BIOTECHNOLOGICAL APPROACH TO THE MANAGEMENT OF EFFLUENTS FROM THE PULP AND PAPER INDUSTRY

L Christopher

WRC Report No. 763/1/07

Water Research Commission

Biotechnological Approach to the Management of Effluents from the Pulp and Paper Industry

Report to the

WATER RESEARCH COMMISSION

by

Prof. L. Christopher[a,b]

[a] Department of Chemical Engineering, Faculty of Engineering, Built Environment and Information Technology, University of Pretoria, Pretoria 0001

[b] Sappi Management Services (Pty) Ltd, Sappi SA Technology Centre, The Innovation Hub, Pretoria, PO Box 12796, 0028 Hatfield

WRC Report No: 763/1/07
ISBN No 978-1-77005-564-3

MAY 2007

DISCLAIMER

This report has been reviewed by the Water Research Commission (WRC) and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the WRC, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

EXECUTIVE SUMMARY

BACKGROUND

Efforts are made by the pulp and paper industry to reduce the chloroorganic/chloride discharges by the substitution of chlorine/chlorine dioxide with other more environmentally friendly bleaching agents. In response to the environmental concerns and stringent emission standards, modifications of the production process at the pulping and bleaching stages have been developed. Physical and chemical methods of removing chloroorganics, although quite effective in decolourisation of pulp and paper mills effluents, are unattractive for industrial applications because of the high costs. However, biological methods of effluent treatment have the advantage of being cost-effective and, in addition to colour, removal they can also reduce both BOD and COD of the effluent.

OBJECTIVES

- Development of a novel for the pulp and paper manufacture technology for bio-sulfite pulping of wood by using white-rot fungi.
- Development of a novel for the pulp and paper manufacture technology for biobleaching of sulfite pulp by using hemicellulolytic enzymes and reduced amounts of bleaching chemicals.
- Improving the quality of the effluents produced as a result of the application of the biopulping and biobleaching technologies and as a result of secondary treatment of the effluents.
- Investigation of the possibilities for the complex utilisation of the effluents as substrates for generating xylanases and/or for production of valuable by-products.

RESEARCH APPROACH

Decolourization and remediation of industrial waste waters from the pulp and paper industry was investigated following microbial pretreatment or bleaching with enzymes. The effluents under study were derived from the pulping and bleaching stages of pulp production. Acid sulfite pulping and elemental chlorine-free bleaching experiments were carried out under standardized conditions. Two industrial pulp types were examined for their bleachability with enzymes: hardwood acid sulfite pulp and hardwood soda-AQ (anthraquinone) pulp. Following pulping and bleaching, pulp properties such as brightness, viscosity and kappa number were determined according to the Standard Methods of the Technical Association of the Pulp and Paper Industry (TAPPI, Atlanta, USA). Most of the waste water analyses such as chemical oxygen demand and colour were carried out as described in the Standard Methods for Examination of Water and

Waste Water (APHA, American Public Health Association, Washington, DC, USA). The microbial pre-treatment of wood chips for biopulping was performed under solid-state fermentation conditions. The submerged fermentation experiments for enzyme production were carried out in shake flasks and evaluation of the cultivation conditions was based on the levels of xylanase activity produced. The efficiency of biosorption and microbial decolourization was evaluated mainly based on their impact on colour, chemical load and chlorinated organic matter.

RESEARCH

Biosulfite pulping

- Among a group of ten fungal species tested *Ceriporiopsis subvermispora* SS-3 was found the most promising isolate for future optimization studies of biosulfite pulping of *Eucalyptus grandis* wood chips as judged by results obtained on kappa number, alkali solubility S_{10} and S_{18}, brightness and pulp yield. Kappa number is used as a criterion for the oxidative potential and lignin content of pulps and is determined as the volume of 0.1 M potassium permanganate (ml) consumed by 1 g moisture-free pulp (corrected to 50% consumption of the permanganate). Alkali solubilities S_{10} and S_{18} provide information on the low molecular weight carbohydrates (degraded cellulose and hemicellulose) in pulp: a 10% sodium hydroxide solution dissolves both degraded cellulose and hemicellulose (S_{10}) whereas hemicellulose is soluble in 18% sodium hydroxide solution (S_{18}).
- Pretreatment with *C. subvermispora* SS-3 for ten days resulted in a substantial decrease in kappa number (by 29%) and increase in brightness (by 12%). On the other hand 5% of pulp yield was lost, and both viscosity and alkali solubility (S_{10} and S_{18}) were retained at levels comparable to the control.
- When the fungal incubation time was reduced to five days, a 5% reduction of kappa number, 11% increase in brightness and no loss of pulp yield were observed. Alternatively, cooking time or amount of pulping chemicals could possibly be reduced at a given pulp yield and savings in energy or chemicals obtained respectively.
- Beneficial biosulfite pulping could be performed without use of additional carbon source during the fungal pretreatment of chips. However, the use of inoculum levels of as high as 1% on chips (dry weight basis) may preclude the scale-up of the process.
- Fungal pretreatment of wood could improve the selectivity not only of pulping but of the bleaching process as well. As a result, the higher brightness of pulp could be translated in savings of bleaching chemicals which in turn would reduce the chemical load and AOX levels in the bleach plant effluent as well.

Biobleaching

- The impact of enzyme pretreatment of pulp on brightness was dependent on the particular xylanase preparation used. No relation seems to exist between the enzymatic release of reducing sugars from pulp, on one hand, and xylan removal from pulp or bleachability of pulps, on another. However, bleachability of sulfite pulp appears to improve in samples where xylan was enzymatically hydrolyzed and removed to a great extent.
- Brightness gain obtained as result of xylanase pretreatment of pulp seems to decline as the number of chemical bleaching steps increase. The impact of enzyme pretreatment on brightness was dependent on the particular bleaching sequence and regardless of the enzyme dose used.
- Chemical savings of up to 51% (as active chlorine) could be achieved when sulfite pulp was biobleached with *Aureobasidium pullulans* xylanases in sequence X-O$D_1E_0D_2$P. The dose of chlorine dioxide at D_1 stage could be reduced by up to 80% whereas at D_2 stage it was lowered by up 50%.
- Recent results indicate that the economics of the biobleaching process could be improved since enzyme charges of as low as 5 U xylanase/g pulp appeared to produce a brightness gain in bleaching of sulfite and soda pulps. Approximately 4.5 tonnes of pulp could be treated with 1 litre of Iogen HS-90 (22 731 U xylanase/ml). The charge per tonne of pulp would be about 0.220 ℓ or 0.250 kg of enzyme (density 1.15 kg/ℓ) and based on a price of US$12.0/kg of enzyme, the cost to treat a tonne of pulp would be US$3.0.
- The use of accessory xylan-degrading enzymes such as acetyl xylan esterases did not aid xylanase action on pulp, suggesting that these enzymes are not required in biobleaching. Furthermore, another enzyme, α-glucuronidase, was found inefficient on sulfite pulp, indicating that this enzyme does not play an important role in xylan degradation.

Effluent biotechnology

- As result of the reduction of the active chlorine charges during biobleaching of dissolving pulp, the chloride and AOX levels of the total bleach pulp effluent were reduced respectively by 31% and 38% whereas COD decreased by 22.5% at D_1, 7% at Eo and 20% at D_2.
- The bleach plant effluent from the alkali extraction stage (E_o) contained a fraction of coloured compounds (approximately 50% of total) which was adsorbable onto *Rhizomucor pusillus* biomass. The biosorption of colour was a rapid process which could remove approximately 90% of the adsorbable colour in the first few hours of effluent treatment. Treatment with *R.*

pusillus decolourised the effluent by 45-55% after 6-8 h and by 43% only after 2 h. On the other hand, treatment of E_O with the white-rot fungus *Trametes nivosa* 208 required a minimum of 3 days for efficient colour degradation and reduction of about 65-70%. A prolonged treatment for up to 7 days did not decrease further colour of effluent.

- It was demonstrated that the fungal biomass could be completely regenerated and reused on the effluent. This would be beneficial because of the large volumes of wastewater emanating from pulp and paper mills that require remediation.
- Conditions would have to be monitored to prevent microbial contamination, especially when prolonged treatment periods are used at lower temperatures. To avoid this, decontamination of Eo prior to fungal treatment would be necessary. *Rhizomucor pusillus* could grow and tolerate temperatures of up to 55°C and retain 85% of its decolouring abilities at 55°C as compared to 30°C. The use of elevated temperatures for effluent treatment would reduce the chances of microbial contamination and eliminate the need of cooling the effluent.
- Fungal treatment of E_O could substantially reduce the COD (20-30%), toxicity (80-130%) and AOX (30%) levels in the effluent. Results from fungal biotreatment of E_O were sensitive towards variation in the E_O composition and may vary from batch to batch depending on the initial pH, temperature and colour of E_O.
- This is the first report of using a mucoralean fungus in decolourisation of pulp and paper mill effluents. Biomass of *R. pusillus* proved to be efficient and compared well with other physico-chemical adsorbents in uptake of colour from the effluent. Under the conditions used in this study, the following descending order of colour removal was established: biomass (51%) > strong anion exchanger (48%) > activated carbon (37%) > chitosan (34%) > chitin (7%) > strong cation exchanger (4%).
- Results from preliminary experiments indicated that the bleach plant effluent could be used as a sole carbon source for xylanase production. In non-optimised conditions the effluent induced the production of cellulase-free enzymes in most of the strains tested. To the best of our knowledge, this is the first report of utilising a bleaching effluent from the pulp and paper industry for enzyme (xylanase) production.

CONCLUSIONS

- Biopulping of wood chips with selected white-rot fungi as well as biobleaching of sulfite pulp with xylanases are promising in terms of improving the quality of pulp and bleach plant effluent.

- Fungal pretreatment of the bleach plant effluent was shown to be effective in colour reduction of the effluent.
- The eventual implementation of these biotechnologies may lead to savings in chemicals and considerable reductions of the AOX/chloride-levels, toxicity and chemical load of the effluent.
- This work indicates the potential usefulness that some of the effluents have in the production of valuable by-products, in particular xylanases.

ACKNOWLEDGEMENTS

The research results presented in this report emanated from a project funded by the Water Research Commission and entitled:

"BIOTECHNOLOGICAL APPROACH TO THE MANAGEMENT OF EFFLUENTS FROM THE PULP AND PAPER INDUSTRY"

The Steering Committee responsible for this project consisted of the following persons:

Mr GN Steenveld	Water Research Commission (Chairman)
Mr FP Marais	Water Research Commission (Secretary)
Ms APM Oelofse	Water Research Commission
Prof CA Buckley	University of Natal
Prof BA Prior	University of the Orange Free State
Dr AL Kühn	IWQS Department of Water Affairs & Forestry
Prof LP Christov	Sappi Management Services
Mr NS Jory	Sappi Management Services
Mr A du Plooy	Sappi Management Services
Mr DA Weightman	Sappi Saiccor

The financing of the project by the Water Research Commission and the contribution of the members of the Steering Committee is gratefully acknowledged.

This project was only possible with the co-operation of many individuals and institutions. The author therefore wishes to express his sincere gratitude to the following:

- The Water Research Commission, for funding this project,
- Sappi Saiccor and Sappi Management Services, for the continuous technical and financial support,
- Sappi Management Services, for giving permission to publish this work,
- Greg Steenveld (Water Research Commission), for his fruitful discussions and critical comments made throughout the duration of this project,

- Derek Weightman and John Thubron (Sappi Saiccor), for the technical advice and assistance in the pulping experiments and pulping related analyses,
- Neville Jory, Attie du Plooy, Mike Birkett and Ron Braunstein (Sappi Management Services), for technical advice and assistance in analysing pulp viscosity and effluent AOX,
- Prof. James du Preez (University of the Orange Free State), for his assistance in performing the fermentation experiments and critical reviewing of the manuscript,
- The research group of the Sappi Biotechnology Laboratory, for the excellent technical execution of the work programme.

TABLE OF CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Meaning	Units
AOX	Absorbable Organic Halides	$g\ l^{-1}$
BOD	Biochemical Oxygen Demand	$g\ l^{-1}$
C	Chlorine	
COD	Chemical Oxygen Demand	$g\ l^{-1}$
D	Chlorine Dioxide	
DP	Degree of Polymerisation	
E	Alkaline Extraction	
ECF	Elemental Chlorine-Free	
Eo	Alkaline Extraction in Oxygen Atmosphere	
H	Sodium Hypochlorite	
LiP	Lignin Peroxidase	
MnP	Manganese-Dependent Peroxidase	
O	Oxygen	
P	Hydrogen Peroxide	
PCU	Platinum Cobalt Units	
RS	Reducing Sugars	$g\ l^{-1}$
S_{10}/S_{18}	Alkali solubility of pulp	%
SS	Suspended Solids	$g\ l^{-1}$
SSL	Spent Sulfite Liquor	
TCF	Totally Chrorine-Free	
TDS	Total Dissolved Solids	$g\ l^{-1}$
TOCl	Total Organically Bound Chlorine	$g\ l^{-1}$
TOX	Total Organic Halides	$g\ l^{-1}$
TS	Total Solids	$g\ l^{-1}$
TSS	Total Suspended Solids	$g\ l^{-1}$
X	Xylanase	

GLOSSARY OF SPECIALIZED TERMS

Adsorbable organic halogen (AOX): Measure of the organically bound halides (chlorides, bromides, etc) in a wastewater sample.

Bleaching: A chemical process applied to cellulosic materials to increase their brightness.

Biobleaching: Use of biological means (enzymes or microorganisms) to pretreat pulp prior to chemical bleaching with the primary objective of increasing brightness.

Brightness: The reflectance of visible light at 457 nm from cellulosic pulp fibres formed into sheets.

Chemical oxygen demand (COD): Chemical test to determine the oxygen demand of all organic matter present in a sample of wastewater.

Polyoxometalates (POM): Hetreropolyacids with a metal-oxygen octahedral as the basic structural units.

Pulping: A mechanical or chemical process applied to fibrous lignocellulosic materials to separate fibres or degrade and remove lignin from the fibres. Chemical pulping produces alkaline (kraft) and acid (sulfite) pulps.

Spent sulfite liquor (SSL): Liquor separated from the pulp following a sulfite pulping, containing the residual cooking chemicals and dissolved constituents of the wood (predominantly lignosulfonates, monocarbohydrates, volatile organic acids).

Xylan: Heteropolysaccharides consisting of xylose units forming a xylan backbone and side chains of arabinose, acetic and methylglucuronic acids; xylan content in hardwoods and softwoods varies between 20 to 35%.

Xylanase: Carbohydrate hydrolyzing enzymes with high specificity towards xylan.

Xylanase activity: One unit (U) of xylanase activity is defined as that amount of enzyme which catalyses the release of one µmol of xylose equivalents per min of reaction.

LIST OF TABLES

Page

LIST OF FIGURES

CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

South Africa is a major international producer of pulp and paper products. With a production of 1,860 million tonnes of pulp during 1991 it ranked twelfth amongst the world's top producers. (Gerischer 1993). In 1992 South Africa was the 10th largest producer of pulp and 22nd largest producer of paper and board in the world (Bethlehem 1995). The industry contributes at least 3% of the gross national product and is a major employer.

Of the five major pulp and paper companies in South Africa, the South African Pulp and Paper Industry (Sappi) is supplying half South Africa's requirements and is already the largest papermaker on the African continent. Sappi Saiccor produces 15% of the world's dissolving pulp. The end uses of dissolving pulp include cellophane and rayon (after regeneration of cellulose from cellulose xanthate), cellulose esters (acetates, nitrates, propionates and butyrates), cellulose ethers (carboxymethyl cellulose, methyl and ethyl cellulose), graft and cross-linked cellulose derivatives (Biermann 1993). For instance, the cellulose acetates are widely used in films, eyeglass frames, cigarette filters, etc. whereas the carboxymethyl cellulose can find applications as a thickener, detergent, in cosmetics, etc. In contrast to pulps used for papermaking, the hemicellulose in dissolving pulp is undesirable and currently removed together with lignin during pulping and bleaching. However, part of hemicellulose (mainly xylan in hardwoods and mannan in softwoods) remains in pulp after bleaching causing certain problems in the viscose process (Hinck et al., 1985). The strength properties of the viscose end product are strongly influenced by the hemicellulose content of dissolving pulp. The higher the degree of polymerisation (DP) of pulp the higher the end product strength. The efficiency of conversion of cellulose into the specific derivative is dependant upon the xylan and mannan content of dissolving pulp as well. High viscose yields require low hemicellulose levels in cellulose. Also, a reduction in the fibre swelling and penetration rate of alkali into the cellulose fibres is caused by excessive amounts of hemicellulose present in pulp. The optical properties of cellulose acetates and nitrates can be affected negatively by the hemicellulose contaminants in cellulose. On the other hand high brightness levels are required in dissolving pulp production indicating that the cellulose pulp must be virtually lignin-free. Thus, the lignin and hemicellulose in dissolving pulp processing have to be degraded and removed substantially to ease the conversion of cellulose to its end-use product.

1.2 ENVIRONMENTAL ASPECTS

Unfortunately, the pulp and paper industry is also a major contributor to the pollution problems of this country. Chlorination is generally one of the stages of pulp bleaching. It involves addition of chlorine or chlorine dioxide and oxidation to remove residual lignin in the pulp. Chlorine and chlorine dioxide react with lignin in pulp to form chlorinated organic compounds (Sjöström 1981). The effluents containing the chlorolignins are highly coloured and can cause serious environmental problems because some of these compounds are in part toxic and mutagenic. In addition a high concentration of chlorides in the effluents contributes to their corrosiveness. The absence or reduction of chlorides allows the use of recovery-type processes with recycling wash waters. The colour is mainly due to the presence of solubilised lignin and its derivatives. Kraft bleach plant effluents originating from the first alkali extraction stage (E) contribute 80% of the colour, 30% of biochemical oxygen demand (BOD) and 60% of chemical oxygen demand (COD) to the mill's total pollution load, although their volume is very low (Mehna et al., 1995). Low molecular weight components of E effluent comprise various chlorinated organics of which the chlorinated phenols are the major component. Some of the chlorolignins of low molecular mass have mutagenic and toxic properties and are known to be highly resistant to biodegradation and to accumulate in aquatic organisms (Ander et al., 1977).

1.3 ALTERNATIVES FOR THE PULP AND PAPER INDUSTRY

The release of the bleach plant effluents with high absorbable organic halogen (AOX) levels into the receiving waters has become one of the major environmental problems for the pulp and paper industry in recent years. Efforts are made to reduce the chloroorganic/chloride discharges by the substitution of chlorine/chlorine dioxide with other more environmentally friendly bleaching agents. In response to the environmental concerns and stringent emission standards, modifications of the production process at the pulping and bleaching stages have been developed. These imply extending the cooking time for additional lignin removal, introduction of oxygen delignification as a pretreatment step or elemental chlorine-free (ECF) and totally chlorine-free (TCF) bleaching. Physical and chemical methods of removing chloroorganics, although quite effective in decolourization of pulp and paper mills effluents, are unattractive for industrial applications because of the high costs. However, biological methods of effluent treatment (e.g. with microorganisms) have the advantage of being cost-effective and in addition to colour removal they can also reduce both BOD and COD of the effluent (Eaton et al., 1980).

1.4 BIOTECHNOLOGICAL APPROACH

Another alternative to pulping and bleaching (hemicellulose and lignin removal from wood and pulp, respectively) is to apply enzymes (xylanases or lignin-degrading enzymes) or microorganisms (white-rot fungi) to alleviate the heavy chemical loads during pulping and bleaching. Some of the advantages resulting from the possible implementation of the biotechnological methods in the manufacture of bleached pulp would be: savings in chemicals for pulping and bleaching; improvements in pulp quality; improvements in effluent quality.

Extensive research on biopulping and biobleaching of sulfite pulps to produce dissolving pulp has been conducted at the Sappi Biotechnology Laboratory, Department of Microbiology and Biochemistry, University of the Orange Free State in Bloemfontein for the last six years (Christov and Prior 1993; 1994; 1995; 1996; 1997; 1998; Christov and Wolfaardt 1995; Christov and Akhtar 1996; Christov et al., 1996; 1997; 1998; Ferraz et al., 1998; Mosai et al., 1998). The major aim of our biotechnology programme at the Sappi Biotechnology Laboratory was to develop a new bioprocess for production of dissolving pulp, the implementation of which would lead to positive changes in the dissolving pulp properties as well as mill effluent quality in a cost-effective way. The original objectives of the project are as follows:

- Development of a novel for the pulp and paper manufacture technology for bio-sulfite pulping of wood by using white-rot fungi.
- Development of a novel for the pulp and paper manufacture technology for biobleaching of sulfite pulp by using hemicellulolytic enzymes and reduced amounts of bleaching chemicals.
- Improving the quality of the effluents produced as a result of the application of the biopulping and biobleaching technologies and as result of secondary treatment of the effluents.
- Investigation of the possibilities for the complex utilization of the effluents as substrates for generating xylanases and/or for production of valuable by-products.

The outline of the report follows the structure as indicated below:

1. Biosulfite pulping of eucalyptus wood

- screening of white-rot fungi for biosulfite pulping

- optimization of biopulping treatment conditions
- effect of biopulping on bleaching of dissolving pulp

2. Biobleaching of pulp with xylanases

- reduction of active chlorine charges in biobleaching
- optimization of xylanase charges on pulp
- studies on other hemicellulolytic enzymes involved in biobleaching

3. Modifying effluent quality

- effect of xylanase pretreatment on the AOX/chloride content of bleach plant effluents
- screening for microorganisms and optimization of treatment conditions for colour removal from Eo effluent
- effect of fungal treatment on properties of Eo effluent
- effluent utilization for xylanase production

CHAPTER 2
BIOTECHNOLOGY IN THE PULP AND PAPER INDUSTRY

2.1 BIOPULPING

2.1.1 Introduction

Biopulping is the fungal pretreatment of wood chips, designed as a solid state fermentation process, for production of mechanical or chemical pulp. The concept of biopulping is based on the ability of some white-rot fungi to colonize and degrade selectively lignin in wood thereby leaving cellulose relatively intact.

2.1.2 Mechanism of biopulping

The biochemical mechanism of biopulping is not yet understood, but it is suggested that the extracellular lignin-degrading enzymes (lignin peroxidase, manganese peroxidase and laccase) secreted by the white-rot fungi are involved. As the delignification process with preferential lignin-degrading white rot fungi proceeds, lignin and hemicelluloses in the secondary cell walls and subsequently the middle lamellae are solubilised and removed (Messner and Srebotnik 1994). However, it is not clear yet how the ligninases penetrate from the lumen through the cell wall to the middle lamella, as the pore sizes in wood do not permit any access of enzymes at all. Therefore, it is possible that low molecular weight compounds (mediators) are also secreted and activated by the fungus or enzymatically, which could be responsible for the attack, modification and solubilisation of lignin (Call 1993; Messner and Srebotnik 1994).

2.1.3 Evaluation of biopulping

One of the main criteria used in selection of a microoorganism suitable for biopulping is the ratio of weight loss of wood to lignin loss (Otjen et al., 1987). The smaller this ratio is, the higher the selectivity in lignin degradation is. Important factors in selection are the percentage losses of cellulose and hemicellulose, expressed as glucose and xylose loss, respectively (Blanchette et al., 1988). The first one should be kept as low as possible, whereas the latter may vary depending on the desired pulp properties. The morphological and chemical changes in the cell walls after fungal pretreatment have been evaluated using staining techniques (Srebotnik and Messner 1994) and scanning electron microscopy (Blanchette 1995).

2.1.4 Requirements in biopulping

There are certain process conditions and design requirements necessary to gain a biopulping effect (Wall et al., 1993). Biopulping can be carried out in bioreactors of different type such as rotating drums, stationary trays, and static bed depending on the requirements the particular microorganism would have for optimal results. Temperature has to be controlled at the level of the temperature optimum for the particular fungal strain used in biopulping. Aeration with humidified air is necessary in biopulping, as a solid state fermentation process, because white-rot fungi degrade lignin by oxidation. High moisture content (at about the fiber saturation point) should be kept in wood chips during biopulping to ensure an optimal colonization and penetration of fungal hyphae (Blanchette et al., 1991); however, very high moisture content would slow down the rate of the mass transfer between oxygen and chips. In some cases the degree of asepsis should be controlled (e.g. by autoclaving, steaming, chemical pretreatment of chips) or monitored to ensure a preferential lignin degradation by the particular fungal strain used depending on its resistance against contamination and ability to compete with the microbial flora available in the chips. Some nutrient supplementation including glucose, nitrogen, minerals and vitamins could be provided to the chips in order to protect against non-specific cell wall degradation, especially of cellulose, and therefore to increase the selectivity of lignin degradation (Labosky et al., 1991); however, some results indicate a positive biopulping effect on nutrient-unsupplemented wood chips as well (Dawson-Andoh et al., 1991; Wall et al., 1993). The time of fungal treatment needs also to be optimized as the selectivity of lignin degradation tends to decline with increase of incubation periods (Otjen and Blanchette 1986). The type (spores, disrupted mycelia, colonized chips, solid inocula) and size of inoculum can be a factor in determining the efficiency and economic feasibility of biopulping.

2.1.5 Biopulping for papermaking

2.1.5.1 Biomechanical pulping

Biomechanical pulping is directed towards production of mechanical pulps for papermaking with decreased energy requirements for fibrillation and refining and improved strength properties. This process can save energy and increase paper strength properties by up to 50% as compared to the traditional mechanical pulping accomplished without use of fungi (Akhtar et al., 1993). However, the disadvantages of the biopulping process are in the reduction of the optical properties (up to 20%) and yield of the paper product (up to 10%). For instance, treatment of Loblolly pine (*Pinus taeda*) with the white-rot fungus *Ceriporiopsis subvermispora* strain Fp-90031 for two to four weeks resulted in production of paper with reduced refining requirements by 42% in a stationary type bioreactor and 32% in a rotating bioreactor (Blanchette et al., 1991). At the same time, burst and tear index were increased by 32 and 67% (stationary reactor), and 29 and 71% (rotating reactor). When *C. subvermispora* strain CZ-3 was used, energy savings in production of paper from Loblolly pine amounted at 30% (Fischer et al., 1994).

2.1.5.2 Biokraft pulping

Very little information on biokraft pulping is available. It was reported, however, that biokraft pulping of *Phanerochaete chrysosporium*-degraded aspen can increase pulp yield at a given kappa number by 3-6%, reduce the beating time, and improve the tensile strength, burst and fold properties over the control. At the same time there were distinct reductions in tear and brightness for handsheets of fungally degraded wood (Oriarian et al., 1990). Significant improvement of the physicomechanical properties of the handsheets was observed for both Douglas-fir and red-alder kraft pulps pretreated with *P. chrysosporium* (Dawson-Andoh et al., 1991). Using the same microorganism to treat aspen, Chen and Schmidt (1995) studied a novel technology of for fungal pretreatment without prior sterilization of wood chips. These authors also reported substantial improvements in certain paper properties (burst strength) and refiner energy savings. Kappa number reduction but also pulp yield loss and increase in alkali consumption in biokraft pulping of pine with a number of South African isolates was also reported (Wolfaardt et al., 1996).

2.1.5.3 Biosulfite pulping

Production of paper was also studied using biosulfite pulping with *C. subvermispora* TUB 51 (Messner et al., 1992). After fungal pretreatment of birch and spruce wood chips for 2 weeks and chemical pulping, kappa number was reduced by 33 and 30%, respectively. Brightness of paper sheets from birch was boosted by approximately 4% whereas paper strength (tensile and tearing resistance) decreased by 8-12%. Compared to *C. subvermispora*, *P. chrysosporium* was not so effective in biosulfite pulping of birch (12% kappa number reduction in 2 weeks pretreatment time) and spruce (0%). When *C.subvermispora* CZ-3 was applied on spruce chips for 2 weeks, kappa number after the sulfite cook was reduced by 22% (Fischer et al., 1994). No significant changes in paper strength properties after sulfite pulping were observed as compared with the control. Biosulfite pulping of loblolly pine with *C. subvermispora* CZ-3 resulted in a 48% reduction of kappa number at the same pulp yield and amount of chemicals consumed as the control (Scott et al., 1996). A 30 min shorter cooking time was necessary for the biotreated samples to reach the kappa number of the control which could be translated in some energy savings for pulping.

2.1.5.4 Biopulping for dissolving pulp production

Due to the selective modification of lignin in wood by the white-rot fungi its degradation and removal using pulping and bleaching chemicals becomes easier. This advantage of biopulping could be used not only in the papermaking but in dissolving pulp production as well (Christov et al., 1998). The first experiment on fungal pretreatment of eucalyptus wood chips (*Eucalyptus grandis*) for biosulfite pulping was carried out using *C. subvermispora* L-14807 SS-3 as part of the collaborative work between the Forest Products Laboratory in Madison, Wisconsin, USA and Sappi Biotechnology Laboratory in Bloemfontein, South Africa (Christov and Akhtar 1996).

2.2 BIOBLEACHING

2.2.1 Introduction

The bleaching of kraft and sulfite pulps for dissolving pulp manufacture can be regarded as a purification process aimed at final delignification, and increase in the α-cellulose content and brightness. It involves the use of a number of bleaching chemicals which with regard to environmental aspects can be classified into non-chlorine containing reagents such as oxygen, ozone, hydrogen peroxide or chlorine-containing reagents such as elemental chlorine, chlorine dioxide and sodium or calcium hypochlorite (Fengel and Wegener 1984). Bleaching of pulp with the latter group causes the formation of chlorolignins as well as low molecular weight aromatic and aliphatic compounds, many of which, especially the chlorinated phenols, catechols and guaiacols are toxic to the receiving waters.

In order to reduce the negative environmental impact of bleaching, new pulping and bleaching techniques are being developed. The aim is to minimize or eliminate the use of chlorine-containing agents thereby implying extended cooking time or non-chlorine bleaching. An alternative approach is to use biological systems such as enzymes and microorganisms which can selectively remove hemicelluloses and/or lignin from pulp without affecting the cellulose.

2.2.2 Biobleaching of kraft pulps

Most of the research effort has been focused on the biobleaching of kraft pulps. For instance, xylanases have been shown to improve pulp properties such as breaking length, fibrillation, porosity and beatability, viscosity, brightness (Viikari et al., 1994). Pretreatment with xylanases was reported to enhance kraft pulp bleachability and reduce the amount of total active chlorine needed in bleaching by approximately 15%.

According to the proposed mechanism of biobleaching of kraft pulps with xylanases (Kantelinen et al., 1993) the enzyme can hydrolyze that fraction of xylan that has been reprecipitated on the fiber surface during the kraft pulping. This enables the bleaching chemicals to attain an improved accessibility to residual lignin resulting in enhanced lignin degradation and diffusion from pulp. In addition, the size of the lignin-carbohydrate complexes will be reduced by the enzyme action which would then improve their diffusion in the alkali extraction step (Paice et al., 1992). Only about 20% of xylan in birch kraft pulps could be removed even when using very high loadings of *Trichoderma reesei* xylanases (Kantelinen et al., 1993). Possible reasons for this could be the modifications in the structure and rearrangements and aggregations of the xylan chains during its readsorption on the cellulose fibers.

2.2.3 Biobleaching of sulfite pulps

Little attention has been paid to enzymatic prebleaching of sulfite pulps for dissolving pulp production in the past (Paice and Jurasek 1984). It was found that the enzymatic hydrolysis of xylan in dissolving pulp is limited by the poor accessibility of the substrate (Christov and Prior 1993). When unbleached sulfite pulp of eucalyptus wood was treated with *Aureobasidium pullulans* xylanases in non-optimized conditions, the pentosan content and kappa number decreased by up to 35 and 24%, respectively, if very high enzyme charges and prolonged incubation times were applied (Christov and Prior 1994). Using triple-repeated consecutive xylanase-oxygen treatments the pentosan content of sulfite pulp was reduced two-fold, the kappa number decreased by 60%, brightness was enhanced by 18 points, and the α-cellulose content was enriched by 3 points (Christov and Prior 1996).

Significant amounts of hemicellulose (xylans and glucomannans) are dissolved in the sulfite pulping process. The bonds between the pentose units (arabinose and xylose) are hydrolyzed much more rapidly than the glycopyranosidic ones. On the other hand the glucuronic acid-xylose and xylose-acetic acid linkages are relatively more resistant to the acid hydrolysis conditions and little cellulose is lost in the sulfite pulping (Sjöström 1981). Therefore the degradation products of the hemicellulose acid hydrolysis would appear in the cooking liquor in the following approximate order: *arabinose*> *galactose*> *xylose*> *mannose*> *glucose*> *acetic acid*> *glucuronic acid.* In the above order glucose would be the hydrolysis product originating mostly from the glucomannan rather than cellulose polymer. Also, the glucomannans are more resistant to acid dissociation than xylans. Hence, in sulfite cooking of birch only 45% of the original xylan remains in pulp after 20 min cooking and its original DP of 200 is reduced to less than 100.

During acid sulfite pulping, redeposition of xylans onto the fiber surface has not been observed (Annergren and Rydholm 1959). The possible reasons for this would be that the harsh cooking conditions and presence of acid-resistant residual acetyl and 4-*O*-methylglucuronic acid groups function as a barrier against the adsorption and intercrystallisation of xylan onto the cellulose micromolecules. Therefore the remaining xylan in sulfite pulp is less accessible and the effects produced on hemicellulose by xylanases can be regarded as a result of changes in the pulp fibers rather than on the fiber surface only (Christov and Prior 1996). Hence, the xylan localised in the primary and mainly secondary cell walls as well as the xylan fraction involved in lignin-carbohydrate formations may be enzymatically attacked (Christov et al., 1996). Major factors influencing the degree of enzymatic removal of xylan from sulfite pulp would be the size, i.e. penetration capabilities of xylanases and their substrate specificity on one hand, and the accessibility, physical and chemical state of the xylan substrate, on the other.

2.2.4 Benefits, problems and trends in biobleaching on large scale

Since biobleaching with xylanases was first reported in 1986 (Viikari et al., 1986), a number of mill trials both in Europe and North America have been conducted (Turner et al., 1992). The first industrial application of xylanases was carried out in Finland in 1991. In 1994 it was reported that 18 bleaching mills in Canada have run xylanase trials and six are regular users of the enzyme to treat 750,000 tonnes of pulp representing 8% of Canada's bleached kraft pulp production (Tolan et al., 1996). The benefits of using xylanases have been mostly the economic and environmental advantages which include: 1) savings in bleaching chemicals; 2) increased throughput; 3) improved pulp properties such as brightness and strength properties; 4) marketing advantage; 5) easy adaptation to different bleaching sequences with minimal investment costs; 6) improved effluent with reduced AOX content. On the other hand, some problems have been encountered associated mainly with corrosion of equipment, maintenance of residence time of xylanase treatment, pulp yield loss and bleach plant control. However, the xylanase bleaching technology at present is being further developed towards increasing the pH and temperature stability of xylanases, use of enzyme mixtures with supplementary hemicellulolytic accessory enzymes to enhance xylanase efficiency; improving the competitiveness of xylanases with respect to enzyme price, optimization of both ECF and TCF bleaching in conjunction with xylanases (Viikari et al., 1994).

2.3 EFFLUENT BIOTECHNOLOGY

2.3.1 Introduction

The pulp and paper industry is one of the major users of water and their effluent streams usually contain halogenated organic materials that pose environmental problems (Wang et al., 1992; Kallas and Munter 1994). Chlorinated organic compounds are produced during the chlorination bleaching phase. These are made soluble in dilute alkali and extracted from the pulp in the subsequent extraction phase (Prasad and Joyce 1991). Chloroorganics tend to persist in nature because of their inherent recalcitrance; they are often toxic to aquatic live; many are genotoxic and have the potential to migrate widely throughout the ecosystem, ultimately accumulating in the fatty tissues of organisms (Suntio et al., 1988). Legislation has been introduced to limit the effluent loads of these toxic compounds (Sierka and Bryant 1994). Furthermore during bleaching treatments chromophoric, highly oxidised, polymeric lignin derivatives are formed that give rise to a dark colourisation in the effluent (Livernoche et al., 1983; Bergbauer et al., 1991). The colour poses an aesthetic problem and contributes to BOD (Bajpai and Bajpai 1994). Conventional biological treatments cannot effectively remove the colour and current research efforts are directed into solving this problem (Boman and Frostell 1988; Prasad and Joyce 1991). The reason why traditional methods have failed can be attributed to the recalcitrance of chlorolignins and the difficulty in mineralising these compounds (Mehna et al., 1995). Fortunately white rot fungi have the ability to degrade lignin and its chlorinated derivatives effectively (Eriksson 1991; Bajpai and Baipai 1994). However lignin cannot serve as the sole carbon source for these fungi because its degradation is apparently a very energy intensive process (Leisola et al., 1983; Boman and Frostell 1988). The specific cultivation conditions necessary for white rot fungi to degrade lignin (Boman and Frostell 1988), have led to different strategies to overcome these difficulties. Various cultivation methods have been devised. For instance: the patented Mycor process, continuous flow systems (Prasad and Joyce 1992), immobilisation of white rot fungi (Livernoche et al., 1983; Kirkpatrick et al., 1990), etc. Other organisms , such as soil inhibiting fungi imperfecti (Rodriques et al. 1996), algae (Lee et al., 1978), and streptomyces strains (Hemandez et al., 1996) have also been studied for their ability to decolourise chromophoric substances. White rot fungi produce a variety of ligninolytic enzymes, including peroxidases and laccases (Lamar 1992). The mechanism whereby these fungi degrade lignin is still not fully understood (Manzanares et al., 1995). It has been demonstrated that these enzymes can convert/biotransform phenolic/polyphenolic substances and this has led to interest in the use of these enzymes to treat waste waters (Paice and Jurasek 1984; Roy-Arcand and Archibald 1991; Al-Kassim et al., 1994; Limura et al., 1996). The use of membrane reactors for the immobilisation of biomass and/or enzymes are a new and promising area in bioremediation (Van der Walt et al., 1997). Immobilisation leads to increased stability, and allows continuous use of enzymes. Many studies indicated that adsorption of coloured compounds on biomass occurred during cultivation (Livemoche et al., 1983; Royer et al., 1985; Feijoo et al., 1995). Of interest is also the observation that oxidised phenolic compounds have a high affinity for chitosan, a compound of certain fungal cell walls (Muzzarelli 1994). Chitosan exhibits polycationic characteristics at acidic pH-levels that could facilitate sorption of anionic substances. Attention has been focused recently on the use of a chitosan coated hollow-fibre membrane process for the bioremediation of a phenolic containing effluent (Edwards et al., 1997). Recent studied indicate that certain physical/chemical methods have improved cost-wise and therefore hold promise for the treatment/pre-treatment of effluent (Kallas and Munter 1994). A variety of physical and chemical treatments have been tested. These include: adsorption using ion exchange resins or activated carbon, ultra-filtration, chemical precipitation (Boman and Frostell 1988), oxidation (ozonation, hydrogen peroxide, ect.) using chemical methods (Feijo et al., 1995), and photo-catalysis, involving irradiation of titanium oxide (Sierka and Bryant 1994). Manzanares et al., (1995) observed that the problem involving the colourisation of paper mill effluent has not been solved. This reflects, to a great extent, the technical difficulties encountered when cultivating white rot fungi and the problems of setting up an effective but also an economic process for the bioremediation of paper and pulp effluents.

2.3.2 Chemical composition of effluents

The content of chlorinated substances in effluent is usually measured as total organically bound chlorine (TOCl) or adsorbable organic halogen (AOX) (Eriksson 1993). Chloroorganic material from pulp mill effluent is generally described in terms of their molecular mass. The molecular weight of these materials has serious implications in terms of biodegradability, toxicity and colourisation (Martin et al., 1995; Jokela and Salkinoja-Salonen 1995).

2.3.2.1 High molecular mass compounds

More than one-half of the colour load from bleach plant effluent originates from high molecular weight lignin-derived material in the alkaline extraction stage (Paice and Jurasek 1984). Effluent from the extraction stage accounts for 80% of the colour, 30% of the BOD and 60% of the COD of the mill pollution load (Prasad and Joyce 1991). The high molecular mass substances are generally believed to be stable and therefore biologically inert (Jokela and Salkinoja-Salonen 1992). However, Eriksson et al., (1985) reported that high molecular mass material in spent liquors from the chlorination and alkali extraction stages of bleachery softwood kraft pulp were chemically unstable under conditions prevailing in receiving water systems. These materials slowly released chlorinated catechols and guaiacols (Eriksson et al., 1985). Martin et al., (1995) concluded that conflicting results on the aromatic nature of high molecular mass compounds are prevalent, but literature reveals both association by adsorption and chemical bonds might account for the presence of some chloroaromatics in high molecular weight material. Therefore there seem to be evidence both for adsorption, as well as, chemical decomposition, as sources of chloroaromatics from high molecular mass compounds. The ultimate predestination of high molecular weight compounds in effluent, during biological treatment, is still unknown. Working with high molecular mass fractions (> 30000 Dalton), Bergbauer et al., (1991) observed that sequential depolyrnerisation does not necessarily lead to complete degradation of these aromatic compounds. Furthermore once degradation of lignin derivatives by *Trametes versicolor* had reached a certain level, neither additional co-substrates, nor inoculation of the culture filtrate with fresh mycelium, could induce any further degradation. Thus, it seems that the end result of using this fungus could be the accumulation of highly recalcitrant compounds in the ecosystem.

2.3.2.2 Low molecular mass compounds

Compounds with a relative molecular mass lower than l000g/mol are acutely toxic to aqueous organisms (Roy-Arcand and Archibald 1991). These substances include chlorinated phenols, quaiacols, catechols (Limura et al., 1996) as well as chlorosyringols and chloroaliphatics (Roy-Arcand and Archibald 1991). Suntio et al., (1988), in a review covering the nature and properties of chemicals in pulp mill effluents of relative low molecular weight (< l000g/mol), divided these compounds in the following categories: I. acidic compounds, II. phenolic compounds, III. neutral compounds. Chlorinated phenols in bleach effluents are typical chlorinated lignin-degradation products that contain methoxyl groups. The predominant compounds of the neutral fraction are chloroform, chlorodimethylsulfones, and chloroacetones, while minor concentrations of additional chlorinated hydrocarbons, ethers, ketones, aldehydes, lactones and thiophenes have been detected (Smith et al., 1994). Chlorinated acidic compounds present in bleaching effluent are largely aliphatic acids, mainly chloroacetic acid. Although a variety of low molecular substances do occur in the effluent streams, most organic chlorine present is associated with high molecular mass material (Smith et al, 1994).

2.3.3 Cultivation strategies for decolourisation of effluents

None of the traditional biological methods can degrade high molecular mass chromophoric compounds effectively. These methods include aerated lagoons, activated sludge systems and anaerobic cultivation (Boman and Frostell 1988; Archibald et al., 1990). Aerated lagoons can reduce TOCl by about 25%, whereas activated sludge systems can reduce chlorinated compounds by 40%. However aeration costs are high and in the case of activated sludge systems, it seems as if a substantial part of the chloroorganics are adsorbed onto the surface of the biomass, which could lead to disposal problems of excess sludge. Ultra-filtration of the E-stage effluent seems promising, but this will also result in a disposal problem (Boman and Frostell 1988). Anaerobic treatment have gained popularity in the pulp and paper industry because of low energy consumption and the low production levels of well stabilised excess sludge. However bleach plant effluents are more diluted than desired for a low retention time and varying effluent composition can lead to toxic shock conditions, detrimental for the efficient functioning of the anaerobic systems. By combining ultra-filtration of the E-stage effluent and incorporating the permeate with the C/D effluent, followed by anaerobic filter treatment, an overall reduction of 62% in chloroorganics was achieved (Boman and Frostell 1988). However an effective biotreatment system must degrade both high and low molecular weight compounds and this can be achieved by white rot fungi. One major drawback is the need for the addition of an easily degradable, inexpensive carbon source and the complicated physiological demands of some of these fungi when degrading lignin (Boman and Frostell 1988). The requirements for high oxygen tension and a growth substrate impede the practical implementation of fungal decolourisation (Eaton et al., 1982). Various parameters and cultivation strategies need to be considered in an effort to develop a successful treatment process.

2.3.3.1 The Mycor process

In the Mycor-process the fungus *P. chrysosporium* is immobilised on the surface of rotating disks that can be enclosed for additional oxygen supply. A growth stage is necessary before decolourisation commences, during this stage nutrient nitrogen is depleted and the fungus become ligninolytic. The Mycor process reduced colour in an alkaline stage spent liquor by 80% and furthermore converted 70% of the organically bound chlorine into chloride at a hydraulic retention time of 2 days (Boman and Frostell 1988). Eaton et al., (1982) described a system, called a FPL/NCSU Mycor method and proposed that a fixed film Mycor reactor could be charged with nutrients which could include primary sludge. Although steam treatment initially might be beneficial, further aseptic procedures would not be required with fungi like *P.*

chrysosporium. Effluents with temperatures varying between 28°C to 40°C and pH 4.5 could be treated. In laboratory scale bench reactors operation was achieved for over 60 days. Some limitations of the Mycor process seem to be the poor surface to volume ratio since mycelium is in constant contact with substrate to an extent of only about 40%. Moreover the mycelium is present in a thick layer that could result in deficient oxygen and nutrient supply and a lower productivity in general.

2.3.3.2 Continuous-flow systems

Three continuous-flow laboratory decolourisation strategies were examined (Prasad and Joyce 1991). Alternative I consisted of a set up similar to a conventional oxidation basin with a surface area of 5l0 cm^2 for fungal cultivation. Fungal mats of *Trichoderma* sp, were transferred to the reactor and decolourisation was carried out without aeration. Alternative II utilised a vessel in which the height was increased by decreasing the surface area and four baffles were introduced to divide the vessel into compartments. *Trichoderma* packed in synthetic wire bags were suspended in the middle of each zone and continuous aeration supplied. Alternative III was similar to the Mycor process in that fungal mats were clasped between circular wires, supported by outer central rings and securely fixed in a metallic flame. The discs were rotated continuously. These systems all could reduce colour by at least 50% for the first 6 days, but alternative III gave the best results with a greater than 78% total colour removal from extraction-stage effluent as well as a COD reduction of 25%. Colour removal targets in excess of 57%, was sustained for 18 days with alternative III.

2.3.3.3 Fungal pellets

Royer et al., (1985) used *Coriolus versicolor* in the form of mycelial pellets to decolourise lignin-containing kraft E-stage effluent. Both adsorption and oxidation generally proceeded best between pH 4 and pH 5 and at temperatures of 25 °C to 30°C. Decolourisation was practically non-existent at 40°C, which corresponded to the temperature of the liquor effluent at the E-stage. Magnesium ions improved the consumption of chromophores which might indicate that an enzymatic mechanism take place since magnesium ions are activators of many enzymes. At an initial colour level of 7000 colour units, the mean colour removal proceeded at a rate of 300 CU g^{-1} mycelium h^{-1}. The authors suggested the use of airlift reactors employing the pelleted form of the fungus with the possibility of minimising operational costs. This strategy would facilitate recycling and the use of large amounts of fungal biomass. Mehna et al., (1995) were able to achieve a colour reduction of 92% with a COD elimination of 69% using *T. versicolor* after 7 days.

2.3.3.4 Flask cultivation

Trichoderma sp, was initially cultivated in shake flasks and washed before experiments were conducted under specific conditions to determine optimal pH and carbon sources. Under optimal conditions (pH 4.0 and glucose as carbon source) this fungus decreased the colour of kraft bleach plant effluent by 85% and reduced the COD by 25%, after 3 days incubation in shake flasks. The maximum total decolourization at pH 4.0 without an additional carbon source was 68.6% after 3 days cultivation and therefore glucose stimulated decolourisation. Other carbon sources such as pulp and pith which are abundant and inexpensive, increased the decolourisation after six days, since they were not metabolised immediately. The results of these studies emphasise the importance of carrying out the treatments under strictly defined conditions (Prasad and Joyce 1991).

2.3.3.5 Fermentor cultivation

Trametes versicolor was cultivated in a laboratory fermentor with 0.8% glucose plus 12 mM ammonium sulphate and at a controlled pH level of 5.0 under optimal aeration. This resulted in an 88% reduction in the colour units within 3 days, whereas an 80% reduction was observed in flask cultures after 6 days. This emphasises the importance of culture conditions on the efficiency of decolourisation (Bergbauer et al., 1991).

2.3.3.6 Immobilised culture treatment strategies

It would seem that for industrial purposes a process using biomass support would be more stable. The application of immobilised filamentous fungi, in continuous culture, would alleviate the inherent problems of biomass adherence, steady state instability, non-Newtonian behaviour (Anselino and Novias 1992). In liquid cultures *C. versicolor* removed over 60% of the colour of a combined bleach kraft effluent within 6 days in the presence of sucrose. This contrasted drastically with the results obtained when the same effluent was treated with fungus immobilised in beads of calcium alginate gel. In the latter situation , 80% decolourisation was attained after 3 days in the presence of sucrose. Recycled beads could remove colour efficiently in air repeatedly but not under anaerobic conditions (Livernoche et al., 1983). A fluidized bioreactor containing polyurethane immobilised *T. versicolor*, operated at a residence time of 9 hours, reduced colour of a bleach plant effluent by 65%. Simultaneous reduction in AOX-levels of 57% was recorded. Biological activity and mechanical stability were maintained for 45 days. In the Mycopor-process white rot fungi were immobilised in polyurethane foam and the system was operated continuously using a trickling filter reactor set-up. Long term treatment of bleaching effluent from the alkaline stage of a sulphate mill by this process resulted in a maximum of 80% decolouring activity. This was accompanied by 50% COD reduction and 80% toxicity elimination after one passage through a trickling filter of l m length (Jaklin-Farcher et al., 1992).

2.3.4 Ligninolytic enzymes and their possible role in decolourisation of effluents

The study of ligninolytic enzymes have attracted intense interest as more researchers investigated these catalytic agents of delignification in an effort to understand the mechanisms involved in this complex process. White rot fungi produce a variety of key ligninolytic enzymes composed of lignin peroxidase (LiP), manganese dependent peroxidase (MnP), and multiple iso-enzymes of laccase. Confusion was caused when mixtures of peroxidases and/or laccases were studied to demonstrate *in vitro* depolymerization of high molecular mass lignin. Depolyrnerization was usually accompanied by repolymerisation of the low molecular weight fractions (Lamar 1992). Further evidence indicated that a mixture of MnPs and laccases from *Rigidoporus lignosus* could degrade *Herea* lignin (Lamar 1992). Mutants of *P. chysosporium* lacking the ability to produce MnP and LiP did not show significant decolourisation activity when grown under nitrogen limiting conditions. Direct evidence linking MnP with decolourisation was provided by the *in vitro* depolymerisation of high molecular weight chlorolignin by MnP in the presence of Mn^{2+} and peroxide (Lamar 1992). Jaspers et al. (1994) indicated the importance of manganese peroxidase in the decolourisation of kraft pulp bleach plant effluent, since *in vivo* studies with purified MnP and LiP showed that only MnP had a decolourising activity. Also MnP but not LiP activity was detected when growing *P. chrysosporium* on the effluent. Many aspects of the mechanisms and functions of the enzymes involved in lignin mineralisation are scarcely known (Manzanares et al., 1995). Current knowledge is insufficient to allow construction of an enzyme mixture that prevents the repolymerisation reactions from taking place in a cell-free state (Eriksson 1993). Therefore processes based on the whole fungus are still preferable (Ritter et al., 1990).

2.3.5 Adsorption studies using microbial biomass

Many researchers reported adsorption of compounds on biomass. Boman and Frostell (1988) reported that 40%-50% of chlorinated phenols and l0% of the adsorbable chloroorganics are first adsorbed onto the fungal biomass before actual breakdown starts. Evidence has been presented that the initial colour decrease (up to 30%, after 3 days), using *P. chrysosporium,* of Kraft pulp mill effluent could possibly be attributed to adsorption of chromogenic compounds during fungal growth (Feijoo et al., 1995). In subsequent work Jaspers and Penninckx (1996) reported that depending on the conditions of incubation, pellets of *P. chysosporium* strongly adsorbed colour and AOX from kraft bleach plant effluent. Royer et al. (1985) regarded the decolourisation process by *T. versicolor* of kraft bleach effluent to consist of two parts, namely: adsorption, completed after 24 hours, and secondly, subsequent oxidation. About l0 % of TOX removal, obtained after treatment of a mixture of first chlorination stage and first alkaline stage effluent with *Ganoderma lacidum* and *C. versicolor*, was estimated to be due to adsorption (Wang et al., 1992). Biosorption of high-molecular weight organochlorines was studied using municipal waste water sludge as sorbent. The maximum percentage removal of high-molecular weight organo-chlorine was 70%. Only 8% of the organo-chlorines desorbed from live biomass, after 24 hours, indicating that the process was not readily reversible (Srinivasan and Unwin 1995). Hernandez et al. (1994) used *Streptomyces* strains to decolourise paper mill effluent obtained after semi-chemical alkaline pulping of wheat straw. They estimated that about 20% of the initial colour was lost due to adsorption by the mycelia. Furthermore only strains in which previous adsorption have been observed, did transformation of the major chromophoric groups in the effluent. Therefore this evidence clearly illustrates that adsorption and transformation of coloured compounds were linked and decolourisation required initial adsorption of the colour to the mycelia.

2.4 CONCLUSIONS

1. The fungal pretreatment of wood with selected white-rot fungi as well as biobleaching with xylanases can offer some apparent benefits to the pulp and paper industry. In the sector of dissolving pulp production these would be the potential increase in pulp yield, higher brightness ceiling, savings in pulping and bleaching chemicals, increased pulp mill capacity.
2. Biopulping and biobleaching are also promising in terms of improving of effluent quality thereby reducing the AOX and chemical load of effluent.
3. The high volumes of effluent produced by the pulp and paper industry present serious problems for the treatment of these waste waters. Biological treatment regimes that include lignolytic microorganisms, especially white rot fungi, have potential, but the necessity of including a cosubstrate and the need for aeration would seriously hamper their utilisation from an economic point of view.
4. Cultivation conditions have an important impact on the efficiency of white rot fungi during biological treatment of bleach plant effluent. Higher efficiencies are called for in order to develop a practical biotreatment process.

5. Biosorption of chromophores from bleach effluent has several potential advantages. These include the fast rate of the adsorption process and the option of incineration of the spent biomass after repeated cycles of adsorption/desorption and/or use as a supplement in the paper-making process.
6. Because of the complexity of treating paper mill effluent, combinations of physical/chemical/biological treatment strategies might lead to a synergistic beneficial outcome that would facilitate the development of economic and efficient treatment procedures.
7. Fungal pretreatment of the bleach plant effluent was shown to be effective in colour reduction of the effluent. Another alternative to the secondary biotreatment of effluent would be its utilization for valuable by-products such as enzymes. The eventual implementation of these biotechnologies may lead to considerable reductions of the AOX/chloride levels, toxicity and chemical load of the effluent.

CHAPTER 3
BIOSULFITE PULPING

3.1 INTRODUCTION

In the last few years it has been demonstrated that pretreatment of wood with lignin-degrading fungi could be beneficial not only to the process of mechanical pulping (Akhtar et al., 1992) but also in chemical pulping (Messner and Srebotnik 1994). Reports on biochemical pulping indicate reduction in kappa number at a given pulp yield (Oriaran et al., 1990; Scott et al., 1996) as well as improvements in certain physicochemical properties of paper handsheets such as brightness (Messner et al., 1992) and strength properties (Dawson-Andoh et al., 1991; Chen and Schmidt 1995).

The biochemical mechanism of biopulping is not yet understood. However, it is suggested that the secreted extracellular lignin-degrading enzymes and/or low molecular weight active compounds (mediators) generated by the fungus might be responsible for lignin depolymerisation and/or modification (Call 1993; Messner and Srebotnik 1994). The biopulping effect apparently is dependent on the particular wood species, microorganism, biotreatment and pulping conditions used. The most studied organism for biokraft pulping so far is *Phanaerochaete chrysosporium* (Oriaran et al., 1991; Dawson-Andoh et al., 1991; Chen and Schmidt 1995) whereas best results in biosulfite pulping were obtained with *Ceriporiopsis subvermispora* (Messner and Srebotnik 1994; Scott et al., 1996).

Most of the research effort regarding biochemical pulping has been focused on improving the quality and economics of paper production (Messner and Srebotnik 1994). However, our previous results (Christov and Wolfaardt 1995; Christov and Akhtar 1996; Ferraz et al., 1998) indicated that biopulping could also offer some benefits in the sector of dissolving pulp manufacture. In contrast to pulps used for papermaking, the residual hemicellulose and lignin in dissolving pulp are undesirable since they could cause certain problems in the conversion process to viscose rayon and other cellulose derivatives (Hinck et al., 1985). A selective lignin- and hemicellulose-directed degradation by white-rot fungi would facilitate the entire process of dissolving pulp production and its processing to end-uses.

3.2 MATERIALS AND METHODS

3.2.1 Fungal strains used for biopulping

Ten strains of white-rot fungi were used in this study. *Phanerochaete chrysosporium* BKMF-1767, *Ceriporiopsis subvermispora* SS-3 and *Ceriporiopsis subvermispora* CZ-3 were obtained from the Mycological Culture Collection, USDA Forest Products Laboratory, Madison, WI, USA. *Trametes versicolor* ATCC 20869 and *Trametes versicolor* 52J were a kind gift from Dr. M.G. Paice, PAPRICAN, Pointe Claire, QC, Canada. *Phanerochaete sordida* YK-624 was provided by Dr. R. Kondo, Kyushu University, Fukuoka, Japan. *Laetiporus sulphureus* SCC-180*,* *Pycnoporus sanguineus* SCC-92, *Stereum hirsutum* SCC-74, and *Sclerotinia sclerotiorum* UFHRF are local South African isolates: the first three cultures were obtained from the Sappi Culture Collection (SCC), University of the Orange Free State (UOFS), Bloemfontein, South Africa whereas the last one was kindly donated to us by Dr. J. Mildenhall, University of Fort Hare, Fort Hare, South Africa. Five strains of the white-rot fungus *Ceriporiopsis subvermispora* (strains SS-1, SS-3, SS-4, SS-5 and SS-10) were obtained from the Mycological Culture Collection, Center for Forest Mycology Research, USDA Forest Products Laboratory, Madison, WI, USA. All cultures were maintained on potato dextrose agar (PDA) slants and kept refrigerated until use. PDA plates were inoculated from these slants and incubated at 29±1°C for 7 days.

3.2.2 Wood chips

Wood chips from a clone of *Eucalyptus grandis* GU A380 (6 years old) were obtained from Sappi Forests, Howick, South Africa.

3.2.3 Inoculum preparation

Fungal inoculum was prepared by suspending agar discs from seven-day old cultures in 2L Erlenmeyer flasks containing 500 mL of sterilized PMY medium (4% glucose, 1% malt extract, 1% peptone and 0.4% yeast extract). Flasks were shaken at 150 rpm for seven days at 29±1°C. The culture broth was then centrifuged at 8000 rpm for 30 min and sedimented mycelium was transferred to 5 L Erlenmeyer flasks containing 2 L PMY medium. The fungal mycelium was homogenized into fungal pellets for 15 s at 20500 rpm (Diax 600, Heidolph, Kelheim, Germany) and thereafter incubated for seven days at 29±1°C and 150 rpm. The cultures were again centrifuged and the biomass was homogenized as above in sterile distilled water.

3.2.4 Fungal pretreatment of wood chips

Samples of wood chips (each 600 g dry weight) were autoclaved in autoclavable bags for 30 min at 121°C. After cooling to room temperature, a 0.1% inoculum of *C. subvermispora* (dry weight basis) was added to the chips and held at room temperature for 2 h with mixing every 30 min for better distribution of inoculum and medium. The chip moisture content was adjusted to 55% (wet weight basis). The fungal treatment of chips was performed in static bed bioreactors at 27°C for 2 weeks. During incubation, humidified air was aerated aseptically through the bioreactor with a specific aeration rate of 0.05 l $l^{-1}min^{-1}$ to maintain a relative humidity of about 65%. Treatments were performed in duplicate. Controls were run under the same conditions without fungal inoculation of wood chips.

Laboratory experiments were carried out also in bioreactors (2 L) containing 169 g wood chips (dry weight). After autoclaving at 121°C for 20 min. and cooling the bioreactors, 303 mL of sterile distilled water containing 2 g of mycelium (dry weight) was introduced into each of the jars to bring the final moisture of wood chips to about 60% (wet weight basis). Wood chips and fungal biomass were mixed and incubated at 29°C for 5, 7 and 10 days. Controls were prepared under the same conditions but without fungal inoculation.

3.2.5 Acid sulfite pulping

Acid sulfite pulping under standardized conditions with calcium as a base was performed at Sappi Saiccor in a pilot plant digester. The cooking liquor contained 8.25% total SO_2 and 1.05% CaO. The total pulping time was 405 min with the maximum temperature of 140°C kept for 85 min.

3.2.6 Bleaching of pulp

Bleaching of biosulfite pulp was carried out in three bleaching sequences: $OD_1E_oD_2H$, $OD_1E_oD_2P$ and $XOD_1E_oD_2P$. The following treatment stages were used in bleaching: xylanase (X), oxygen (O), chlorine dioxide (D_1 and D_2), sodium hydroxide with reinforced oxygen (E_o), sodium hypochlorite (H), and hydrogen peroxide (P). The charges of chemicals (% on dry weight pulp) were as follows: O, oxygen 0.8 and NaOH 1.8; D_1, 0.9 as active chlorine (act.Cl); E_o, NaOH 3.3 and oxygen 0.8; D_2, 0.6 as act.Cl; H, 0.5 as act.Cl.; P, H_2O_2 0.6 and NaOH 0.4. The pulp consistency (%, w/w) and temperature (°C) used were respectively 11 and 85, for O; 10 and 60, for D_1; 11 and 100, for E_o; 11 and 65, for D_2; 12 and 50, for H; 12 and 100, for P. Treatment time (min) was: 60 (O); 40 (D_1); 140 (E_o); 180 (D_2); 120 (H); 60 (P). Prior to use and after each treatment step pulp was thoroughly washed with distilled water until a neutral pH of the wash waters was attained.

3.2.7 Pulp tests

Kappa number, brightness, alkali solubilities (S_{10} and S_{18}) and viscosity were determined according to Tappi Test Methods (1991) T 236 cm-87, T 452 cm-87, T 235 cm-85, and T 206 os-63 respectively. Pulp yield was measured based on the loss of wood material during pulping. All tests were carried out in duplicate. The maximum difference between each two values relative to the corresponding average value was less than 7%.

3.2.8 Experimental design and statistical analysis

A factorial experiment was designed with three treatment times as one factor and treatments with different fungi as a second. Wood chips were treated for 5, 7 and 10 days with 10 fungal isolates and a control. Each treatment was repeated twice. One-way analysis of variance was performed and treatment means were compared with Tukey's method at a 90% confidence level (Winer 1971). Data representing kappa number, brightness, alkali solubility S_{10} and S_{18}, viscosity, and yield of pulp were statistically analysed.

3.3 RESULTS AND DISCUSSION

3.3.1 Screening of white-rot fungi for biopulping

The acid sulfite pulping qualities of *E. grandis* wood chips pretreated for 10 days with 10 fungal isolates are shown in Table 3.1. In all instances the biopulped samples had a lower pulp yield compared to the control. Maximum loss of yield (11%) was observed in the *T. versicolor* ATCC 20869 pretreated sample whereas only 2% pulp was lost relative to the control after pretreatment with *P. sordida.* Both strains of *C. subvermispora* as well as *P. sanguineus* reduced kappa number of pulp over the control by 29% (SS-3), 17% (CZ-3) and 6% (SCC-92), respectively. In the rest of the treated samples the relative percentage of lignin in pulp apparently increased at the expense of reduced pulp (cellulose) yield (Table 3.1). Only *C. subvermispora* SS-3 was able to brighten the pulp further by 6.3 points if compared to the control level of 51.5%. In all other instances brightness was lowered by 6.2 (*T. versicolor* ATCC 20869) to 16.9 (*S. hirsutum* SCC-74) points (Table 3.1). Appreciable amounts of degraded cellulose (S_{10}) were detected in the *T. versicolor* 52J (15.4%) and *C. subvermispora* CZ-3 (15.2%) pretreated pulp whereas the hemicellulose content (S_{18}) was affected to a lesser extent. Viscosities of treated pulp were comparable to the control (Table 3.1).

Table 3.1. Biosulfite pulping of *Eucalyptus grandis* wood chips with selected white-rot fungi[a]

Fungi	Yield (%)	Kappa number (%)	Brightness (cP)	Viscosity (%)	S_{10} (%)	S_{18}
Control	47.7	8.9	51.5	81.1	11.8	8.8
Phanerochaete sordida YK-624	46.6	10.1	44.2	84.3	11.1	10.4
Trametes versicolor 52J	46.4	10.2	43.6	79.0	15.4	9.1
Laetiporus sulphureus SCC-180	45.7	9.8	43.0	74.6	12.4	10.4
Phanerochaete chrysosporium BKMF-1767	45.6	12.6	43.6	74.5	12.9	9.8
Ceriporiopsis subvermispora SS-3	45.3	6.3	57.8	80.6	12.3	9.4
Ceriporiopsis subvermispora CZ-3	45.2	7.4	37.0	78.1	15.2	8.7
Stereum hirsutum SCC-74	45.1	11.8	34.6	90.9	13.5	8.9
Pycnoporus sanguineus SCC-92	44.0	8.5	40.1	73.1	12.5	9.8
Sclerotinia sclerotiorum UFHRF	43.4	11.8	42.0	96.8	11.9	10.0
Trametes versicolor ATCC 20869	42.6	10.9	45.3	97.8	13.3	10.2

[a] Wood chips were pretreated with fungi for 10 days. Values are averages of two replications.

3.3.2 Optimization of biopulping conditions

Based on the results obtained *C. subvermispora* SS-3 was selected for further studies. Changes in pulp yield (Fig. 3.1), kappa number (Fig. 3.2) and brightness (Fig. 3.3) were followed when the fungal pretreatment time was reduced from 10 days to 7 and 5 days. The loss in pulp yield comparative to the control (Fig. 3.1) was reduced from 4% (10 days fungal treatment time) to 2% (7 days) and 0% (5 days). On the other hand kappa number (Fig. 3.2) increased from 6.3 (10 days) to 6.5 (7 days) and 8.2 (5 days). Statistical analysis showed significant differences for kappa number between the control and the samples pretreated with *C. subvermispora* SS-3 for 10 days (Fig. 3.2). However, the brightness gain obtained as a biopulping effect was retained at the same level (57.2-57.8%) for the three treatment times (Fig. 3.3). The degraded cellulose and hemicellulose content in pulp decreased slightly by up to 4% (S_{10}) and 1% (S_{18}) when treatment time was reduced from 10 to 5 days. At the same time a slight increase of viscosity (by 9%) was observed.

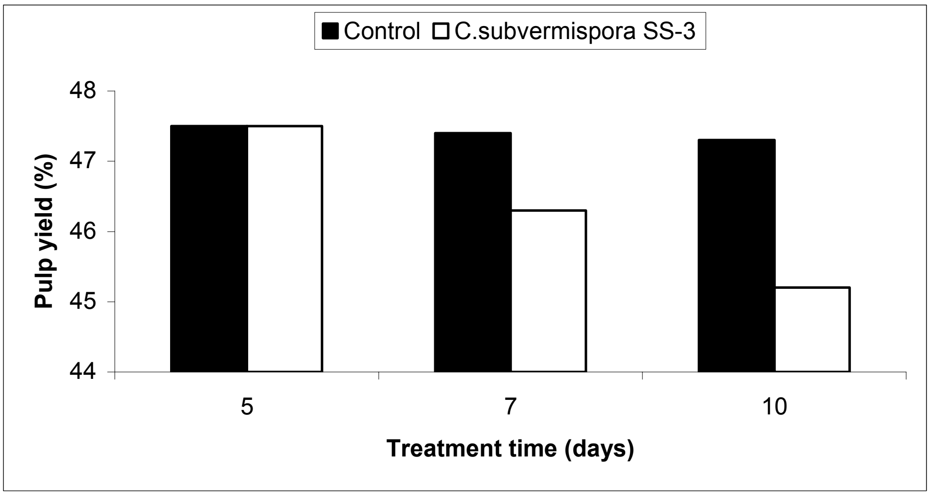

Fig. 3.1. Effect of time of fungal pretreatment of eucalyptus wood with *Ceriporiopsis subvermispora* SS-3 on yield of biosulfite pulp

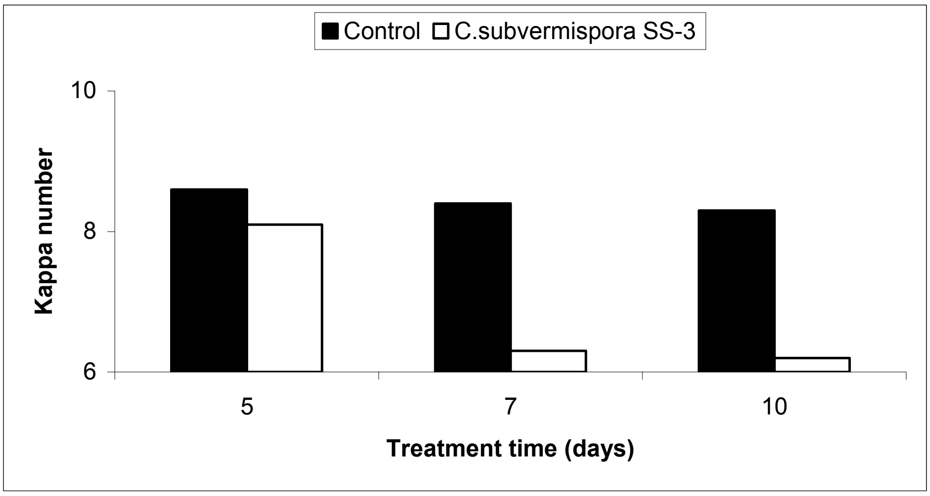

Fig. 3.2. Effect of time of fungal pretreatment of eucalyptus wood with *Ceriporiopsis subvermispora* SS-3 on kappa number of biosulfite pulp

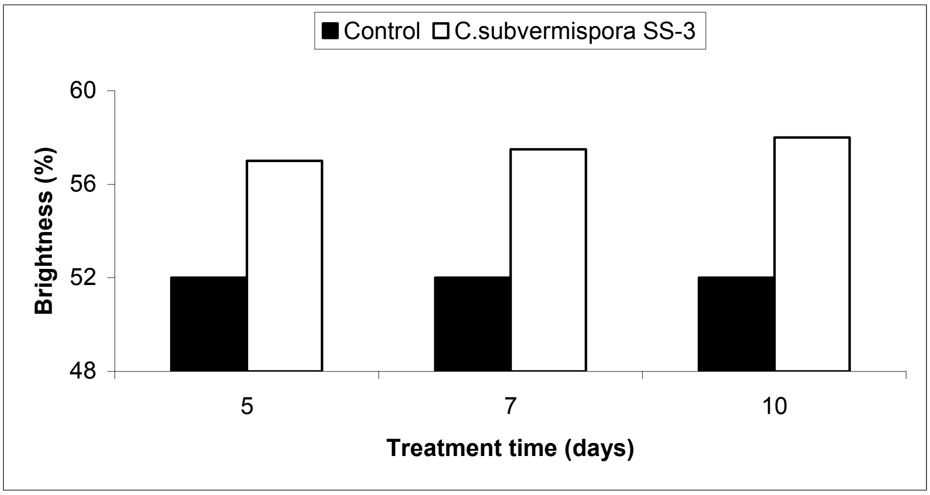

Fig. 3.3. Effect of time of fungal pretreatment of eucalyptus wood with *Ceriporiopsis subvermispora* SS-3 on brightness of biosulfite pulp

3.3.3 Effect of biopulping on bleaching of dissolving pulp

Tables 3.2-3.5 compile results from biopulping of eucalyptus wood chips in conjunction with bleaching/biobleaching of sulfite pulp to dissolving pulp. Five strains of *C. subvermispora* have been used to decay the chips for 2 weeks prior to pulping. Table 3.2 shows the properties of pulp produced by fungal pretreatment and acid sulfite pulping of chips using calcium as a base. In all instances brightness of the treated chips was reduced over to the control. Yield-wise only SS-3 and SS-5 pretreated samples were comparable to the control level of 47%. Significant yield loss was observed in the SS-1 treated pulp (40.8%) which however correlates with the deterioration of

Table 3.2. Biosulfite pulping of eucalyptus wood with five strains of *Ceriporiopsis subvermispora*[a]

Strains	S_{10}[b] (%, w/w)	S_{18} (%, w/w)	α-Cellulose (%, w/w)	Kappa number	Brightness (%, ISO)	Viscosity (cP)	Yield (%, w/w)
Control	9.2	6.8	90.7	8.9	51.5	31.1	47.4
SS-1	10.2	6.9	90.0	8.0	47.7	29.6	40.8
SS-3	9.2	6.7	90.6	8.3	49.3	30.4	47.1
SS-4	9.8	6.8	90.5	8.7	49.4	30.6	45.2
SS-5	9.4	6.7	90.7	8.9	48.6	30.2	46.9
SS-10	9.6	6.9	90.3	8.7	46.8	30.4	45.6

[a]Fungal pretreatment of wood chips: 2 weeks
[b]Values are averages of duplicate determinations

the S_{10} value (10.2%) indicating non-selective cellulose degradation. In most cases the α-cellulose content (the long DP fraction of cellulose) of the specimens was directly proportional to their yield (Table 3.2). There was no significant variation in the viscosity and alkali solubility S_{18} (hemicellulose content) of the samples. Kappa number was reduced by up to 10% compared to the control and did not correlate with the corresponding brightness of the samples.

Table 3.3. Properties of dissolving pulp produced by biosulfite pulping of eucalyptus wood with five strains of *Ceriporiopsis subvermispora*[a] and $OD_1E_0D_2H$-bleaching

Strains	S_{10}[b] (%, w/w)	S_{18} (%, w/w)	α-Cellulose (%, w/w)	Brightness (%, ISO)	Viscosity (cP)
Control	7.9	4.7	92.8	93.1	17.8
SS-1	8.7	4.9	92.2	93.0	19.7
SS-3	7.8	4.8	92.8	94.2	20.4
SS-4	8.4	4.7	92.8	92.8	20.8
SS-5	7.9	4.7	92.9	93.1	20.0
SS-10	8.2	4.6	92.8	92.7	20.6

[a]Fungal pretreatment of wood chips: 2 weeks
[b]Values are averages of duplicate determinations

Tables 3.3-3.5 present results from bleaching of the biosulfite pulp in 3 different bleaching sequences: $OD_1E_0D_2H$ (Table 3); $OD_1E_0D_2P$ (Table 4); and X-$OD_1E_0D_2P$ (Table 3.5). The major difference in the results between the fungally pretreated samples and the respective controls was in the brightness of the handsheets, especially in the bleaching sequences $OD_1E_0D_2P$ and X-$OD_1E_0D_2P$. In most instances a brightness gain over the control was observed. For example, in sequence $OD_1E_0D_2P$ (Table 3.4)

Table 3.4. Properties of dissolving pulp produced by biosulfite pulping of eucalyptus wood with five strains of *Ceriporiopsis subvermispora*[a] and $OD_1E_0D_2P$-bleaching

Strains	S_{10}[b] (%, w/w)	S_{18} (%, w/w)	α-Cellulose (%, w/w)	Brightness (%, ISO)	Viscosity (cP)
Control	7.7	4.5	92.7	93.2	17.2
SS-1	9.1	4.8	92.0	96.2	19.0
SS-3	8.3	5.4	92.6	92.9	19.9
SS-4	8.4	4.6	92.7	95.1	19.5
SS-5	8.5	4.6	92.2	93.6	17.2
SS-10	8.3	4.7	92.3	93.7	17.8

[a]Fungal pretreatment of wood chips: 2 weeks
[b]Values are averages of duplicate determinations

maximum brightness of 96.2% and 95.1% was obtained with the SS-1 and SS-4 pretreated samples. In sequence X-$OD_1E_0D_2P$ (Table 3.5) most successful were the SS-3 and SS-10 strains: 1.7 and 2.3 brightness points increase over the control. Interestingly, the biobleaching effect due to xylanase was not observed in all instances if Tables 3.4 and 3.5 are compared. For example, brightness of the SS-1 pretreated sample bleached in sequence $OD_1E_0D_2P$ (Table 3.4) was almost 3 brightness points higher than the SS-1 pretreated sample prebleached with xylanase (Table 3.5). No brightness increase was observed in the SS-4 pretreated samples from Tables 3.4 and 3.5 as well. On the other hand, a biobleaching gain in brightness was detected in the SS-3 (2.6 points) and SS-10 (1.2 points) pretreated samples. This phenomenon might be explained with the modifications in the composition and ultrastructure of the samples which took place during biopulping.

Table 3.5. Properties of dissolving pulp produced by biosulfite pulping of eucalyptus wood with five strains of *Ceriporiopsis subvermispora*[a] and X-$OD_1E_0D_2P$-bleaching

Strains	S_{10}[b] (%, w/w)	S_{18} (%, w/w)	α-Cellulose (%, w/w)	Brightness (%, ISO)	Viscosity (cP)
Control	8.1	4.5	92.5	93.2	13.7
SS-1	9.4	4.7	91.5	93.4	15.5
SS-3	8.1	4.5	92.7	95.5	18.5
SS-4	8.3	4.5	92.7	93.8	19.5
SS-5	8.4	4.5	92.3	94.0	17.1
SS-10	8.1	4.7	92.6	94.9	17.1

[a]Fungal pretreatment of wood chips: 2 weeks
[b]Values are averages of duplicate determinations

Figure 3.4 illustrates the final yields obtained to produce dissolving pulp from *C. subvermispora*-decayed eucalyptus wood. The results are indicative for the different modes of action of the fungal strains on wood resulting in different pulp yields after bleaching. The yields obtained by the means of fungal pretreatment with the SS-3 and SS-10 strains were identical in all bleaching sequences and comparable to the control. The best yields of dissolving pulp relative to the controls were achieved by fungal pretreatment with the SS-5 strain. Although the yield of the SS-5 treated sample was lower than the control after biosulfite pulping (Table 3.2), it was the highest after (bio)bleaching. This led to an yield increase of 0.8% in the $OD_1E_0D_2H$, 1.0% in the $OD_1E_0D_2P$ and 1.0% in the X-$OD_1E_0D_2P$ bleaching, respectively (Fig. 3.4). Therefore the fungal pretreatment of wood chips can not only increase the extent of lignin removal during pulping and bleaching, resulting in higher brightness of dissolving pulp, but also improve the selectivity of the bleaching process thereby increasing the final pulp yield.

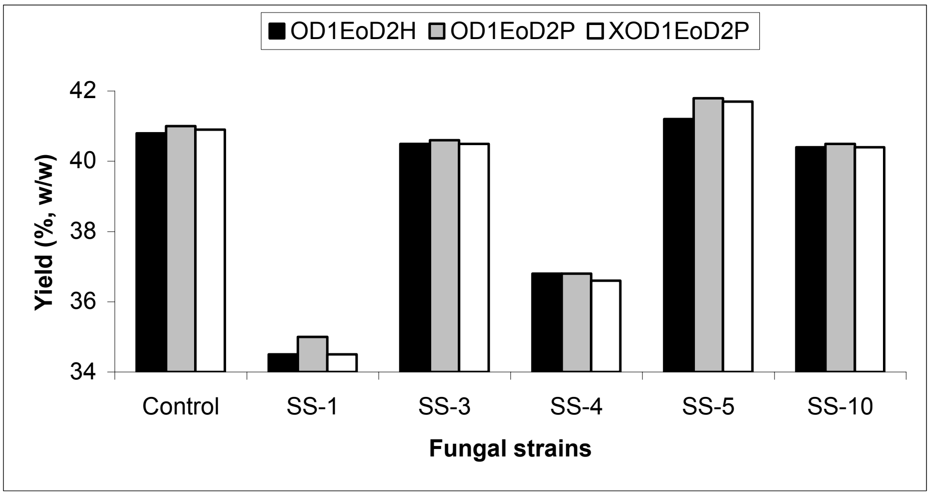

Fig. 3.4. Yield of dissolving pulp produced by biosulfite pulping of eucalyptus wood with five strains of *Ceriporiopsis subvermispora* using three bleaching sequences

3.4 CONCLUSIONS

- Among 10 fungal species tested *Ceriporiopsis subvermispora* SS-3 was found the most promising isolate for future optimization studies of biosulfite pulping of *Eucalyptus grandis* wood chips. Pretreatment with *C. subvermispora* SS-3 for ten days resulted in a substantial decrease in kappa number (by 29%) and increase in brightness (by 12%). On the other hand 5% of pulp yield was lost, and both viscosity and alkali solubility (S_{10} and S_{18}) were retained at levels comparable to the control.
- When the fungal incubation time was reduced to five days, a 5% reduction of kappa number, 11% increase in brightness and no loss of pulp yield was observed. Alternatively, cooking time or amount of pulping chemicals could possibly be reduced at a given pulp yield and savings in energy or chemicals obtained respectively.
- Beneficial biosulfite pulping could be performed without use of additional carbon source and with a fungal pretreatment time of as short as five days. However, the use of inoculum levels of as high as 1% on chips (dry weight basis) and the need of decontamination of wood chips prior to biopulping may however preclude the large-scale implementation of the process.
- When biopulping with different white-rot fungi is to be evaluated in a process where further bleaching is required (as in the case of dissolving pulp production), the assessment of the effectiveness of biopulping would depend also on the properties of the bleached pulp produced. For example, pulp yield obtained after biopulping would not be the final selection criterion because it does not always correlate with the pulp yield after bleaching of pulp.

- Fungal pretreatment of wood could improve the selectivity not only of pulping but of the bleaching process as well. As a result, the higher brightness of pulp could be translated in savings of bleaching chemicals which in turn would reduce the chemical load and AOX levels in the bleach plant effluent as well. Further research is needed as to optimise the fungal pretreatment conditions for biosulfite pulping.

CHAPTER 4
BIOBLEACHING

4.1 INTRODUCTION

The composition and structure of xylan are more complicated than that of cellulose and can vary in various wood species. In hardwoods, the major hemicellulose component is the *O*-acetyl-4-*O*-methylglucuronoxylan whereas in the softwood species the *O*-acetyl-galactoglucomannan is the predominant one (Fengel and Wegener 1984). High xylan content in dissolving pulp could have some undesirable detrimental effects on the swelling and xanthation reactions during the conversion process to viscose rayon (Hinck et al., 1985). Reduction of the hemicellulose is achievable at the alkali extraction stage of bleaching or later in the alkalization of dissolving pulp. However, due to the non-selectivity of these chemical processes pulp yield would also be negatively affected.

The enzymatic degradation of xylan would require the use of endo-xylanases, enzymes which can randomly split and solubilise xylan polymer. As a result, the penetration of bleaching chemicals into pulp fibers could be improved and therefore lignin removal facilitated. This approach, called biobleaching, provides the possibility of hydrolyzing selectively xylan in pulp thereby retaining the cellulose intact as well as minimizing the amounts of chlorine-containing bleaching chemicals. Recently new and more environmentally-friendly elemental chlorine-free (ECF) and totally chlorine-free (TCF) bleaching techniques (including biobleaching with enzymes) are being developed in the pulp and paper industry to alleviate the acute pollution problems (Viikari et al., 1994).

Biobleaching of kraft pulps with xylanases has been extensively reported since 1986 (Viikari et al., 1986). The enzymatic hydrolysis reaction takes place mainly on the fibre surface with the alkali-resistant xylan that was reprecipitated during the kraft process. The latter forms a physical barrier to the extraction of residual lignin from the fibers (Kantelinen *et al.*, 1993). The enzymatic removal of xylan improves the extractability of lignin in the subsequent chemical bleaching resulting in a higher pulp brightness.

Little attention has been paid to enzymatic prebleaching of sulfite pulps in the past (Paice and Jurasek 1984; Noe et al., 1986). However, recent reports indicate that xylanase treatment of sulfite pulps can also produce the "bleach-boosting" effect (Christov and Prior 1996). It was shown that xylan in dissolving pulp can be reduced two-fold in a totally chlorine-free bleaching.

4.2 MATERIALS AND METHODS

4.2.1 Crude xylanase preparations

A primary screening of over 100 mesophilic fungi for xylanase production was carried out at the Technical University of Budapest in Hungary. The best seven strains which showed less than 0.1 U cellulolytic activity/ml were selected for further screening (Table 4.1). These included: *Aspergillus foetidus* (ATCC 14916); *Aspergillus oryzae* (NRRL 1808); *Aspergillus niger* (ATCC 10864); *Aspergillus niger* (NRRL 3536); *Aspergillus phoenicis* (ATCC 15555); *Gliocladium viride* (CBS 658.70) and *Aureobasidium pullulans* (NRRL Y-2311). These strains were maintained on potato-dextrose agar plates with subculturing every three months.

Table 4.1. Characterisation of crude xylanases used in the biobleaching experiments

Strain	Carbon source	Xylanase (U/ml)	CMC-ase (U/ml)	pH optimum	T optimum (°C)
Aspergillus foetidus	Corn cobs	547.4	6.6	5.0	60
Aspergillus oryzae	Xylan	182.5	N.D.[a]	6.0	60
Aspergillus kawachii	Xylan	18.0	N.D.	3.0	60
Gliocladium viride	Xylan	49.5	N.D.	6.0	50
Cephalosporium	Wheat bran	20.0	N.D.	6.5-9.0	40
Actinomycetes	Wheat bran	25.0	0.4	4.5-6.0	60
Aureobasidium pullulans	Xylose	20.0	N.D.	4.7	55

[a] Not detected

4.2.2 Commercial xylanase preparations

The characteristics of the commercial xylanases used in the biobleaching experiments are shown in Table 4.2. Prior to chemical bleaching, never-dried unbleached sulfite pulp was treated with the xylanase preparations under the pH and temperature optima of each enzyme at four different enzyme charges (5, 15, 50 and 150 U xylanase/g dry pulp equivalent) for 3 h. Thereafter, pulp was bleached in two sequences ($OD_1E_OD_2H$ and $OD_1E_OD_2P$) and brightness was measured and compared against the controls. Commercial xylanases were evaluated also in biobleaching of soda-aq pulp. Enzymes were applied on the oxygen-delignified pulp under their optimum pH and temperature conditions (Table 4.2) at 9% pulp consistency for 1 h. A charge of 5 U xylanase/g pulp was used. Pulp was thereafter bleached in sequence DED under conditions currently used by the mill. Brightness of bleached pulp was measured and compared.

Table 4.2. Characterisation of commercial xylanase preparations used in the biobleaching experiments

Enzyme preparation	Company	Optimum pH	Optimum T (^oC)	Xylanase (U/ml)
Iogen HS-90	Iogen (Canada)	5.0	50	22 731
Ecopulp X-200	Primalco (Finland)	5.0	50	12 647
Ecopulp TX-200	Primalco (Finland)	7.0	70	13 593
Cartazyme SR-10	Clariant (USA)	4.0	50	28 288
Cartazyme NST-10	Clariant (USA)	6.5	60	5 133
Cartazyme NS-10	Clariant (USA)	7.5	65	12 948
Cartazyme HS-10	Clariant (USA)	4.0	50	30 239
Cartazyme 9704-E	Clariant (USA)	6.5	60	15 442

4.2.3 Purified xylanolytic enzymes

Two different endoxylanases (EX), purified to homogeneity and representatives of families 10 and 11, namely EX of *Thermomyces lanuginosus* (family 11) and *Thermoascus aurantiacus* (family 10), were used on pulp in the absence or presence of the acetyl xylan esterases (AcXE) from *Schizophyllum commune* (Table 4.3). The suspension of carefully washed pulp was supplied with 0.01 % NaN_3 (w/v) to prevent microbial contamination. Buffer was not used because the presence of salts would interfere with the chromatographic analysis of enzyme-released products in concentrated pulp slurry filtrates. All enzymes were used at a charge of 4 U/g and pulp was bleached with hydrogen peroxide following the enzyme pretreatment. Additional hydrolysis experiments with *S. commune* α-glucuronidase were performed. This enzyme was used at a charge of 6 U/g, 40°C and pH of 5.7 (100 mM acetate buffer) for 48 h.

Table 4.3. Characterisation of purified enzymes used in the biobleaching experiments

Enzyme	Family kDa	Mol. mass	pI value	Optimum pH	T(°C)
EX *Thermomyces lanuginosus*	11	25-26	3.7	6.5	75
EX *Thermomyces aurantiacus*	10	33	6.3	4.5	70
AcXE *Schizophyllum Commune*	-	30-31	3.4	7.7	40

4.2.4 Enzyme assays

Xylanase and carboxymethyl cellulase (CMC-ase) activities were determined spectrophotometrically at 540 nm by the dinitrosalicylic acid method based on the release of reducing sugars from oat spelts xylan and CMC, respectively (Miller 1959). One unit (U) of enzyme activity was defined as that amount of enzyme which catalyses the release of 1 µmol of xylose or glucose reducing sugar equivalents per minute of reaction. The acetyl xylan esterase was assayed on 4-nitrophenyl acetate as described by Johnson et al. (1988).

4.2.5 Pulps

Unbleached pulp was obtained from Sappi Saiccor, Umkomaas. It was produced by acid sulfite pulping of *Eucalyptus grandis* wood chips with a cooking liquor containing 1.1% CaO and 8.5% SO_2 for 405 min with the maximum temperature of 140°C held for 85 min. Soda-aq pulp was obtained from Sappi Enstra, Springs. Pulps were thoroughly washed before and after each treatment step until wash waters attained neutral pH.

4.2.6 Enzyme treatment of pulp

Pulp was treated with the xylanase under the optimum pH and temperature conditions of each enzyme. Charges in the range of 5-150 U/g pulp and incubation times of up to 3 h were used. After treatment, samples of the enzyme filtrate were withdrawn for determination of the residual xylanase activity. The enzymic reaction with pulp was terminated by boiling the samples for 10 min and filtrates collected for analysis of RS (Nelson 1944; Somogyi 1952) and absorbance at 280 nm. Controls were prepared under the same conditions but without the use of enzymes.

4.2.7 Chemical bleaching of pulp

Bleaching of biosulfite pulp was carried out in three bleaching sequences: $OD_1E_oD_2H$, $OD_1E_oD_2P$ and $XOD_1E_oD_2P$. The following treatment stages were used in bleaching: xylanase (X), oxygen (O), chlorine dioxide (D_1 and D_2), sodium hydroxide with reinforced oxygen (E_o), sodium hypochlorite (H), and hydrogen peroxide (P). The charges of chemicals (% on dry weight pulp) were as follows: O, oxygen 0.8 and NaOH 1.8; D_1, 0.9 as active chlorine (act. Cl); E_o, NaOH 3.3 and oxygen 0.8; D_2, 0.6 as act. Cl; H, 0.5 as act .Cl.; P, H_2O_2 0.6 and NaOH 0.4. The pulp consistency (%, w/w) and temperature (°C) used were respectively 11 and 85, for O; 10 and 60, for D_1; 11 and 100, for E_o; 11 and 65, for D_2; 12 and 50, for H; 12 and 100, for P. Treatment time (min) was: 60 (O); 40 (D_1); 140 (E_o); 180 (D_2); 120 (H); 60 (P). Prior to use and after each treatment step pulp was thoroughly washed with distilled water until a neutral pH of the wash waters was attained. Pulp samples were also bleached with hydrogen peroxide in sealed polyethelene bags in a water bath kept at a constant temperature. The following treatment conditions were used: 0.6% H_2O_2, 0.4% NaOH, 0.5% $MgSO_4.7H_2O$, 70°C, 3 h, 10% pulp consistency. After bleaching, pulp was acidified with 0.1 N sulphuric acid, washed with distilled water, dried at 45°C and then analysed.

4.2.8 Tests on pulp properties

Pulp tests were carried out according to the Standard Methods of the Technical Association of the Pulp and Paper Industry (TAPPI, Atlanta, GA, USA): kappa number (T 236 cm-85); brightness (T 452 om-92); viscosity (T 230 om-89); α-cellulose (T 203 om-88); alkali solubility S_{10} and S_{18} (T 235 cm-85). Kappa number is used as a criterion for the lignin content of pulps and is determined as the volume of 0.1 M potassium permanganate (ml) consumed by 1 g of moisture-free pulp (corrected to 50% consumption of the permanganate). Alkali solubilities S_{10} and S_{18} provide information on the low molecular weight carbohydrates (degraded cellulose and hemicellulose) in pulp. A 10% sodium hydroxide solution dissolves both degraded cellulose and hemicellulose (S_{10}) whereas hemicellulose is soluble in an 18% sodium hydroxide solution (S_{18}). All pulp properties were analysed in duplicate.

4.3 RESULTS AND DISCUSSION

4.3.1 Biobleaching using crude xylanases

The enzyme-pretreated pulp was fully bleached to dissolving pulp (Table 4.4). Two xylanase preparations (from *Cephalosporium* and *A. pullulans*), which were previously shown to hydrolyse pulp xylan to the greatest extent, produced a bleach-boosting effect as compared to control. This was in agreement with the brightness results obtained after XP bleaching of pulp: the same two enzymes produced the highest brightness increase over the control of approximately 3 brightness points (*Cephalosporium*) and 2 brightness points (*A. pullulans*) in one-stage peroxide bleaching. However, the brightness gain over the control was reduced to only 1 brightness point with both xylanase preparations when pulp was bleached to dissolving pulp in a 5-stage bleaching sequence. Apparently the more chemical bleaching steps involved after the xylanase pretreatment step, the less pronounced the biobleaching effect on pulp. Therefore, no relation seems to exist between the enzymatic release of reducing sugars from pulp and xylan removal from pulp or bleachability of pulp. Bleachability of sulfite pulp appears to improve in samples where xylan was hydrolyzed and removed to a great extent whereas the brightness gain obtained as result of xylanase pretreatment of pulp seems to decline as number of chemical bleaching steps increase.

Table 4.4. Biobleaching of sulfite pulp in sequence XOD_1EoD_2P using crude xylanase preparations

Xylanase producer	E_o-kappa number	S_{18} (%)	S_{10} (%)	Brightness (%)
Aspergillus foetidius	1.3	5.5	8.8	90.9
Aspergillus oryzae	1.3	5.5	8.6	90.0
Aspergillus kawachii	1.4	5.2	8.6	90.4
Gliocladium viridae	1.3	5.3	8.2	90.1
Cephalosporium	1.4	5.6	8.9	91.0
Actinomycetes	1.5	5.5	9.1	88.8
Aureobasidium pullulans	1.3	5.3	8.6	91.2
Control	1.4	5.7	8.8	90.0

4.3.2 Biobleaching using commercial xylanases

A brightness increase over the control was observed in both bleaching sequences (Table 4.5). The impact of enzyme pretreatment on brightness was dependent on the particular xylanase preparation used. For instance, in sequence $XOD_1E_OD_2H$ Iogen HS-90, Ecopulp TX-200 and Cartazyme SR-10 caused some brightness increase of about 1.0 brightness point. In sequence $XOD_1E_OD_2P$ all enzyme preparations produced a biobleaching effect. Brightness was increased by 0.8-2.8 brightness points. Again, best results were obtained with Iogen HS-90, Ecopulp TX-200 and Cartazyme SR-10. Interestingly, there was no correlation between enzyme doses used and brightness increase.

Table 4.5. Brightness of dissolving pulp pretreated with commercial xylanase preparations

		Brightness (%)	
Enzyme	Charge (U/g)	$XOD_1E_OD_2H$	$XOD_1E_OD_2P$
Iogen HS-90	5	93.2	93.8
	15	93.1	93.7
	50	92.7	93.7
	150	92.7	92.8
Ecopulp X-200	5	91.5	92.6
	15	91.3	92.0
	50	91.3	92.5
	150	91.5	92.7
Ecopulp TX-200	5	93.0	93.4
	15	92.6	93.6
	50	92.4	93.6
	150	93.0	93.8
Cartazyme SR-10	5	92.7	93.7
	15	92.3	94.0
	50	93.1	93.6
	150	93.1	93.6
Cartazyme NS-10	5	91.9	92.7
	15	91.9	93.1
	50	91.9	92.6
	150	92.2	92.7
Cartazyme HS-10	5	92.9	93.3
	15	92.7	92.9
	50	93.1	93.6
	150	92.7	93.1
Control	-	92.1	91.2

Commercial xylanases were also evaluated in biobleaching of soda-aq pulp (Table 4.6). In all biobleached samples, a brightness increase of between 0.8 and 1.7 brightness points was observed. Best results were obtained with Iogen HS-90: a gain of 1.7 brightness points over the control. It should be noted that this xylanase preparation, when used in biobleaching of sulfite pulp, did improve brightness by 1.1 points in $XOD_1E_OD_2H$-sequence and by 1.6 points in $XOD_1E_OD_2P$-sequence, respectively.

Table 4.6. Brightness of O-X-D_1ED_2-bleached[a] soda-aq pulp

Treatment	Brightness (%)
Iogen HS-90	89.7
Ecopulp X-200	89.4
Ecopulp TX-200	89.2
Cartazyme SR-10	89.2
Cartazyme NST-10	89.3
Cartazyme NS-10	89.3
Cartazyme HS-10	88.8
Cartazyme 9704-E	89.2
Control	88.0

[a] X treatment: 5 U xylanase/g pulp; Treatment time: 1 h; Consistency: 9%, D_1: 2.63% act Cl; 113 min; 67°C; 9% consistency, E: 0.7% NaOH; 67 min; 67°C; 10% consistency, D_2: 1.315% act Cl; 180 min; 67°C; 10% consistency

4.3.3 Reduction of active chlorine charges in biobleaching

Table 4.7 indicates the major differences in the properties of dissolving pulps bleached in the sequences $OD_1E_OD_2$, $OD_1E_OD_2P$ and $OD_1E_OD_2H$ with or without enzyme pretreatment with *A. pullulans* xylanases. Notably, the bleaching combinations X-$OD_1E_OD_2P$ and $OD_1E_OD_2$ ending with hydrogen peroxide proved to be the most efficient in terms of S_{10}, S_{18} and brightness. The brightness of the X-$OD_1E_OD_2P$-bleached dissolving pulp (94.2%) was by approximately 2 and 3 units higher than that of the X-$OD_1E_OD_2H$ and X-$OD_1E_OD_2$ pulp, respectively. The brightness gain over the controls, due to the biobleaching with xylanases, was the highest also with the X-$OD_1E_OD_2P$ pulp (1.7 units) as compared to the other two pulps: 1.6 units for the X-$OD_1E_OD_2$ and 1.1 units for the X-$OD_1E_OD_2H$ pulp. The introduction of P or H as a final bleaching step in the bleaching sequence $OD_1E_OD_2$ did increase slightly S_{10} and S_{18}. This was more obvious in the case of sodium hypochlorite where there was a significant drop in the viscosity as well. In addition to its bleaching function, the hypochlorite is used also to control the DP of the final dissolving pulp since conversion to cellulose derivatives and some resultant properties are dependent upon the molecular chain length of the cellulose. This is especially needed if the dissolving pulp is used for viscose rayon manufacture. If a higher viscosity of dissolving pulp is desired (higher than 25 cP), as it is required for example in the cellulose acetate production, then the bleaching sequence $OD_1E_oD_2P$ would be more appropriate. In both bleaching combinations, however, the xylanase pretreatment would improve the quality of the final dissolving pulp. Thus, when a xylanase charge of 15 U/g pulp was applied to pulp, the total savings of active chlorine to bleach dissolving pulp to a 92% brightness were approximately 60% for the X- $OD_1E_oD_2P$-bleached pulp (Fig. 4.1).

Table 4.7. Biobleaching of sulfite pulp with *A. pullulans* xylanase (X)[a]

Bleaching sequence	S_{10} (%)	S_{18} (%)	Brightness (%)	Viscosity (cP)
$OD_1E_oD_2$	9.1	4.6	89.8	27.1
$X\text{-}D_1E_oD_2$	7.8	4.1	91.4	29.2
$OD_1E_oD_2P$	9.3	4.8	92.5	30.9
$X\text{-}D_1E_oD_2P$	7.8	4.4	94.2	33.2
$OD_1E_oD_2H$	9.5	5.2	90.9	18.0
$X\text{-}D_1E_oD_2H$	8.8	4.9	92.0	19.8

[a] 15 U xylanase/g pulp; values represent the mean of duplicate determinations

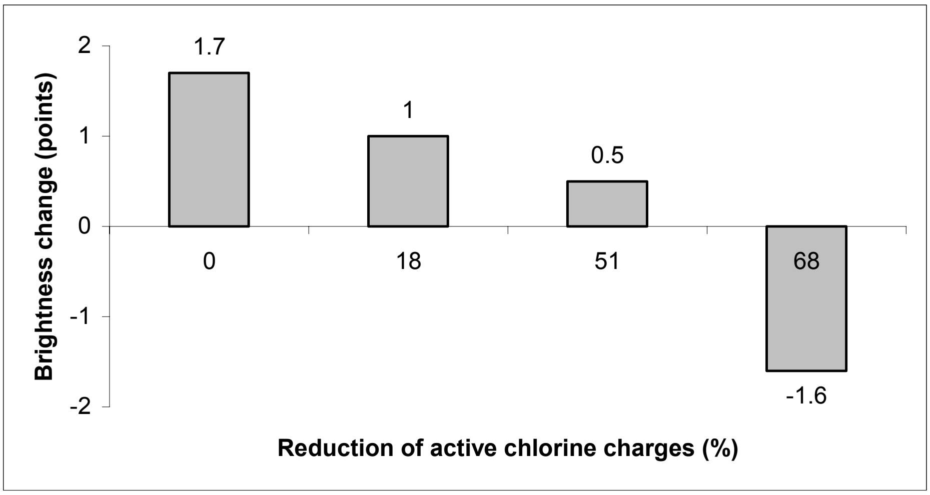

Fig. 4.1. Reduction of active chlorine charges during biobleaching of dissolving pulp with *Aureobasidium pullulans* xylanase. Brightness is expressed as change of percentage points over control.

4.3.4 Optimisation of xylanase charges in biobleaching

Optimization of the amount of enzyme required in the biobleaching process is necessary as to make the process cost-effective. For an economically feasible process, the expenses related to the introduction of the xylanase pretreatment step should be lower than the savings of bleaching chemicals obtained as a result of biobleaching (mainly chlorine dioxide, sodium hydroxide and sulphuric acid). Recent results indicate that charges of as low as 4 U xylanase/g pulp could be used in biobleaching (Table 4.8). Since Iogen HS-90 contained 22 731 U xylanase/ml, approximately 4.5 tonnes of pulp could be treated with 1 litre of the preparation. The charge per tonne of pulp would be about 0.220 l or 0.250 kg of enzyme (density 1.15 kg/l). Based on a price of US$ 12.0/kg of enzyme, the cost to treat a tonne of pulp would be US$ 3.0.

4.3.5 Use of accessory xylanolytic enzymes in biobleaching

The high degree of acetylation of xylan in the unbleached sulfite pulp (0.6%) was the main reason for use of two purified acetyl xylan esterases (AcXE) in conjunction with two purified endoxylanases (EXs) to examine the role of acetyl xylan esterases (AcXE) in the hydrolysis of xylan and xylanase-facilitated pulp bleaching (Table 4.8). Kappa numbers did not change significantly as compared to control although there was a decrease in the pulp hemicellulose by up to 0.7 points (Table 4.8). Treatment of the pulp with both EX enzymes resulted in about the same increase in the pulp brightness. However, the addition of *S. commune* AcXE did not cause any further brightness increase. Although the two endoxylanases (EXs) belonged to two different family groups and their mode of action on sulfite pulp was different in terms of release of sugars from pulp, their biobleaching abilities were similar. However, the enzymatic release of acetic acid by acetyl xylan esterases (AcXE) did not enhance biobleaching of pulp by endoxylanases (EX). This indicates that the esterases may not play an important role in this bioprocess.

Table 4.8. Hydrogen peroxide bleaching of sulfite pulp pretreated with purified enzymes

Sample	Kappa number	S_{18} (%)	Brightness (%)
Control	4.5	7.9	69.8
EX_1 [a]	4.3	7.5	70.9
EX_1 + AcXE [b]	4.2	7.5	70.4
EX_2 [c]	4.3	7.3	70.6
EX_2 + AcXE	4.4	7.2	70.3

[a] 4 U xylanase/g pulp; EX_1: *Thermomyces lanuginosus*
[b] 4 U acetyl xylan esterase; AcXE: *Schizophillum commune*
[e] 4 U xylanase/g pulp; EX_2: *Thermoascus aurantiacus*

The content of 4-*O*-methylglucuronic acid in pulp was determined to be 0.2%. However, hydrolysis of pulp with *S. commune* α-glucuronidase did not release any glucuronic acid from pulp suggesting that this enzyme would not aid in the biobleaching process.

4.4 CONCLUSIONS

- No relation seems to exist between the enzymatic release of reducing sugars from pulp and xylan removal from pulp
- No relation seems to exist between the enzymatic release of reducing sugars from pulp and bleachability of pulp.
- Bleachability of sulfite pulp appears to improve in samples where xylan was hydrolyzed and removed to a great extent.
- Brightness gain obtained as result of xylanase pretreatment of pulp seems to decline as number of chemical bleaching steps increase.
- The impact of enzyme pretreatment on brightness was dependent on the particular xylanase preparation and particular bleaching sequence used. Best results were obtained with Iogen HS-90, Ecopulp TX-200 and Cartazyme SR-10. A greater brightness gain was obtained when pulp was bleached in sequence $XOD_1E_OD_2P$ than $XOD_1E_OD_2H$. It should be noted that this xylanase preparation of Iogen HS90 when used in biobleaching of sulfite pulp did improve brightness by 1.1 points in $XOD_1E_OD_2H$-sequence and by 1.6 points in $XOD_1E_OD_2P$-sequence, respectively.
- Chemical savings of up to 51% (as active chlorine) could be achieved when sulfite pulp was biobleached with *A. pullulans* xylanases in sequence $X\text{-}OD_1E_oD_2P$. The dose of chlorine dioxide at D_1 stage could be reduced by up to 80% whereas at D_2 stage it was lowered by up 50%. However, a complete elimination of D_1 and/or D_2 was unattainable. The reduction of the chlorine dioxide loadings at D_1 and D_2 stages of bleaching had no apparent impact on the viscosity and S_{10}/S_{18} values of dissolving pulp.
- A comparison of the biobleaching sequences $XOD_1E_oD_2P$ and $XOD_1E_oD_2H$ applied in dissolving pulp production indicates that the first one is superior to the second one in terms of brightness and alkali solubility (S_{10} and S_{18}). Moreover, a replacement of the hypochlorite with peroxide as a final bleaching step would result in a further reduction of the AOX-levels of the bleach plant effluent.

- Recent results indicate that charges of as low as 5 U xylanase/g pulp could be used in biobleaching. Approximately 4.5 tonnes of pulp could be treated with 1 litre of Iogen HS-90 (22 731 U xylanase/ml). The charge per tonne of pulp would be about 0.220 l or 0.250 kg of enzyme (density 1.15 kg/l) and based on a price of US$ 12.0/kg of enzyme, the cost to treat a tonne of pulp would be US$ 3.0.
- Although the two purified xylanases (*Thermomyces lanuginosus* and *Thermoascus aurantiacus*) belonged to two different family groups (Family 10 and 11) and their mode of action on sulfite pulp was different in terms of release of sugars from pulp, their biobleaching abilities were similar.
- The enzymatic release acetic acid by acetyl xylan esterases did not enhance biobleaching of pulp by xylanases, indicating that the esterases may not play an important role in this bioprocess.
- Although the content of 4-*O*-methylglucuronic acid in pulp was determined to be 0.2%, hydrolysis of pulp with *S. commune* α-glucuronidase did not release any glucuronic acid from pulp, suggesting that this enzyme would not aid the biobleaching process.

CHAPTER 5
EFFLUENT BIOTECHNOLOGY

5.1 INTRODUCTION

Bleach plant effluents from the pulp and paper industry contain coloured as well as toxic chloroorganic materials that pose environmental problems and necessitate remediation of the waste waters (Bajpai and Bajpai 1994). The colour in these effluents is mainly due to lignin and lignin derivatives such as chlorolignins, which are resistant to degradation because of the presence of carbon to carbon linkages of biphenyl type. These compounds inhibit the natural process of photosynthesis in the stream due to absorbance of sunlight. Although physico-chemical treatments have been used to decolourise bleach plant effluents, it is generally accepted that such strategies are too expensive for practical implementation. Biological decolourization methods use several classes of microorganisms (bacteria, algae and fungi) to degrade the polymeric lignin derived chromophoric material. Among these, wood degrading white-rot fungi have been shown to efficiently and completely degrade and metabolise lignin, resulting in rapid decolourisation of the effluents. Biodegradation of colour by white-rot fungi is a relatively inexpensive, but slow process (Bajpai and Bajpai 1994). This is especially problematic for application in the pulp and paper industry since large volumes of effluent are produced continuously. Furthermore, the requirements for high oxygen tension and a growth substrate constrain the practical implementation of effluent decolourisation using white-rot fungi.

Recently interest has been shown in fungal adsorption of colour compounds present in bleach plant effluents (Jaspers and Penninckx 1996). This phenomenon has been seen in the past as a technological problem associated with fungal treatment where biodegradation and not bioadsorption of coloured compounds from effluents was the objective (Marton et al., 1969). In general terms, adsorption has been shown to be a good method to remove colour contaminants (Liversidge et al., 1997). Although adsorbents such as activated carbon are effective in removing colour from effluents, they are too expensive from an economical perspective. Therefore a considerable amount of work has been done to test alternative adsorbents. Fungi are easily grown and produce high yields of biomass. Furthermore, fungi are extensively used in a variety of large-scale industrial fermentation processes and represent a potential source of adsorbent material (Kapoor and Viraraghavan 1995).

5.2 MATERIALS AND METHODS

5.2.1 Effluents

The spent sulfite liquor (SSL) used in this study was derived from the acid sulfite pulping of *Eucalyptus grandis* at the Sappi Saiccor dissolving pulp producing mill in Umkomaas. The SSL effluent had a pH of 1.6, COD of 228 g l^{-1} and a colour of 22000 Platinum Cobalt Units (PCU). Bleach plant effluent Eo was obtained from the alkali extraction stage (E_o) of the bleach plant of Sappi Saiccor. The E_o-effluent had a pH of 7.6, COD of 14.8 g l^{-1} and colour of 15700 Platinum Cobalt Units (PCU) and was stored in closed containers at 4^oC.

5.2.2 Screening experiments

A number of white-rot fungi and thermotolerant *Rhizomucor* strains from the Sappi Culture Collection (SCC) and Department of Microbiology and Biochemistry, UOFS have been screened for their abilities to decolourise bleach plant effluent Eo and SSL. Inocula were grown for 2 days at 50°C, cells were harvested and introduced into the effluent together with 1% glucose, 0.16% $(NH_4)_2SO_4$, 0.6% K_2HPO_4 and 0.4% KH_2PO_4. Effluents were previously diluted 1:1 with water, sterilized and effluent pH was adjusted to 5.0. The treatment was carried out at 28°C (white-rot fungi) and 50°C (mucoralean fungi) for 7 days and 4 days, respectively. The experiments were repeated and results averaged in terms of colour.

5.2.3 Adsorbents

Activated carbon was obtained from Aldrich (Steinheim, Germany). The strong anion-exchange resin Dowex-1 (Cl^--form, 100-200 mesh) was purchased from Sigma (St. Louis, MO, USA) and the strong cation-exchange resin AG 50WX12 (H^+-form, 100-200 mesh) was from Bio-Rad (Richmond, CA, USA). Chitosan was purchased from Fluka Biochemika (Neu-Ulm, Switzerland) whereas chitin was obtained from Nutritional Biochemicals Corporation (Cleveland, OH, USA).

5.2.4 Biomass production and preparation

The mucoralean fungus *Rhizomucor pusillus* strain RM7 was obtained from the culture collection of the Department of Microbiology and Biochemistry, University of the Orange Free State and was maintained on potato dextrose agar. For inoculum preparations, four 1 x 1 x 1 cm cubes were cut from a fresh plate and transferred to a 1000 ml sterile Erlenmeyer flask containing 200 ml growth medium of the following composition (g/l): glucose, 20; malt extract, 20; peptone, 20; yeast extract, 8. The pH of the medium was adjusted to 5.0 prior to sterilisation. After a 2 day incubation period at 30^{o}C on an orbital shaker (150 rpm), mycelium was transferred aseptically into flasks (1000 ml) containing 200 ml sterile growth medium. Incubation was carried out for another 2 days at 30^{o}C and thereafter cells were harvested. Before use in adsorption experiments, fungal biomass was washed thoroughly with distilled water on Buchner funnels.

5.2.5 Adsorption experiments

Adsorption experiments were performed using a charge of 0.5 g dry biomass equivalents/100 ml E_o-effluent in Erlenmeyer flasks on orbital shakers at 30^{o}C or 55°C and 100 rpm for up to 24 h. Adsorption experiments using activated carbon, ion exchange resins, commercial chitin and chitosan were conducted at 30°C in a similar way as described for the use of fungal biomass. Samples for colour determination were withdrawn regularly. To test the effect of effluent recycling on colour adsorption, fresh biomass was employed to treat the same batch of effluent in five consecutive adsorption cycles of 1 h treatment time each. To test the effect of biomass recycling on colour adsorption, the same batch of biomass was employed to treat fresh amounts of effluent in five consecutive one-hour cycles. Colour changes were monitored during each treatment cycle.

5.2.6 Isotherm determinations

The biosorption isotherm of coloured material was prepared by incubating 0.5 g of fungal biomass from *R. pusillus* in 50 ml of effluent of initial colour in the range 1075-15700 PCU. The experiments were conducted in sealed tubes on a rotary shaker at 30°C and 100 rpm. Following a contact time of 24 h the biomass was removed by filtration and the colour of the filtrates determined.

5.2.7 Desorption experiments

The sorbent materials used in this study were tested in desorption experiments in order to establish whether they could be regenerated. Treatment with 1 N NaOH was carried out at 30°C for 4 h on an orbital shaker at 100 rpm. Colour desorption was measured and compared against the amount of colour adsorbed onto the respective adsorbent.

5.2.8 Analyses

Colour readings of effluent samples were performed using a Hach spectrophotometer (Loveland, CO, USA) at 455 nm after appropriate dilution and pH adjustments to pH 7.6 (APHA 1989). Colour of samples was expressed as Platinum Cobalt Units (PCU). Conductivity and pH were measured using HANNA - HI8733 conductivity meter and HACH EC30, respectively. Magnesium, calcium, chemical oxygen demand (COD), chlorides and phenols were determined using Hach DR/2000 spectrophotometer. Total solids (TS), total dissolved solids (TDS) and total suspended solids (TSS) were measured according to the APHA methods (1989). Reducing Sugars (RS) in the effluent were determined using the Somogyi-Nelson method (Nelson 1944; Somogyi 1952), whereas gas chromatography was used to measure acetic acid, ethanol and methanol present in effluents.

5.3 RESULTS AND DISCUSSION

5.3.1 Effluent characterization

General analyses of the Saiccor's effluents have been completed with three effluents: the spent sulfite liquor (SSL), E_O-effluent from the bleach plant (BPE) and total effluent (TE). The SSL apparently had the highest colour (22 000 PCU), total solids (TS; 164 750 mg/l), total dissolved solids (TDS; 149 200 mg/ml), total suspended solids (TSS; 15 500 mg/ml) and COD (228 000 mg/ml). The high COD and the presence of some inhibiting microbial growth compounds such as acetic acid, phenols, etc. indicate that the success in the eventual production of xylanases on the SSL would be dependent upon some appropriate pretreatment of the SSL. On the other hand the bleach plant effluent Eo contained lower amounts of acetic acid and no furfural but greater amounts of phenols as compared to SSL.

5.3.2 Effect of biobleaching on the AOX/chloride content of effluent

As a result of biobleaching of sulfite pulp with xylanase the active chlorine charges at D_1 and D_2 could be reduced by 33% at D_1 and by 50% at D_2 thereby maintaining the same brightness level of 92% as the control. This in turn would lead to changes in the composition of the bleach pulp effluents. The impact of xylanase pretreatment on COD and AOX/chloride content of bleach pulp effluents D_1, Eo and D_2 is shown in Table 5.1. The COD, AOX and chloride content of all three bleach pulp effluents released from xylanase-pretreated pulp was reduced due to the reduction of the charges at D_1 and D_2. COD decreased by 22.5% at D_1, 7% at Eo and 20% at D_2. The total reduction of COD in these three effluents was 8%. However, due to the introduction of the enzyme pretreatment step the COD of the total bleach pulp effluent would increase by approximately 30-35% depending on the xylanase dose applied. The Cl^- content was reduced by 21% at D_1, 30% at Eo and 47% at D_2, whereas AOX content of D_1-effluent was by 45% lower, of Eo-effluent by 24% lower and of D_2-effluent by 37% lower than the corresponding control effluents (Table 5.1).

Table 5.1. Analysis of bleach pulp effluents following xylanase pretreatment[a]

Bleaching stage	Charge on pulp (% act. Cl)	COD ($mg\ l^{-1}$)	Cl^- ($mg\ l^{-1}$)	AOX ($mg\ l^{-1}$)
Control				
D_1	0.9	560	98	22.2
D_1Eo	-	10210	180	11.8
D_1EoD_2	0.6	220	70	11.2
Xylanase-pretreatment				
XD_1	0.6	434	77	12.1
XD_1Eo	-	9490	125	9.0
X D_1EoD_2	0.3	175	37	7.1

[a] *A. pullulans* xylanase, 15 U/g pulp, values are averages of two determinations

Therefore, as a result of the 40% total reduction of the active chlorine charges during biobleaching of dissolving pulp, the chloride and AOX levels of the total bleach pulp effluent were reduced respectively by 31% and 38%.

Table 5.2. Colour and lignin removal from bleach plant effluent (Eo) with selected white-rot fungi[a]

Fungi	Decolourisation[b]	Delignification[c]
Control	**0.0**	**0.0**
Ceriporiopsis subvermispora SS-3	26.9	2.6
Ceriporiopsis subvermispora CZ-3	29.4	18.8
Phanerochaete chrysosporium BKMF-1767	11.7	12.5
Phanerochaete sordida YK-624	34.0	27.0
Trametes versicolor 52J	64.0	50.7
Trametes versicolor ATCC 20869	55.8	37.9
Trametes nivosa SCC-208	70.0	33.0
Pycnoporus sanguineus SCC-92	22.8	17.4
Rhizomucor pusillus RM 7	74.9	20.5

[a] Effluent pretreated with fungi at a dose of 5 g dry biomass equivalents/l effluent for 7 d
[b] Absorbance at 455 nm
[c] Absorbance at 280 nm

5.3.3 Screening of microorganisms for effluent decolourisation

Table 5.2 lists results obtained following screening of a number of fungal strains in terms of colour and lignin removal from the bleach plant effluent E_o. Surprisingly, *Ceriporiopsis subvermispora*, that appeared as one of the most efficient lignin degraders of eucalyptus wood, did not significantly affect lignin in effluent. A substantial lignin and colour reduction in the effluent was achieved with the *R. pusillus* and *Trametes* strains. For instance, *T. nivosa* 208 was able to reduce colour by 70% whereas *R. pusillus* RM 7 removed approximately 75% of effluent colour. These two isolates were selected for further optimisation studies.

5.3.4 Effluent decolourisation

5.3.4.1 Optimisation of treatment conditions

A number of factors which may influence colour reduction by the *Rhizomucor* strains have been studied: time; pH; temperature; carbon source; inoculum size, initial colour.

5.3.4.1.1 Effect of treatment time

It appears that colour reduction of E_O increased with time up to 96 h and thereafter it declined slightly (Table 5.3). With *R. pusillus* RM7 the maximum colour removal achieved was 68% (after 4 days) whereas *T. nivosa* 208 reduced colour by 71% after 4 days. It should be noted that only after 2 h RM7 was able to remove 43% of colour indicating the strong adsorption capacity of this fungus.

5.3.4.1.2 Effect of carbon source

The colour reduction of Eo by the *Rhizomucor* and *Trametes* strains in the presence/absence of glucose and mineral salts was studied. It was shown that in both instances additional carbon source was required only for incubation periods longer than 24 h whereas the supplement of effluent with minerals was not essential for colour reduction (data not shown).

Table 5.3.. Effect of time of fungal treatment[a] on colour reduction of bleach plant effluent (Eo)

Time (h)	Colour reduction (%)	
	RM7	208
0	15	23
2	43	33
8	48	36
24	51	45
48	62	66
72	66	71
96	68	71
120	63	68
144	62	67
168	57	65

[a]Biomass dose 5 g/L; 30°C (208) and 50°C (RM7); pH 4 (8 h) and 5 (7 days)

5.3.4.1.3 Effect of pH

The maximum colour removal in relation to pH of effluent was attained in the acidic pH range. It appears that pH between 3 and 4 was the optimum for both RM7 and 208 after 6 h incubation at 50°C and 30°C, respectively (Fig. 5.1).

5.3.4.1.4 Effect of temperature

The effect of temperature of treatment of Eo on the decolourisation abilities of *Rhizomucor* and *Trametes* was studied in the range 25-65°C (Fig. 5.2). The highest colour reduction (46-48% for RM7 and 37-40% for 208) was produced at relatively low temperatures: 25 and 30°C. When temperature was increased above 30°C and up to 65°C colour reduction decreased and stabilized to about 40% (RM7) and 35% (208).

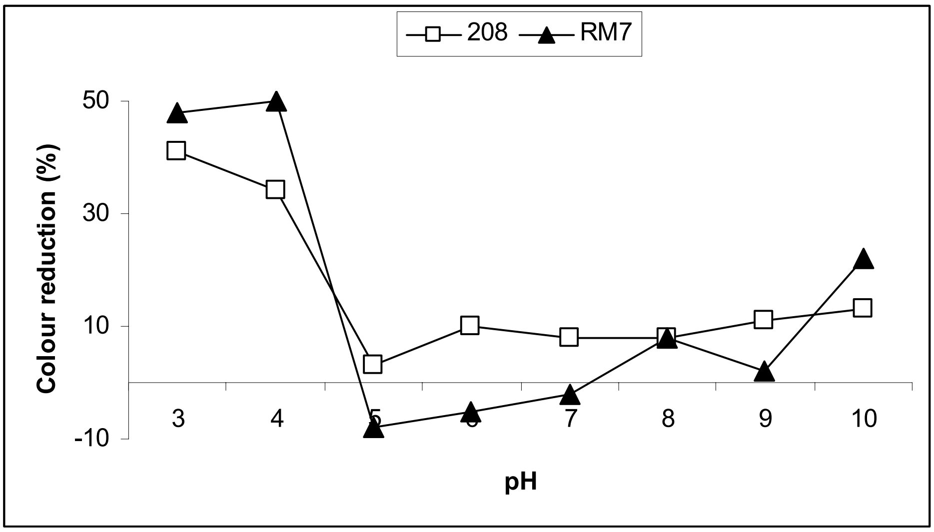

Fig. 5.1. Effect of pH on colour reduction of bleach plant effluent (Eo) by *Rhizomucor pusillus* RM7 and *Trametes nivosa* 208

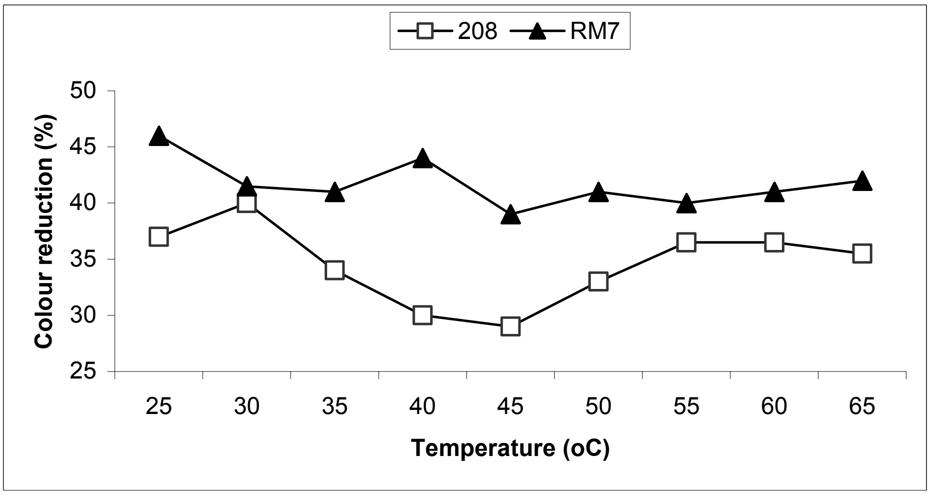

Fig. 5.2. Effect of temperature on colour reduction of bleach plant effluent (Eo) by *Rhizomucor pusillus* RM7 and *Trametes nivosa* 208

5.3.4.1.5 Effect of biomass charge and age

Results indicate that when the biomass size of RM7 was increased from 5 g l^{-1} to 13 g l^{-1} no significant change in colour was observed (Fig. 5.3). Apparently only a fraction of the total color (approximately 50%) is adsorbable. If the adsorption capacities of RM7 and 208 used under the same conditions are compared, it is obvious that RM7 could decolourize E_O effluent to a greater extent. It was observed that the age of the biomass used for effluent treatment also can influence the colour of E_O over a short treatment period. A maximum colour reduction of 38% was achieved when using a 67 h old biomass. Dead cells could adsorb colour as good as living fungal biomass (data not shown).

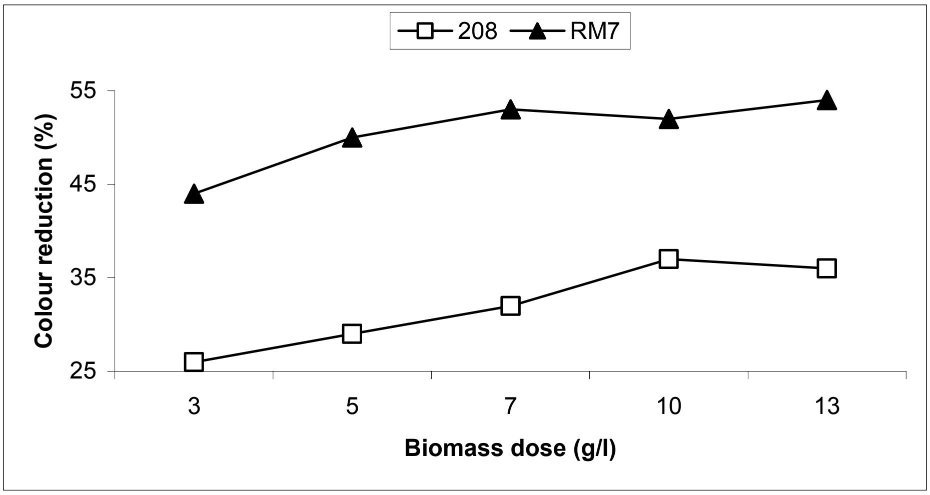

Fig. 5.3. Effect of biomass dose on colour reduction of (Eo) by *Rhizomucor pusillus* RM7 and *Trametes nivosa* 208

5.3.4.1.6 Effect of aeration

It appeared that aeration was not important in terms of colour reduction of E_O by RM7: 65% reduction was observed after a 7 days treatment with RM7 and 66% without aeration (Table 5.4). Colour decrease with RM7 over an 8 h period was also independent of aeration. On the other hand, aeration was needed for efficient fungal growth and oxidation of coloured compounds in effluent by 208 (data not shown).

Table 5.4. Effect of aeration on colour reduction of bleach plant effluent (Eo) by *Rhizomucor pusillus*[a]

Treatment	Colour reduction (%)		
	0 h	8 h	7 days
Aeration	16	52	65
No aeration	17	55	66

[a]Biomass dose 5 g/L; 50°C; pH 4 (8 h) and 5 (7 days)

5.3.4.1.7 Effect of initial colour of effluent

A maximum color reduction of 48% was observed at an initial colour of E_O of about 3 000 PCU as compared to the control of 30% reduction obtained on undiluted E_O with initial 11400 PCU (Table 5.5). Apparently colour adsorption by biomass has an optimum depending on the concentration of the coloured components in the effluent.

5.3.4.1.8 Effect of effluent presterilisation

Although the effluent was not autoclaved before inoculation with RM7, no contamination was observed over a 7 day period (Fig. 5.4). Apparently this was due to the elevated incubation temperature of 50°C which the mucoralean fungus could tolerate but not the white-rot fungus. When unsterilised E_O was used in the treatment experiment with 208, contamination problems occurred (data not shown).

5.3.4.1.9 Effect of effluent recycling

Adsorption of colour from E_o-effluent was conducted with fresh batches of *R. pusillus* biomass to repeatedly treat the same effluent sample in five one-hour cycles, resulting in 33% colour removal during the first cycle (Fig. 5.5). During the subsequent two cycles this value increased to 44% and then remained constant during the last two cycles. This seems to indicate that the biomass can only remove a certain "adsorbable" fraction of the coloured compounds present in the effluent. Apparently this "adsorbable" fraction possesses certain common molecular properties which would correlate with the sorption capacity of the microbial biomass.

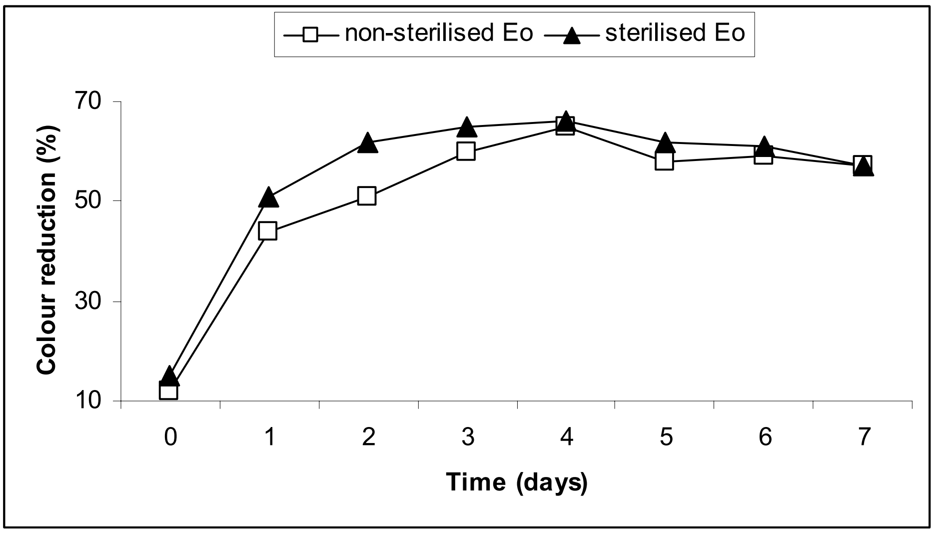

Fig. 5.4. Effect of effluent presterilisation on colour reduction of bleach plant effluent (Eo) by *Rhizomucor pusillus* RM7

Table 5.5. Effect of initial colour of bleach plant effluent (Eo) on colour reduction by *Rhizomucor pusillus*[a]

Initial colour (PCU)	Colour reduction (%)
11400	30(±3)
7706	26(±6)
5825	36(±2)
4671	43(±2)
3875	36(±2)
3510	35(±1)
2925	48(±2)
1875	41(±3)
1350	35(±2)

[a]Biomass dose 5 g/L; 50°C; pH 4; 1 h

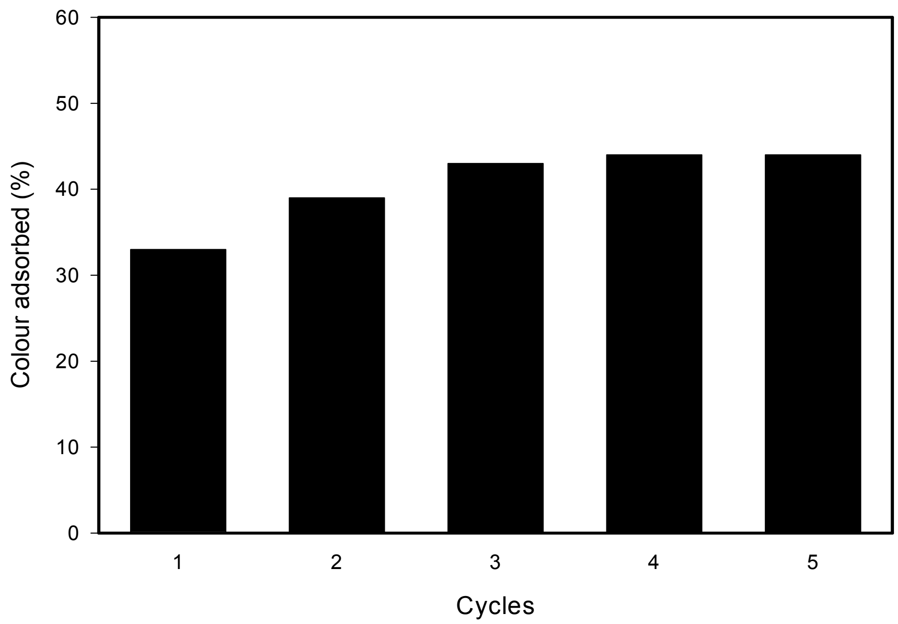

Fig. 5.5. Effect of effluent recycling on colour adsorption by *Rhizomucor pusillus* biomass at 30°C (charge: 0.5 g biomass/100 ml effluent; cycle incubation time 1 h)

5.3.4.1.10 Effect of biomass recycling

A gradual decrease in the decolouring activity was observed from 34%, after the first cycle, to 12% in the last, when the same batch of biomass was employed to treat fresh effluent samples in five cycles (Fig. 5.6). Thus, saturation of the active biomass adsorption sites probably occurred as the treatment cycles progressed. This once again indicates that the colour reduction by the fungal biomass of RM7 in these conditions is a process of sorption of chromophores from the effluent to the biomass. The linear adsorption isotherm indicates that the maximum adsorption capacity of biomass was not reached for the colour densities tested (Fig. 5.7). There was however a fairly strong affinity of this biosorbent material for the coloured compounds of the effluent as judged by the slope of the isotherm. Apparently the use of *Rhizomucor* would be most efficient in the first 2-4 hours of effluent treatment, when 84-88% of the total adsorbable colour was already removed from the effluent.

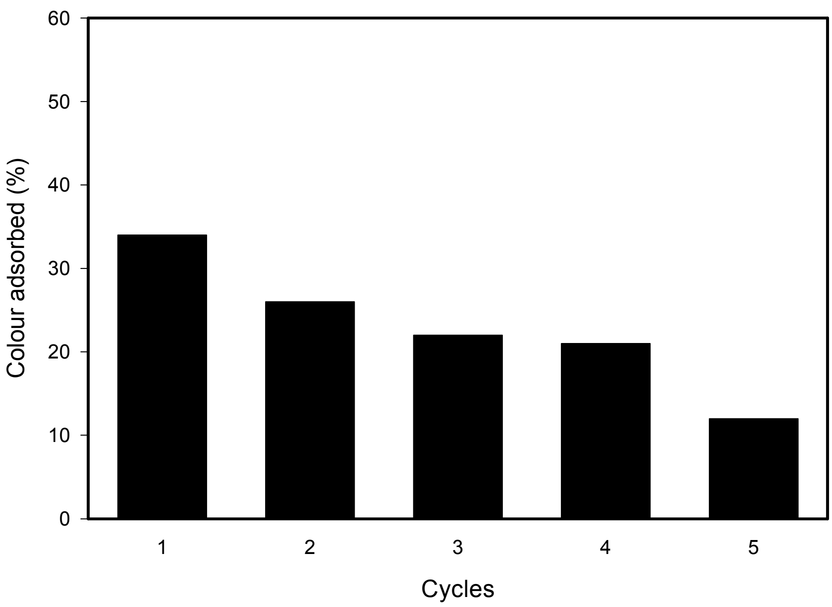

Fig. 5.6. Effect of biomass recycling on colour adsorption by *Rhizomucor pusillus* biomass at 30°C (charge: 0.5 g biomass/100 ml °effluent; cycle incubation time 1 h)

5.3.4.2 *Effect of fungal treatment on effluent properties*

Both *R. pusillus* and *T. nivosa* removed a significant portion of color from E_O within 3 days of treatment: 66% (RM7) and 71% (208). COD of effluent was reduced by 23% and 29% by RM7 and 208, respectively, when compared to COD of untreated E_O (Table 5.6).

RS were almost completely depleted by both treatments. A 12% reduction in the absorbance at 280 nm (phenolics) was detected on the 208-pretreated effluent. At the same time, a 19% increase in the absorbance at 254 nm (aromatics) was observed when effluent was treated with RM7. Both treatments reduced toxicity of E_O. RM7 was much more efficient than 208 in this regard: 132% (RM7) compared to 80% (208) decrease of the toxic impact of E_O, respectively. At the same time, AOX was reduced by 32% and 34% after treatment with RM7 and 208, respectively (Table 5.6). For comparison, the AOX levels of E_O effluent decreased by 23% only after 1 h treatment with RM7 and by 35% after 1 week incubation with 208 (data not shown).

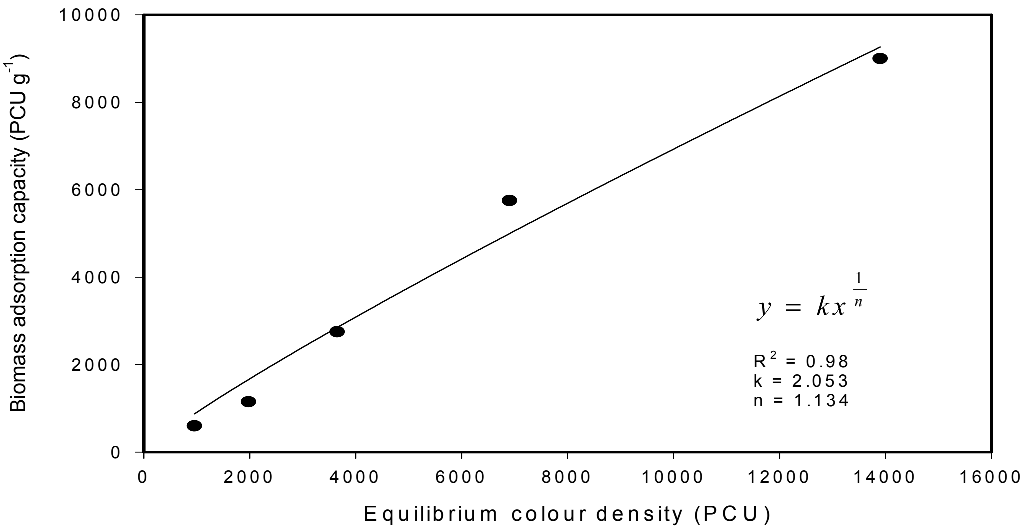

Fig. 5.7. Adsorption isotherm of coloured compounds from bleach plant effluent (Eo) on *Rhizomucor pusillus* biomass.

Table 5.6. Effect of fungal treatment with *Rhizomucor pusillus* RM7 and *Trametes nivosa* 208 on properties of bleach plant effluent (Eo)[a]

Analysis	Control	Reduction as compared to control (%)	
		RM7	208
Colour (PCU)	12000	66	71
COD (mg/l)	19530	23	29
RS (mg/ml)	0.4	98	97
A_{280} (nm)	39	2	12
A_{254} (nm)	48	-19	5
Toxicity (%)	-100	132	80
AOX mg/l)	16	32	34

biomass dose 5 g/L; 50^{o}C; pH 5; 3 days

5.3.4.3 Colour adsorption using physico-chemical adsorbents

For comparative purpose, the colour uptake from effluent by various adsorbents was studied. Results from the adsorption experiments with activated carbon indicated that 37% of colour was removed following treatment with this adsorbent for 24 h (Fig. 5.8). The level of colour removal achieved after 2 h treatment was 24%. Dowex (strong anion exchanger) removed 48% of colour during 24 hours of treatment (Fig. 5.8) whereas the Bio-Rad resin (strong cation exchanger) could retain only 4% of the chromophoric material present in E_o (data not shown). This seems to indicate that most of the coloured compounds found in the effluent exhibit a negative charge. Commercial chitin and chitosan decolourised E_o-effluent by up to 7% and 34%, respectively (Fig. 5.8). From these results it was evident that chitosan had a greater affinity for colour than chitin. Interaction between anionic-based compounds and chitin has been attributed to van der Waals forces as well as electrostatic attraction between the coloured molecules and the cationic protonated amino groups (Rippon 1984). Because chitosan contains a higher proportion of amino groups than chitin, the electrostatic interactions would be expected to be higher for chitosan than chitin (Rippon 1984). Chitin and chitosan are found in the cell walls of mucoralean fungi. These substances were reported to exhibit cationic characteristics and function as anionic exchange materials (Anderson et al., 1997).

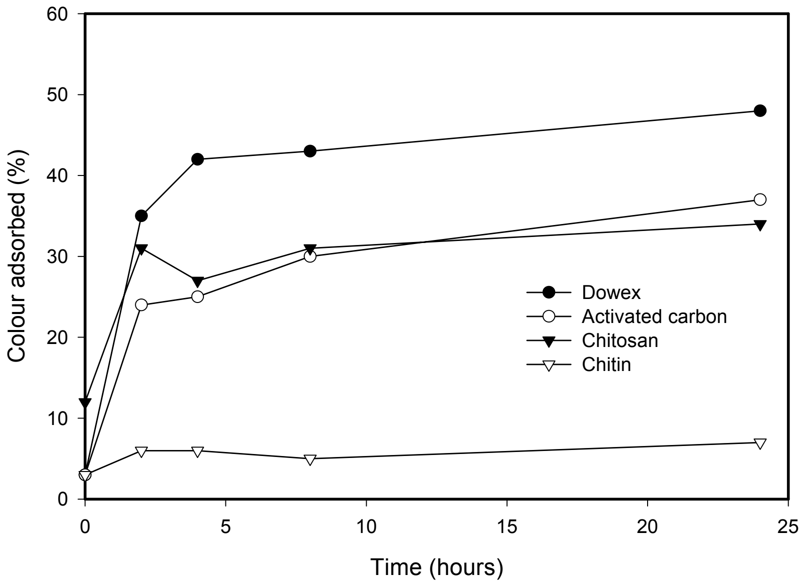

Fig. 5.8. Adsorption of colour from bleach plant effluent (Eo) at 30°C by various physico-chemical adsorbents (charge: 0.5 g adsorbent/100 ml effluent).

5.3.4.4 Colour desorption

Of practical interest is the extent to which adsorbed colour could be released and sorbent materials regenerated for subsequent reuse. The decolouring activity of *R. pusillus* biomass was completely regenerated using 1 N NaOH (Fig. 5.9). Therefore it seems that colour adsorption by biomass is a reversible process and colour-containing material could be extracted back from the biomass. On the other hand, a 50% restoration of the adsorption abilities of chitin was obtained. The amount of colour desorbed from chitosan and activated carbon decreased to 18% and 16%, respectively, whereas very little colour could be removed from Dowex. Thus, chemisorption may be responsible for the inability of the sorbent materials to release colour.

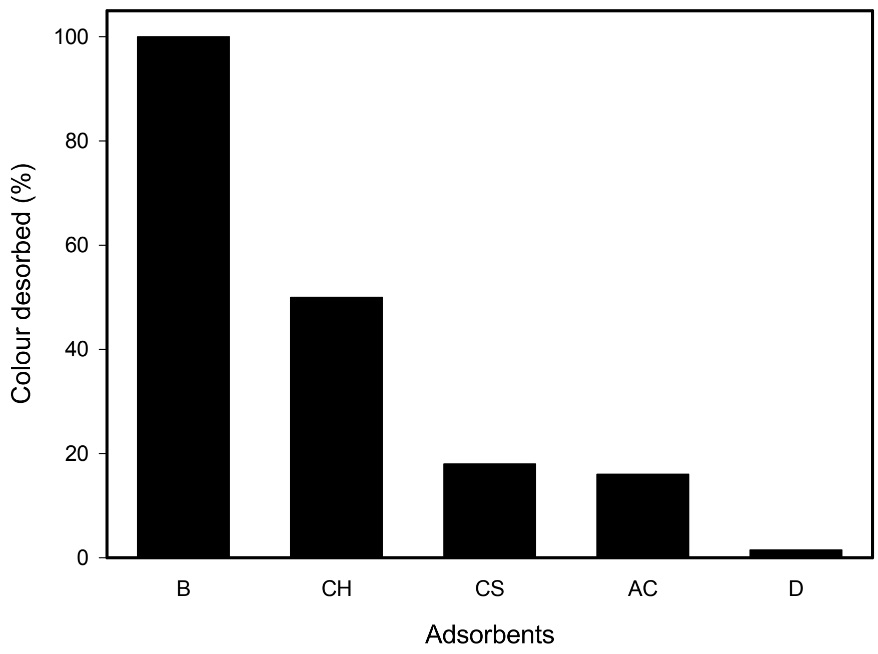

Fig. 5.9. Desorption of colour from various adsorbents using 1 N NaOH: B, biomass; CH, chitin; CS, chitosan; AC, activated carbon; D, Dowex.

5.3.5 Effluent utilization

5.3.5.1 Carbohydrate composition of spent sulfite liquor

The carbohydrate composition of the SSL effluent indicated the presence of a number of monosugars as determined by the HPLC analysis (Fig. 5.10). The predominant carbohydrate was xylose which constituted 71% of the total amount of monosugars or 25 g l^{-1} of effluent (Table 5.7). In addition, some appreciable amount of glucose (17%) and galactose (7%) were detected. The total monosugar content of the effluent was about 36g l^{-1}. This shows that SSL could be a potential cheap carbon source for many biotechnical applications.

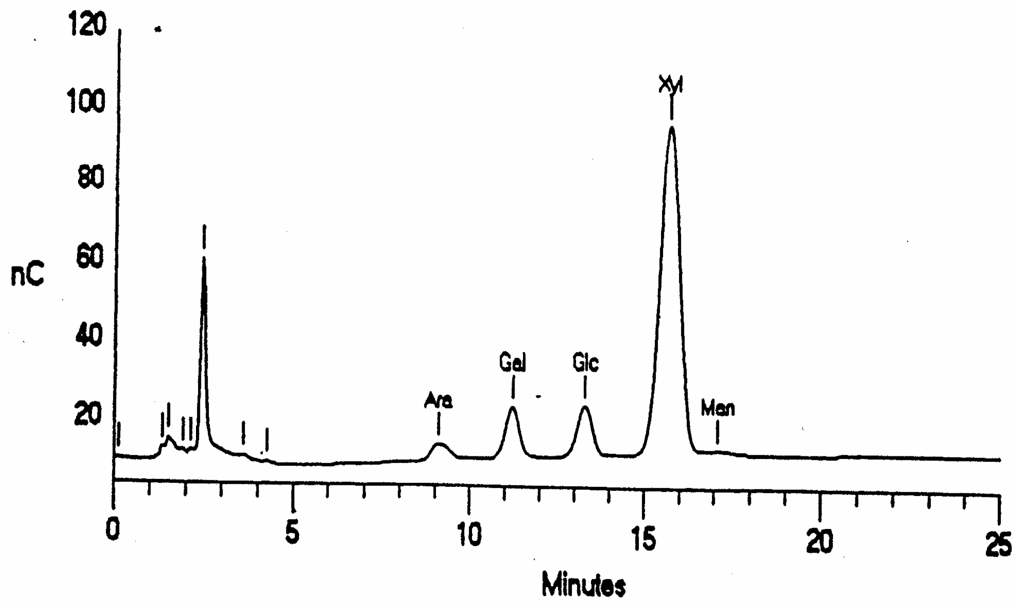

Fig. 5.10. HPLC chromatogram of the monocarbohydrate composition of spent sulfite liquor (SSL)

Table 5.7. Monocarbohydrate composition of spent sulfite liquor (SSL)

Sugar		Concentration		
		mg ml^{-1}	% (w/v)	% (relative)
Arabinose	(Ara)	1.30[a]	0.13	3.65
Galactose	(Gal)	2.44	0.24	6.85
Glucose	(Glu)	6.10	0.61	17.13
Xylose	(Xyl)	25.29	2.53	71.04
Mannose	(Man)	0.47	0.05	1.33
Total		35.6	3.56	100.00

[a] Values are averages of duplicate determinations

5.3.5.2 Carbohydrate composition of bleach plant effluent

Most of the carbohydrates of the bleach plant effluent Eo were in a form of solubilised oligosaccharides as a result of the alkaline extraction. Eo can be enriched tremendously in monocarbohydrates (approximately 45 times) after acid hydrolysis with sulphuric acid (Table 5.8). The amount of arabinose was 2.5 times and that of glucose approximately 15 times greater after hydrolysis than before, however, xylose concentration increased 380 times which rendered it the predominant sugar in Eo with 86% of the total sugar content. Furthermore, xylose was shown to enhance xylanase production in certain microorganisms.

5.3.5.3 Utilisation of spent sulfite liquor

5.3.5.3.1 Pretreatment

The presence of other components in SSL such as acetic acid, furfural, phenols, sulfites, etc. represents an inhibition barrier towards the efficient utilization of the carbohydrates. Therefore some partial removal of some of these components is regarded necessary prior to the use of SSL.

Table 5.8. Monocarbohydrate composition of bleach plant effluent (Eo)

Sugar	Raw Eo% (mg/100ml)	of total	Hydrolysed Eo (mg/100ml)	% of total
Mannose	ND[a]	0.0	0.03	0.2
Galactose	ND	0.0	0.07	0.4
Arabinose	0.22	57.9	0.55	3.1
Glucose	0.12	31.6	1.85	10.4
Xylose	0.04	10.5	15.20	85.9
Total	0.38	100	17.70	100

ND[a] , not detected

Table 5.9 presents results from the steam-stripping of SSL aimed at removal of some of the volatile components in the effluent. As indicated, this pretreatment was very effective in reduction of the acetic acid (by 67%) and sulfite (by 86%) content of SSL. Some decrease of phenols (by 17%) and COD (by 8%) was detected as well (Table 5.10). However, the COD of the effluent after steam-stripping was still very high (211 g l^{-1}) for any microbial growth, thus a 10-fold dilution was carried out to bring the COD down to about 21 g l^{-1}.

5.3.5.3.2 Utilisation for xylanase production

The SSL effluent pretreated as described above was then used as a sole carbon source in xylanase production by the yeast *Aureobasidium pullulans*. This enzyme has proved to be very efficient in the biobleaching experiments on sulfite pulp and its eventual production on a cheap carbon source would contribute further to make the entire biotechnological process more cost-effective. Table 5.10 shows data from the experiments on enzyme production. The effect of pH in combination with some mineral supplements on the xylanase biosynthesis by *A. pullulans* was studied and the xylanase activity after 24 h and 48 h incubation was assayed.

Table 5.9. Steam-stripping of spent sulfite liquor (SSL)

Components	Concentration (g l^{-1})		Reduction
	Initial	Steam-stripping	(%)
Acetic acid	21[a]	7	67
Sulfites	1.75	0.25	86
Phenols	0.0012	0.0010	17
COD	230	211	8

[a] Values are averages of two determinations

Xylanase activity produced on pure xylose (15 g l^{-1}) served as control. Results indicated that a higher activity was produced on SSL (both supplemented and non-supplemented) at pH 4.25 than pH 5.0 or 6.0. Maximum xylanase activity on SSL at pH 4.25 after both 24 h and 48 h was obtained when some minerals (H_3PO_4, KH_2PO_4 and $MgSO_4$) were added: 23 Uml^{-1} or 71% of control (at 24 h) and 19 U ml^{-1} or 38% of control (at 48 h). In most instances xylanase production on SSL peaked at the 24 h and after that declined whereas maximum enzyme activity on xylose was produced after 2 days: 50 U ml^{-1}. The non-supplemented effluent also produced promising results: 53% of control activity after 24 h and 31% after 48 h, respectively (Table 5.10).

5.3.5.4 *Utilisation of bleach plant effluent for xylanase production*

All three bleach plant effluents when used as a sole carbon source produced some xylanase activity which ranged between 14 and 29% of the control of 10 g l^{-1} xylose, or 175% and 362% when compared to the control of 1 g l^{-1} xylose with the best results obtained with the Eo effluent (Table 5.11). Eo effluent was also membrane-treated and both the permeate and retentate were tested separately in the fermentation experiments.

Table 5.10. Utilisation of spent sulfite liquor (SSL) as a sole carbon source[a] for xylanase production by *Aureobasidium pullulans*

Supplements (g l^{-1})	pH	Xylanase activity (U ml^{-1})			
		24 h	% of control[b]	48 h	% of control
Control	5.0	33	100	50	100
None	4.25	17	53	15	31
None	5.0	9	26	5	10
H_3PO_4 (0.3)	4.25	5	16	10	20
H_3PO_4 (0.3)	6.0	6	19	9	19
H_3PO_4 (0.3) KH_2PO_4 (0.1) $MgSO_4.7H_2O$ (0.5)	4.25	23	71	19	38
H_3PO_4 (0.3) KH_2PO_4 (0.1) $MgSO_4.7H_2O$ (0.5)	5.0	16	47	12	24

[a] Spent sulfite liquor (SSL) was first steam-stripped at 94°C for 0.5 h and then diluted 10 times
[b] Control (g l^{-1}): xylose, 15; yeast extract, 1; KH_2PO_4, 5; $(NH_4)_2SO_4$, 2.5 (pH 5.0)

Both SSL and Eo effluents have been treated with the ultrafiltration Osmonics membrane BQ01 (molecular weight cut-off of about 1000 Da) and colour of the permeate and retentated were compared to colour of control. Results indicated that the BQ01 membrane filter was very effective in colour removal: the permeate of Eo contained 92% less colour than the feed whereas the SSL permeate was almost colourless (98.3-100%; data not shown). Another two membranes were tested on the SSL effluent: Desal-5L (MW cut-off about 300 Da) and B006 (less than 1000

Da MW cut-off). Both of them produced colourless permeate (data not shown). Therefore this pretreatment, although useful in separation of colour, did not lead to any further enhancement in the activity titres obtained (Table 5.11). Encouraging results were obtained with the yeast *A. pullulans*: xylanase levels of as high as 50% of the control (fermentation on 1% xylose) were produced on Eo under non-optimized conditions. Screening of seven fungal isolates on the bleach plant effluent in shake flasks indicated that the production of cellulase-free xylanase enzymes was pH-dependent. The economical feasibility of the entire biobleaching technology using xylanases would be improved by utilizing Eo which at present is discarded as a waste product. In addition, this would alleviate to a degree the environmental pressure on the industry.

Table 5.11. Utilisation of bleach plant effluents as a sole carbon source[a] for xylanase production by *Aureobasidium pullulans*

Effluent	Reducing sugars	Relative xylanase activity (% of control)	
	($g\ l^{-1}$)	Control: xylose ($10\ g\ l^{-1}$)	Control: xylose ($1\ g\ l^{-1}$)
Total effluent	2.10	26	325
O-effluent	0.11	14	175
Eo-effluent	0.17	29	362
Eo-effluent[b] (permeate)	0.01	13	162
Eo-effluent (retentate)	0.20	20	250

[a] *A. pullulans* was grown in shake flasks at 28°C for 3 days in a medium containing: ($g\ l^{-1}$): carbon source (as indicated); citric acid, 0.25; yeast extract, 5; $(NH_4)_2SO_4$, 5; KH_2PO_4, 3; $MgSO_4.7H_20$, 0.4; $CaCl_2.2H_20$, 0.02
[b] Eo-effluent was treated by ultrafiltration with Osmonics membrane BQ01 (1000 Da molecular weight cut-off)

5.4 CONCLUSIONS

- As a result of the total reduction of the active chlorine charges of 40% during biobleaching of dissolving pulp, the chloride and AOX levels of the total bleach pulp effluent were reduced respectively by 31% and 38%. COD decreased by 22.5% at D_1, 7% at Eo and 20% at D_2. The Cl^- content was reduced by 21% at D_1, 30% at Eo and 47% at D_2. The AOX content of D_1-

effluent was reduced by 45%, of Eo-effluent by 24% and of D_2-effluent by 37% when compared to the corresponding controls, respectively.

- Treatment of E_O with the white-rot fungus *Trametes nivosa* 208 and the mucoralean fungus *Rhizomucor pusillus* RM7 required a minimum of 3 days for efficient colour degradation and reduction of about 65-70%. A prolonged treatment for up to 7 days did not decrease further colour of effluent.
- Colored compounds in E_o-effluent were mainly negatively charged (>90%) and of hydrophilic nature. The bleach plant effluent from the alkali extraction stage (E_o) contained a fraction of coloured compounds (approximately 50% of total) which was adsorbable onto *Rhizomucor pusillus* biomass. The biosorption of colour was a rapid process which could remove approximately 90% of the adsorbable colour in the first few hours of effluent treatment.
- Adsorption of colour from effluent by the fungal biomass was favoured by relatively low temperatures (30°) and pH (4). The biomass from *Rhizomucor* RM7 proved to be a stronger bioadsorbent of colour than that of *Trametes*. Treatment with RM7 required 6-8 h to decolourize the effluent by 45-55%. After only 2 h RM7 was able to reduce color by 43%. At the same time *Trametes* could remove about 35% of colour from E_O.
- It was demonstrated that the fungal biomass could be completely regenerated and reused on the effluent. This would be beneficial because of the large volumes of wastewater emanating from pulp and paper mills that require remediation. Only partial restoration of the decolouring activities of the commercial physico-chemical adsorbents was obtained, suggesting chemisorption of coloured compounds onto the sorbent material.
- The use of *Trametes* required aeration for efficient fungal growth and biodegradation of coloured compounds. Aeration was found not important in the decolourization process by *Rhizomucor*.
- Conditions would have to be monitored to prevent microbial contamination, especially when prolonged treatment periods are used at lower temperatures. To avoid this, decontamination of Eo prior to fungal treatment would be necessary. *Rhizomucor pusillus* could grow and tolerate temperatures of up to 55°C and retain 85% of its decolouring abilities at 55°C as compared to 30°C. The use of elevated temperatures for effluent treatment would reduce the chances of microbial contamination and eliminate the need of cooling the effluent.
- Fungal treatment of E_O could substantially reduce the COD (20-30%), toxicity (80-130%) and AOX (30%) levels in the effluent. Results from fungal biotreatment of E_O were sensitive

towards variation in the E_O composition and may vary from batch to batch depending on the initial pH, temperature and colour of E_O.

- To the best of our knowledge, this is the first report of using a mucoralean fungus in decolourisation of pulp and paper mill effluents. Biomass of *R. pusillus* proved to be efficient and compared well with other physico-chemical adsorbents in uptake of colour from the effluent. This biosorbent material was found to have a relatively strong affinity for the coloured compounds in the effluent as judged by the slope of the adsorption isotherm. Under the conditions used in this study, the following descending order of colour removal was established: biomass (51%) > strong anion exchanger (48%) > activated carbon (37%) > chitosan (34%) > chitin (7%) > strong cation exchanger (4%).
- Results from preliminary experiments indicated that the bleach plant effluent could be used as a sole carbon source for xylanase production. Under non-optimised conditions, the effluent induced the production of cellulase-free enzymes in most of the strains tested. To the best of our knowledge, this is the first report of utilising a bleaching effluent from the pulp and paper industry for enzyme (xylanase) production.
- Screening of seven fungal isolates on the bleach plant effluent in shake flasks indicated that the production of cellulase-free xylanase enzymes was pH-dependent.
- Encouraging results were obtained with the yeast *Aureobasidium pullulans*: xylanase levels of as high as 50% of the control (fermentation on 1% xylose) were produced on Eo under non-optimized conditions. Membrane pretreatment of effluent, although useful in separation of colour, did not lead to any further enhancement in the xylanase activity titres obtained.
- The SSL and bleach plant effluents represent soluble substrates and potential cheap and abundant carbon sources. Bleach plant effluent Eo can be enriched in monocarbohydrates (approximately 45 times) following acid hydrolysis with sulphuric acid. The amount of arabinose was 2.5 times and that of glucose approximately 15 times greater after hydrolysis than before. However, the xylose concentration increased 380 times which rendered it the predominant sugar in the treated Eo with 86% of the total sugar content. Xylose was shown to enhance xylanase production in certain microorganisms.
- The economical feasibility of the entire biobleaching technology using xylanases would be improved by utilising both SSL and Eo which at present are discarded as industrial waste. This would in turn alleviate to a degree the environmental pressure on the industry. Therefore attention should be focused on optimization of enzyme production using pulp mill effluents.

CHAPTER 6
GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 GENERAL CONCLUSIONS

- Biopulping of wood chips with selected white-rot fungi as well as biobleaching of sulfite pulp with xylanases are promising technologies in terms of improving the quality of pulp and bleach plant effluent thereby resulting in considerable savings in chemicals and reductions of the AOX/chloride-levels, colour, toxicity and chemical load of the effluent.
- It has been demonstrated that effluents may be useful in the production of valuable by-products, in particular xylanases, which in turn can be applied in the biobleaching process. This would further contribute to the overall reduction of the environmental impact of the waste waters.

6.2 RECOMMENDATIONS

- Further research is needed as to optimise the fungal pretreatment conditions for biosulfite pulping. The need for decontamination of wood chips prior to biopulping may however preclude the large-scale implementation of the process.
- The costs for the enzyme bleaching process could be further reduced by using new xylanase products with improved pH and temperature characteristics at low enzyme doses.
- The economical feasibility of the entire biobleaching technology using xylanases would be improved by utilising both the SSL and Eo which at present are discarded as industrial waste. This would in turn alleviate the environmental pressure on the industry. Therefore attention should be focused on optimisation of enzyme production using pulp mill effluents.

REFERENCES

Akhtar, M., Attridge, M. C., Myers, G. C., Kirk, T. K. and Blanchette, R. A. (1992) Biomechanical pulping of Loblolly pine with different strains of the white-rot fungus C*eriporiopsis subvermispora. Tappi J.,* **75**, 105-109.

Akhtar, M., Attridge, M.C., Myers, G.C. and Blanchette, R.A. (1993) Biomechanical pulping of Loblolly pine chips with selected white-rot fungi. *Holzforschung,* **47,** 36-40.

Al-Kassim, L., Taylor, K.E., Bewtra, J.K. and Biswas, N. (1994) Optimization of phenol removal by a fungal peroxidase from *Coprinus macrorhizus* using batch, continuous, and discontinuous semibatch reactors. *Enzyme Microb. Technol*., **16**, 120-l24.

Ander P., Eriksson K.-E., Kolar M.-C., Kringstad K.P., Rannung U. and Ramel C. (1977) Studies on the mutagenic properties of bleaching effluents. *Sven. Papperstidn.* **80**, 454-859.

Anderson, J. E., Beelman, R. B. and Doores, S. (1997) Enhanced production and thermal stability of staphylococcal enterotoxin A in the presence of chitin. *J. Food Protec.*, **60**, 1351-1357.

Annergren, G.A. and Rydholm, S.A. (1959) On the bahavior of hemicelluloses during sulfite pulping. *Svensk Papperstidning*, **62**, 737-746.

Anselmo, A.M. and Novais, J.M. (1992) Biological treatment of phenolic wastes: comparison between free and immobilized cell systems. *Biotechnol. Lett*., **14**, 239-244.

APHA (1989) *Standard Methods for the Examination of Water and Wastewater*. Ed. L.S. Clesceri, A.E. Greenberg and R.R. Trussel. American Public Health Association, Washington, DC.

Archibald, F., Piace, M.G. and Jurasek, L. (1990) Decolorization of kraft bleachery effluent chromophores by *Coriolus* (*Trametes*) *versicolor Enzyme Microb. Technol*., **12**, 846-853.

Bajpai, P., and Bajpai, P.K. (1994) Biological colour removal of pulp and paper mill wastewaters. *J. Biotechnol.,* **33**, 211-220.

Bergbauer, M., Eggert, C. and 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.

Bethlehem L. (1995) *An Industrial Strategy for the Pulp and Paper Sector.* UCT Press, Cape Town.

Biermann C.J. (1993) *Essentials of Pulping and Papermaking.* Academic Press, New York.

Blanchette, R.A., Burnes, T.A., Leatham, G.F. and Effland, M.J. (1988) Selection of white-rot fungi for biopulping. *Biomass*, **15**, 93-101.

Blanchette, R.A., Leatham, G.F., Attridge, M., Akhtar, M. and Myers, G.C. (1991) Biomechanical pulping with *Ceriporiopsis subvermispora. US Patent* 5,055,159.

Blanchette, R.A. (1995) Degradation of lignocellulose complex in wood. *Can. J. Bot.*, **73,** 999-1010.

Boman, B. and Frostell, B. (1988) Some aspects on biological treatment of bleached pulp effluents. *Nordic Pulp and Paper Res. J.*, 13-18.

Call, H. P. (1993) Process for producing cellulose from lignin containing raw materials using an enzyme or microorganism while monitoring and maintaining the redox potential. *US Patent* 5,203,964.

Chen, Y.-R. and Schmidt, E.L. (1995) Improving aspen kraft pulp by a novel, low-technology fungal pretreatment. *Wood Fiber Sci.,* **27**, 198-204.

Christov L.P. and Prior B.A. (1993) Esterases of xylan-degrading microorganisms: Production, properties, and significance. *Enzyme Microbial. Technol.* **15**, 460-475.

Christov L.P. and Prior B.A. (1993) Xylan removal from dissolving pulp using enzymes of *Aureobasidium pullulans*. *Biotechnol. Lett.* **15**, 1269-1274.

Christov L.P. and Prior B.A. (1994) Enzymatic prebleaching of sulphite pulps. *Appl. Microbiol. Biotechnol.* **42**, 492-498.

Christov L.P. and Prior B.A. (1995) Role of hydrolysis conditions in the enzyme-mediated release of reducing sugars from sulphite pulp. *Biotechnol. Lett.* **17**, 821-826.

Christov L.P. and Wolfaardt J.F. (1995) Use of biological methods in production of dissolving pulp from eucalyptus wood. *All African Conference on Biotechnology.* Pretoria, November 13-15.

Christov L.P. and Akhtar M. (1996) Use of *Ceriporiopsis subvermispora* in dissolving pulp production. *ACS Symp. Environmentally Benign Pulping Methods: Fungal Biotechnology and Biopulping.* New Orleans, March 25-28.

Christov L.P. and Prior B.A. (1996) Biobleaching of sulphite pulp for producing dissolving pulp. *S.A. Patent* 96/4589.

Christov L.P. and Prior B.A. (1996) Biopulping and biobleaching of sulphite pulp for producing dissolving pulp. *S.A. Patent* 96/4590.

Christov L.P. and Prior B.A. (1996) Repeated treatments with *Aureobasidium pullulans* hemicellulases and alkali enhance biobleaching of sulphite pulps. *Enzyme Microb. Technol.* **18**, 244-250.

Christov L.P. and Prior B.A. (1996) Reduction of active chlorine charges in bleaching of xylanase-pretreated sulfite pulp. In *Enzymes for Pulp and Paper Processing*, ed. T.W. Jeffries and L. Viikari, chap. 16, pp. 208-218. American Chemical Society, Washington, DC.

Christov L.P., Akhtar M. and Prior B.A. (1996) Biobleaching in dissolving pulp production. In *Biotechnology in the Pulp and Paper Industry*, ed. E. Srebotnik and K. Messner, pp. 625-628. Facultas-Universitätsverlag, Vienna.

Christov L.P., Akhtar M. and Prior B.A. (1996) Impact of xylanase and fungal pretreatment on alkali solubility and brightness of dissolving pulp. *Holzforschung* **50**, 579-582.

Christov, L.P. and Prior, B.A. (1997) Biobleaching of sulphite pulp for producing dissolving pulp. *S.A. Patent* 96/4589.

Christov, L.P. and Prior, B.A. (1997) Biopulping and biobleaching of sulphite pulp for producing dissolving pulp. *S.A. Patent* 96/4590.

Christov L.P. and Prior B.A. (1997) Bleaching response of sulfite pulps to pretreatment with xylanases. *Biotechnol. Progr.* **13**, 695-698.

Christov L.P., Myburgh J, van Tonder A. and Prior B.A. (1997) Hydrolysis of extracted and fibre-bound xylan with *Aureobasidium pullulans* enzymes. *J. Biotechnol.* **55**, 21-29.

Christov, L.P. and Prior, B.A. (1998) Research in biotechnology for the pulp and paper industry in South Africa. *SA J. Sci.* 94: 695-698.

Christov, L.P., Akhtar, M. and Prior, B.A. (1998) The potential of biosulphite pulping in dissolving pulp production. *Enzyme Microb. Technol*. 23: 70-74.

Christov, L.P., Szakacs, G. and Balakrishnan, H. (1999) Production, partial chracterisation and use of fungal cellulase-free xylanases in pulp bleaching. *Proc. Biochem.* 34: 511-517.

Dawson-Andoh, B.E., Morrell, J.J., Biermann, C.J. and Hull, J.L. (1991) Effect of fungal pretreatment on strength and optical properties of softwood and hardwood kraft pulps. *Tappi J*., **74,** 187-189.

Eaton D., Chang H.M. and Kirk T.K. (1980) Fungal decolorization of kraft bleach plant effluents. *Tappi* **63**, 103-106.

Eaton, D.C., Chang, H-M., Joyce, T.W., Jeffries, T.W. and Kirk, T.K. (1982) Method obtains fungal reduction of the color of extraction-stage kraft bleach effluents. *Tappi J*., **65**, 89-92.

Edwards, W., Leukes, W.D., Jacobs, E.P., Sanderson, R., Burton, S.G., and Rose, P.D. (1997) Chitosan-coated hollow-fibre membranes for phenolic effluent bioremediation. *Biotech SA '97,* p. 75, Programme and Abstracts.

Eriksson K-E., Kolar, M-C., Ljungquist, P.O. and Kringstad, K.P. (1985) Studies on microbial and chemical conversions of chlorolignins. *Environ. Sci. Technol*., **19**, 1219-1224.

Eriksson, K-E. L. (1991) Biotechnology: three approaches to reduce the enviromental impact of the pulp and paper industry. *Sci. Prog. Oxford* **75**, 175-189.

Eriksson, K-E. L. (1993) Concluding remarks: where do we stand and where are we going? Lignin biodegradation and practical utilization. *J. Biotechnol*., **30**, 149-158.

Feijoo, G., Vidal, G., Moreira, M.T., M⎾ndez R. and Lema, J.M. (1995) Degradation of high molecular weight compounds of kraft pulp mill effluents by a combined treatment with fungi and bacteria. *Biotechnol. Lett*., **17,** 1261-l266.

Fengel, D. and Wegener, G. (1984) *Wood: Chemistry, Ultrastructure, Reactions*. Walter de Gruyter, Berlin.

Ferraz A., Christov L.P. and Akhtar M. (1998) Fungal pretreatment for organosolv and dissolving pulp production. In *Environmentally Friendly Technologies for the Pulp and Paper Industry*, ed. R.A. Young and M. Akhtar, chap. 13, pp. 421-447. John Wiley & Sons, Inc., New York.

Fischer, K., Akhtar, M., Blanchette, R.A., Burnes, T.A., Messner, K. and Kirk, T.K. (1994) Reduction of resin content in wood chips during experimental biological pulping processes. *Holzforschung*, **48**, 285-290.

Gerischer G.F.R. (1993) Pulp and paper manufacture in South Africa. In *Forestry Handbook*, ed. H.A. van der Sijde, pp. 498-519. South African Institute of Forestry, Pretoria.

Hernandez, M., Rodriquez, J., Soliveri, J. and Copa, J.L. (1994) Paper mill effluent decolorization by fifty *Streptomyces* strains. *Appl. Environ. Microbiol*., **60**, 3909-3913.

Hinck J.F., Casebier R.L. and Hamilton J.K. (1985) In *Pulp and Paper Manufacture*, ed. M.G. Kocurek, O.V. Ingruber and P.E. Wong, pp. 213-243. Joint Textbook Committee of the Paper Industry, Montreal.

Jaklin-Farcher, S., Szeker, E., Stifter, U. and Messner, K. (1992) Scale up of the mycopor reactor. In *Proceedings of the Fifth International Conference on Biotechnology in the Pulp and Paper Industry,* ed. Kuwahara, M., and Shimada, pp. 81, Uni Publishers Co., Tokyo.

Jaspers, C.J., Jimenez, G. and Penninckx, M.J. (1994) Evidence for the role of manganese peroxidation in the decolorization of kraft pulp bleach plant effluent by *Phanerochaete chrysosporium*: Effects of initial culture conditions on enzyme production. *J. Biotechnol*., **37**, 229-234.

Jaspers, C..J. and Penninckx, M.J. (1996) Adsorption effects in the decolorization of a kraft bleach plant effluent by *Phanerochaete chrysosporium. Biotechnol. Lett*., **18**, 1257-1260.

Johnson, K.G., Fontana, J.D. and MacKenzie, C.R. (1988) Measurement of acetylxylan esterase. *Methods Enzymol*., **160**, 551-560.

Jokela, J.K. and Salkinoja-Salonen, M. (1992) Molecular weight distributions of organic halogens in bleached kraft pulp mill effluents. *Environ. Sci. Technol*., **26**, 1190-1197.

Kallas, J. and Munter, R. (1994) Post-treatment of pulp and paper industry wastewater using oxidation and adsorption processes. *Water. Sci. Technol*., **29**, 259-272.

Kantelinen, A., Hortling, B., Sundquist, J., Linko, M. and Viikari, L. (1993) Proposed mechanism of the enzymatic bleaching of kraft pulp with xylanases. *Holzforschung*, **47**, 318-324.

Kapoor, A. and Viraraghavan, T. (1995) Fungal biosorption - an alternative treatment option for heavy metal bearing wastewaters: A review. *Biores. Technol.*, **53**, 195-206.

Kirkpatrick, N., Reid, I.D., Ziomek, E. and Paice, M.G. (1990) Biological bleaching of hardwood kraft pulp using *Trametes* (*coriolus) versicolor* immobilized in polyurethane foam. *Appl. Microbiol Biotechnol*., **33**, 105-108.

Labosky, P., Zhang, J. and Royce, D.J. (1991) Lignin biodegradation of nitrogen supplemented red oak (*Quercus rubra*) wood chips with two strains of *Phanerochaete chrysosporium*. *Wood Fiber Sci*., **23**, 533-539.

Lamar, R. T. (1992) The role of fungal lignin-degrading enzymes in xenobiotic degradation. *Environ. Biotechnol*., **3**, 261-266.

Lee, E. G-H., Mueller, J.C. and Walden, C.C. (1978) Decolorization of bleached kraft mill effluents by algae. *Tappi J*., **61**, 59-62.

Leisola, M., Ulmer, D., Haltmeier, T. and Fiechter, A. (1983) Rapid solubilization and depolymerization of purified kraft lignin by thin layers of *Phanerochaete chrysosporium.* Eur. J. Appl. Microbiol. Biotechnol., **17**, 117-120.

Limura, Y., Hartikainen, P. and Tatsumi, K. (1996) Dechlorination of tetrachloroguaiacol by laccase of white-rot basidiomycete *Coriolus versicolor*. *Appl. Microbiol. Biotechnol*., **45**, 434-439.

Livernoche, D., Jurasek, L., Desrochers, M., Doric, J. and Veliky, I.A. (1983) Removal of color from kraft mill wastewaters with cultures of white-rot fungi and with immobilized mycelium of *Coriolus versicolor*. *Biotechnol. Bioeng*., **25**, 2055-2065.

Liversidge, R. M., Lloyd, G. J., Wase, D. A. J. and Forster, C. F. (1997) Removal of basic blue 41 dye from aqueous solution by linseed cake. *Proc. Biochem.*, **32**, 473-477.

Manzanares, P., Fajardo, S. and Martin, C. (1995) Production of ligninolytic activities when treating paper pulp effluents by *Trametes versicolor*. *J. Biotechnol*., **43**, 125-132.

Martin, V.J.J., Burnison, B.K., Lee, H. and Hewitt, M. (1995) Chlorophenolics from high molecular weight chlorinated organics isolated from bleached kraft mill effluents. *Holzforschung*, **49**, 453-461.

Marton, J., Stern, A. M. and Marton, T. (1969) Decolorization of kraft black liquor with *Polyporus versicolor*, a white rot fungus. *Tappi*, **52**, 1975-1981.

Mehna, A., Bajpai, P. and Bajpai, P.K. (1995) Studies on decolorization of effluent from a small pulp mill utilizing agriresidues with *Trametes versicolor*. *Enzyme Microb. Technol*., **17**, 18-22.

Muzarelli, A.A.R., Ilari, P., Xia, W., Pinotti, M. and Tomasetti M. (1994) Tyrosinase-mediated quinone tanning of chitinous materials. *Carbohyd. Polym*. **24**, 295-300.

Messner, K., Masek, S., Srebotnik, E. and Techt, G. (1992) Fungal pretreatment of wood chips for chemical pulping. In *Biotechnology in Pulp and Paper Industry*, ed. M. Kuwahara & M. Shimada. Uni Publishers Co., Ltd, pp. 9-13, Tokyo.

Messner, K. and Srebotnik, E. (1994) Biopulping: an overview of developments in an environmentally safe paper-making technology. *FEMS Microbiol. Rev.,* **13**, 351-364.

Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem*., **31**, 426-428.

Mosai, S., Wolfaardt, J.F., Prior, B.A. and Christov, L.P. (1998) Evaluation of selected lignin-degrading fungi for biosulphite pulping. *Biores. Technol.* (in press)

Nelson, N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.***, 153**, 375-380.

Noe, P., Chevalier, J., Mora, F., Comtat, J. (1986) Action of xylanases on chemical pulp fibers. Part II: Enzymatic beating. *J. Wood Chem. Technol.***, 6**, 167-184.

Oriaran, T.P., Labossky, Jr., P. and Blankenhorn, P.R. (1990) Kraft pulp and papermaking properties of *Phanerochaete chrysosporium*-degraded aspen. *Tappi J.,* **73**, 147-152.

Oriaran, T.P., Labossky, Jr., P. and Blankenhorn, P.R. (1991) Kraft pulp and papermaking properties of *Phanerochaete chrysosporium* degraded red oak. *Wood Fiber Sci.,* **23**, 316-327.

Otjen, L. and Blanchette, R.A. (1986) A discussion on microstructural changes in wood during decomposition by white-rot basidiomycetes. *Can. J. Bot.,* **64,** 905-911.

Otjen, L., Blanchette, R., Effland, M. and Leatham, G. (1987) Assessment of 30 white-rot basidiomycetes for selective lignin degradation. *Holzforschung,* **41,** 343-349.

Paice, M.G. and Jurasek, L. (1984) Removing hemicellulose from pulps by specific enzymic hydrolysis. *J. Wood Chem. Technol.***, 4**, 187-198.

Paice, M.G., N. Gurnagul and L. Jurasek. (1992) Mechanism of hemicellulose-directed prebleaching of kraft pulps. *Enzyme Microb. Technol.,* **14**, 272-276.

Rodriques, A., Perestelo, F., Carnicero, A., Regalado, V. Perez, R., De la Fuente, G. and Falcon, M.A. (1996) Degradation of natural lignins and lignocellulosic substrates by soil-inhabiting fungi imperfecti. *FEMS Microbiology Ecology*, **21**, 213-219.

Prasad, D. Y. and Joyce, T.W. (1991) Color removal from kraft bleach plant effluents by *Trichoderma sp*. *Tappi J*., 165-169.

Rippon, J. A. (1984) Improving the dye coverage of immature cotton fibres by treatment with chitosan. *J. Soc. Dyers Colour.*, **100**, 298-303.

Ritter, D., Jaklin-Farcher, S., Messner, K. and Stachelberger, H. (1990) Polymerization and depolymerzation of lignosulfonate by *Phanerochaete chrysosporium* immobilized on foam. *J. Biotechnol*., **13**, 229-241.

Roy-Arcand, L., and Archibald, F.S. (1991) Direct dechlorination of chlorophenolic compounds by laccases from *Trametes* (*Coriolus*) *versicolor*. *Enzyme Microb. Technol*., **13**, 194-203.

Royer, G., Desrochers, M., Jurasek, L., Rouleau, D. and Mayer, R.C. (1985) Batch and continuous decolorisation of bleached kraft effluents by a white-rot fungus. *J. Chem. Biotechnol*., **358**, 14-22.

Scott, G.M., Akhtar, M., Lentz, M., Sykes, M. and Abubakr, S. (1996) Biosulfite pulping using *Ceriporiopsis subvermispora.* In *Biotechnology in the Pulp and Paper Industry*, ed. E. Srebotnik and K. Messner. Facultas-Universitätsverlag, pp. 217-220, Vienna.

Sierka, R.A. and Bryant, C.W. (1994) Enhancement of biotreatment of effluent quality by illuminated titanium dioxide and membrane pretreatment of the kraft extraction waste stream and by increased chlorine dioxide substitution. *Water Sci. Technol*., **29**, 209-218.

Sjöström E. (1981) *Wood Chemistry: Fundamentals and Applications.* Academic Press, New York.

Smith T.J., Wearne, R.H. and Wallis, A.F.A. (1994) Characteristics of the chlorinated organic substances in filtrates from bleaching of oxygen-delignified eucalypt kraft pulp. *Water. Sci. Technol*., **29**, 61-71.

Srebotnik, E. and Messner, K. (1994) A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white-rot fungi. *Appl. Environ. Microbiol.,* **60,** 1383-1386.

Srinivasan, D. and Unwin, J.P. (1995) Pulp and paper effluent management*. Water Environ. Res.*, **67**, 531-544.

Somogyi, M. (1952) Notes on sugar determination. *J. Biol. Chem.***,** **195**, 19-23.

Suntio, L.R., Shiu, W.Y. and Mackay, D. (1988) A review of the nature and Properties of chemicals present in pulp mill effluents. *Chemosphere,* **17**, 1249-1290.

Tappi Test Methods (1991) Technical Association of the Pulp and Paper Industry (TAPPI), Atlanta, Georgia.

Tolan J.S., Olson D. and Dines R.E. (1996) Survey of mill usage of xylanase. In *Enzymes for Pulp and Paper Processing*, ed. T.W. Jeffries and L. Viikari, chap. 3, pp. 25-35. American Chemical Society, Washington, DC.

Turner, J.C., Skerker, P.S., Burns, B.J., Howard, J.C., Alonso, M.A. and Andres, J.L. (1992) Bleaching with enzymes instead of chlorine - mill trials. *Tappi J.*, **75**, 83-89.

Van der Walt, A., Jacobs, E.P. and Nel, C. (1997) Design, development and characterization of a transverse flow membrane bioreactor. *Biotech SA '97,* p. 80, Programme and Abstracts.

Viikari, L., Ranua, M., Kantelinen, A., Sundquist, J. and Linko, M. (1986) Bleaching with enzymes. *Proceedings of the Third International Conference on Biotechnology in the Pulp and Paper Industry*, pp. 67-69, Stockholm.

Viikari, L., Kantelinen, A., Sundquist, J. and Linko, M. (1994) Xylanases in bleaching: from an idea to the industry. *FEMS Microbiol. Rev.***,** **13**, 335-350.

Wall, M.B., Cameron, D.C. and Lightfoot, E.N. (1993) Biopulping process design and kinetics. *Biotechnol. Adv.,* **11,** 645-662.

Wang S-H., Ferguson, J.F. and McCarthy J.L. (1992) The decolorization and dechlorination of kraft bleach plant effluent solutes by use of three fungi: *Ganoderma lacidum*, *Coriolus versicolor* and *Hericium erinaceum*. *Holzforschung*., **46**, 219-223.

Wolfaardt J.F., Bosman J.L., Jacobs A., Male J.R. and Rabie C.J. (1996) Bio-kraft pulping of softwood. In *Biotechnology in the Pulp and Paper Industry*, ed. E. Srebotnik and K. Messner, pp. 211-216. Facultas-Universitätsverlag, Vienna.

ANNEXURE

PRODUCTS EMANATING FROM THE PROJECT

1. Book chapters

i. Christov, L.P., Akhtar, M. and Prior, B.A. (1996) Biobleaching in dissolving pulp production. In: ***Biotechnology in the Pulp and Paper Industry*** (Srebotnik, E. and Messner, K., eds.). Facultas-Universitätsverlg, Vienna, Austria, pp. 625-628.

ii. Christov, L.P. and Prior, B.A. (1996) Reduction of active chlorine charges in bleaching of xylanase-pretreated sulfite pulp. In: ***Enzymes for Pulp and Paper Processing*** (Jeffries, T.W. and Viikari, L., eds.). American Chemical Society, Washington, D.C., USA, pp. 208-218.

iii. Ferraz, A., Christov, L.P. and Akhtar, M. (1998) Fungal pretreatment for organosolv pulping and dissolving pulp production. In: ***Environmentally Friendly Pulping and Bleaching Methods*** (Young, R.A. and Akhtar, M., eds.), chap. 13, John Wiley & Sons, Inc., New York, pp. 421-447.

2. Peer-reviewed journals

i. Christov, L.P. and Prior, B.A. (1996) Repeated treatments with *Aureobasidium pullulans* hemicellulases and alkali enhance biobleaching of sulphite pulps. ***Enzyme and Microbial Technology*** 18: 244-250.

ii. Christov, L.P., Akhtar, M. and Prior, B.A. (1996) Impact of xylanase and fungal pretreatment on alkali solubility and brightness of dissolving pulp. ***Holzforschung*** 50: 579-582.

iii. O'Neill, F.H., Christov, L.P., Botes, P.J. and Prior, B.A. (1996) A rapid and simple assay for feruloyl and p-coumaroyl esterases. ***World J. Microbiol. Biotechnol.*** 12: 239-242.

iv. Christov, L.P. and Prior, B.A. (1997) Bleaching response of sulfite pulps to pretreatment with xylanases. ***Biotechnol. Progr***. 13: 695-698.

v. Christov, L.P., Myburgh, J., Van Tonder, A. and Prior, B.A. (1997) Hydrolysis of extracted and fibre-bound xylan with *Aureobasidium pullulans* enzymes. ***J. Biotechnol***. 55: 21-29.

vi. Christov, L.P. and Prior, B.A. (1998) Research in biotechnology for the pulp and paper industry in South Africa. ***S.A. J. Sci***. 94: 695-698.

vii. Christov, L.P., Akhtar, M. and Prior, B.A. (1998) The potential of biosulphite pulping in dissolving pulp production. ***Enzyme Microb. Technol.*** 23: 70-74.

viii. Mosai, S., Wolfaardt, J.F., Prior, B.A. and Christov, L.P. (1998) Evaluation of selected lignin-degrading fungi for biosulphite pulping. ***Biores. Technol***. (in press)

ix. Christov, L.P., Szakacs, G. and Balakrishnan, H. (1998) Production, partial chracterisation and use of fungal cellulase-free xylanases in pulp bleaching. ***Proc. Biochem***. (in press).

x. Christov, L.P., Myburgh, J., O'Neill, F.H., Van Tonder, A. and Prior, B.A. Modification of the carbohydrate composition of sulfite pulp by purified and charaterized ß-xylanase and ß-xylosidase of *Aureobasidium pullulans*. ***Biotechnol. Progr***. (submitted).

xi. Christov, L.P., van Driessel, B. and du Plessis, C.A. Fungal biomass from *Rhizomucor pusillus* as adsorbent of chromophores from a bleach plant effluent. ***Proc. Biochem.*** (submitted).

xii. Christov, L.P. and Steyn, M.G. (1998) Modifying the quality of a bleach plant effluent using mucoralean and white-rot fungi. ***J. Pulp Pap. Sci.*** (submitted).

xiii. Balakrishnan, H., Szakacs, G., Rele, M.V., Prior, B.A. and Christov, L.P. (1998) Impact of fungal xylanases on bleachability of sulphite pulp. ***Pulp Pap. Can.*** (submitted).

3. Patents

i. Christov, L.P. and Prior, B.A. (1997) Biobleaching of sulphite pulp for producing dissolving pulp. ***S.A. Patent*** **96/4589**.

ii. Christov, L.P. and Prior, B.A. (1997) Biopulping and biobleaching of sulphite pulp for producing dissolving pulp. ***S.A. Patent*** **96/4590**.

4. Presentations

i. Prior, B.A. and Christov, L.P. (1996) Application of biotechnology in the pulp and paper industry in South Africa. ***Regional Conference on the Impact of Microbiology on Socio-Economic Development and Environment in Sub-Sahara Africa***. Dar es Salaam, Tansania, September 10-15.

ii. Christov, L.P. (1996) Biobleachability of sulphite pulps. ***Cellulases and Their Application.*** Seminar supported by PROBRAIN, Mie University-School of Bioresources, Kamihama-cho, Japan, December 5

iii. Christov, L.P. and Prior, B.A. (1997) Effect of xylanase pretreatment on bleachability of sulphite pulp. ***Biotech SA' 97: Second Grahamstown Conference Biotechnology and Development in Southern Africa***. Grahamstown, South Africa, January 21-24.

iv. Du Plessis, L., Wolfaardt, J.F., Christov, L.P. and Prior, B.A. (1997) Fungal decolourization of bleach plant effluent from a sulphite pulp mill. ***Biotech SA' 97: Second Grahamstown Conference Biotechnology and Development in Southern Africa***. Grahamstown, South Africa, January 21-24.

v. Steyn, M.G., Botha, A. and Christov, L.P. (1997) Colour reduction of pulp mill effluents using *Rhizomucor* strains. ***Biotech SA' 97: Second Grahamstown Conference Biotechnology and Development in Southern Africa***. Grahamstown, South Africa, January 21-24.

vi. Wills, K.E., Tivchev, G.N., Christov, L.P. and Kasan, H.C. (1997) Preliminary investigation of the cultivation of *Aureobasidium pullulans* on spent sulphite liquor. ***Biotech SA' 97: Second Grahamstown Conference Biotechnology and Development in Southern Africa***. Grahamstown, South Africa, January 21-24.

vii. Mosai, S., Wolfaardt, J.F., Christov, L.P. and Prior, B.A. (1997) Evaluation of lignin degrading fungi for biosulphite pulping. ***Biotech SA' 97: Second Grahamstown Conference Biotechnology and Development in Southern Africa***. Grahamstown, South Africa, January 21-24.

viii. O'Neill, F.H., Christov, L.P. and Prior, B.A. (1997) Production of hemicellulolytic enzymes by microorganisma. ***Biotech SA' 97: Second Grahamstown Conference Biotechnology and Development in Southern Africa***. Grahamstown, South Africa, January 21-24.

ix. Mosai, S., Wolfaardt, J.F., Christov, L.P. and Prior, B.A. (1997) Sulphite biopulping of *Eucalyptus grandis* wood chips with white-rot fungi. ***Fifth Brazilian Symposium on the Chemistry of Lignins and Other Wood Components/Workshop of the International Energy Agency on Biotechnology Applied to Lignocellulosic Materials***. Curitiba, Brazil, August 31-September 4.

x. Christov, L.P. and Prior, B.A. (1997) Assessment of xylanase pretreatment of sulfite pulps in one-stage and multi-stage bleaching to dissolving pulp. ***Tappi Biological Sciences Symposium***. San Francisco, USA, October 20-23.

xi. Balakrishnan, H., Szakacs, G., Rele, M.V., Prior, B.A. and Christov, L.P. (1998) Screening of cellulase-free fungal xylanases and their evaluation of their performance on sulfite pulp. ***Seventh International Conference on Biotechnology in the Pulp and Paper Industry,*** Vancouver, Canada, June 16-19, Abstract book, vol. C, pp. C7-C10.

xii. Christov, L.P. and Akhtar, M. (1998) Evaluation of selected *Ceriporiopsis subvermispora* strains for biosulfite pulping and bleaching to prepare dissolving pulp. ***Seventh International Conference on Biotechnology in the Pulp and Paper Industry,*** Vancouver, Canada, June 16-19, Abstract book, vol. B, pp. C15-B17.

xiii. Christov, L.P. and Steyn, M.G. (1998) Modifying the quality of a bleach plant effluent using mucoralean and white-rot fungi. ***Seventh International Conference on Biotechnology in the Pulp and Paper Industry,*** Vancouver, Canada, June 16-19, Abstract book, vol. C, pp. C203-C206.

xiv. Van Driessel, B., du Plessis, C.A., Prior, B.A. amd Christov, L.P. (1998) The role of fungal biomass as adsorbent of chromophores from bleach plant effluent. ***Seventh International Conference on Biotechnology in the Pulp and Paper Industry,*** Vancouver, Canada, June 16-19, Abstract book, vol. C, pp. C207-C210.

xv. Christov, L.P. and Steyn, M.G. (1998) Fungal biotreatment of industrial pulp mill effluents. ***Tenth Biennual Congress of the South African Society of Microbiology***, Durban, South Africa, July 5-8. Abstract book, P39.

xvi. Balakrishnan, H., Szakacs, G., Rele, M.V., Prior, B.A. and Christov, L.P. (1998) Impact of fungal xylanases on bleachability of sulphite pulp. ***Tenth Biennual Congress of the South African Society of Microbiology***, Durban, South Africa, July 5-8. Abstract book, P113.

xvii. Cronje, M., van Biljon, P., Prior, B.A. and Christov, L.P. (1998) Carbohydrate analysis of sulphite pulp. ***Second International Congress of the Federation of African Societies of Biochemistry and Molecular Biology and Fifteenth Congress of the South African Society of Biochemistry and Molecular Biology***, Potchefstroom, South Africa, September 30-October 3.

5. Annual Reports

i. Annual Reports to the NRF (THRIP)

6. Degrees

This project funded by the Water Research Commission had enabled:

- Mr Sipho Mosai to obtain his MSc degree (1997) from the University of the Orange Free State (Title of thesis: "Biosulfite pulping of Eucalyptus wood with white-rot fungi").

- Ms M.V. Nhlapo to obtain her BSc (Hons) degree (1999) from the University of the Orange Free State (Title of thesis: "Depletion of carbon source in bleach plant effluent by selected fungi").

- *Mr Brian van Driessel to complete part of his PhD thesis (University of the Orange Free State) on "Bioremediation of a bleach plant effluent".*