7	50,4	51,0	52,4	53,1	52,5	52,1	51,6	51,3	51,0	50,8	
	2	51,3	52,4	51,6	50,7	50,1	50,0	49,9	49,8	49,7	49,5	49,2	49,3	
	3	54,6	55,7	54,9	54,0	53,5	53,3	53,3	53,3	53,3	53,2	53,2	53,2	
	4	52,8	53,2	53,3	52,8	52,7	52,5	52,4	52,2	52,0	51,9	51,9	51,9	
	5	31,3	32,3	32,3	31,6	31,3	31,3	30,9	30,6	30,2	29,9	29,5	29,4	
	6	31,2	32,4	32,2	31,3	31,1	31,0	30,8	30,6	30,3	30,1	29,8	29,8	
	7	27,4	28,8	28,4	27,9	27,9	27,9	27,8	27,6	27,3	27,1	26,8	26,7	
	8	15,8	16,0	16,4	15,7	15,5	15,4	15,2	15,0	14,7	14,5	14,3	14,2	
	9	4,3	4,8	6,2	6,1	6,2	6,1	5,5	5,0	4,1	3,3	2,5	2,0	
	10	6,3	8,8	18,5	18,7	17,5	10,7	6,5	3,1	0,6	-0,2	-1,1	-1,3	

APPENDIX E: LEVELS OF CO_2 OBSERVED IN A COMMERCIAL CHIP PILE

Concentration of CO_2 (%) at different positions, times and at different ages of the experimental chip pile.

Age of pile	Position	Time												
		08:00	10:00	12:00	14:00	16:00	18:00	20:00	22:00	00:00	02:00	04:00	06:00	08:00
1 Day	1				0,4	0,4	0,6	0,1	0,8	0,8	0,8	0,3	0,6	1,0
	2			0,0	0,0	0,2	0,7	0,9	1,5	2,3	3,0	3,5	3,7	3,7
	3			0,1	0,1	0,6	1,3	1,8	2,0	2,6	3,2	3,9	4,2	5,1
	4		0,1	1,1	1,1	2,0	3,0	4,2	4,6	5,9	6,7	8,4	8,6	9,7
	5				1,1	1,1	0,9	1,5	2,0	0,9	1,2	1,4	1,6	1,7
	6			1,6	1,7	1,7	1,6	2,0	2,7	1,7	1,5	2,0	2,1	2,7
	7			1,3	1,7	2,1	2,6	3,1	3,6	3,9	3,4	4,6	4,2	6,5
	8					0,8	0,5	1,3	0,7	0,4	0,3	0,4	0,5	0,6
	9						0,1	0,0	0,2	0,1	0,1	0,2	0,2	0,2
	10						0,0	0,0	0,1	0,1	0,1	0,1	0,1	0,1
1 Week	1	1,3	1,5	1,8	1,8	1,6	1,3	1,2	1,4	1,5	1,3	1,3	1,3	
	2	2,3	2,0	2,4	2,5	2,2	2,1	1,9	1,9	1,9	1,8	1,7	1,7	
	3	6,0	5,4	6,2	6,0	5,4	6,2	3,8	4,2	3,6	3,4	3,2	3,0	
	4	7,3	8,5	12,7	10,8	9,4	8,9	7,1	5,5	4,7	4,1	3,6	3,7	
	5	0,7	0,8	0,8	0,8	0,8	0,8	0,7	0,9	0,8	0,8	0,8	0,8	
	6	0,9	1,0	1,2	1,2	1,2	1,1	1,1	1,0	0,9	0,8	0,8	0,8	
	7	0,7	0,8	0,9	1,0	0,9	0,9	0,8	0,8	0,7	0,7	0,7	0,6	
	8	0,3	0,3	0,3	0,4	0,3	0,4	0,4	0,4	0,4	0,3	0,3	0,3	
	9	0,0	0,2	0,2	0,2	0,2	0,5	0,3	0,3	0,2	0,2	0,2	0,2	
	10	0,0	0,1	0,1	0,2	0,1	0,2	0,2	0,2	0,2	0,1	0,2	0,2	
2 Weeks	1	1,2	1,2	1,4	1,4	1,4	1,6	1,4	1,1	0,9	1,0	1,2	1,4	
	2	1,8	1,5	2,1	1,8	2,2	2,0	1,9	1,8	1,6	1,7	1,6	1,6	
	3	2,4	2,1	2,8	2,7	2,7	2,8	2,6	2,4	2,3	2,3	2,3	2,4	
	4	3,1	2,8	2,9	2,8	3,6	3,2	3,1	3,0	2,9	2,9	2,9	3,0	
	5	0,6	0,8	0,8	0,8	0,8	0,8	0,8	0,7	0,6	0,6	0,6	0,6	
	6	0,6	0,7	0,8	0,8	0,8	0,8	0,7	0,6	0,6	0,6	0,6	0,5	
	7	0,4	0,5	0,5	0,5	0,5	0,5	0,5	0,4	0,4	0,4	0,4	0,4	
	8	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,2	
	9	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	
	10	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	
3 Weeks	1	1,1	0,7	1,0	0,9	0,9	0,9	0,8	0,7	0,6	0,5	0,5	0,9	
	2	1,2	1,5	1,0	1,0	1,0	1,2	1,1	1,0	1,0	1,0	1,0	1,2	
	3	1,9	1,9	1,1	1,2	1,1	1,6	1,5	1,5	1,5	1,6	1,6	1,6	
	4	1,9	1,4	2,9	2,9	2,3	2,1	1,7	1,7	1,7	1,6	1,5	1,5	
	5	0,5	0,4	0,5	0,5	0,5	0,3	0,3	0,2	0,2	0,2	0,1	0,1	
	6	0,5	0,5	0,6	0,6	0,6	0,4	0,3	0,2	0,2	0,2	0,1	0,2	
	7	0,4	0,3	0,4	0,4	0,3	0,2	0,1	0,1	0,1	0,0	0,0	0,0	
	8	0,3	0,1	0,2	0,2	0,2	0,0	0,0	0,0	0,0	0,0	0,0	0,0	
	9	0,2	0,1	0,2	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	
	10	0,2	0,1	0,1	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	

APPENDIX F INFLUENCE OF CO_2 ON THE BIOPULPING EFFICIENCY OF SELECTED STRAINS OF WHITE-ROT FUNGI.

Methods

Inoculum and wood chips were prepared as described previously (Chapter 3). The wood chips were inoculated with *Coriolus hirsutus* (WR 83), *Stereum hirsutum* (WR 95), *Pycnoporus sanguineus* (WR 124) and sterile growth medium as a control. Treatments were incubated for three weeks at 28 °C under atmospheric CO_2 concentration for comparison with treatments incubated at 10 %, 15 % and 21,5 % CO_2. Only one CO_2 incubator was available, therefore, three factorial experiments with completely randomized designs were conducted where each experiment consisted of four fungal treatments and two CO_2 treatments. Treated chips were harvested, dried and pulped according to the methods described for screening (Chapter 3). Kappa numbers of treated chip samples were determined and the data subjected to one-way analysis of variance. Means for the fungal treatments were compared with Tukey's test.

Results

All three fungal strains were able to reduce kappa number significantly at 10 % CO_2 when compared to the control inoculation. Furthermore, the mean kappa number for all three strains and the control did not differ significantly between the incubation at atmospheric CO_2 and incubation at 10 % CO_2 (Table 1). All three fungal strains were also able to reduce kappa number significantly at 15 % CO_2 when compared to the control treatment. No significant difference was found between the mean kappa number of any treatments incubated at atmospheric CO_2 and the treatments incubated

at 15 % CO_2 (Table 2). At 21,5 % CO_2, all three fungal strains were again able to reduce the kappa number significantly. However, the treatments incubated at 21,5 % CO_2, resulted in a significantly higher kappa number when compared to the treatments incubated at atmospheric CO_2 (Table 3).

Table 1. Effect of different fungal strains on kappa number in an environment with 10 % CO_2.

Treatment		Control	10 % CO_2
Control		26,8a	28,0a
Coriolus hirsutus	**WR 83**	23,9c	23,6c
Stereum hirsutum	**WR 95**	25,0b	25,8b
Pycnoporus sanguineus	**WR 124**	22,3c	22,3c
	Treatment means	24,5z	24,9z

a, b, c Means of three replications. Means in the same column followed by the same letter do not differ significantly ($p \leq 0,05$; Tukey's test)

z Means of four treatments. Means in the same row followed by the same letter do not differ significantly ($p \leq 0,05$; Tukey's test)

Table 2. Effect of different fungal strains on kappa number in an environment with 15 % CO_2.

Treatment		Control	15 % CO_2
Control		30,7a	30,6a
Coriolus hirsutus	**WR 83**	28,4b	28,3b
Stereum hirsutum	**WR 95**	27,5bc	27,4bc
Pycnoporus sanguineus	**WR 124**	27,1c	25,5c
	Treatment means	28,4z	28,0z

a, b, c Means of three replications. Means in the same column followed by the same letter do not differ significantly ($p \leq 0,05$; Tukey's test)

z Means of four treatments. Means in the same row followed by the same letter do not differ significantly ($p \leq 0,05$; Tukey's test)

Table 3. Effect of different fungal strains on kappa number in an environment with 21,5 % CO_2.

Treatment		Control	21,5 % CO_2
Control		31,5a	32,3a
Coriolus hirsutus	**WR 83**	29,4b	31,3b
Stereum hirsutum	**WR 95**	27,6c	28,7c
Pycnoporus sanguineus	**WR 124**	25,8d	26,9d
	Treatment means	28,6y	29,8z

a, b, c, d Means of three replications. Means in the same column followed by the same letter do not differ significantly ($p \leq 0,05$; Tukey's test)

y, z Means of four treatments. Means in the same row followed by the same letter do not differ significantly ($p \leq 0,05$; Tukey's test)

Conclusion

All tree fungal strains were able to degrade lignin, as reflected by kappa number, at concentrations of CO_2 as high as 15 %.

APPENDIX G: INFLUENCE OF α-PINENE ON THE GROWTH OF FUNGAL STRAINS

Growth of different fungal strains in environments saturated with α-pinene in comparison with growth under atmospheric conditions.

Taxonomic affiliation and habitat	Strain	Colony diameter (mm)		Relative growth (%)[a]	Group means
		α-pinene	Control		
Ascomycetes Live hardwoods	*Botryosphaeria dothidea* CHS52	41,9	50,0	0,84	
	Ceratocystis virescens CMW3276	18,4	40,8	0,45	
	Cylindrocladium candelabrum CMW3911	30,6	42,3	0,72	
	Ophiostoma piliferum CMW2524	17,3	22,1	0,78	
	Ophiostoma quercus CMW2519	29,0	37,8	0,77	
	" " CMW2542	25,1	31,2	0,80	0,73
Ascomycetes Live softwoods	*Ceratocystis minuta* CMW1477	17,7	23,8	0,75	
	Ophiostoma ips CMW84	22,3	37,7	0,59	
	Ophiostoma minus CMW1993	30,9	13,8	2,25	
	Ophiostoma piceae CMW1367	21,0	20,1	1,09	
	Ophiostoma piliferum CMW2506	38,8	43,4	0,89	
	Sphaeropsis sapinea CWS30	18,3	29,3	0,63	1,03
Basidiomycetes Live softwoods	*Amylostereum aureolatum* A11	34,0	46,4	0,73	
	" " M7W	22,2	30,1	0,74	
	" " SN13.3B	32,1	28,6	1,12	
	Armillaria heimii CMW2717	8,6	11,3	0,77	
	" " CMW2742	10,4	19,8	0,53	0,78
Basidiomycetes Live hardwoods	" " CMW3164	10,7	10,2	1,05	
	Armillaria mellea CMW3179	13,9	14,3	0,97	
	" " CMW3974	6,7	11,3	0,59	
	Chondrostereum purpureum WR66	12,6	48,9	0,26	
	Cylindrobasidium laeve SCC282	18,5	36,3	0,51	
	Unidentified sp. WR251	6,5	39,9	0,16	0,59
Basidiomycetes Dead wood	*Ceriporiopsis subvermispora* CZ-3	7,8	42,4	0,18	
	Pycnoporus sanguineus WR330	12,8	49,6	0,26	
	" " WR124	8,1	38,7	0,21	
	Phanerochaete chrysosporium ATCC32629	6,0	49,2	0,12	
	Stereum hirsutum WR3	19,7	37,9	0,52	
	" " WR95	23,1	46,3	0,50	0,30

a Growth rate in an α-pinene saturated environment relative to growth under atmospheric conditions.