FURTHER DEVELOPMENT OF A BIOTECHNOLOGICAL APPROACH TO THE MANAGEMENT OF WASTE WATERS FROM THE PULP AND PAPER INDUSTRY

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Final Report to the Water Research Commission

by

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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 waste waters, are unattractive for industrial applications because of the high costs. However, biological methods of waste water treatment have the advantage of being cost-effective and in addition to colour removal, they can also reduce the chemical oxygen demand and adsorbable organic halogen of the waste water.

OBJECTIVES

- Development of a modern biobleaching technology to improve the existing chemical bleaching processes of pulp and paper production in an environmentally-friendly and cost-effective way.
- Reduction of the environmental impact of waste waters from the pulp and paper industry by employing microorganisms with special emphasis of waste water biodecolourisation, biodegradation and bioremediation.
- Utilisation of waste waters from the pulp and paper industry to obtain valuable products such as enzymes (xylanases), single cell protein (scp) and/or high-value fatty acids (gamma-linolenic acid)

RESEARCH APPROACH

Remediation of industrial waste waters from the pulp and paper industry was investigated using biological methods such as pretreatment with enzymes, white-rot and mucoralean fungi. The waste waters under study were derived from the extraction stage of the bleach plant as well as the spent sulphite liquor from the pulping stage of pulp production. Two industrial pulp types were examined for their bleachability with enzymes: hardwood soda-aq pulp and acid sulphite dissolving pulp. Following biological treatments, pulp properties such as brightness, viscosity, kappa number, etc. 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, colour, solid content, etc. were carried out as described in the Standard Methods for Examination of Water and Waste Water (American Public Health Association, Washington, DC, USA). Fermentation experiments for enzyme and gamma-linolenic production were carried out in shake flasks. Screening for best microbial sources was assessed according to levels of xylanase activity and single cell protein attained. The efficiency of various approaches of waste water bioremediation was evaluated mainly based on the extent of waste water decolourisation, however, the impact on chemical load, chloride content and chlorinated organic matter was also accounted for.

RESEARCH

Biobleaching-based waste water bioremediation

- It has been demonstrated that implementation of the enzyme bleaching technology in the pulp and paper industry could improve the existing technology of pulp and paper manufacture in a cost-effective and environmentally friendly way.
- Chemical savings of up to 2.5 kg of ClO_2/ton of pulp could be achieved when dissolving pulp was bleached with xylanase whereas biobleaching of soda-aq pulp effected a 30% reduction (4.5 kg of ClO_2/ton of pulp) of the total amount of chlorine dioxide used.

- The major impact of the reduction of the active chlorine charges during biobleaching was on the chloride, adsorbable organic halogen and bacterial growth levels of the total bleach pulp waste water whereas colour and chemical oxygen demand of waste water were impacted to a lesser extent. For instance, a 39% reduction of the chlorine dioxide use reflected in a corresponding decrease of 36% of the chlorides, 34% of adsorbable organic halogen and 30% of bacterial growth inhibition levels of the total waste water derived from the $D_1E_oD_2$-bleaching of dissolving pulp.

Waste water *bioremediation using microorganisms*

- Remediation of bleach plant waste water was studied using different biological treatment systems: activated sludge, trickling filters and rotating biological contactor. Activated sludge treatment removed chemical oxygen demand efficiently (58%) and decreased bacterial growth inhibition (43%) and adsorbable organic halogen (20%) in the waste water, however, only 10% of colour could be removed. On the other hand, waste water treatment in trickling filters demonstrated the potential of white-rot fungi for the decolourisation of bleach plant waste water (61%). However, the most efficient treatment system proved to be the rotating biological contactor where colour, bacterial growth inhibition, adsorbable organic halogen and chemical oxygen demand were decreased by significant levels, i.e. up to 74%, 69%, 55% and 64%, respectively.
- It was demonstrated that using a laboratory rotating biological contactor reactor both *Coriolus versicolor*, a white-rot fungus, and *Rhizomucor pusillus* strain RM7, a mucoralean fungus were able to decolourise the waste water within three days of continuous treatment and could in principle be used for bioremediation of industrial waste waters.
- It was shown that decolourisation of 53 to 73% could be attained using a hydraulic retention time of 23 h. The relatively high decolourisation levels (32-55% decolourisation) achieved at low hydraulic retention times (0.67-6 h) may be due to a rapid adsorption of colour by the biomass initially.
- Adsoption seems to be one of the first steps in waste water decolourisation by fungi. All colour adsorbed by *R. pusillus* during waste water decolourisation was released from the fungal biomass following alkali treatment, thus, biomass could be

completely regenerated and reused. Therefore, the decolourisation mode of *R. pusillus* appeared to be due to physical adsorption.

- Cell wall fractions extracted from the biomass of *R. pusillus* (alkali-resistant, residual and chitosan fractions) appeared to be principally responsible for the decolouring of waste water by this fungus. The residual and chitosan fractions, representing combined only approximately 12% (w/w) of the *R. pusillus* biomass, were able to removed more colour from the bleach plant waste water (about 41%) than the intact biomass (39%). The major compound(s) of the cell wall fractions could be either chitosan and/or chitin, based on the release of glucosamine upon acid hydrolysis of these fractions. The mechanism of colour removal by *R. pusillus* might involve ionic interactions between aminoglucans from the cell wall of this fungus and the negatively charged compounds found in the bleach plant waste water.
- Only part of the colour removed by *C. versicolour* could be ascribed to adsorption (45%), suggesting the presence of two mechanisms of colour removal in this fungus: 1) bioadsorption and 2) biodegradation. The latter was also supported by changes in high molecular weight distribution profile of waste water as well as presence of lignolytic enzymes following treatment with *C. versicolor*.
- Decolourisation by both fungi was directly proportional to initial colour intensities. Initial colour levels were directly proportional to decolourisation rate expressed as change in colour/g.d in both fungi. Although this phenomenon has been described using white-rot fungi, as far as known, this is the first report where it has been demonstrated to occur using a mucorelean fungus for waste water treatment.
- The decrease in bacterial growth inhibition following fungal pretreatment with both *R. pusillus* and *C. versicolor* correlated well with the adsorbable organic halogen removal from waste water: *R. pusillus* removed 55% adsorbable organic halogen and also showed the greatest reduction of 69% in bacterial growth inhibition. Similarly, *C. versicolor* caused a 40% reduction in AOX accompanied by a 38% stimulation in bacterial growth. It also was demonstrated that chloroorganic removal from waste water was accompanied by stimulation in bacterial growth.
- Additional carbon source is required for lignin degradation by white-rot fungi. This has been verified in this work because supplementation of glucose to decolourisation

media enhanced decolourisation by *C. versicolor*. In contrast, no such stimulation in colour removal by *R. pusillus* was noted upon glucose additions to decolourisation media.

- Manganese peroxidase and laccase activities were detected in the waste water treated by *C. versicolor*, however, no ligninolytic enzyme activities were found in waste water treated by *R. pusillus*. The presence of these enzymes in *C. versicolor* treated waste water indicates that enzymatic action could be responsible for part of colour removal.
- Differences in the decolouring mechanisms between the white-rot fungus (adsorption + biodegradation) and the mucoralean fungus (adsorption) have been observed. This aspect needs to be investigated in greater detail to verify the mode responsible for the decolourisation activity in both types of fungi. Long term studies are necessary to evaluate the suitability and potential of these fungi to treat pulp and paper mill waste waters on an industrial scale.

Waste water utilisation for xylanase production

- High xylanase activities of up to 320 U/ml were obtained with *Aspergillus foetidus* ATCC 14916 using xylan as carbon source. *A. oryzae* NRRL 3485 and 1808, *A. niger* ATCC 13497 and *A. phoenicis* 13157 also yielded relatively high xylanase activities of up to 115, 130, 127 and 109 U/ml, respectively.
- Relatively low xylanase activities were obtained using other carbon substrates, namely D-xylose, molasses and wheat bran. The pH and temperature optima of the xylanases produced by the selected fungi were typical of fungal xylanases.
- High xylanase activities were also obtained using the spent sulphite liquor concentrate as carbon source, with *A.* oryzae NRRL 3485 and *A. phoenicis* ATCC 13157 yielding activities of up to 209 and 140 U/ml, respectively. These results indicated the potential of the spent sulphite liquor to serve as carbon feedstock for microbial growth and enzyme production.
- The application of the crude xylanase preparations in the pretreatment of the post-oxygen soda-aq pulp at relatively low enzyme dose of 10 U/g resulted in a brightness increase of 1.4 points over the control using full chlorine dioxide charges in a X-DED

bleaching. Alternatively, biobleaching could reduce the chlorine dioxide consumption by up to 30% while retaining brightness at the control level.

Tall oil utilisation for gamma-linolenic acid production

- Tall oil, which is a by-product derived from kraft mill spent liquor, could be utilised by selected fungi for production of high-value fatty acids such as gamma-linolenic acid.
- In the presence of acetate, *Mucor circinelloides f. circinelloides* CBS 108.16 produced significantly more biomass (6 g/l) containing high concentrations crude oil (43.3%) compared to when only tall oil was used as sole carbon substrate (biomass of 2.0 g/l and lipid content of 7.5%).
- The presence of acetate caused enhanced tall oil utilisation leading to production of high concentrations of gamma-linolenic acid (5.2%) by *Mucor circinelloides f. circinelloides* CBS 108.16.
- Enhanced tall oil utilisation and high concentrations of gamma-linolenic acid were obtained also in the crude cellular lipids in *Mucor circinelloides f. circinelloides* CBS SAS 045 both in presence (3.7% gamma-linolenic acid) and absence (5.7% gamma-linolenic acid) of acetate.
- No general pattern was observed concerning the influence of acetate on growth and production outputs. No gamma-linolenic acid could be detected in any growth experiments with yeast.

CONCLUSIONS

- The economical feasibility of the entire biobleaching technology using xylanases would be improved by utilising pulp mill waste waters which at present are discarded as industrial waste. The use of xylanases in bleaching as a means of rendering the waste waters more environmentally friendly has been demonstrated. Biobleaching can reduce the amount of chlorine-containing chemicals used in bleaching. This impacts directly on the levels of chlorides and adsorbable organic halogen of the waste waters. The implementation of the enzyme bleaching technology in the pulp and paper

industry could improve the existing technology of pulp and paper manufacture in a cost-effective and environmentally friendly way.

- The possibility of waste water bioremediation prior to waste water discharge has been demonstrated. The use of continuous biosystems such as trickling filters, rotating biological contactors and activated sludge systems have been shown to reduce the environmental impact of these waste waters. The most efficient treatment system proved to be the rotating biological contactor where colour, bacterial growth inhibition levels, adsorbable organic halogen and chemical oxygen demand were decreased to a significant extent. Definite differences in the decolouring mechanisms between the white-rot fungi (adsorption + biodegradation) and the mucoralean fungi (adsorption) have been observed.

- The potential of pulp and paper mill waste waters and by-products derived from waste waters for biotechnical utilisation has been demonstrated. The bioutilisation of these waste waters would alleviate problems associated with their discharge. Results indicated that the spent sulphite liquor has certainly potential as a carbon feedstock and inducer for xylanase production and could improve the overall economics of the biobleaching technology.

- It has been demostrated that tall oil which is a by-product derived from kraft pulp and paper mill waste waters could be utilised by selected fungi for production of high-value fatty acids such as gamma-linolenic acid.

PRODUCTS EMANATING FROM THE PROJECT

1. Publications

1. Christov, L.P. and Steyn, M.G. (1999) The use of the white-rot fungus *Trametes nivosa* in bioremediation of pulp effluents. *African International Environmental*

Protection Symposium, 2-8 July, 1999, Pietermaritzburg, South Africa. ISBN 0-620-239-45-X.

2. Van Driessel, B., du Plessis, C.A. and Christov, L.P. (1999) Adsorption of colour from a bleachery effluent using a mucoralean fungus. *African International Environmental Protection Symposium*, 2-8 July, 1999, Pietermaritzburg, South Africa. ISBN 0-620-239-45-X.
3. Christov, L.P., van Driessel, B. and du Plessis, C.A. (1999) Fungal biomass from *Rhizomucor pusillus* as adsorbent of chromophores from a bleach plant effluent. *Proc. Biochem.* 35: 91-95.
4. Madlala, A.M., Bissoon, S., Singh, S. and Christov, L. (2001) Xylanase-induced reduction of chlorine dioxide consumption during elemental chlorine-free bleaching of different pulp types. *Biotechnol. Lett.* 23: 345-351.
5. Van Driessel, B. and Christov, L. (2001) Decolorization of bleach plant effluent by mucoralean and white-rot fungi in a rotating biological contactor reactor. *J. Biosc. Bioeng.* 92 (3): 271-276.
6. Bissoon, S., Christov, L. and Singh, S. (2002) Bleach boosting effects of purified xylanase from *Thermomyces lanuginosus* SSBP on bagasse pulp. *Process Biochem.* 36 (7): 567-672.
7. Van Driessel, B. and Christov, L. (2002) Adsorption of colour from a bleach plant effluent using biomass and cell wall fractions from *Rhizomucor pusillus*. *J. Chem. Technol. Biotechnol.* 77: 155-158.

2. Presentations

1. Christov, L.P. and Steyn, M.G. (1999) The use of the white-rot fungus *Trametes nivosa* in bioremediation of pulp effluents. *African International Environmental Symposium '99* (AiEPS '99), Pietermaritzburg, South Africa, July 4-8, 1999.
2. Van Driessel, B., du Plessis, C.A. and Christov, L.P. (1999) Adsorption of colour from a bleachery effluent using a mucoralean fungus. *African International Environmental Symposium '99* (AiEPS '99), Pietermaritzburg, South Africa, July 4-8, 1999.

3. Chipeta, Z.A., Du Preez, J.C. and Christov, L.P. (1999) Evaluation of xylanase production by fungi grown on pulp mill effluents. *IEA Bioenergy Workshop on Biotechnology for the Conversion of Lignocellulose*. Itala Game Reserve, South Africa, August 22-26, 1999.
4. Van Driessel, B., du Plessis, C.A. and Christov, L.P. (1999) Treatment of bleach plant effluent employing a tubular rotating bioreactor containing immobilised *Coriolus versicolour*. *IEA Bioenergy Workshop on Biotechnology for the Conversion of Lignocellulose*. Itala Game Reserve, South Africa, August 22-26, 1999.
5. Bareetseng, A.S., Christov, L.P., Coetzee, D.J. and Kock, J.L.F. (1999) Utilisation of tall oil derived from kraft mill effluents by fungi in the presence of acetate. *IEA Bioenergy Workshop on Biotechnology for the Conversion of Lignocellulose*. Itala Game Reserve, South Africa, August 22-26, 1999.
6. Van Driessel, B., du Plessis, C.A. and Christov, L. (2000) Decolourisation of bleach plant effluent using immobilised fungi in rotating biological contactor reactors. *BIOY2K Combined Millenium Meeting 2000, Workshop "Biotechnology in the Pulp and Paper Industry"*, Grahamstown, South Africa, January 23-28, 2000.
7. Chipeta, Z.A., du Preez, J.C. and Christov, L. (2000) Evaluation of xylanase production by fungi grown on pulp mill effluents. *BIOY2K Combined Millenium Meeting 2000, Workshop "Biotechnology in the Pulp and Paper Industry"*, Grahamstown, South Africa, January 23-28, 2000.
8. Bareetseng, A.S., Kock, J.L.F., Christov, L. and Coetzee, D.J. (2000) Tall oil utilisation by fungi in the presence of acetate. *BIOY2K Combined Millenium Meeting 2000*, Grahamstown, *Workshop "Biotechnology in the Pulp and Paper Industry"*, South Africa, January 23-28, 2000.
9. Van Driessel, B. and Christov, L. (2001) Treatment of bleach plant effluent using immobilised fungi in novel rotating biological contactor reactors. *8th International Conference on Biotechnology in the Pulp and Paper Industry (ICBPPI)*, June 4-8, 2001, Helsinki, Finland.
10. Chipeta Z.A., du Preez, J.C. and Christov, L. (2001) The evaluation of xylanase-producing fungi grown on pulp mill effluents. *8th International Conference on*

Biotechnology in the Pulp and Paper Industry (ICBPPI), June 4-8, 2001, Helsinki, Finland.

3. Reports

1. Annual reports to NRF (THRIP)

4. Degrees

This project funded by the Water Research Commission had enabled:

- Ms Marlese Cronje to complete part of her thesis and obtain her MSc degree (2000) from the University of the Orange Free State (Title of thesis: "Carbohydrate composition and enzymatic hydrolysis of sulphite pulp").
- Mr Andreas Madlala to complete part of his thesis and obtain a MTech degree (2000) from the ML Sultan Technikon (Title of thesis: "Aplication of xylanases in biobleaching of industrial pulps").
- Mr Brian van Driessel to complete part of his PhD thesis (University of the Orange Free State) on "Bioremediation of a bleach plant effluent".

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"FURTHER DEVELOPMENT OF A BIOTECHNOLOGICAL APPROACH TO THE MANAGEMENT OF WASTE WATERS 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)
Dr G Offringa	Water Research Commission
Mrs CM Smit	Water Research Commission (Co-ordinator)
Prof LP Christov	Sappi Management Services
Prof JC du Preez	University of the Free State
Prof CA Buckley	University of Natal
Mr J Hunt	Paper Manufacturers Association of SA
Mr P Herbst	Department of Water Affairs and Forestry
Mr NS Jory	Sappi Management Services
Mr A du Plooy	Sappi Management Services
Mr DA Weightman	Sappi Saiccor (Pty) Ltd

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Meaning	Units
AOX	Adsorbable Organic Halogen	g/l
BOD	Biochemical Oxygen Demand	g/l
C	Chlorine	
COD	Chemical Oxygen Demand	g/l
D	Chlorine Dioxide	
DP	Degree of Polymerisation	
E	Alkaline Extraction	
E_o	Alkaline Extraction in Oxygen Atmosphere	
ECF	Elemental Chlorine-Free	
GLA	Gamma-linolenic acid	
LiP	Lignin Peroxidase	
MLSS	Mixed Liquor Suspended Solids	
MnP	Manganese-Dependent Peroxidase	
H	Sodium Hypochlorite	
O	Oxygen	
P	Hydrogen Peroxide	
PCU	Platinum Cobalt Units	
RS	Reducing Sugars	g/l
SS	Suspended Solids	g/l
SSL	Spent Sulphite Liquor	
TCF	Totally Chrorine-Free	
TOCl	Total Organically Bound Chlorine	g/l
TOX	Total Organic Halogen	g/l
TDS	Total Dissolved Solids	g/l
TS	Total Solids	g/l
TSS	Total Suspended Solids	g/l
X	Xylanase	

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CHAPTER I
INTRODUCTION

1. Introduction

South Africa is a major international producer of pulp and paper products. In 2000, South Africa was the 16th largest producer of pulp, the 24th biggest producer of paper and the 29th biggest consumer of paper in the world (Pulp & Paper International, July 2001). 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, Sappi is supplying half South Africa's requirements and is already the largest papermaker on the African continent. In year 2000, Sappi was ranked the 16th biggest pulp and paper company by sales in the world (Pulp & Paper International, September 2001). 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 efficiency of conversion of cellulose into the specific derivative is dependent upon the xylan and mannan content of dissolving pulp. 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.

2. Environmental considerations

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 waste waters 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 waste waters contribute 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 waste waters 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 waste water 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).

3. Biotechnology in the pulp and paper industry

The release of the bleach plant waste waters with high adsorbable 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 decolourisation of pulp and paper mills waste waters, are unattractive for industrial applications because of the high costs. However, biological methods of waste water 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 waste water (Eaton et al. 1980).

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 waste water quality.

The major aim of the biotechnology programme at the Sappi Biotechnology Laboratory is to develop new bioprocesses for the pulp and paper industry to improve the existing technology of pulp and paper manufacture in environmentally-friendly and cost-effective way. The original objectives of the project were as follows:

- Development of a modern biobleaching technology to improve the existing chemical bleaching processes of pulp and paper production in an environmentally-friendly and cost-effective way.
- Reduction of the environmental impact of waste waters from the pulp and paper industry by employing microorganisms with special emphasis of waste water biodecolourisation, biodegradation and bioremediation.

- Utilisation of waste waters from the pulp and paper industry to obtain valuable products such as enzymes (xylanases), single cell protein (scp) and/or high-value fatty acids (gamma-linoleinc acid)

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

1. Biobleaching-based waste water bioremediation
- reduction of active chlorine charges in biobleaching with xylanase
- impact of biobleaching on COD, colour, chloride, AOX and bacterial growth levels in bleach waste waters
2. Waste water bioremediation using microorganisms
- physico-chemical methods for colour removal from bleach plant waste water
- biological methods for waste water bioremediation
- evaluation of trickling filters for waste water bioremediation
- evaluation of activated sludge for waste water bioremediation
- evaluation of rotating biological contactors for waste water bioremediation
3. Waste water utilisation for xylanase production
- screening of microorganisms on waste waters for xylanase production
- optimisation of xylanase production on waste waters in shake flasks
- pH and tempetrature optima of xylanases produced on waste waters
- evaluation of xylanases produced on waste waters in biobleaching
4. Tall oil utilisation for gamma-linolenic acid production
- screening of microorganisms on tall oil for gamma-linolenic acid production
- impact of acetate on growth and gamma-linolenic acid production

CHAPTER II

WASTE WATER BIOTECHNOLOGY

1. Waste water bioremediation using microorganisms

1.1. Introduction

The pulp and paper industry is one of the major users of water and the waste water 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 life; 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). Furthermore during bleaching, chromophoric and highly oxidised, polymeric lignin derivatives are formed that give rise to a dark colourisation in the waste water (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 1991), 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 (Livernoche 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 waste water (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 waste water (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, etc.) using chemical methods (Feijoo 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 waste water 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 waste waters.

1.2. Chemical composition of waste waters

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

1.2.1. High molecular mass compounds

More than one-half of the colour load from bleach plant waste water originates from high molecular weight lignin-derived material in the alkaline extraction stage (Paice and Jurasek 1984). Waste water 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 waste water, during biological treatment, is still unknown. Working with high molecular mass fractions (> 30000 Dalton), Bergbauer et al. (1991) observed that sequential depolymerisation does not necessarily lead to complete degradation of these aromatic compounds. Furthermore once degradation of lignin derivatives by *Trametes versicolour* 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.

1.2.2. Low molecular mass compounds

Certain compounds in pulp and paper mill waste waters with a relative molecular mass lower than 1000g/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 waste waters of relative low molecular weight (< 1000g/mol), divided these compounds in the following categories: I. acidic compounds, II. phenolic compounds, III. neutral compounds. Chlorinated phenols in bleach waste waters 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 waste water are largely aliphatic acids, mainly chloroacetic acid. Although a variety of low molecular substances do occur in the waste water streams, most organic chlorine present is associated with high molecular mass material (Smith et al. 1994).

1.3. Cultivation strategies for decolourisation of waste waters

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. Ultrafiltration of the E-stage waste water 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 waste waters are more diluted than desired for a low retention time and varying waste water composition can lead to toxic shock conditions, detrimental for the efficient functioning of the anaerobic systems. By combining ultrafiltration of the E-stage waste water and incorporating the permeate with the C/D waste water, 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 (Boman and Frostell 1988). 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. 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.

1.3.1. The Mycor process

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. 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). Although steam treatment initially might be beneficial, further aseptic procedures would not be required with fungi like *P. chrysosporium*. Waste waters 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 mycelia is in constant contact with substrate to an extent of only about 40%. Moreover

the mycelia is present in a thick layer that could result in deficient oxygen and nutrient supply and a lower productivity in general.

1.3.2. 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 510 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 waste water as well as a COD reduction of 25%. Colour removal targets in excess of 57%, was sustained for 18 days with alternative III.

1.3.3. Fungal pellets

Royer et al. (1985) used *Coriolus versicolour* in the form of mycelial pellets to decolourise lignin-containing kraft E-stage waste water. 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 waste water at the E-stage. Magnesium ions improved the consumption of chromophores which might indicate that an enzymatic mechanism took place since magnesium ions are activators of many enzymes. At an initial colour level of 7000 colour units (CU), the mean colour removal proceeded at a rate of 300 CU/g mycelium.h. 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% after 7 days using *T. versicolour*.

1.3.4. 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 waste water by 85% and reduced the COD by 25%, after 3 days incubation in shake flasks. The maximum total decolourisation 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).

1.3.5. Fermentor cultivation

Trametes versicolour 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 a 88% reduction in the colour units within 3 days, whereas a 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).

1.3.6. 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 (Anselmo and Novias 1992). In liquid cultures, *C. versicolour* removed over 60% of the colour of a combined bleach kraft waste water within 6 days in the presence of sucrose. This contrasted drastically with the results obtained when the same waste water 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. versicolour*, operated at a residence time of 9 h, reduced colour of a bleach plant waste water 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 waste water from the alkaline stage of a sulphate mill by this process resulted in a maximum of 80% decouloring 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). The principle applicability of capillary membrane technology using immobilised white-rot fungi in the bioremediation of industrial waste waters and aromatic pollutants has been demonstrated (Ryan et al. 2000).

1.4. Ligninolytic enzymes and their possible role in decolourisation of waste waters

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* depolymerisation of high molecular mass lignin. Depolymerisation 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 waste water, 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 waste water. 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).

1.5. 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 10% 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 waste water 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 waste water. Royer et al. (1985) regarded the decolourisation process by *T. versicolour* of kraft bleach waste water to consist of two parts, namely: adsorption, completed after 24 hours, and secondly, subsequent oxidation. About 10% of TOX removal, obtained after treatment of a mixture of first chlorination stage and first alkaline stage waste water with *Ganoderma lacidum* and *C. versicolour*, 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 waste water 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 waste water. 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. Biobleaching-based waste water bioremediation

2.1. Introduction

The bleaching of pulps 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. 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 hydrolyse that fraction of xylan that has been reprecipitated on the fibre 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 fibres.

2.3. Biobleaching of sulphite pulps

Little attention has been paid to enzymatic prebleaching of sulphite 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 sulphite 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 sulphite 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 sulphite 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 sulphite 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 sulphite pulping, redeposition of xylans onto the fibre 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 fibres rather than on the fibre 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.

2.4. Scale-up of biobleaching

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 waste water 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).

3. Conclusions

1. Biobleaching of pulp and paper 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. Biobleaching can be used as a means of waste water bioremediation by reducing the amounts of bleaching chemical used, especially chlorine containing chemicals.
3. Fungal pretreatment of the bleach plant waste water was shown to be effective in colour reduction of the waste water. Another alternative to the secondary biotreatment of waste water would be its utilisation 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 waste water.
4. Cultivation conditions have an important impact on the efficiency of white rot fungi during biological treatment of bleach plant waste water. Higher efficiencies are called for in order to develop a practical biotreatment process.
5. The high volumes of waste water 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.

6. Biosorption of chromophores from bleach waste water have 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.
7. Because of the complexity of treating paper mill waste water, 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.

CHAPTER III

WASTE WATER BIOREMEDIATION

1. Biobleaching-based waste water bioremediation

1.1. Abstract

The effect of biobleaching using xylanase of both unbleached sulfite dissolving pulp and post-oxygen soda pulp on the reduction of chlorine dioxide charges during chemical bleaching and waste water properties was investigated. Xylanase pretreatment caused a 39% reduction (2.5 kg ClO_2/ton pulp) and a 30% reduction (4.5 kg ClO_2/ton pulp) in the active chlorine charges used in bleaching of sulfite and soda-aq pulp, respectively, thereby producing a pulp with a brightness equal to the control. It was found that the major impact of the reduction of the active chlorine charges during biobleaching was on the chloride, AOX and toxicity levels of the bleach waste waters at the D_1, E_o and D_2 stages. At the same time, a limited decrease in the colour and COD of the E_o-waste water was detected. For instance, when dissolving pulp was bleached with 39% less chlorine dioxide than the control, the chloride, AOX and bacterial growth inhibition levels of the total waste water, derived from the $D_1E_oD_2$ bleaching, decreased by 36%, 34%, and 30% respectively, however, the overall reduction in colour and COD was only 2% and 9%.

1.2. Introduction

Bleach plant waste waters from the pulp and paper industry are highly coloured, contain chlorinated organic material and can cause serious environmental problems because some of these compounds are in part toxic and mutagenic (Bajpai and Bajpai 1994). In addition, a high concentration of chlorides in the waste waters contribute to their corrosiveness. These characteristics are of environmental concern and legislation is becoming stricter regarding the impact of waste waters on the environment. In response to the environmental concerns and stringent emission standards, the pulp and paper industry is making efforts to reduce the chloroorganic/chloride discharges by introduction of more environmentally-benign bleaching agents. These imply extending the cooking time for additional lignin removal, the use of oxygen as a prebleaching step, elemental

chlorine-free and totally chlorine-free bleaching. Physico-chemical methods of removing chloroorganics (e.g. membrane treatment), although quite effective in decolourisation of pulp and paper mill waste waters, can be unattractive for industrial applications because of the high costs.

Biotechnology can offer some apparent benefits to the pulp and paper industry. These include potential increase in pulp yield, heigher brightness ceiling, reduced chemical loads, improved waste water quality, establishment of environmetally-friendly bleaching sequences, increased pulp efficiency and capacity. Biotechnological alternatives to reduce the environmental impact of the industry involve: 1) the use of enzymes (xylanases) prior to bleaching to alleviate the heavy chemical charges (especially chlorine-containing chemicals) in the bleaching process and therefore minimize pollution, or 2) the use of microorganisms for waste water utilisation or secondary biotreatment of waste waters, which has the advantage of being cost-effective and can also reduce BOD, COD and AOX of the waste water (Kondo 1998).

1.3. Materials and methods

1.3.1. Enzyme assay

Xylanase activity was determined spectrophotometrically at 540 nm by the dinitrosalicylic acid method based on the release of reducing sugars from oat spelts xylan (Miller 1959). One unit (U) of enzyme activity was defined as that amount of enzyme which catalyses the release of 1 μmol of xylose reducing sugar equivalents per min of reaction.

1.3.2. Pulps

Unbleached sulfite 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. Post-oxygen soda-aq pulp was obtained from Sappi Enstra mill in

Springs. Pulps were thoroughly washed before and after each treatment step until wash waters attained neutral pH.

1.3.3. Enzyme treatment of pulp

Pulp was treated with xylanase at pH of 5.0 and temperature of 60°C and charge of 5 U xylanase/g pulp for 3 h. The enzymatic reaction with pulp was terminated by boiling the samples for 15 min. Controls were prepared under the same conditions but without the use of enzymes.

1.3.4. Chemical bleaching of pulp

Bleaching of enzymatically-pretreated dissolving pulp and post-oxygen soda pulp was carried out in sequences $OD_1E_oD_2H$ and DED, respectively, under standardized bleaching conditions. The following treatment stages were used in bleaching: oxygen (O), chlorine dioxide (D_1 and D_2), sodium hydroxide with reinforced oxygen (E_o) or without (E), sodium hypochlorite (H).

1.3.5. Analyses

Brightness of pulp was measured according to the Standard Methods of the Technical Association of the Pulp and Paper Industry (TAPPI, Atlanta, GA, USA) using Elrepho 2000 (Zeiss, Dresden, Germany). Samples for colour determinations were filtered through a 0.45 μm membrane filter before the pH was adjusted to pH 7.6 with sulphuric acid. The colour was determined with a Hach DR/2000 spectrophotometer (Loveland, Colorado, USA) at 455 nm using APHA Platinum-Cobalt standard with a range of 0-500 colour units (PCU). One colour unit equals 1 mg/l platinum as chloroplatinate ion. Chemical oxygen demand (COD) was also determined using the Hach spectrophotometer according to instructions set out in the manual for the instrument. Adsorbable organic halogen (AOX) analyses were performed using an Euroglas AOX EC S 1000 analyzer (Delft, Netherlands) according to the manufacturer's instructions. Prior to analysis, samples were diluted 100 to 500 times to obtain an AOX concentration within the range 10 to 100 μg Cl/l. The chlorinated organic matter is burned in oxygen in a sealed

environment thereby the chloride anions formed are determined by a microcoulometric titration (Odendahl et al. 1990).

Bacterial growth inhibition tests on waste water were performed (Slabbert 1986; Slabbert 1988). *Pseudomonas putida* strain Berlin 33/2 (DSM, Teilsammlung, Bayreuth, Germany) was used as a test organism. Tests were performed in 25 ml minimal medium (1.05 g K_2HPO_4, 0.45 g KH_2PO_4, 0.047 g sodium citrate, 0.1 g $(NH_4)_2SO_4$, 0.01 g $MgSO_4.7H_2O$ and 0.25 g glucose per liter of deionised water (pH 7.15) in 50 ml medicine bottles. A culture of *P. putida*, grown, as static cultures in an incubator, overnight at 27°C in minimal medium, was diluted with fresh minimal medium (to an absorption level of approximately 0.8), 30 min prior to inoculation of filter-sterilised (Millex, Millipore, Molsheim, France) test samples. The cell suspension was added at a ratio of 1:4 to a 12.5-times concentrate of the minimal medium and used as 2.5 ml volumes for inoculation of 22.5 ml test samples. Test samples were incubated at 27°C for 6 h after which growth was measured in terms of adsorption. Absorption determinations were carried out spectrophotometrically at 600 nm. Solutions containing samples and medium only (without inoculum) were used to blank the spectrophotometer. Sterile deionised water was used for control tests. Results were expressed as percentage inhibition or stimulation (%) over control. Values represented the mean of at least two determinations.

1.4. Results and discussion

1.4.1. Effect of biobleaching on reduction of active chlorine charges

Biobleaching of sulfite and soda-aq pulps with xylanase using reduced charges of chlorine dioxide has been studied (Table 1). Maximum brightness of 93.5% was obtained with a xylanase pretreatment pulp without reduction in the active chlorine charges. The brightness gain over the control was 1.5 points. The active chlorine charges at D_1 and D_2 could be reduced by 33% at D_1 and by 50% at D_2, respectively, while still maintaining the control brightness level of 92%. This resulted in total savings of active chlorine of 39% or 2.5 kg of ClO_2/ton of pulp (Table 1). Similarly, a brightness gain of 1.5 brightness points over the control was obtained without reducing the D charges in biobleaching of soda alkaline pulp (Table 2). Alternatively, the amount of ClO_2 could be reduced by 30%

and 4.5 kg of ClO_2/ton of pulp could be saved while still keeping brightness at the control level.

Table 1. Impact of biobleaching of dissolving pulp on reduction of chlorine dioxide charges

Reduction at D_1 (%)	Reduction at D_2 (%)	Total reduction		Brightness (%)
		%	kg ClO_2/t pulp	
0 (Control)	**0**	**0**	**0.0**	**92.0**
0	0	0	0.0	93.5
0	50	18	1.1	93.1
0	100	35	2.3	92.4
33	0	21	1.4	92.9
33	**50**	**39**	**2.5**	**92.0**
33	100	57	3.7	91.0
50	0	32	2.1	92.3
50	50	50	3.2	91.1
50	100	68	4.4	90.3

Table 2. Impact of biobleaching of soda-aq pulp on reduction of chlorine dioxide charges

Reduction at D_1 (%)	Reduction at D_2 (%)	Total reduction		Brightness (%)
		%	kg ClO_2/t pulp	
0 (Control)	**0**	**0.0**	**0.0**	**86.7**
0	0	0.0	0.0	88.2
10	10	10.0	1.5	88.0
15	10	13.3	2.0	87.9
15	20	16.7	2.5	87.7
20	20	20.0	3.0	87.6
20	30	23.3	3.5	87.5
25	30	26.7	4.0	87.1
30	**30**	**30.0**	**4.5**	**87.0**
35	30	33.3	5.0	86.1
40	30	36.7	5.5	85.3

1.4.2. Effect of biobleaching on waste water properties

The reductions in the amounts of chlorine dioxide used in bleaching did lead to changes in the composition of the bleach pulp waste waters. The impact of xylanase pretreatment on COD, AOX/chloride content, colour and bacterial growth levels in bleach pulp waste

waters D_1, E_o and D_2 has been investigated (Table 3). The COD, AOX and chloride content of all three bleach pulp waste waters, released during bleaching of the xylanase-pretreated pulp, was reduced as a consequence of minimizing the charges at D_1 and D_2. Thus, COD decreased by 25% at D_1, 7% at Eo and 25% at D_2. The total reduction of COD in these three waste waters was 9%. The Cl^- content was lowered by 31% at D_1, 33% at E_o, and 49% at D_2, whereas AOX content decreased by 36% (D_1), 22% (E_o) and 42% (D_2) (Table 3). At all three stages, the decrease in the bacterial growth inhibition levels correlated well with the reductions in the AOX and chloride content (Table 3). Colour of the D_1-waste water decreased by 9% and of D_2-waste water by 42%, respectively. However, the overall reduction of colour was only 2% since most of the colour originated from the E_o-waste water, where no reduction was observed. Therefore, the major impact of the reduction of the active chlorine charges during biobleaching was on the chloride, AOX and bacterial growth levels of the total bleach pulp waste water.

Table 3. Impact of biobleaching on properties of bleach plant waste waters

Bleaching stage	Reduction over controls (%)					
	Active chlorine	COD	Cl^-	AOX	Bacterial growth inhibition[a]	Colour
D_1	33	25	31	36	30	9
E_o	-	7	33	22	18	0
D_2	50 (39)[b]	25 (9)[a]	49 (36)	42 (34)	41 (30)	42 (2)

[a] See Materials and methods for detailed description of bacterial growth inhibition test
[b] Values in brackets indicate overall reduction over the three bleaching stages

1.5. Conclusions

1. Chemical savings of up to 2.5 kg of ClO_2/ton of pulp could be achieved when dissolving pulp was bleached with xylanase whereas biobleaching of soda-aq pulp effected a 30% reduction (4.5 kg of ClO_2/ton of pulp) of the total amount of chlorine dioxide used.
2. The major impact of the reduction of the active chlorine charges during biobleaching was on the chloride, AOX and bacterial growth levels of the total bleach pulp waste water whereas colour and COD of waste water were impacted to a lesser extent. For

instance, a 39% reduction of the chlorine dioxide use reflected in a corresponding decrease of 36% of the chlorides, 34% of AOX and 30% of bacterial growth inhibition levels of the total waste water derived from the $D_1E_oD_2$-bleaching of dissolving pulp.

3. The implementation of the enzyme bleaching technology in the pulp and paper industry could improve the existing technology of pulp and paper manufacture in a cost-effective and environmentally friendly way.

2. Waste water bioremediation using microorganisms

2.1. Abstract

In this study, applications of physico-chemical treatment methods as well as biological methods were investigated to reduce colour of waste water from the alkali extraction stage of a bleach plant waste water from a dissolving pulp mill. The aim was to develop an effective and economically viable bioremediation process for the pulp and paper industry.

The following physical-chemical methods were used: adsorption, desorption and coagulation. It was demonstrated that biomass from the mucoralean fungus *Rhizomucor pusillus* can be used efficiently as a bioadsorbent of colour from industrial waste waters. The biosorption of colour was a rapid process which could remove approximately 90% of the adsorbable colour (which was approximately 50% of total colour) in the first few hours (2-4 h) of waste water treatment. Chitosan solutions when used as coagulation and adsorption agents decolourised the waste water by between 57%-75%. With commercial adsorbents, a smaller decolourisation activity was observed. Almost all of adsorbed colour could be removed from *R. pusillus* by alkali treatment, in contrast to the commercial adsorbents, where chemisorption was suggested to play a major role.

The use of biological methods for waste water treatment involved: 1) Trickling filters 2) Rotating biological contactor reactors (RBC) 3) Activated sludge reactors. With trickling filters containing immobilised white-rot fungi, maximum decolourisation (61%) was obtained with *Coriolus versicolor*. This fungus required a cosubstrate to efficiently decolourise the waste water.

In RBC waste water decolourisation was studied using *C. versicolor*, a white-rot fungus and *R. pusillus* strain RM7, a mucoralean fungus. Decolourisation rate was directly proportional to initial colour intensities in both fungi. Decolouring activity was not adversely affected by colour intensity, except at the lowest levels tested (1550 PCU). It could be shown that decolourisation activity levels of between 61 to 74% were possible

using a retention time of 23 h. With *R. pusillus*, 55% of AOX were removed compared to 40 % by *C. versicolor*. Following treatment, there was a reduction in bacterial growth inhibition levels of 69 percentage points when *R. pusillus* was used for waste water treatment, whereas *C. versicolor* reduced bacterial growth inhibition by a margin of 38 percentage points. Addition of glucose to decolourisation media stimulated colour removal by *C. versicolor*, however this was not observed with *R. pusillus*. Ligninolytic enzyme activities were only detected in waste water treated by *C. versicolor*. It seems that there are definite differences in the decolouring mechanisms between the white-rot fungus (adsorption + biodegradation) and the mucoralean fungus (adsorption). Desorption experiments conducted under alkaline conditions indicated that almost all adsorbed coloured compounds could be removed from *R. pusillus*. Although the major portion of adsorbed colour could be desorbed from *C. versicolor* by alkaline treatment there seems to be a fraction that could not be removed from the cell wall of this fungi under these conditions. These results indicated that adsorption could play a significant role in the removal of colour from waste water containing media by both fungi under these conditions. Waste water was also treated in activated sludge reactors. Treatment in RBC overall improved waste water quality to a higher degree when compared to treatment in an activated sludge reactor. The activated sludge system did not remove colour to a significant extent and AOX levels were decreased by 20%.

2.2. Introduction

Bleach plant waste waters from the pulp and paper industry are highly coloured and also partly toxic due to the presence of chloroorganics, hence the need for pretreatment prior to discharge (Royer et al. 1991). Because of the complexity of these waste waters, various treatment methods have been tested with limited success. Physico-chemical treatments methods yield variable results and are also generally too expensive for application commercially (Royer et al. 1991). Conventional biological treatment options such as activated sludge and aerated lagoons reduce COD load and toxicity of bleach plant waste waters but cannot remove colour, which is due mainly to high molecular weight chloroorganic materials in the waste water (Royer et al. 1991). In contrast, biological methods employing white-rot fungi can reduce colour, toxicity and to some

extent COD/BOD (Royer et al. 1991). However, treatment with white-rot fungi usually requires prolonged retention times for sufficient decolourisation (Bajpai and Bajpai 1994).

In this study, applications of physico-chemical as well as biological treatment methods for waste water were investigated. The emphasis was mainly on colour removal however, impact on COD, bacterial growth and AOX of a bleach plant waste water derived from the alkali extraction stage of a dissolving pulp mill were also studied.

2.3. Materials and methods

2.3.1. Maintenance and cultivation

Coriolus versicolor strain SCC-63, *Stereum illudens* SCC-31, *Pycnoporus sanguineus* SCC-92 and *Rhizomucor pusillus* RM7 were maintained on potato dextrose agar at 4°C. The fungal strains were obtained from the Sappi Culture Collection at the University of the Free State. For inoculum preparations, four 1x1 cm cubes were cut from fresh plates and transferred to sterile 500 ml Erlenmeyer flasks containing 200 ml growth medium of the following composition in g/l: glucose, 20; malt extract, 20; peptone, 20; yeast extract, 8. The pH of the medium was adjusted to 5 prior to sterilisation. After 4 days incubation on an orbital shaker at 30°C, 40 ml culture broth was aseptically transferred to sterile 1l Erlenmeyer flasks containing 400 ml growth medium. The flasks were incubated for 4 days before homogenization.

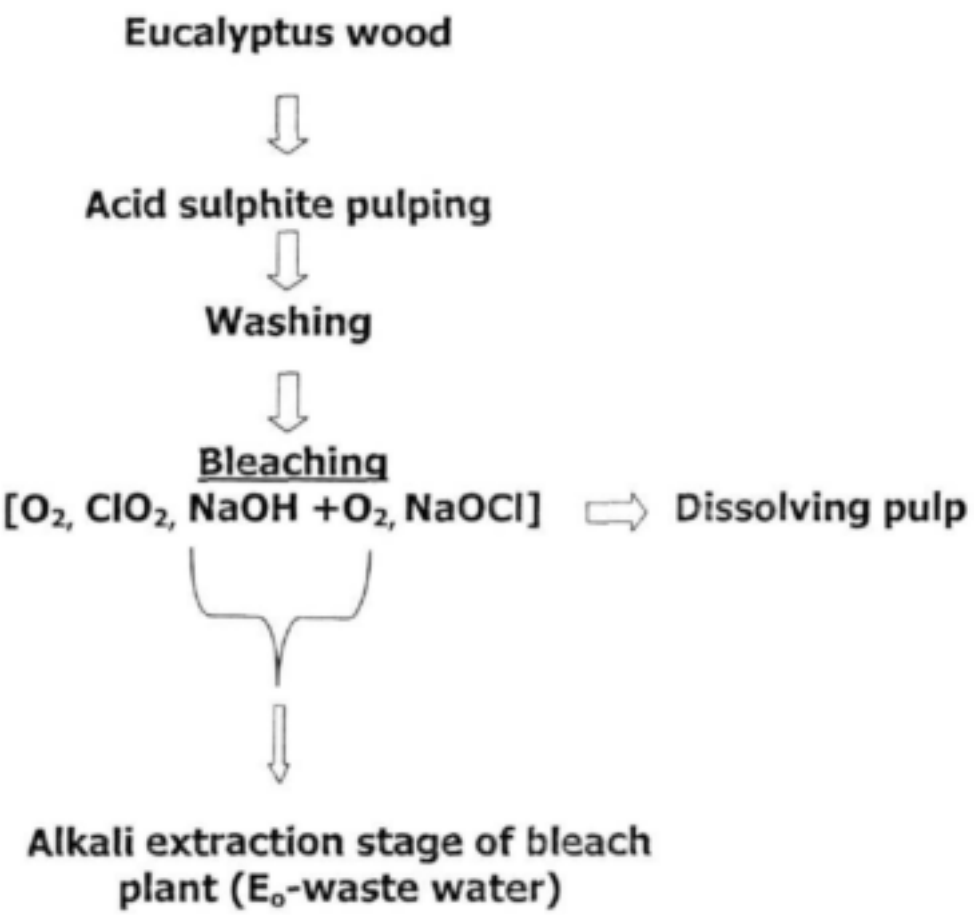
2.3.2. Waste water

The waste water used in this study was obtained from the alkaline extraction stage (E_o) of the bleach plant of a sulfite dissolving pulp mill (Diagram 1). Waste water was stored in closed containers at 4°C prior to treatment. The waste water had a colour of 12200 PCU, COD of 11000 mg/l and a pH of 8.15.

2.3.3. Coagulation

A stock solution of chitosan (0.8 g/l; 1% acetic acid solution used as solvent) was mixed with different volumes of E_o-waste water and the decolouring activity determined after the flocs that formed by coagulation have settled out under gravity after 20 min. The effect of concentration and molecular mass (M_r) of chitosan dissolved in acetic acid solutions on decolouring activity during treatment of E_o-waste water was also investigated. A 1:1 mixture of chitosan: E_o was employed. Colour was determined after the flocs that formed upon mixing, settled out under gravity after 20 min.

Diagram 1. Dissolving pulp production



2.3.4. Cell wall fractionation and chitosan extraction

Cell wall fractionation and chitosan extraction was performed as follows: Dry biomass (4 g) was treated with 100 ml 1 N NaOH at 121°C for 30 min. The alkali-resistant fractions were subsequently treated for chitosan extraction. This method involves the homogenisation of alkali-extracted material with 2% acetic acid followed by reflux in 2% acetic acid for 1 h. Thereafter the pH of the supernatant was adjusted to 8.5 with NaOH. The resultant precipitate (chitosan fraction) was washed with deionised water. The alkali-resistant, chitosan and the residual fractions, obtained after chitosan extraction was completed, were washed, dialysed against deionised water, dried at 50°C to a constant weight and used in adsorption experiments. Charges used for the cell wall fractions were 0.332 g per 100 cm^3 waste water, for the residual fraction, 0.145 g per 100 cm^3 waste water, for the chitosan fraction and 0.615 g per 100 cm^3 waste water, for the alkali-resistant fraction. These charges were based on the amount of cell wall material extracted from 4 g dry *R. pusillus* biomass (Diagram 2).

2.3.5. Adsorption experiments

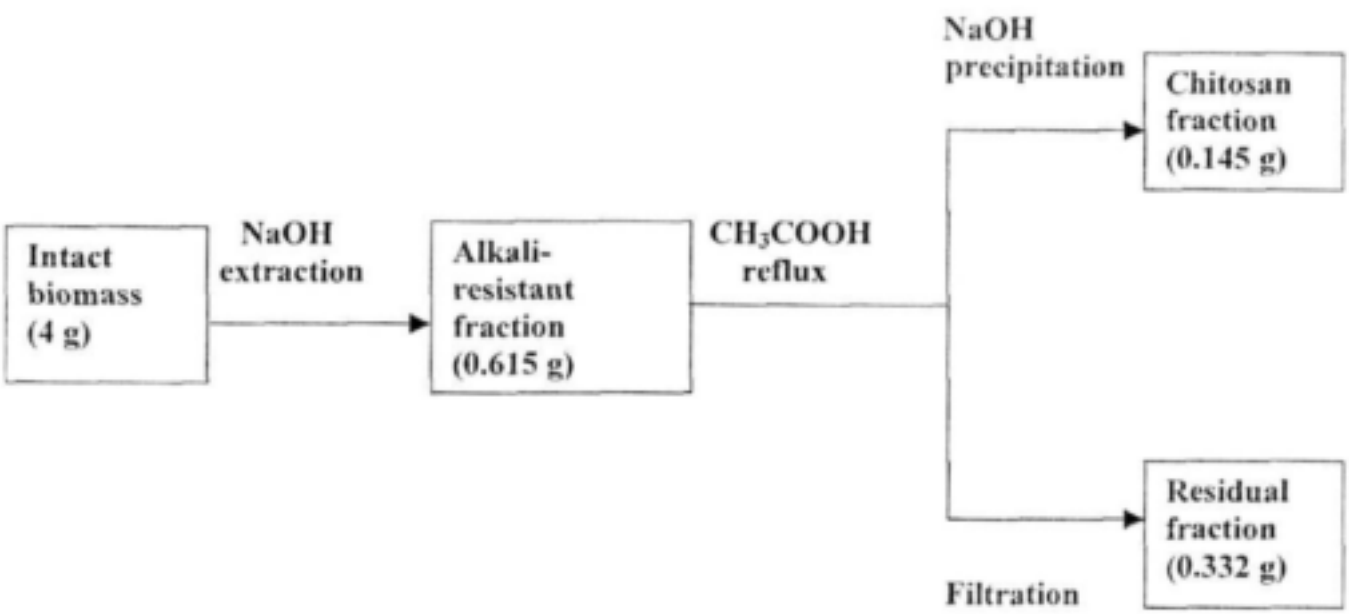
Dried *R. pusillus* RM7 biomass used at a charge of 4 g/100 ml E_o-waste water and alkali resistant fractions obtained from RM7 biomass (4 g) by alkali extraction (1N NaOH, 121°C, 0.5 h) were used in adsorption experiments conducted in shake flasks at 30°C to treat 100 ml of E_o-waste water. Wet RM7 biomass was employed as adsorbent at a charge of 0.5g per 100 ml waste water in shake flasks at 30°C. Adsorption experiments using activated carbon, ion exchange resin, commercial chitin and chitosan were conducted in a similar way as described for the wet fungal biomass. To test the effect of waste water recycling on colour adsorption, fresh biomass was employed to treat the same batch of waste water in five consecutive adsorption cycles of 1 h treatment time each. To test the effect of biomass recycling on colour adsorption the same biomass was employed to treat fresh amounts of waste water in five consecutive 1-h cycles.

2.3.6. Desorption experiments

The sorbent materials were tested in desorption experiments to establish whether they could be regenerated. Treatment with 1N NaOH was carried out at 30°C for 4 h on an

orbital shaker. Colour desorbed was measured and compared against the amount of colour initially adsorbed onto the respective adsorbent.

Diagram 2. Fractions from the cell wall of *R. pusillus*



2.3.7. Trickling filters

A trickling filter system comprising 1 m columns into which were packed urethane foam containing immobilised white-rot fungi (*Trametes versicolour* (column I), *Stereum*

illudens (column II) and *Pycnoporus sanguinens* (column III) were utilised for the treatment of waste water. Fungi were inoculated after homogenisation in jars containing 1% molasses solution and sterilised foam cubes. Twice a day the contents of the jars were mixed to allow immobilisation to proceed. After 4 weeks, visual inspection revealed that immobilisation occurred and immobilised foam cubes were aseptically packed into columns. Attached to the tubes were reservoirs containing bleach plant waste water diluted 1: 1 with 1% molasses solution and the system was run in a recirculating mode. One column was packed only with foam and served as control. The reactors were run at a contact time of 30 min (Diagram 3).

2.3.8. Operation of activated sludge reactor

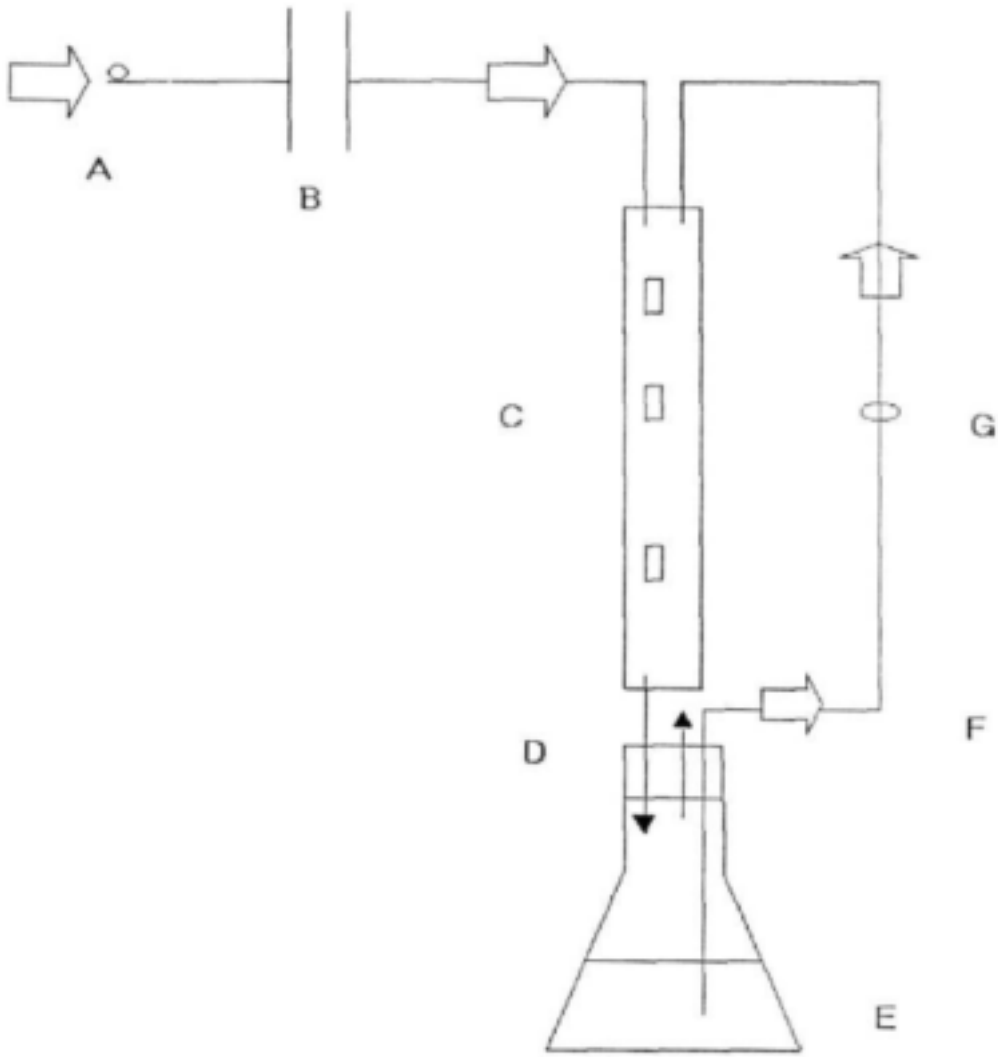
An OECD (Organisation for Economic Cooperation and Development) laboratory scale activated sludge reactor was used for the treatment of E_0-waste water (Diagram 4). The reactor was seeded with activated sludge from an activated sludge facility treating bleach plant waste water. Mixed liquor suspended solids (MLSS) was maintained at about 4 g/l. Air was supplied at a rate of 2.3 l/min. Sludge age was kept at 20 days. Sludge was returned from clarifier at a controlled rate with the use of a timer. The reactor was operated at a temperature of 25°C.

2.3.9. Rotating biological contactor

2.3.9.1. Design of rotating biological contactor

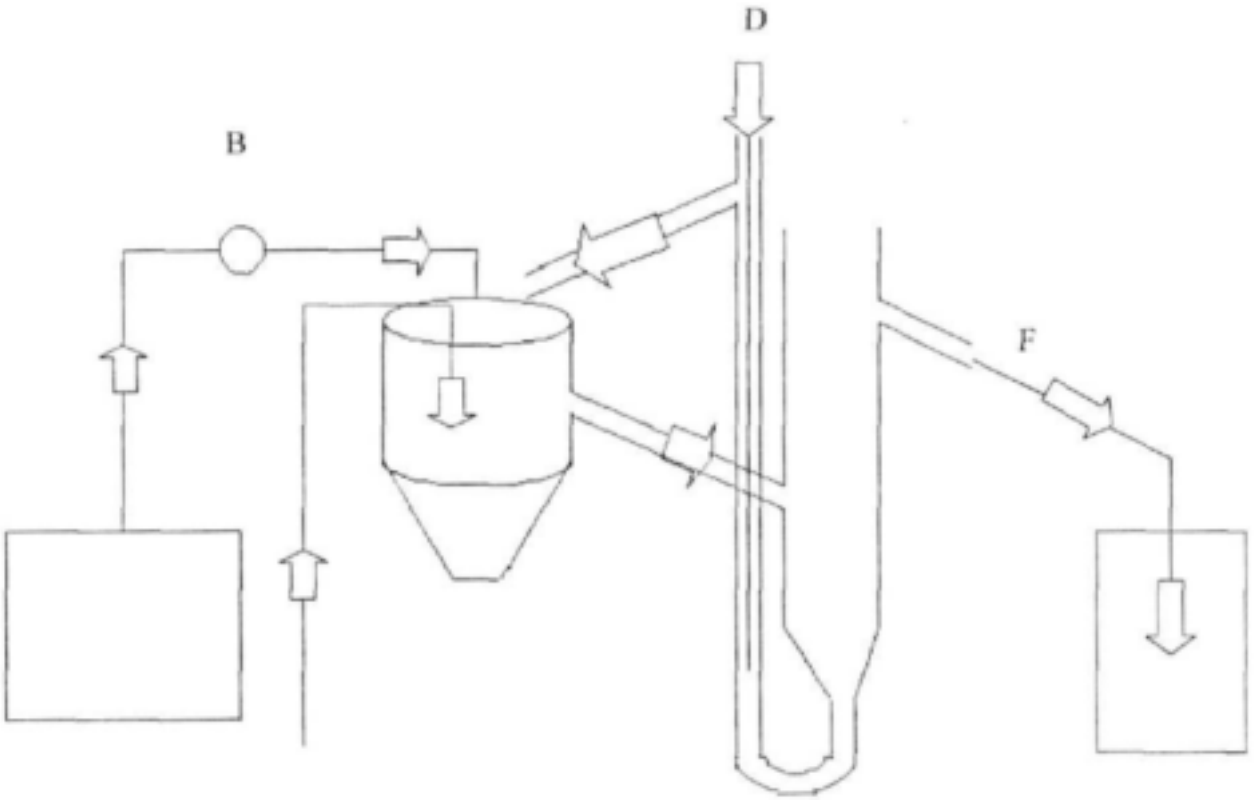
The RBC reactors consisted of 100 cm plastic tubes with a diameter of 7.5 cm (Diagram 5). The tubes could be disassembled into 24 cm sections and each section contained 12 plastic disks with a spacing of about 1.5 cm between them. The disks had an opening in the middle with a diameter of 2.5 cm. They were packed vertically into the tubes and held in place by o-ring containing sections. Each disk was fitted with porous plastic material to serve as support for microbial growth. Tubes were mounted on rollers, powered by an electric motor and rotated at 4 rpm. Rollers were mounted in a frame that could be

Diagram 3. Trickling filter reactor



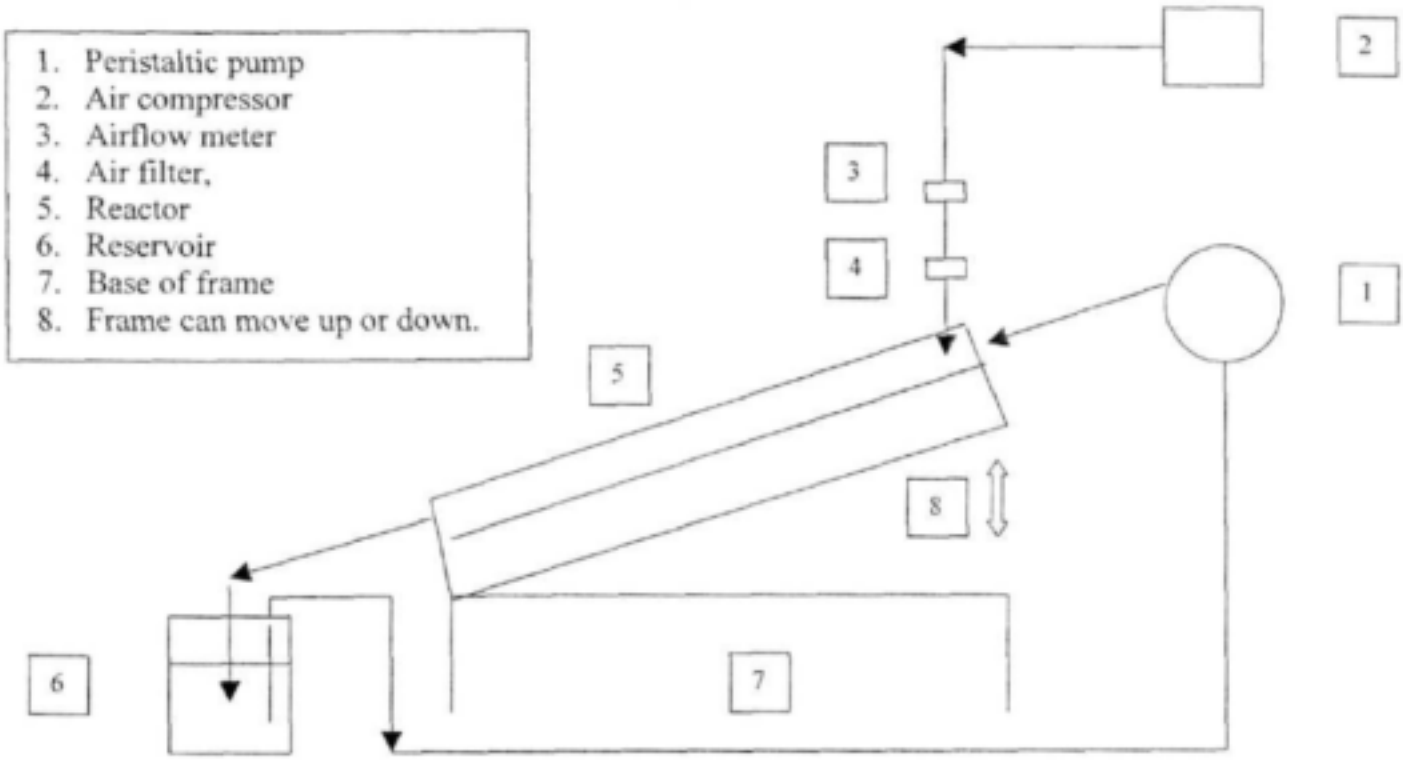
A) Air pump
B) $CuSO_4$ trap
C) Reactor with foam cubes and fungi
D) Waste water
E) Reservoir
F) Waste water
G) Peristaltic pump

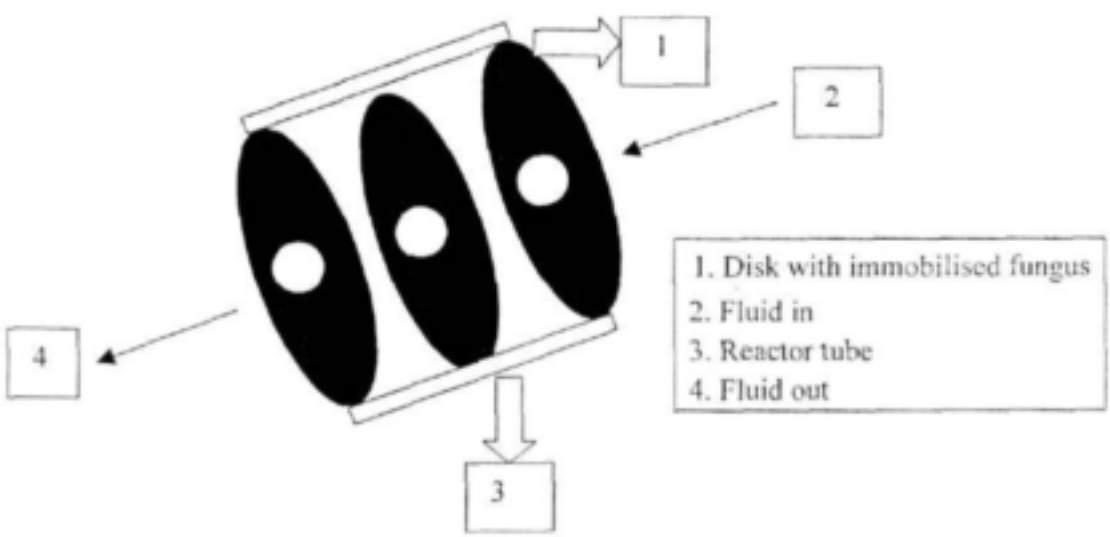
Diagram 4. Activated sludge reactor



A) Container with influent
B) Pump
C) Aeration basin
D) Air pumped into glass tube
E) Clarifier
F) Effluent overflow
G) Vessel containing effluent
H) Air inlet

Diagram 5. Rotating biological contactor reactor





elevated to independently vary the volume hold capacity of the RBC system. Tubes were sealed at each end with caps containing inlet and outlet pipes. Inlet pipes were supplied with glass fittings. Reactors were connected to a peristaltic pump by silicon tubing attached to the glass fittings. Air was supplied to the reactors through side tubes, connected to the glass fittings.

2.3.9.2. Immobilisation of fungi in rotating biological contactor

Cell cultures (1.2 l) were homogenized for 60 s under aseptic conditions at a rate of 13,500 rpm. Prior to immobilisation, the RBC reactors were treated with 70% (v/v) ethanol for 1 h and then washed with sterile distilled water. The homogenized culture was pumped into the RBC reactors at a rate of 500 ml/h with a peristaltic pump. Thereafter growth medium (pH 3.5) was supplied to the reactors using a peristaltic pump. Following a week of incubation at 25°C, freshly prepared and homogenized culture was pumped into the reactors and this procedure was repeated after another week.

2.3.9.3. Reactor volume determination

After immobilisation of the fungi in the RBC, the volume of the reactors was determined. This was accomplished by dosing the inlet feed to the reactors with 50 ml of a sterile NaCl solution (5 g/l). Samples (20 ml) were continuously taken during and after dosing and dispensed in vials that were numbered according to the order in which sampling proceeded. The volume required to elute NaCl from each RBC represent the volume hold capacity of each reactor. Conductivity of samples was measured with a Hanna conductivity meter (Hanna instruments, Padova, Italy) in order to follow NaCl elution from the reactors. NaCl was eluted from the tubes when the conductivity of samples reached a maximum level. Reactor volume was used to calculate hydraulic retention time. Residence time was varied between 0.67 to 23 h to determine the effect of this parameter on decolourisation. The hydraulic retention time used during investigations on the effect of initial colour intensities on decolourisation was 24 h.

2.3.9.4. Decolourisation medium

Media with the following composition were used during decolouring experiments (g/l): glucose, 10; NH_4NO_3, 0.5; KH_2PO_4, 0.1; $MgSO_4$, 0.5; $FeSO_4.7H_2O$, 0.01; $CaCl_2$, 0.5. The pH of the medium was adjusted to pH 3.5 with a Hanna pH-meter (Hanna instruments, Padova, Italy). E_o-waste water was used at various strengths to determine the effect of initial waste water colour on decolourisation. Subsequently it was decided to supply waste water at 50% of full strength during decolourisation experiments conducted under optimized conditions. Glucose, citrate buffer (0.01 M) and E_o-waste water were heat-sterilised (121°C, 20 min). The other constituents of the decolourisation medium were filter-sterilised using a 0.45 µm sterile Millipore filter (Bedford, MA, USA) and then aseptically added to the heat-sterilised portion to bring the final volume of the medium to 2 l in the RBC system.

2.3.9.5. Operation of rotating biological contactor

Following immobilisation, the fungi were adapted to the waste water-containing decolourisation medium employing four residence times before decolourisation experiments commenced. Sterile decolouring medium contained in a rubber stoppered 2 l Erlenmeyer flask was positioned at the outflow end of each reactor and served as reservoir (Scheme 5). Reservoirs were connected to the reactors by silicon tubes. Medium was circulated between reservoirs and the reactors at a fixed rate by a peristaltic pump. Content of reservoirs were agitated by magnetic stirrers (80 rpm). Samples were taken and then analysed as treated medium emerged from the reactors before mixing occurred in the reservoirs. Samples were also taken from the reservoirs after 72 h for bacterial growth inhibition and AOX determinations after steady state ocurred. Air was supplied to the reactors by an air pump through in-line sterile Hepa-vent Whatman air filters (Ann Arbor, MI, USA) at a flow rate of 2.3 l/min. Reactors were operated at 25°C.

2.3.9.6. Estimation of colour adsorbed to biomass

Colour adsorbed to biomass after decolourisation in RBC was determined by treatment of fungal cell mass with 1N NaOH for 24 h. A magnetic stirrer was used to agitate the mixture. Colour desorbed by 1N NaOH was measured. Prior to alkali treatment, the

fungal biomass was washed with distilled water on Buchner funnels. Desorption experiments were also performed after treatment of decolourisation media with fungal biomass for 5 min in shake flasks, using the same method as described above.

2.3.9.7. Effect of glucose addition on decolourisation

Glucose was added (2 g/l up to 10 g/l) to decolourisation media during decolourisation experiments to assess the influence thereof on colour removal by both fungi.

2.3.9.8. Enzyme activity determinations

The presence of manganese peroxidase and laccase were assessed according to the method of Mester et al. (1997). Lignin peroxidase activity was determined according to procedures of Addleman and Archibald (1993). The substrate 2,6-dimethoxyphenol was used. Enzyme activity was expressed in U/l.

2.3.10. Analytical methods

Reactor tubes were washed with distilled water before biomass was scraped from the disks upon completion of experiments. Biomass was dried to a constant weight at 105°C and then determined gravimetrically. Samples for colour determinations were filtered through a 0.45 µm membrane filter before the pH was adjusted to pH 7.6 with sulphuric acid. The colour was determined with a Hach DR/2000 spectrophotometer (Loveland, Colorado, USA) at 455 nm using APHA Platinum-Cobalt standard with a range of 0-500 colour units (PCU). One colour unit equals 1 mg/l platinum as chloroplatinate ion. Chemical oxygen demand (COD) was also determined using the Hach spectrophotometer according to instructions set out in the manual for the instrument. Conductivity of samples was measured with a Hanna conductivity meter and expressed in mS/m. Adsorbable organic halogen (AOX) analyses were performed using an Euroglas AOX EC S 1000 analyzer (Delft, Netherlands) according to the manufacturer's instructions. Prior to analysis, samples were diluted 100 to 500 times to obtain an AOX concentration within the range 10 to 100 µg Cl/l. The chlorinated organic matter is burned in oxygen in a sealed environment thereby the chloride anions formed are determined by a microcoulometric titration (Odendahl et al. 1990).

Bacterial growth inhibition tests on waste water were performed (Slabbert 1986; Slabbert 1988). *Pseudomonas putida* strain Berlin 33/2 (DSM, Teilsammlung, Bayreuth, Germany) was used as a test organism. Tests were performed in 25 ml minimal medium (1.05 g K_2HPO_4, 0.45 g KH_2PO_4, 0.047 g sodium citrate, 0.1 g $(NH_4)_2SO_4$, 0.01 g $MgSO_4.7H_2O$ and 0.25 g glucose per liter of deionised water (pH 7.15) in 50 ml medicine bottles. A culture of *P. putida*, grown, as static cultures in an incubator, overnight at 27°C in minimal medium, was diluted with fresh minimal medium (to an absorption level of approximately 0.8), 30 min prior to inoculation of filter-sterilised (Millex, Millipore, Molsheim, France) test samples. The cell suspension was added at a ratio of 1:4 to a 12.5-times concentrate of the minimal medium and used as 2.5 ml volumes for inoculation of 22.5 ml test samples. Test samples were incubated at 27°C for 6 h after which growth was measured in terms of adsorption. Absorption determinations were carried out spectrophotometrically at 600 nm. Solutions containing samples and medium only (without inoculum) were used to blank the spectrophotometer. Sterile deionised water was used for control tests. Results were expressed as percentage inhibition or stimulation (%) over control. Values represented the mean of at least two determinations.

Chlorophenols were extracted from decolourisation media by using dichlorophenol as organic solvent. Prior to extraction, pH values of the decolouring media were adjusted to 3.0 with concentrated sulphuric acid. The organic phase was concentrated under a nitrogen stream. Chlorophenol analysis was performed using a HP6890 (Palo Alto, CA) gas chromatograph (GC) with flame ionization detector. The GC was equipped with a Chrompac (Middelburg, the Netherlands) 7542 column (10 m x 53 mm) utilising nitrogen as carrier gas (1 ml/min) and a linear temperature program at a rate of 5°C/min up to 240°C after an initial 1 min period at 90°C during operation. To identify chlorophenols in the waste water, extracts were spiked with standard compounds before analysis. An increase in peak area after spiking indicates the presence of the spiked compound in the waste water. Gel permeation chromatography (GPC) was performed using a Waters high performance liquid chromatograph equipped with a 250 Ultrahydrogel column. Sodium nitrate (0.1 M) served as eluent and was delivered to the column at a flow rate of 0.6 ml/min. Peaks were identified by refractive index measurements.

2.4. Results and discussion

2.4.1. Physico-chemical treatment

2.4.1.1. Coagulation

Chitosan dissolved in acetic acid can be utilised to coagulate coloured compounds from bleach plant (E_o) waste water. A 1:1 mixture of chitosan with E_o-waste water resulted in a 66.4 % colour reduction (Table 1). This result was attained after a treatment time of only 20 min. The high decolouring activity obtained over this short treatment time is beneficial for application on industrial scale. Flocs can also be removed easily after settlement. Chitosan is a natural product obtained from chitin, and is therefore biodegradable, an attribute beneficial for its utilisation in environmental applications. It has been indicated that next to cellulose, chitosan and chitin are the most abundant natural compounds (Knorr and Klein 1986). Chitosan has been employed as coagulant in the remediation of an waste water from the pulp and paper industry (Ganjidoust et al. 1997).

Table 1. Chitosan (medium molecular weight) utilised as coagulant to treat E_o-waste water

Ratio Eo:Chitosan	Decolourisation (%)
1:1	66.4
2:1	60.6
3:1	47.4
6:1	35.9
9:1	27.2

Table 2. The effect of concentration and molecular mass (M_r) of chitosan on decolouring activity during treatment of E_o-waste water

Chitosan Concentration (g/l)	Decolourisation (%)		
	Low M_r[a]	Medium M_r[b]	High M_r[c]
0.1	25.2	33.8	24.7
0.2	41.5	57.7	46.0
0.8	53.7	66.7	72.7
1.2	52.4	71.8	74.7
1.6	57.1	66.9	75.3
2.0	43.5	64.1	75.3

a) Low M_r correspond to 70000 g/mol

b) Medium Mr correspond to 750000 g/mol

c) High M_r correspond to 2000000 g/mol

Table 2 indicates that the medium and high molecular weight chitosan used during coagulation decolourised the E_o-waste water maximally at concentration levels of between 1.2 to 1.6 g/l. It was reported that molecular weight and charge density of chitosan played major roles in flocculation as was also confirmed in this study (Agerkivist et al. 1990).

2.4.1.2. Adsorption

Adsorption is a good method to remove colour contaminants from waste waters by taking advantage of the affinity of the adsorption surface for a specific molecular or ionic compound coming into contact with it. Recent developments in environmental biotechnology include the search for micro-organisms as sorbents of contaminants (Kapoor and Viraraghavan 1995).

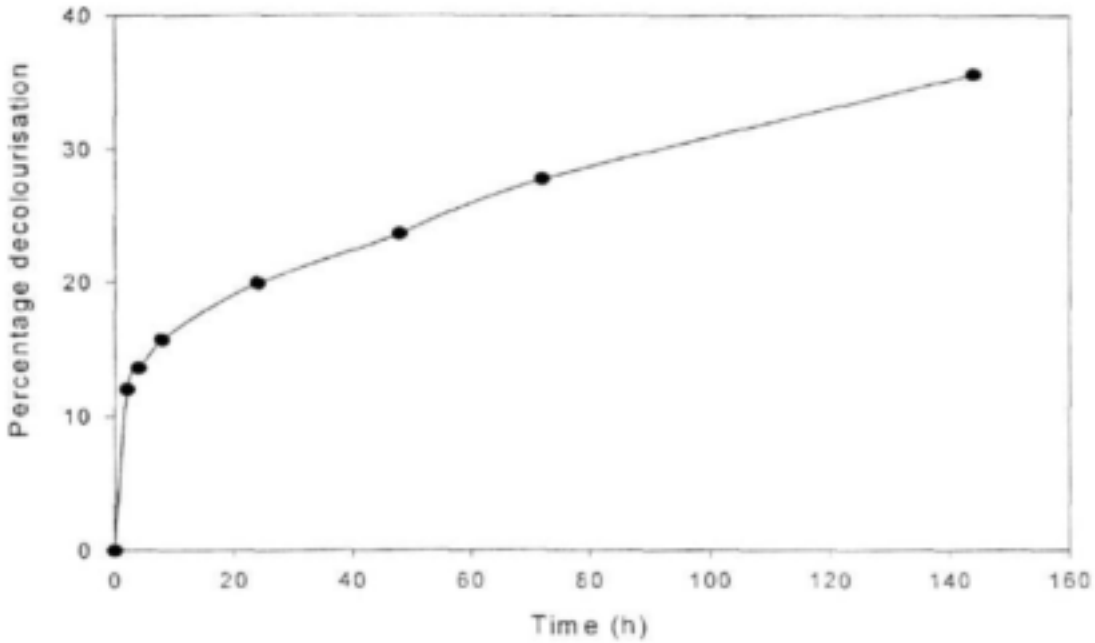


Fig. 1. Colour removal from waste water using an alkali resistant fraction extracted from *Rhizomucor pusillus*

Dried RM7 biomass used at a charge of 4 g/l decolourised E_{40}-waste water to a maximum level of 42%. Alkali resistant fractions obtained from RM7 biomass (4 g) by alkali extraction decolourised the E_{40}-waste water by 36 % (Fig.1). This result suggests that the chitosan-ß-glucan complex found in the cell wall could be mainly responsible for the decolouring activity exhibited by RM7. It was reported that certain fungi yield chitosan-glucan complexes upon NaOH treatment. Chitosan is a polycationic molecule and therefore it possesses polycationic characteristics that could be involved in the decolouring activity shown by this biopolymer.

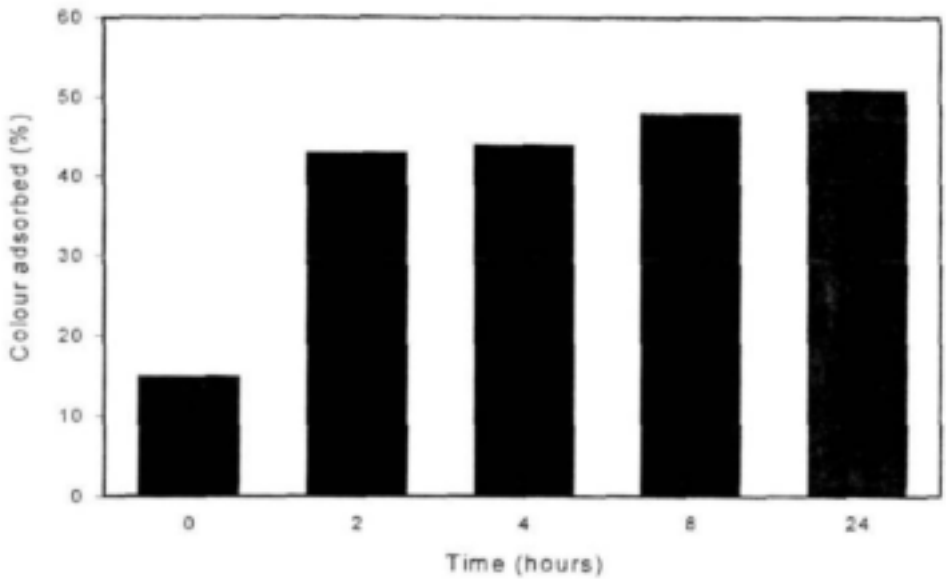


Fig. 2. Decolouring activity attained with *Rhizomucor pusillus* biomass as a function of time

RM7 is a thermotolerant fungus that can grow at temperatures of up to 55°C. It can therefore be used to treat E_o-waste water at elevated temperatures without incurring cooling costs and contamination by other organisms can be limited. Figure 2 shows the decolourisation level that could be attained using RM7 without prior drying of the biomass. A maximum decolouring activity in excess of 50% was attained. When the same biomass was used in five consecutive cycles the decolouring activity decreased after the first cycle as shown in Figure 3.

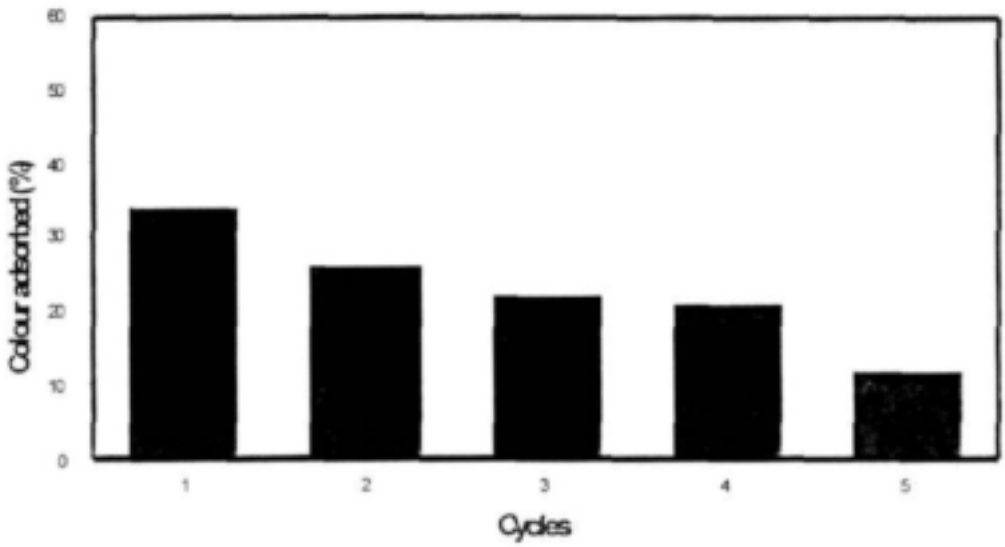


Fig. 3. Decolourisation employing the same *Rhizomucor pusillus* biomass to treat fresh batches of E_o-waste water in five cycles consecutively

The decreasing decolourisation activity exhibited by RM7 as the treatment cycles progress indicate that a level of saturation of the active sites on the biomass takes place. In Figure 4 the adsorption ability of various adsorbents is compared over a 24 h period.

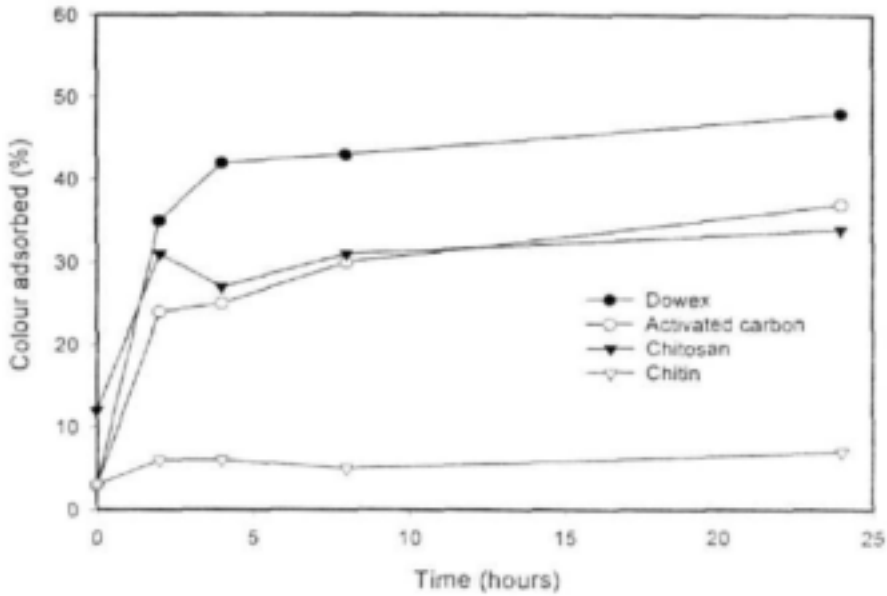


Fig. 4. Decolourisation of E_o-waste water using various adsorbents

Under these conditions Dowex, a strong anion exchange resin, proved to be the best adsorbent, followed by activated carbon and then chitosan. Because of the cationic nature of chitosan, strong anionic resin was also investigated in this study, for comparative purposes. Following the adsorption cycle, the adsorbents were treated with 1N NaOH and the colour desorbed was determined (Fig. 5). Almost all the colour was released from *R. pusillus* biomass, followed by chitin. However, a small percentage of adsorbed colour was released from chitosan, activated carbon and Dowex. Only partial restoration of the decolouring activity of the commercial adsorbents was obtained, suggesting the presence of chemical bonding between these adsorbents and colour compounds from the waste waters that could not be hydrolysed with NaOH. Thus, chemisorption may be responsible for the inability of the sorbent material to release colour.

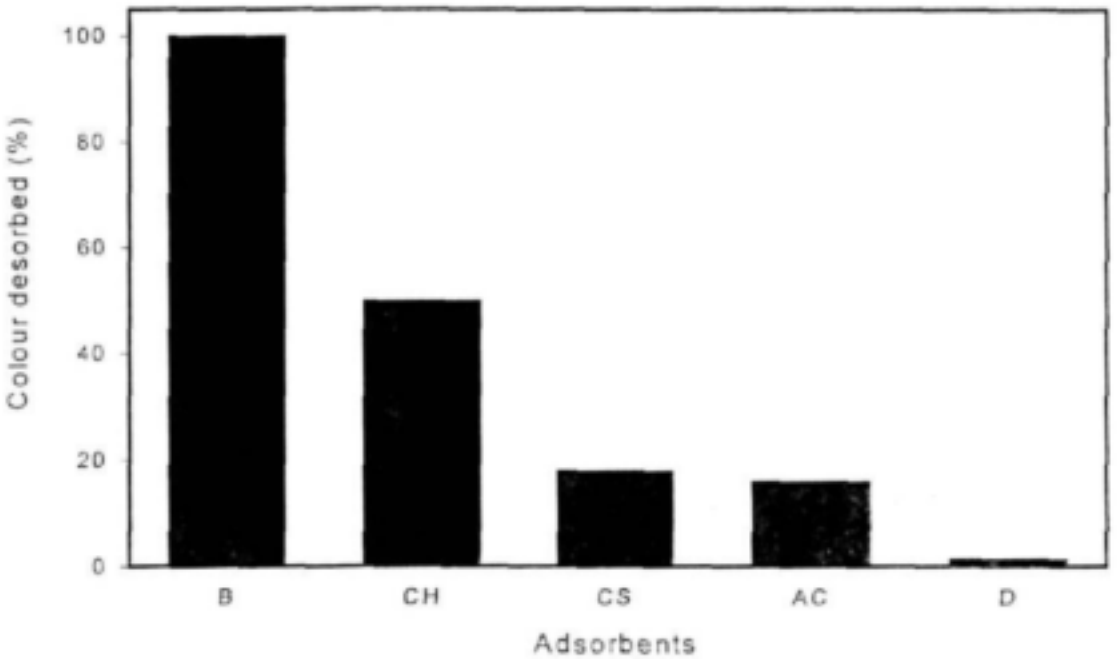


Fig. 5. Desorption profiles of adsorbed colour obtained after treatment of adsorbents with alkali. Symbols used for adsorbents: B, RM7 biomass; CH, chitin; CS, chitosan; AC, activated carbon; D, Dowex.

Table 3. Efficiency of colour removal from bleach plant waste water by biomass and cell wall fractions of *Rhizomucor pusillus*

Fraction	Yield (%)	Charge (g/l)	Decolourisation (%)
Chitosan	3.6	1.45	13.1
Residual	8.3	3.32	27.7
Combined[a]	11.9	4.77	40.8
Alkali-resistant	15.4	6.15	34.0
Intact biomass	100	40.0	39.3

[a] Chitosan and residual fractions combined

Cell wall fractions extracted from the biomass of *R. pusillus* (alkali-resistant, residual and chitosan fractions) appeared to be principally responsible for the decolouring of E_o-waste water by this fungus (Table 3). The residual and chitosan fractions representing combined only approximately 12% (w/w) of the *R. pusillus* biomass, were able to removed more colour from the bleach plant waste water (about 41%) than the intact biomass (39%). The major compound(s) of the cell wall fractions could be either chitosan and/or chitin based on the release of glucosamine upon acid hydrolysis of these fractions. The mechanism of colour removal by *R. pusillus* might involve ionic interactions between aminoglucans from the cell wall of this fungus and the negatively charged compounds found in the bleach plant waste water.

2.4.2. Biological treatment methods

2.4.2.1. Trickling filters

Table 4 shows decolourisation and absorbency readings obtained after treatment with three white-rot fungi. *C. versicolor* (column I) produced the highest decolourisation after 5 days of treatment, followed by *S. illudens* (column II) and *P. sanguineus* (column III). *C. versicolour* also lowered the absorbency readings to the greatest extent.

Table 4. Absorbency readings and decolourisation of E_o-waste water after passage through trickling filter reactors with immobilised white-rot fungi

Decolourisation			Adsorption	
(%)	Column	Time (days)	280 nm	254 nm
61.2	I *(C. versicolor)*	5	1.042	1.577
39.7	II *(S. illudens)*	8	1.182	1.650
26.1	III (*P. sanguineus*)	8	1.196	1.654
0		8	1.353	1.834

a) The control consisted of Eo-waste water diluted 1:1 with distilled water

After 5 days of treatment, the contents of the reservoir of column I was replaced with E_o-waste water diluted 1:1 with distilled water and decolourisation determined after 14 days. Decolourisation dropped to 39.6 % (Table 5). This indicated that *C. versicolour* required an additional carbon source to optimally decolourise bleach plant waste water. Molasses was included initially to serve as an inexpensive source of nutrients.

Table 5. Absorbency readings and decolourisation of E_o-waste water after passage through a trickling filter reactor with *Coriolus versicolor*

Decolourisation			Adsorption	
(%)	Column	Time (days)	280 nm	254 nm
39.6	I	14	1.182	1.707
0	Control	-	1.353	1.834

2.4.2.2. Activated sludge

An OECD laboratory scale activated sludge reactor was used for the treatment of E_o-waste water. Activated sludge plants have gained in popularity for the treatment of waste waters from the pulp and paper industry. After phosphate and nitrogen requirements for optimal COD reduction were determined (data not shown), the effect of hydraulic retention time on COD removal was investigated. Figure 6 indicates that COD removal was maximised at a hydraulic retention time of 15 h.

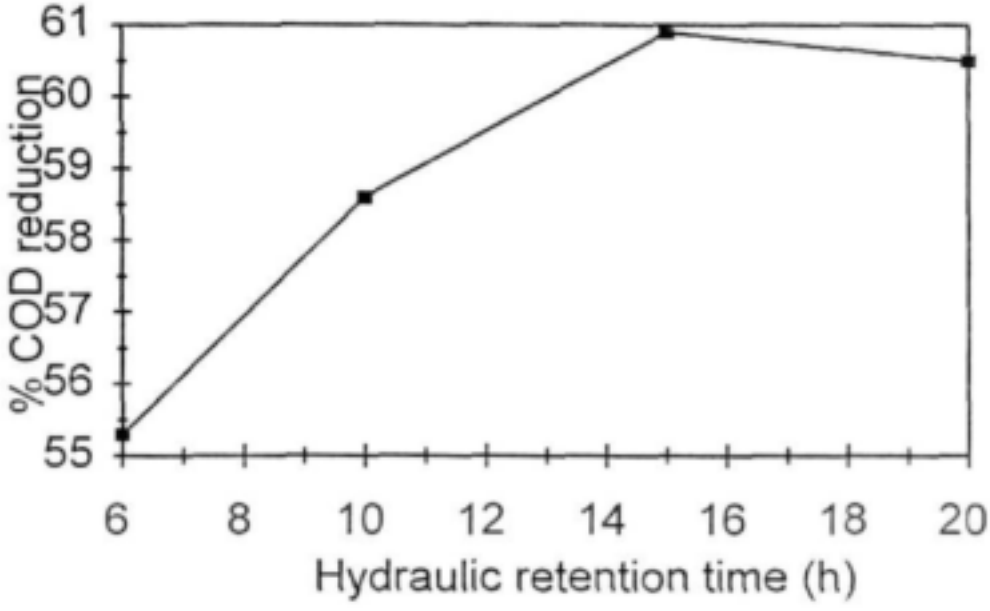


Fig. 6. Effect of hydraulic retention time on COD reduction in activated sludge reactor

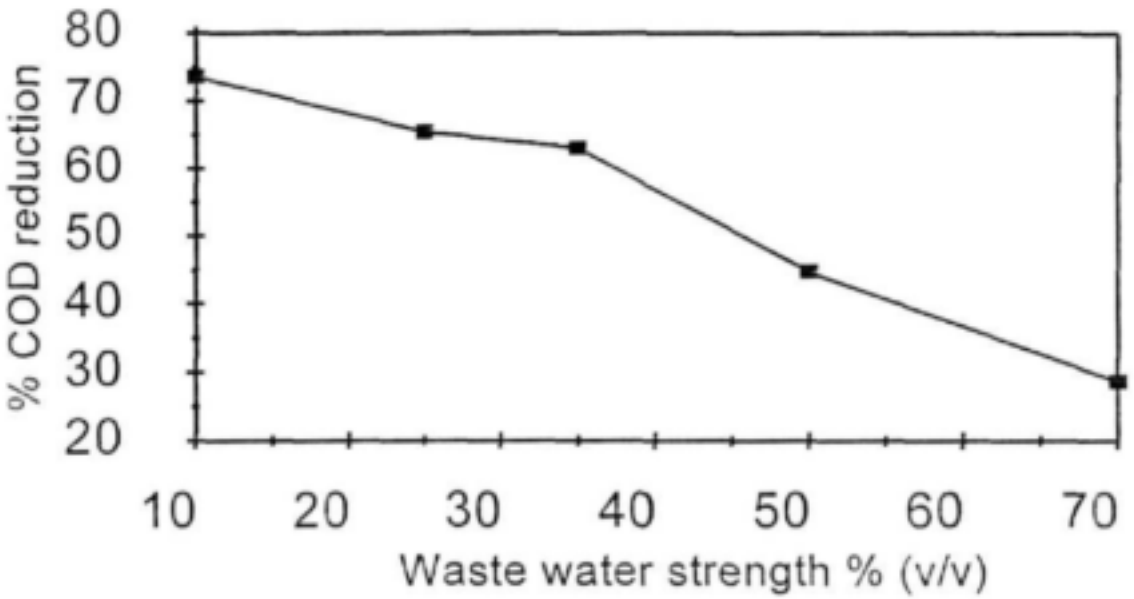


Fig. 7. Effect of waste water loading on COD reduction in activated sludge reactor

Higher retention times are usually required to treat waste waters from the pulp and paper Industry. Waste water was also fed to the reactor at increasing strengths, to test for the effect of COD loading on the system (Fig. 7). Table 6 indicates the improvements in waste water quality that was obtained following treatment in an activated sludge reactor.

Table 6. Improvements in waste water quality following treatment in activated sludge reactor

Parameter	Reduction (%)
AOX	20
COD	58
Colour	10
Bacterial growth inhibition[a]	14
2,4-dichlorophenol	66

a) see Materials and methods for detailed description of bacterial growth inhibition test

COD reduction decreased from 73.6% at an waste water strength of 10 % to 28.8 % at a loading level of 70 % (v/v). Modifications in molecular weight distribution following waste water treatment in activated sludge are given in Table 7. High molecular weight compounds were not degraded and there was minimal change in the colour of the treated waste water.

Table 7. Modification of molecular weight distribution following waste water treatment in activated sludge reactor

Compound reduction (%)		
High molecular weight (1000 g/mol)	**Low molecular weight (450 g/mol)**	**Colour reduction (%)**
0	100	10

2.4.2.3. Rotating biological contactor

Volume capacity of rotating biological contactor

Results indicate that the volume hold capacity in the RBC was 600 ml for *C. versicolor* and 650 ml for *R. pusillus* at the onset of decolourisation experiments conducted under optimized conditions. The biomass immobilized was 42.2 g per tube for *C. versicolor* and 38.9 g per tube in the case of *R. pusillus*. Biomass levels recorded during examination of the effect of colour intensity on decolourisation were 44 g per tube for *C. versicolor* and 68.5 g per tube for *R. pusillus*.

Effect of hydraulic retention time on decolourisation

Residence times varying from 0.67 to 23 h were tested. With *R. pusillus* colour removal leveled off at a residence time of 23 h whereas *C. versicolor* required a hydraulic retention time of 18 h to reach maximum decolourisation (data not shown). For comparative purposes, it was decided to conduct further investigations at a hydraulic retention time of 23 h for both fungi.

Influence of colour intensity

Colour intensities were tested in the range of 1550-10650 PCU. Colour levels did not effect decolourisation significantly except at the lower intensity values tested (Fig. 8). Similar results were reported by other workers (Mehna et al. 1995). The cause for this phenomenon is uncertain at present and needs further investigation. One reason could be that adsorption, the first step of colour removal, is significantly lower at reduced colour levels. Thus the interaction between chromophoric material and mycelia could be diminished affecting efficiency of biodegradation process. Initial colour intensities further influenced the decolourisation rate significantly. Decolourisation rates were also examined as a function of initial colour intensities (Fig. 9). Initial colour levels were directly proportional to decolourisation tempo expressed as change in colour/g.l.

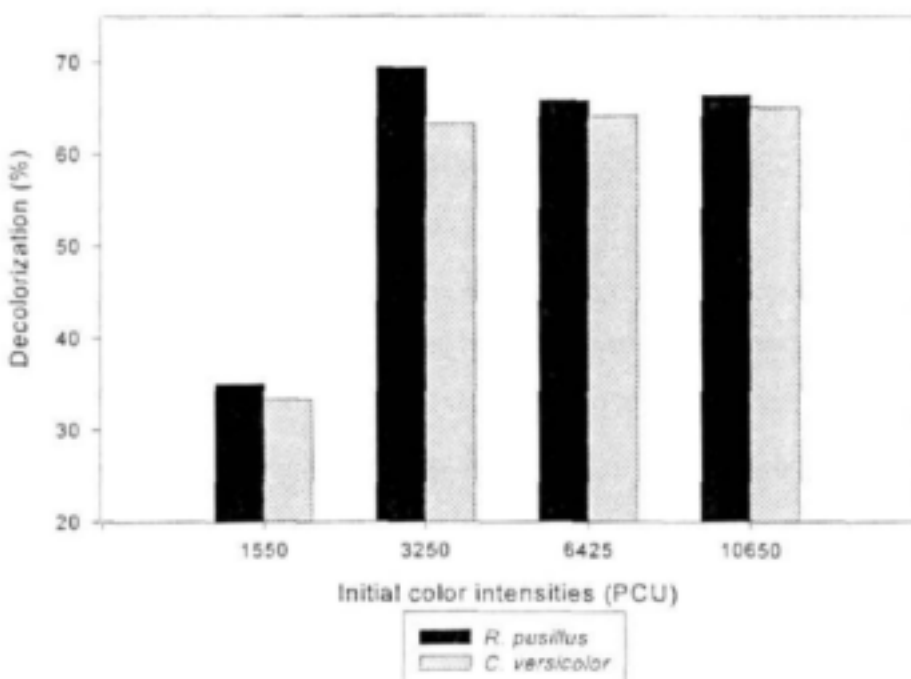


Fig. 8. Impact of initial colour intensities on decolourisation of bleach plant waste water using *Rhizomucor pusillus* and *Coriolus versicolor* immobilised in rotating biological contactor (hydraulic retention time was 24 h).

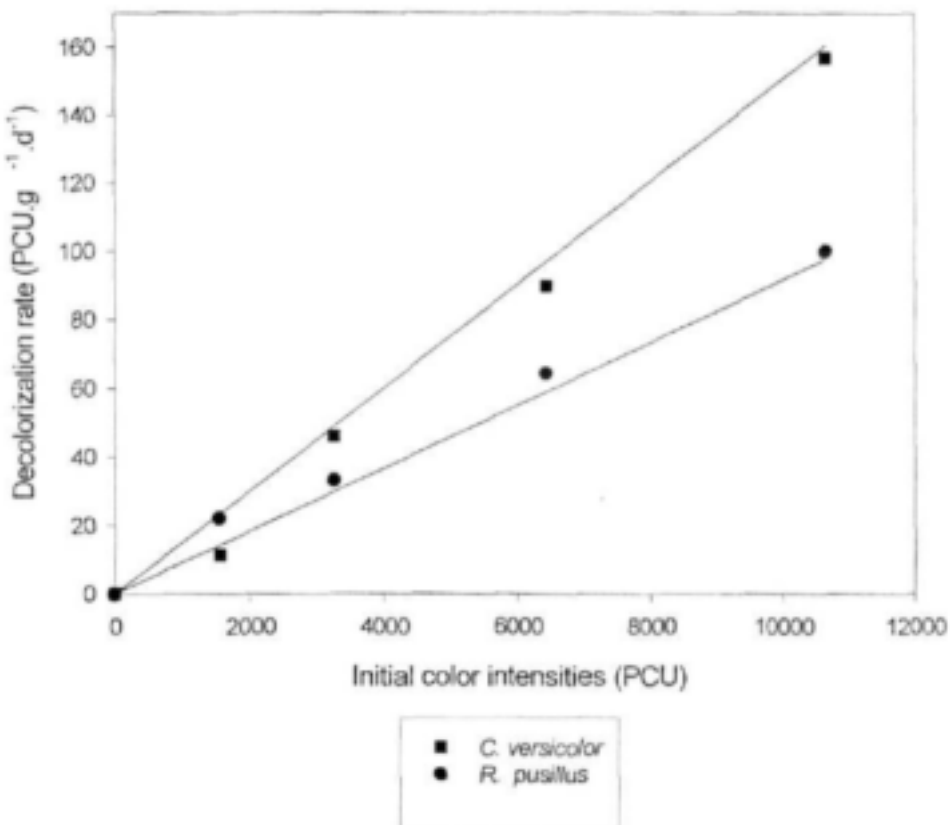


Fig. 9. Decolourisation rate versus initial colour intensities employing *Rhizomucor pusillus* and *Coriolus versicolor* (hydraulic retention time employed was 24 h).

Waste water bioremediation in rotating biological contactor

Decolourisation experiments were conducted using waste water diluted 1:1, at a hydraulic retention time of 23 h. Under these conditions, *R. pusillus* decolourised the waste water by an excess of 73% and *C. versicolor* removed more than 61% of the chromophores from the decolourisation media (Fig. 10). Royer et al. (1985) observed that the process of decolourisation of bleach plant waste water using *C. versicolor* consisted of two phases: adsorption (within 24 h) followed by oxidation (after 48 h). Based on literature and the

findings of this study, it seems that adsorption could be one of the first steps of decolourisation.

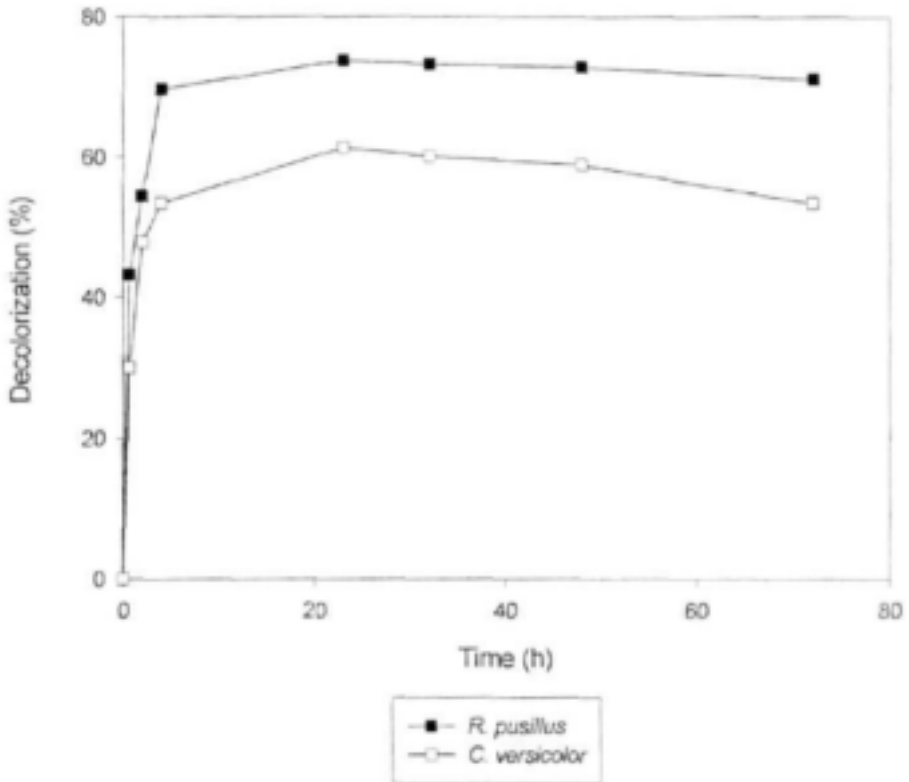


Fig. 10. Decolourisation against time profiles using *Rhizomucor pusillus* and *Coriolus versicolor* (hydraulic retention time used was 23 h at an waste water strength of 50%).

In the case of *C. versicolor*, there was a decrease in the decolourisation activity from 61 % at 23 h to about 53% after 72 h of treatment. On the other hand, *R. pussilus* also showed a slight decrease in colour removal activity from 73.7% (23 h) to 71% (72 h). Decolourisation with *R. pusillus* proceeded fast initially, reaching 43.2% after only 0.67 h, whereas colour removal by *C. versicolor* was 34% for the same time period. In Table 8, the percentage removal of COD and AOX as well as reduction in bacterial growth inhibition levels of waste water are indicated following fungal treatment.

Table 8. Improvements in quality of bleach plant waste water following fungal treatment

Parameter	Reduction (%)	
	Rhizomucor pusillus	***Coriolus versicolor***
AOX	55%	40%
COD	48%	64%
Colour	74%	61%
Bacterial growth inhibition[a]	69%	38%

a) see Materials and methods for detailed description of bacterial growth inhibition test

Overall, greater improvements in waste water quality were obtained using *R. pusillus* compared to *C. versicolor* during treatment with the exception of COD removal where *C. versicolor* exhibited superior efficiency over *R. pusillus*.

Decolourisation mechanism

Colour adsorbed on biomass was estimated after decolourisation by alkaline desorption of chromophoric material from cell mass with 1N NaOH. Table 9 compares decolourisation mechanisms of *C. versicolor* and *R. pusillus*.

Table 9. Mode of decolourisation of bleach plant waste water by *Rhizomucor pusillus* and *Coriolus versicolor* in rotating biological contactor

Decolourisation mode	***Rhizomucor pusillus***	***Coriolus versicolor***
Colour removed (%, total)	63	58
Colour adsorbed (%)	100	47
Colour biodegraded (%)	0	53

Colour removed during waste water treatment with *R. pusillus* could be recovered from fungal biomass by alkali desorption. However, only 47% of the colour removed by *C. versicolor* was released upon treatment of the biomass with NaOH. In shake flasks at a contact time of 5 min, *R. pusillus* removed 35 % of the colour, compared to 32 % by *C.*

versicolor. Almost all the colour removed could be desorbed by treatment with 1N NaOH from the biomass of *R. pusillus*. However, with *C. versicolor* about 13% of the adsorbed colour could not be removed by treatment with NaOH (data not shown). Results obtained here indicated that all colour adsorbed by *R. pusillus* during decolourisation was released following treatment with 1 N NaOH. Therefore, the decolourisation mode of *R. pusillus* appeared to be due to physical adsorption. However, with *C. versicolor* only part of the colour removal could be ascribed to adsorption (47%). Previously it was also shown that the decolouring activity of *R. pusillus* could be completely regenerated by 1N NaOH (Christov et al. 1999). In shake flask experiments, using short contact times of 5 min, the only mechanism of colour reductions should be ascribed to adsorption. It was demonstrated that under these conditions, both *R. pusillus* and *C. versicolor* adsorbed colour that could be quantitatively desorbed with NaOH. However, in the case of *C. versicolor*, a minor fraction of colour could not be desorbed by alkali treatment. These results also indicate that adsorption played a major role during the initial phase of fungal treatment of waste water.

Influence of various glucose concentration levels on decolourisation

Glucose additions to decolourisation media stimulated decolourisation by *C. versicolor*, from 36.7% (no glucose added) up to 57.1% (glucose supplemented at 10 g/l) (Table 10). However, with *R. pusillus* glucose additions did not enhance decolourisation and more than 64% of coloured compounds were removed even when no glucose was added to decolourisation media (data not shown). It is well known that an additional carbon source is required for lignin degradation by white-rot fungi (Nagarathnamma and Bajpai 1999). This has been verified in the current work because supplementation of glucose to decolourisation media enhanced decolourisation by *C. versicolor*. In contrast, no such stimulation in colour removal by *R. pusillus* was noted upon glucose additions to decolourisation media.

Table 10. Effect of glucose addition on decolourisation of bleach plant waste water by *Coriolus versicolor*

Glucose added (g/l)	Decolourisation (%)
0	36.7
2	37.8
4	43.2
6	51.1
8	58.3
10	57.1

Enzyme activities

Ligninolytic enzyme activities present during treatment of waste water in RBC were

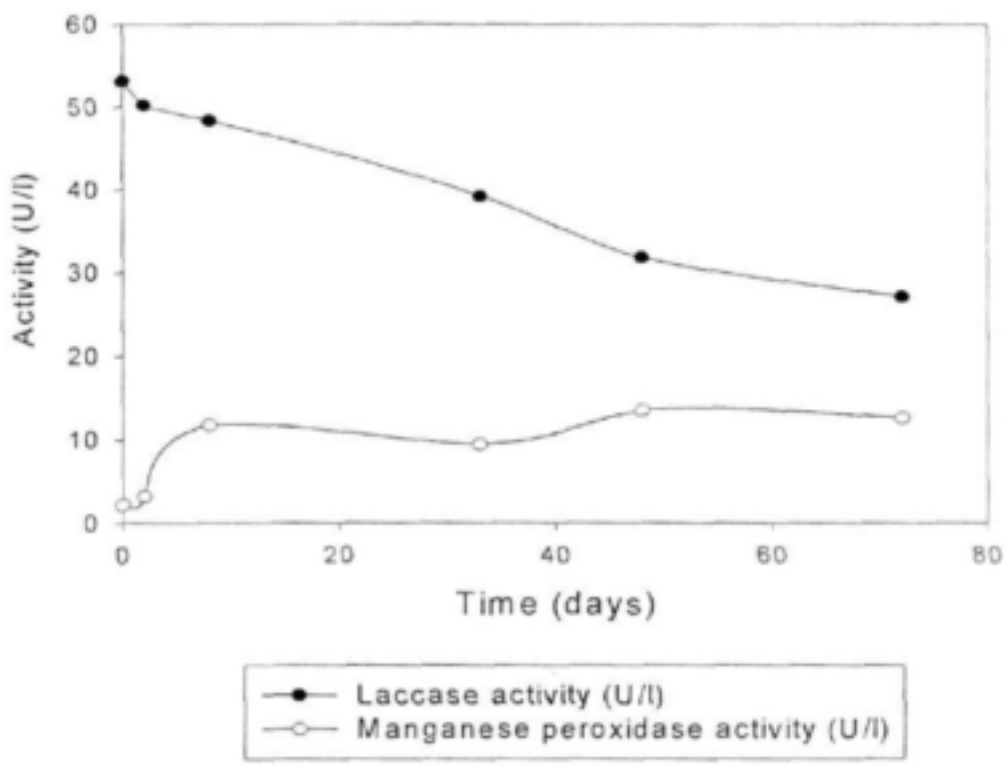


Fig. 11. Laccase and manganese peroxidase activity detected during cultivation of *Coriolus versicolor* in rotating biological contactor

determined (Fig.11). Ligninolytic activities (manganese peroxidase and laccase) were only detected in waste water treated by *C. versicolor*. Ligninolytic enzymes are associated with lignin and lignin derivative depolymerisation in white-rot fungi (Nagarathnamma and Bajpai 1999). The presence of these enzymes in *C. versicolor* treated waste water suggests that enzymatic action could be responsible for part of colour removal.

Chlorophenol removal

2,4-Dichlorophenol was identified in the waste water by GC analysis. Increases in peak area were apparent after spiking of 2,4-dichlorophenol (Table 11). The fate of this compound before and after treatment is shown in Table 12. *Coriolus versicolor* removed a higher percentage of 2,4-dichlorophenol in comparison to *R. pusillus*.

Table 11. Identification of chlorophenols in E_o-waste water

Retention time (min)	Peak area		Compound
	Before[a]	After[b]	
7.078[a] (7.047)[b]	562	913	2.4-dichlorophenol

[a]Before spiking
[b]After spiking

Table 12. Removal of chlorophenols identified in E_o-waste water following treatment in rotating biological contactor

Compound	Reduction of chlorophenols (%)	
	Rhizomucor pusillus	*Coriolus versicolor*
2,4-dichlorophenol	15.7	48.3

Bacterial growth inhibition test

Results on reduction of bacterial growth inhibition demonstrated in this work correlated well with the AOX removal from waste water: *R. pusillus* removed 55% AOX and also

showed the greatest reduction in bacterial growth inhibition (69%). Similarly, with *C. versicolor* a 40% reduction in AOX was accompanied by a 38% improvement in bacterial growth levels. It was demonstrated that chloroorganic removal from waste water was accompanied by toxicity reduction (Nagarathnamma and Bajpai 1999). Overall the RBC was superior to the activated sludge reactor in improving waste water quality.

2.5. Conclusions

1. Remediation of bleach plant waste water was studied using different biological treatment systems: activated sludge, trickling filters and rotating biological contactor (RBC). Activated sludge treatment removed COD efficiently (58%) and decreased bacterial growth inhibition (43%) and AOX (20%) in the waste water, however, only 10% of colour could be removed. On the other hand, waste water treatment in trickling filters demonstrated the potential of white-rot fungi for the decolourisation of bleach plant waste water (61%). However, the most efficient treatment system proved to be the RBC where colour, bacterial growth inhibition, AOX and COD were decreased by significant levels, i.e. up to 74%, 69%, 55% and 64%, respectively.
2. It was demonstrated that using a laboratory RBC reactor both *Coriolus versicolor*, a white-rot fungus, and *Rhizomucor pusillus* strain RM7, a mucoralean fungus were able to decolourise the waste water within three days of continuous treatment and could in principle be used for bioremediation of industrial waste waters.
3. It was shown that decolourisation of 53 to 73% could be attained using a hydraulic retention time of 23 h. The relatively high decolourisation levels (32-55% decolourisation) achieved at low hydraulic retention times (0.67-6 h) may be due to a rapid adsorption of colour by the biomass initially.
4. Adsoption seems to be one of the first steps in waste water decolourisation by fungi. All colour adsorbed by *R. pusillus* during waste water decolourisation was released from the fungal biomass following alkali treatment, thus, biomass could be completely regenerated and reused. Therefore, the decolourisation mode of *R. pusillus* appeared to be due to physical adsorption.
5. Cell wall fractions extracted from the biomass of *R. pusillus* (alkali-resistant, residual and chitosan fractions) appeared to be principally responsible for the decolouring of

waste water by this fungus. The residual and chitosan fractions, representing combined only approximately 12% (w/w) of the *R. pusillus* biomass, were able to removed more colour from the bleach plant waste water (about 41%) than the intact biomass (39%). The major compound(s) of the cell wall fractions could be either chitosan and/or chitin, based on the release of glucosamine upon acid hydrolysis of these fractions. The mechanism of colour removal by *R. pusillus* might involve ionic interactions between aminoglucans from the cell wall of this fungus and the negatively charged compounds found in the bleach plant waste water.

6. However, with *C. versicolor*, only part of the colour removed could be ascribed to adsorption (45%), suggesting the presence of two mechanisms of colour removal in this fungus: 1) bioadsorption and 2) biodegradation. The latter was also supported by changes in high molecular weight distribution profile of waste water as well as presence of lignolytic enzymes following treatment with *C. versicolor*.
7. Decolourisation by both fungi was directly proportional to initial colour intensities. Initial colour levels were directly proportional to decolourisation rate expressed as change in colour/g.d in both fungi. Although this phenomenon has been described using white-rot fungi, as far as known, this is the first report where it has been demonstrated to occur using a mucorelean fungus for waste water treatment.
8. The decrease in bacterial growth inhibition following fungal pretreatment with both *R. pusillus* and *C. versicolor* correlated well with the AOX removal from waste water: *R. pusillus* removed 55% AOX and also showed the greatest reduction of 69% in bacterial growth inhibition. Similarly, *C. versicolor* caused a 40% reduction in AOX accompanied by a 38% stimulation in bacterial growth. It also was demonstrated that chloroorganic removal from waste water was accompanied by stimulation in bacterial growth.
9. Additional carbon source is required for lignin degradation by white-rot fungi. This has been verified in this work because supplementation of glucose to decolourisation media enhanced decolourisation by *C. versicolor*. In contrast, no such stimulation in colour removal by *R. pusillus* was noted upon glucose additions to decolourisation media.

10. Manganese peroxidase and laccase activities were detected in the waste water treated by *C. versicolor*, however, no ligninolytic enzyme activities were found in waste water treated by *R. pusillus*. The presence of these enzymes in *C. vericolor* treated waste water suggested that enzymatic action could be at least partially responsible for of colour removal.
11. There are definite differences in the decolouring mechanisms between the white-rot fungus (adsorption + biodegradation) and the mucoralean fungus (adsorption). This aspect needs to be investigated in greater detail to verify the mechanism responsible for the decolourisation activity in both types of fungi. Long term studies are necessary to evaluate the suitability and potential of these fungi to treat pulp and paper mill waste waters on an industrial scale.

CHAPTER IV

WASTE WATER UTILISATION

1. Waste water utilisation for xylanase production

1.1. Abstract

The use of pulp mill waste waters as carbon feedstock for microbial xylanase production is relatively an unexplored field of research. In this study, the production of xylanases by selected strains of filamentous fungi using spent sulphite liquor (SSL) and bleach plant waste water (E_o) as carbon substrates was investigated. The main sugar in both these waste waters was D-xylose, with D-glucose and D-galactose each amounting to about 9% of the xylose concentration in the SSL. In contrast to the SSL, the carbon content of the E_o waste water was too low to permit its use as a carbon feedstock without using a concentration procedure. No xylanase activity was found using media based on the SSL, raw E_o and concentrated E_o waste waters as carbon feedstocks. Relatively high xylanase activities of up to 209 and 140 U/ml were obtained with *Aspergillus oryzae* NRRL 3485 and *Aspergillus phoenicis* ATCC 13157, respectively, in a medium based on SSL concentrate. By comparison, *A. foetidus* ATCC 14916 produced a xylanase activity of up to 320 U/ml in a medium containing xylan, which is known to be a natural inducer of xylanases. These results indicated that the SSL concentrate, constituted a suitable carbon feedstock for xylanase production by *Aspergillus* species. The xylanases of most of the strains exhibited a maximum activity at an assay pH of 6.0 and at 50°C, whereas the optimum temperature of the xylanase of *A. oryzae* NRRL 3485 was 70°C. Pretreatment of hardwood post-oxygen soda-aq pulp with the crude xylanase from *Aspergillus oryzae* NRRL 3485 grown on xylan and SSL concentrate, respectively, enhanced the brightness by 1.4 and 0.8 brightness points over the control in X-DED bleaching. Alternatively, savings of chlorine dioxide consumption of up to 30% were obtained while retaining brightness at the control level. These results indicated that the SSL concentrate was capable of inducing xylanase production by these fungi, which could be applied in biobleaching to improve the economics of the process and enhance pulp brightness and/or decrease the usage of chlorine dioxide in an environmentally friendly way.

1.2. Introduction

Xylanases are produced by a variety of microorganisms and catalyse the hydrolysis of the β-1,4-glycosidic bonds of xylan. The microbial production of these enzymes may be induced by a wide range of carbon sources including xylan, xylose and hemicellulose-rich substrates, depending on the microbial species. One of the most important biotechnological applications of xylanolytic enzymes in industry is their use in pulp bleaching. The residual lignin in wood fibres is physically and chemically restricted by the hemicellulose in the fibre matrix (Dancault et al. 1994). Internal glycosidic linkages of the heteroxylan backbone are cleaved by β-1,4-endoxylanase, resulting in a decreased degree of polymerisation of the substrate. Xylan degradation and removal would, therefore, be facilitated by the application of these endoxylanases in the bleaching process. This would enhance lignin oxidation and solubilisation by other bleaching chemicals. In this manner, the chemical requirements and waste water load from the bleaching plants would be significantly reduced (Sunna and Antranikian 1997).

Limited research has been conducted on the production of xylanases using pulp mill waste waters as carbon feedstock (Christov et al. 1999). There are, however, a number of factors that negatively affect the production of xylanase using pulp mill waste waters. These may include the presence of inhibitors, such as acetic acid and the degradation products of lignin, in these waste waters. Furthermore, while such waste waters may serve as carbon feedstock for microbial growth, it is unknown whether the waste water would have to be supplemented with an inducer such as xylan for xylanase production.

An investigation of several filamentous fungi for xylanase production using spent sulphite liquor (SSL) and bleaching plant waste water as respective carbon substrates was, therefore, initiated. The xylanases produced in these experiments were also evaluated in terms of their biobleaching efficacy when applied in the pre-treatment of pulp.

1.3. Materials and methods

1.3.1. Preparation of pulp mill waste waters

The waste waters investigated were the raw (unconcentrated) and concentrated SSL (concentrated at Sappi Saiccor, Umkomaas, Kwazulu Natal, RSA) derived from acid sulphite pulping of *Eucalyptus grandis* as well as the raw (unconcentrated) and concentrated E_o (bleach plant) waste waters. Two procedures for concentrating the E_o waste water were used. In one procedure 10 l of raw E_o was concentrated at 60°C to 100 ml using a rotary evaporator. The initial pH of the waste water was pH 8.4 and was not adjusted. The second procedure was similar to the first, except that the initial pH of the raw E_o waste water was adjusted to pH 3.1 prior to evaporation. Evaporation at low pH was aimed at liberating the bound acetic acid from the E_o waste water as acetic acid is known to inhibit microbial growth at high concentrations. Another reason for vacuum concentration was to enrich the waste water with sugars thereby increasing the extent of their efficient microbiological utilisation.

1.3.2. Analytical procedures

The sugar composition of the waste waters was determined using a HPAEC series 4000*i* HPLC (Dionex Corp., Sunnyvale, CA, USA) equipped with a pulse amperometric detector and gold electrode, using a 250 mm x 4 mm (I.D.) PA1 Carbopac column (Dionex Corp.) with inlet and oven temperatures of 30°C and 1.8 mM NaOH as eluent at a flow rate of 1 ml/min. Total reducing sugars were determined by the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952). Acetic acid was determined with a Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Atlanta, GA, USA) equipped with a flame ionisation detector and a 150 mm x 2 mm (I.D.) glass column packed with 80-100 mesh Porapak Q (Waters Associates, Milford, MA, USA), using inlet and oven temperatures of 180°C and a nitrogen carrier gas flow rate of 3 ml/min. The chemical oxygen demand (COD) was determined by a standard spectrophotometric method (HACH Co., 1997). Adsorbable organic halogen (AOX) analyses of E_o samples were performed at the Sappi Technology Centre (Springs, South Africa) using an Euroglas AOX EC S 1000 analyzer (Delft, Netherlands) according to the manufacturer's instructions. Prior to analysis, samples were diluted 100 to 500 times to obtain an AOX

concentration within the range 10 to 100 µg Cl/l. The chlorinated organic matter is burned in oxygen in a sealed environment thereby the chloride anions formed are determined by a microcoulometric titration (Odendahl et al. 1990). The total polyphenols in the waste waters were determined by the Persian blue assay (Graham, 1992). Minerals and PO_4^{3-} were determined by the Institute for Ground Water Studies, University of the Free State, Bloemfontein. Total and suspended solids were determined by drying aliquots of the waste water and its sediment after centrifugation at 10000 rpm for 10 min, respectively, to constant mass at 105°C. The ash content of the E_o and SSL waste waters was also gravimetrically determined after subjecting 20 ml aliquots in pre-weighed ceramic crucibles to 800°C for 12 h. The xylanase activity in the culture supernatants was determined using the 3,5-dinitrosalycylic acid (DNS) assay (Bailey *et al.*, 1992) at pH 6.0 and 50°C with oat spelts or birch wood xylan (Sigma Chemical Co, St Louis, MO, USA) as substrate.

Brightness sheets were prepared after the final bleaching step (D_2, see below) by washing the pulp with tap water and filtering through a 110 mm qualitative Whatman no. 4 filter (Whatman International Ltd, Maidstone, England) prior to drying the pulp discs at 50°C for 24 h. Subsequently the brightness was determined using a Colour Touch 2 brightness meter (Technidyne Corp., New Albany, Indiana, USA).

1.3.3. Evaluation of waste waters as carbon sources for xylanase production

The E_o and SSL waste waters were evaluated as potential carbon sources for fungal growth and the production of xylanases. *Aspergillus oryzae* NRRL 1808 was grown in different media based on the following waste waters:

a) raw SSL waste water
b) concentrated SSL waste water, diluted ten-fold with distilled water
c) concentrated SSL waste water, diluted ten-fold with raw E_o waste water
d) raw E_o waste water
e) concentrated E_o waste water, diluted five-fold with distilled water

Dilutions were selected as to adjust the sugar concentrations in the SSL ad E_o to the same initial level. To the above waste waters the following were added (g/l): citric acid, 0.25; yeast extract, 10; $(NH_4)_2SO_4$, 5; K_2HPO_4, 5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.02 and 1

ml of a trace elements solution (du Preez and van der Walt 1983). These media were adjusted to initial respective pH values of 5.0 and 6.0 prior to autoclaving. The pre-inoculum was prepared by inoculating 500 ml Erlenmeyer shake flasks containing 100 ml of the appropriate medium with 5 ml of a fungal spore suspension from a 5-day old Sabouraud-dextrose agar plate culture and incubated on a rotary shaker at 30°C for 48 h. Erlenmeyer shake flasks (500 ml) containing 100 ml of the appropriate medium were subsequently inoculated with 5 ml of the pre-inoculum and incubated on a rotary shaker at 30°C for 5 days.

1.3.4. Xylanase production using concentrated spent sulphite liquor

A total of eleven strains of filamentous fungi were evaluated for xylanase production using a medium based on the concentrated SSL waste water diluted ten-fold with distilled water and to which was added nutrients as above and adjusted to pH 6.0 prior to autoclaving. A procedure similar to that described above was used for pre-inoculum preparation and culture inoculation, but the final cultures were incubated for 3 or 5 days. The selected fungi used were *Aspergillus niger* ATCC 10864, ATCC 13497, NRRL 2270 and NRRL 3536, *Aspergillus foetidus* ATCC 14916, *Aspergillus phoenicis* ATCC 15555 and ATCC 13157, *Aspergillus oryzae* NRRL 1808, NRRL 3485 and IFO 5238, and *Gliocladium viride* CBS 658.70.

1.3.5. Xylanase production using a mineral salts medium with xylan

The mineral salts medium comprised 10 g oat spelts xylan/l (Sigma) as carbon source with other nutrients as above and was adjusted to pH 6.0 prior to autoclaving. A similar procedure to that described above was used for pre-inoculum preparation and culture inoculation with the same incubation conditions for 3 or 5 days.

1.3.6. Xylanase production using different carbon sources

Xylanase production by *Aspergillus niger* ATCC 10864 and NRRL 3536, *Aspergillus foetidus* ATCC 14916, *Aspergillus phoenicis* ATCC 15555, *Aspergillus oryzae* NRRL 1808 and *Gliocladium viride* CBS 658.70 on different carbon sources was evaluated. Digestive wheat bran (Snowflake, Newtown, South Africa) and D-xylose (Sigma) were used at a concentration of 10 g/l. Sugar cane molasses (courtesy of SMRI, Durban) was

diluted 33-fold with distilled water. All media were adjusted to pH 6.0 prior to autoclaving and the shake flasks were subsequently inoculated with the selected fungal strains and incubated for five days as described above.

1.3.7. Temperature and pH optima of xylanases

The pH and temperature optima of the xylanases produced by the selected filamentous fungi, grown in the waste water-based medium, were determined. The xylanase activity in the culture supernatants were assayed at 50°C at different pH values using 50 mM citrate buffer at pH values of 4.0, 5.0 and 6.0 and 50 mM sodium phosphate buffer at pH values of 7.0 and 8.0. The optimal temperature at pH 6.0 was determined by conducting the enzyme assay of the supernatants in a water bath at temperatures ranging from 40 to 90°C; the reaction was stopped after a 5 min incubation period.

1.3.8. Evaluation of xylanases in biobleaching of pulp

Hardwood oxygen-delignified soda-aq pulp was obtained from Sappi Enstra, Springs, South Africa. Crude xylanase preparations of *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 grown on the concentrated SSL waste water-based medium were applied to pulp. An enzyme charge of 10 U/g dry pulp equivalent was applied at pH 6.0 and 50°C for the xylanase of *A. phoenicis* ATCC 13157 and at pH 7.0 and 70°C in the case of *A. oryzae* NRRL 3485. A pulp consistency of 10% was maintained and the aliquots were incubated for 2 h.

The pulp samples that had been subjected to the above enzyme pretreatments were chemically bleached using the DED bleaching sequence involving two chlorine dioxide steps (D_1 and D_2) and an alkali extraction step (E). Subsequently, brightness sheets were prepared and three brightness determinations were conducted on each sample.

1.4. Results and discussion

1.4.1. Chemical analyses of waste waters

The chemical composition of the waste waters are presented in Tables 1, 2 and 3. The SSL concentrate (designated SSL_c) apparently represented a concentration factor of about

five-fold, judging from the increase in contents of total sugars and total solids (Table 1). Curiously, in the SSL_c the total polyphenol content was about ten-fold higher and the reducing sugars about 100-fold higher than in the SSL waste water. The latter observation suggested hydrolysis of oligomeric sugars during the concentration process. The acetic acid content was 20% higher in the concentrate, whereas in the case of the E_o waste water that had been subjected to evaporation at pH 8.4 the acetic acid content had increased over forty-fold (Table 2). However, by adjusting the E_o waste water to a pH value of 3.1 *prior* to the evaporation procedure, the acetic acid concentration of the E_o concentrate was reduced to 0.01 g/l due to loss of the volatile free acid.

Table 3 shows the sugar composition of the waste waters. The main sugar in all of these waste waters was D-xylose. In the SSL concentrate, the amounts of D-glucose and D-galactose were similar and each amounted to about 9% of the xylose concentration. It is also evident that the carbohydrate content of the E_o waste water (especially xylose content) was comparatively low to permit its use as a carbon feedstock for the cultivation of microorganisms, hence the concentration step was necessary to increase its sugar content. By contrast, the high sugar concentrations of the SSL and concentrated SSL waste waters rendered these waste waters attractive as a carbon feedstock.

Table 1. Chemical properties of waste waters

Property	Waste water	
	SSL	[1]SSL_c
Reducing sugars (g/l)	0.6	58.5
Total sugars (g/l)	29	145
Acetic acid (g/l)	10	12
Total polyphenols (g/l)	14	139
COD (mg/l)	2.0×10^5	1.1×10^6
Suspended solids g/l)	10	18
Total solids (g/l)	145	753
Ash (g/l)	12	41

[1] SSL waste water concentrated at Sappi Saiccor
Results are mean values of duplicate determinations

Table 2. Chemical properties of waste waters

Property	Waste waters		
	E_o	[1]E_{oc}	[2]E_{oc}
Reducing sugars (g/l)	0.04	8.2	nd
Total sugars (g/l)	1	70.5	nd
Acetic acid (g/l)	0.4	17	0.01
Total polyphenols (g/l)	0.1	1	nd
COD (mg/l)	1.0×10^4	2.0×10^5	nd
AOX (mg/l)*	11	707	nd
Suspended solids (g/l)	1	3	nd
Total solids (g/l)	11	191	nd
Ash (g/l)	4	690	nd

[1] Eo waste water concentrated at initial pH 8.4
[2] E_o waste water concentrated at initial pH 3.1
nd, not determined
Results are mean values of duplicate determinations

Table 3. Sugar content of waste waters as determined by high-performance liquid chromatography

Waste water	Constituents (g/l)				
	Arabinose	Galactose	Glucose	Xylose	Total sugars
E_o	0.02	0.02	0.02	0.91	0.97
E_{oc}	0.60	0	3.9	66.0	70.5
SSL	0.60	1.80	1.60	25.1	29.1
SSL_c	4.20	11.10	10.90	119.0	145.2

E_{oc} E_o waste water concentrated at pH 8.4
SSL_c concentrated SSL waste water
Results are mean values of duplicate determinations

1.4.2. Xylanase production on waste water and xylan media

Aspergillus oryzae NRRL 1808 produced no xylanase activity in the raw E_o waste water-based media at both pH 5.0 and 6.0 (Table 4). This could be attributed to the very low sugar concentration in the raw E_o waste water (Tables 2 and 3) and lack of an apparent inducer. Poor growth and an extremely low xylanase activity was detected in the medium with E_o concentrate (concentrated at pH 8.4) at pH 6.0 and no xylanase activity was detected in the unconcentrated SSL waste water at either pH 5.0 or pH 6.0 (Table 4).

144 U/ml at initial pH values of 5.0 and 6.0, respectively. These results indicated that, despite increasing the xylose concentration of the E_o waste water by concentrating the waste water (at pH 8.4), the concomitant increase in acetic acid concentration inhibited growth and xylanase production. However, the results obtained in the SSL concentrate suggested that the concentration of inhibitors may have been decreased during the concentration process, notably acetic acid, which permitted better growth and xylanase production. Xylanase production was also poor in an waste water-based medium where the SSL_c had been diluted with E_o waste water instead of water (Table 4).

Table 4. Xylanase activity (U/ml) produced by *Aspergillus oryzae* NRRL 1808 grown in media based on raw and concentrated waste waters

pH	Carbon substrate				
	E_o	E_{oc}	SSL	SSL_c	$SSL_c + E_o$
5.0	0	0	0	120	nd
6.0	0	0.1	0	144	22

E_{oc} E_o waste water concentrated at pH 8.4
SSL_c Concentrated SSL waste water, diluted 10-fold with distilled water
$SSL_c + E_o$ SSL_c diluted 10-fold with E_o waste water
nd not determined
Results are mean values of duplicate determinations

All of the strains tested produced xylanases in both the SSL concentrate and the xylan based media (Figs. 1 and 2). *A. foetidus* ATCC 14916 produced the highest xylanase activity of up to 320 U/ml on xylan (Fig. 2). Other fungal strains that produced relatively high xylanase activities on xylan were *A. oryzae* NRRL 3485 and 1808, *A. niger* ATCC 13497 and *A. phoenicis* ATCC 13157, which yielded activities of 115, 130, 127 and 109 U/ml, respectively (Fig. 2). It was noteworthy that *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 produced higher xylanase activities with the SSL concentrate as carbon substrate (209 and 140 U/ml, respectively; Figs. 1 and 2) than on xylan.

These results (Fig. 1) indicated that the SSL concentrate constituted a suitable carbon feedstock for xylanase production. Xylan is an expensive substrate, hence the production

of high xylanase activities using a medium based on the SSL concentrate without the addition of an inducer such as xylan is of significance.

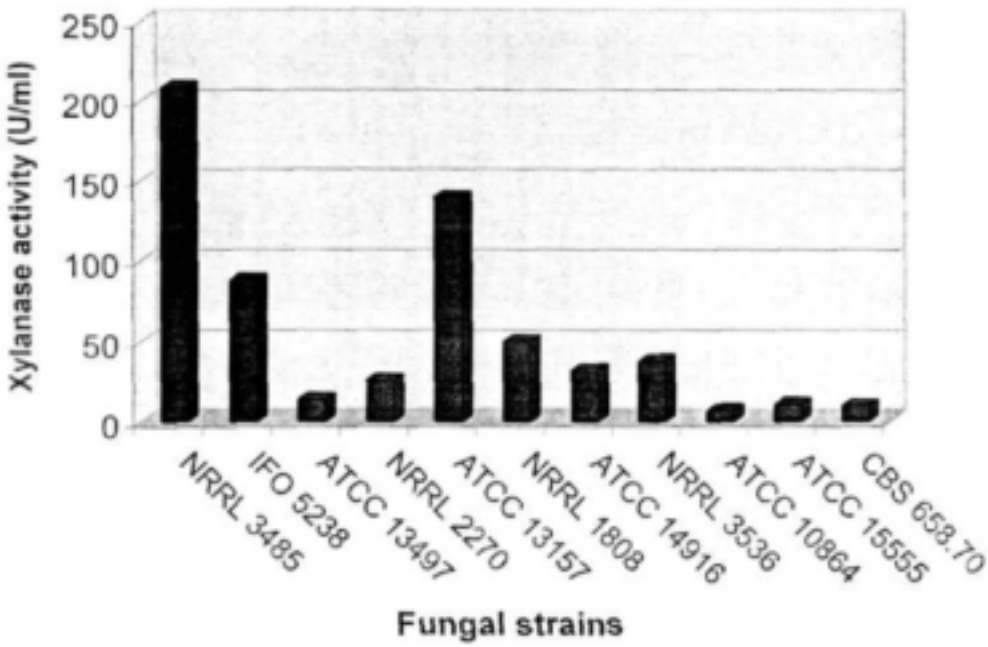


Figure 1. Xylanase activities produced by selected fungi using the SSL concentrate as carbon feedstock

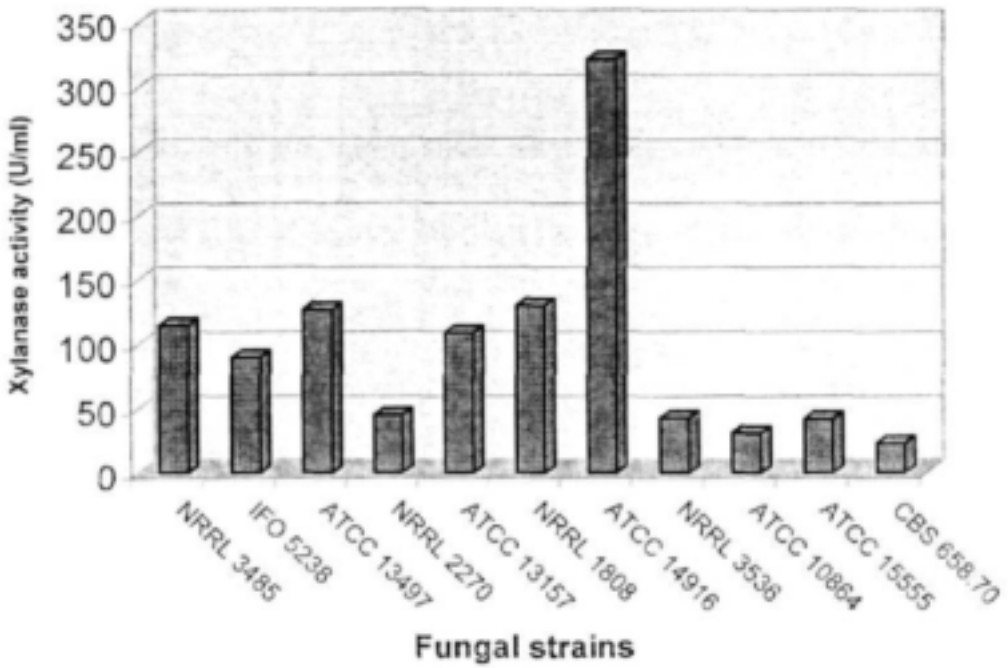


Figure 2. Xylanase activities produced by selected fungi on oat spelts xylan

1.4.3. Determination of the xylanase pH and temperature optima

The xylanases of most of the strains exhibited a maximum activity at an assay pH of 6.0 and at 50°C (Figs. 3 and 4), whereas the temperature optimum for the xylanase of *A. oryzae* NRRL 3485 was 70°C (Fig. 4). *Gliocladium viride* CBS 658.70 and *Aspergillus phoenicis* ATCC 15555 exhibited pH optima at pH 5.0, however, whereas *A. oryzae* NRRL 3485 and IFO 5238 and *A. niger* ATCC 13497 showed maximum xylanase activities at pH 7.0 (Fig. 3). The optimum pH ranges exhibited by these enzymes were typical of fungal xylanases.

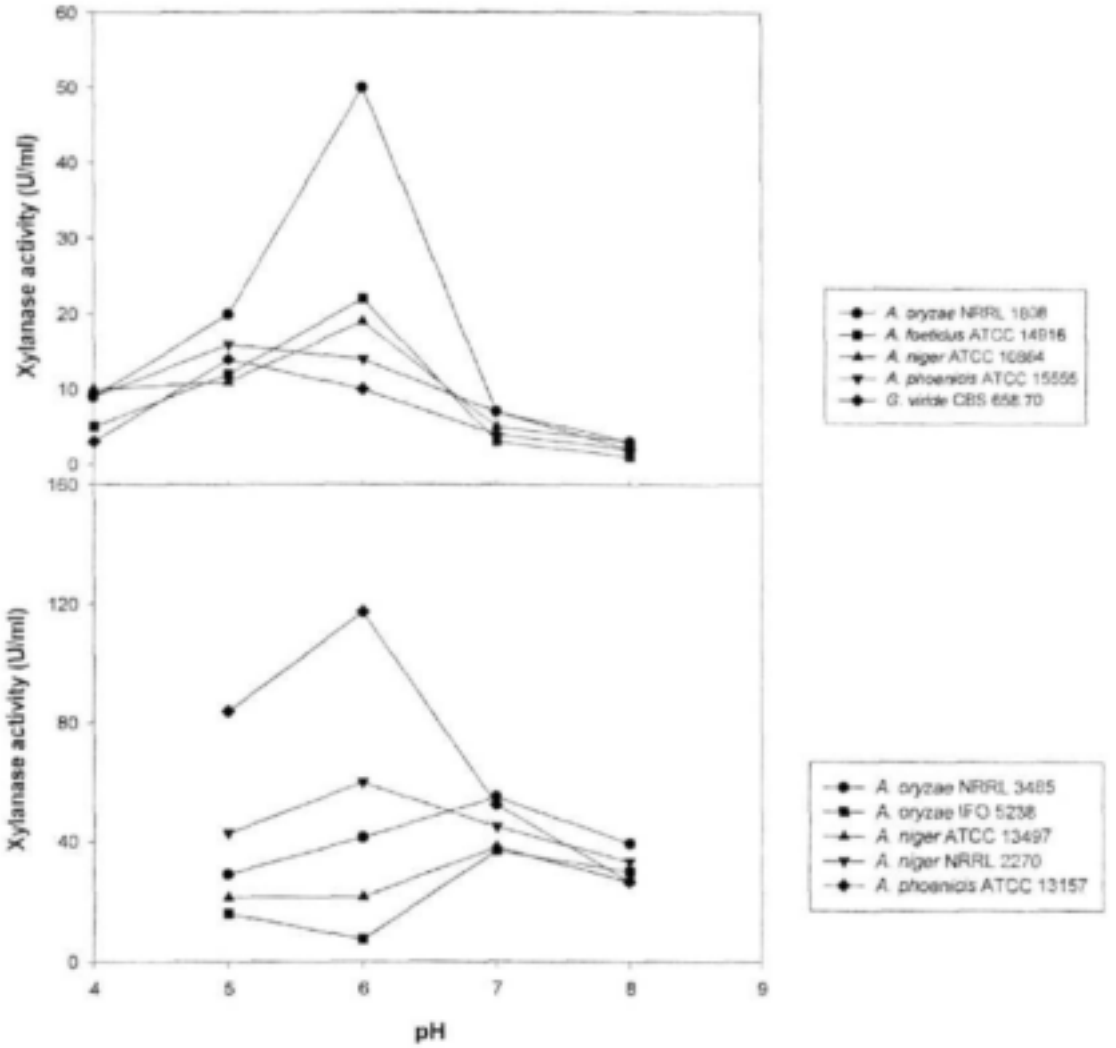


Figure 3. Xylanase activities of selected fungi determined at different pH values and at a constant temperature of 50°C

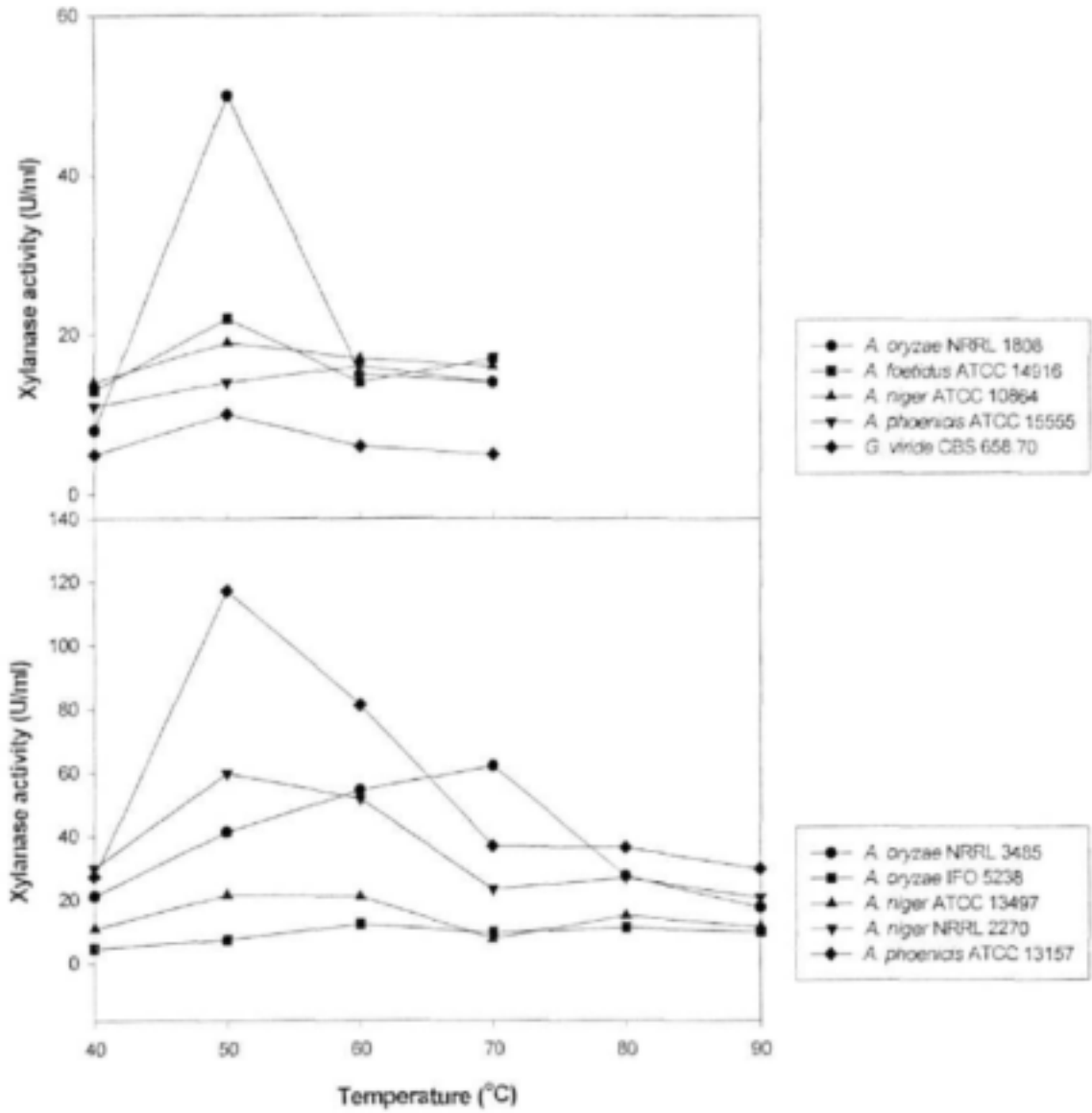


Figure 4. Xylanase activities of selected fungi at pH 6.0 determined at different temperatures

1.4.4. Xylanase production using various carbon sources

Different carbon sources were evaluated in terms of their capacity to induce xylanase production in the selected fungal strains. Xylanase activities obtained on the various carbon sources were generally quite low with the exception of *A. foetidus* ATCC 14916 which yielded a relatively high activity of 60 U/ml on wheat bran (Table 5). With these fungi, therefore, D-xylose failed to act as a significant inducer.

Table 5. Xylanase activities (U/ml) produced by selected fungi on different carbon sources

Fungal strain	Carbon substrate			
	Xylan	**Xylose**	**Molasses**	**Wheat bran**
A. oryzae NRRL 1808	130	4	7	6
A. foetidus ATCC 14916	322	9	10	60
A. niger NRRL 3536	43	8	31	17
A. niger ATCC 10864	31	3	0	11
A. phoenicis ATCC 15555	42	0	11	24
G. viride CBS 658.70	23	0	26	9

Results are mean values of triplicate determinations

1.4.5. Application of xylanases in biobleaching of pulp

Oxygen delignified soda-aq hardwood pulp was pretreated with crude xylanases from *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 grown on xylan and the SSL concentrate (Tables 6 and 7). Using the full chlorine dioxide charges, a brightness gain was achieved with all enzymes tested grown on both xylan and SSL based media. The crude xylanase of *A. oryzae* NRRL 3485 grown on xylan gave the highest gain of 1.4 brightness points over the control (Table 6). Even with a 30% reduction in the chlorine dioxide charge, this enzyme induced a gain of 0.4 brightness points. At the full chlorine dioxide charges, the xylanase of *A. oryzae* NRRL 3485, produced on SSL concentrate, yielded a gain of 0.8 brightness points over the control (Table 7). These results demonstrated that the SSL concentrate had potential to serve as carbon feedstock for the production of fungal xylanases suitable for biobleaching in the pulp and paper industry. The use of this inexpensive carbon substrate could facilitate the industrial production of

enzymes and improve the economics of the biobleaching technology. This in turn would result in a decreased consumption of chlorine based bleaching chemicals and also reduce the level of hazardous chlorinated organics in the mill waste waters.

Table 6. Effect of pre-treatment of post-oxygen soda-aq hardwood pulp with crude xylanases produced with xylan as carbon substrate on brightness and chlorine dioxide consumption during X-DED bleaching

Enzyme source	**Brightness gain over control (points)**		
	Reduction of ClO_2 (%)		
	0	**20**	**30**
Control	0	nd	nd
A. oryzae NRRL 3485	1.4	0.9	0.4
A. phoenicis ATCC 13157	0.1	-1.7	-1.9

Control, 85.1 %; nd, not determined
Results are mean values of duplicate determinations

Table 7. Effect of pre-treatment of post-oxygen soda-aq hardwood pulp with crude xylanases produced with xylan as carbon substrate on brightness and chlorine dioxide consumption during X-DED bleaching

Enzyme source	**Brightness gain over control (points)**		
	Reduction of ClO_2 (%)		
	0	20	30
Control	0	nd	nd
A. oryzae NRRL 3485	0.8	0.4	0.3
A. phoenicis ATCC 13157	0.4	-0.7	-1.5

Control, 85.1 %; nd, not determined
Results are mean values of duplicate determinations

1.5. Conclusions

1. High xylanase activities of up to 320 U/ml were obtained with *Aspergillus foetidus* ATCC 14916 using xylan as carbon source. *A. oryzae* NRRL 3485 and 1808, *A. niger* ATCC 13497 and *A. phoenicis* 13157 also yielded relatively high xylanase activities of up to 115, 130, 127 and 109 U/ml, respectively.
2. Relatively low xylanase activities were obtained using other carbon substrates, namely D-xylose, molasses and wheat bran. The pH and temperature optima of the xylanases produced by the selected fungi were typical of fungal xylanases.
3. High xylanase activities were also obtained using the SSL concentrate as carbon source, with *A.* oryzae NRRL 3485 and *A. phoenicis* ATCC 13157 yielding activities of up to 209 and 140 U/ml, respectively. These results indicated the potential of the L waste water to serve as carbon feedstock for microbial growth and enzyme production. Further research is required to characterise the growth and enzyme production of the most promising strains in order to enhance xylanase production.
4. The application of the crude xylanase preparations in the pre-treatment of the post-oxygen soda-aq pulp at relatively low enzyme dose of 10 U/g resulted in a brightness increase of 1.4 points over the control using full chlorine dioxide charges in a X-DED bleaching. Alternatively, biobleaching could reduce the chlorine dioxide consumption by up to 30% while retaining brightness at the control level.
5. Further work is required to verify and improve the current biobleaching results. Attention should be paid to minimising or eliminating the negative impact the SSL colour introduced during biobleaching on pulp brightness.

2. Tall oil utilisation for gamma-linolenic acid production

2.1. Abstract

When *Mucor circinelloides f. circinelloides* CBS 108.16 was cultivated on 40 g/l tall oil as sole carbon source, this fungus produced only 2.0 g/l biomass containing 7.5 % (m/m) crude oil while 31% (w/w) of the tall oil was utilised after 7 days of growth. Here, only trace amounts of γ-linolenic acid (GLA) were detected as part of the crude oil extract. When acetate was added to this medium, the fungus produced 6.0 g/l biomass containing 43.3% (w/w) crude oil. Here, 68 % (m/m) of the initial 30 g/l tall oil was utilised while 5.2% (w/w) GLA was produced in the crude oil extract. The highest GLA content of 5.7% (w/w) was found in the crude oil of another strain of *Mucor circinelloides f. circinelloides* when cultivated on tall oil. Although up to 19.1 g/l biomass and 40% (w/w) crude oil was accumulated when nine yeasts were grown on tall oil in the presence and absence of acetate, no high value lipids could be detected. This work is of interest since GLA is a high value essential fatty acid produced from plants with the potential of being replaced with single cell oils from fungi.

2.2. Introduction

Recently we described a substantially improved utilisation of sunflower oil by *Mucor circinelloides f. circinelloides* CBS 108.16 in the presence of sodium acetate which was accompanied by a doubling of biomass production and an enhancement of the intracellular polyunsaturated γ-linolenic acid (GLA) content compared to growth conditions with sunflower oil as sole carbon source (Jeffery et al. 1997). A biotechnological process utilising acetate as carbon source has also recently been patented for the production of GLA by *Mucor circinelloides f. circinelloides*. GLA is a high value essential fatty acid produced from plants with the potential of being replaced with single cell oils from fungi (Kock and Botha 1995).

Because of the current interest in the production of polyunsaturated fatty acids (PUFAs) by fungi, we investigated the effect of sodium acetate on changes in the extracellular and intracellular lipid composition during growth on tall oil. It also became the aim of this study to screen other fungal and yeasts capable of

biotransforming tall oil to high essential lipids such as GLA in the presence of acetate. Tall oil was extracted from a kraft mill spent liquor generated during industrial chemical processing of wood to paper.

2.3. Materials and methods

2.3.1. Growth and harvesting

The fungi were transferred from 4 day old YM medium (yeast malt-agar medium; incubated at 25°C) into 10 ml sterile medium present in 100 ml conical flasks. This medium consisted of the following in (g/l): tall oil, 30; sodium acetate, 10; yeast extract, 0.1; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 10.0; $CaCl_2.2H_2O$, 0.05; NH_4Cl, 1.28 and trace elements as described elsewhere (Du Preez and Van der Walt 1983). The pH was adjusted to 5.8 with 2N HCl. These flasks were incubated at 30°C for 7 days while shaking at 160r/min. The cells were then harvested by filtration (Whatman no. 1 filters) washed extensively with distilled water and chloroform (Kendrik and Ratledge 1996), immediately frozen, freeze-dried and weighed. As control the same medium as above was used with the exception that the tall oil was increased to 40 g/l and the sodium acetate omitted. The pH of the medium was determined before harvesting commenced.

2.3.2. Extraction of lipids

These were performed according to the methods described by Kock et al. (1997). Briefly, extracellular lipids, present in the supernatant (pH<4) were immediately extracted after harvesting with hexane until no lipids could be detected. The hexane mixture (hexane and oil) was evaporated until all the hexane was removed. Intracellular lipids were extracted from the freeze-dried cells using chloroform/methanol (2:1; v/v) (Folch et al. 1957). The extracellular and intracellular lipids were dissolved in diethyl ether and transferred to pre-weight vials and dried to a constant weight in a vacuum oven at 50°C over P_2O_5.

2.3.3. Fatty acids analysis

Trans-esterification of extracellular and intracellular lipids was performed by the addition of trimethyl sulphonium hydroxide (TMSH) followed by gas chromatographic analysis (Kock *et al.* 1997).

2.4. Results and discussion

According to Table 1, *Mucor circinelloides f. circinelloides* CBS 108.16 produced higher amounts of biomass, 6.0 g/l containing 43.3% of cellular lipids in the presence of acetate, compared to 2.0 g/l of biomass containing 7.5% cellular lipids in the absence of acetate. During growth of this fungus on tall oil in the presence of acetate, an increase in pH to 7.1 was experienced which was responsible for rapid emulsification of fatty acids. This caused an enhanced lipid utilisation i.e. up to 68%, since the free fatty acids (FFAs) were in the soap form at this pH and rendered the lipids accessible for utilisation.

In the absence of acetate, only 31% (w/w) of the tall oil was utilised within 7 days of growth. In the presence of acetate, 5.2% (m/m) GLA was produced in the crude oil lipids, compared to trace amounts of GLA produced in the absence of acetate. Another strain of *Mucor circinelloides f. circinelloides* i.e. SAS 045 produced, on the other hand, higher amounts of GLA in the absence of acetate, i.e. 5.7% (w/w) compared to 3.7% (w/w) in the presence of acetate. Other fungi such as *Cunninghamella* Cu 001 produced only 1% (w/w) GLA in the absence of acetate, but when acetate was added in the medium no GLA was detected. Although significant growth was experienced with the other Zygomycotan fungi on tall oil, in the presence or absence of acetate, no general pattern could be observed. No GLA could be detected in these fungi.

According to Table 2, all the yeasts tested in this study could grow and produce biomass on tall oil in the presence or absence of acetate and produce cellular lipids. No GLA could be produced by any of the yeasts in the presence or absence of acetate.

2.5. Conclusions

With this as background, the following conclusions can be drawn:

1. Tall oil which is a by-product derived from kraft pulp and paper mill waste waters could be utilised by selected fungi for production of high-value fatty acids such as gamma-linolenic acid (GLA).
2. In the presence of acetate, *Mucor circinelloides f. circinelloides* CBS 108.16 produced significantly more biomass (6 g/l) containing high concentrations crude oil (43.3%) compared to when only tall oil was used as sole carbon substrate (biomass of 2.0 g/l and lipid content of 7.5%).
3. The presence of acetate caused enhanced tall oil utilisation leading to production of high concentrations of GLA (5.2%) by *Mucor circinelloides f. circinelloides* CBS 108.16.
4. Enhanced tall oil utilisation and high concentrations of GLA were obtained also in the crude cellular lipids in *Mucor circinelloides f. circinelloides* CBS SAS 045 both in presence (3.7% GLA) and absence (5.7% GLA) of acetate.
5. No general pattern was observed concerning the influence of acetate on growth and production outputs.
6. No GLA could be detected in any growth experiments with yeast.

Table 1: Growth yield, tall oil accumulation, utilisation, pH and gamma-linolenic acid (GLA) production by fungi grown on tall oil in the presence and absence of acetate

Organism	+ACETATE					- ACETATE				
	Cell yield (g dry wt/l)	Cellular lipid content (% w/w dry wt)	Residual lipids (g/l)	pH	%GLA (w/w)	Cell yield (g dry wt/l)	Cellular lipid content (% w/w dry wt)	Residual lipids (g/l)	pH	% GLA (w/w)
Absidia 200	5.0	13.5	28.0	6.2	0.0	4.0	17.4	27.3	5.7	0.0
A. elegans SAS 218	2.5	45.2	15.8	7.3	0.0	1.0	26.0	24.6	4.2	0.0
Cunninghamella Cu 001	7.0	22.1	12.8	7.5	0.0	8.5	67.9	7.8	3.1	1.0
Gongronella Go 1	7.0	22.1	12.8	7.5	0.0	5.5	13.5	20.2	4.5	0.0
Mucor circinelloides CBS 108. 16	6.0	43.3	9.6	7.1	5.2	2.0	7.5	27.6	4.1	tr
M. circinelloides SAS 045	4.5	37.9	13.6	7.1	3.7	2.0	18.0	26.4	4.1	5.7
Rhizomucor pussillus RM 001	2.0	26.0	25.0	6.6	0.0	5.0	45.0	17.0	3.6	0.0
R. stolonifer R 008	9.5	4.5	25.5	6.9	0.0	1.0	2.0	27.4	5.9	0.0
Thamnostylum SAS 025	7.5	64.5	15.5	7.0	0.0	3.0	38.7	19.7	5.1	0.0

Values represent means of at least two repetitions. Standard deviation was <10% of the mean in all cases. tr = Trace amounts

Table 2: Growth yield, tall oil accumulation, utilisation, pH and gamma-linolenic acid (GLA) production by yeasts grown on tall oil in the presence and absence of acetate

Organism	+ACETATE					- ACETATE				
	Cell yield (g dry wt/l)	Cellular lipid content (% w/w dry wt)	Residual lipids (g/l)	pH	% GLA (w/w)	Cell yield (g dry wt/l)	Cellular lipid content (% w/w dry wt)	Residual lipids (g/l)	pH	% GLA (w/w)
Cryptococcus curvatus **CBS 0570 T**	4.7	14.4	19.8	7.7	0.0	3.8	5.5	21.6	3.4	0.0
Dipodascopsis uninucleata **CBS 0190. 37 T**	4.5	16.3	22.3	7.8	0.0	4.9	9.0	20.9	5.3	0.0
Filobasidiella neoformans **CBS 0132**	5.6	44.6	17.3	7.5	0.0	7.7	18.9	22.2	5.6	0.0
Galactomyces geotrichum **CBS 0772. 71 T**	7.4	12.2	16.3	7.4	0.0	7.1	12.4	19.4	3.9	0.0
Kluyveromyces marxianus **CBS 1556**	2.0	27.6	25.0	7.3	0.0	0.9	24.4	24.4	6.0	0.0
Saccharomyces cerevisiae **CBS 1171 NT**	1.0	13.0	24.6	7.4	0.0	1.6	17.6	25.7	5.9	0.0
Schizosaccharomyces pombe **CBS 0356 T**	0.6	5.5	26.7	6.5	0.0	0.8	7.7	27.3	6.5	0.0
Schwanniomyces occidentalis **CBS 2863**	19.1	39.6	5.8	7.5	0.0	3.9	31.5	18.9	3.9	0.0
Yarrowia lipolytica **CBS 0599**	8.1	17.9	13.8	7.1	0.0	16.7	30.7	7.5	4.1	0.0

Values represent means of at least two repetitions. Standard deviation was <10% of the mean in all cases. tr = Trace amounts

CHAPTER V

GENERAL CONCLUSIONS AND RECOMMENDATIONS

- The use of xylanases in bleaching as a means of rendering the waste waters more environmentally friendly has been demonstrated. Biobleaching can reduce the amount of chlorine-containing chemicals used in bleaching. This impacts directly on the levels of chlorides and adsorbable organic halogen of the waste waters. The implementation of the enzyme bleaching technology in the pulp and paper industry could improve the existing technology of pulp and paper manufacture in a cost-effective and environmentally friendly way.

- The possibility of waste water bioremediation prior to waste water discharge has been demonstrated. The use of continuous biosystems such as trickling filters, rotating biological contactors and activated sludge systems have been shown to reduce the environmental impact of these waste waters. Activated sludge treatment efficiently removed chemical oxygen demand (58%) and decreased bacterial growth inhibition (43%) and adsorbable organic halogen (20%) in the waste water, however, only 10% of colour could be removed. Waste water treatment in trickling filters demonstrated the potential of white-rot fungi for the decolourisation of bleach plant waste water (61%). However, the most efficient treatment system proved to be the rotating biological contactor where colour, bacterial growth inhibition levels, adsorbable organic halogen and chemical oxygen demand were decreased by up to 74%, 69%, 55% and 64%, respectively.

- Definite differences in the decolouring mechanisms between the white-rot fungi (adsorption + biodegradation) and the mucoralean fungi (adsorption) have been observed. This aspect needs to be investigated in greater detail to verify the mode responsible for the decolourisation activity in both types of fungi. Long term studies are necessary to evaluate the suitability and potential of these fungi to treat pulp and

paper mill waste waters on an industrial scale. Techno-economical evaluation has to be made before further research in this direction can be planned.

- The potential of pulp and paper mill waste waters and by-products derived from waste waters for biotechnical utilisation has been demonstrated. The bioutilisation of these waste waters would alleviate problems associated with their discharge. The potential end-products of the microbial fermentation of these waste waters could be: enzymes (xylanases); single cell protein; high value fatty acids (gamma-linolenic acid)

- Results indicated that the spent sulphite liquor certainly has potential as a carbon feedstock and inducer for xylanase production and could improve the overall economics of the biobleaching technology. Further bioreactor studies and optimization experiments are needed to establish the economical feasibility of the process.

- It has been demonstrated that tall oil, which is a by-product derived from kraft pulp and paper mill waste waters, could be utilised by selected fungi for production of high-value fatty acids such as gamma-linolenic acid.

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