Bio-remediation and Bio-utilization of Pulping and Bleaching Waste Waters

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 and chloride discharges by the substitution of chlorine-containing chemicals with other more environmentally friendly bleaching agents such as hydrogen peroxide, oxygen and ozone. In response to the environmental concerns and stringent emission standards, modifications of the production process at the pulping and bleaching stages have been developed that can reduce the levels of absorbable organic halogen and toxic effect of the pulping and bleaching waste waters. The bio-utilization of industrial waste waters in production of high-value products such as enzymes and the use of enzymes in bio-bleaching to reduce the chemical consumption of chlorine-based bleaching agents present new environmentally sound technologies that can significantly minimize the environmental impact of the pulp and paper industry.

OBJECTIVES

- Development of a bioremediation and biobleaching technology to minimize the use of hazardous chlorine-based bleaching chemicals which would produce certain economical benefits as well as reduce the environmental impact of the pulp and paper industry.
- Development of a microbial fermentation technology of pulping waste waters to obtain high-value products such as enzymes to be utilized in a biobleaching process for environmental clean-up and upgrading the quality of pulp and paper products.

RESEARCH APPROACH

Remediation of industrial waste waters from the pulp and paper industry was investigated using bleaching with enzymes, biomimetic systems (polyoxometalates) and microbial fermentation processes. The waste waters under study were derived from the pulping and bleaching stages of pulp production. Two industrial pulp types were examined for their bleachability with enzymes: hardwood soda-aq pulp and bagasse soda-aq pulp. Following enzymatic 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 (APHA, American Public Health Association, Washington, DC, USA). Fermentation experiments for enzyme production were carried out in shake flasks and bioreactors in batch and fed-batch cultures. Evaluation of cultivation conditions was based on the levels of xylanase activity produced. The efficiency of various approaches of waste water bioremediation was evaluated mainly based on the impact on chemical load, toxic effect, chloride content and chlorinated organic matter.

RESEARCH

Polyoxometalate-based waste water bioremediation

- Polyoxometalate (POM) pretreatment of hardwood soda-aq pulp enabled chlorine dioxide savings of 10% compared to alkaline oxygen control and 50% compared to the acid oxygen control while retaining brightness at the control level.
- POM pretreatment of bagasse soda pulp could replace the entire chlorine bleaching step without loss in final pulp quality. In addition, up to 50% of the hypochlorite charge could be reduced without deterioration of bagasse pulp properties.
- The use of POM in pulp bleaching represents an alternative bleaching technology that can offer a significant reduction of the consumption of chlorine-containing bleach chemicals with reduced environmental impact. However, further studies are required to demonstrate the reusability and economical viability and determine the environmental impact of this technology.

Xylanase-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.
- Biobleaching of bagasse pulp with xylanase (X) afforded reductions in sodium hypochlorite (32%) and sodium hydroxide (20%). Similarly, reductions of chlorine

dioxide (30%) and sodium hydroxide (20%) were possible during biobleaching of soda-aq pulp.

- Overall improvements in the bleach filtrate quality could be achieved when xylanase treatment was employed prior to bleaching. The major impact of the reduction of the active chlorine charges during biobleaching was on the chloride, adsorbable organic halides (AOX) and toxic effect levels (as determined by the bacterial growth inhibition test) of the bleach waste waters. Overall, the AOX content correlated well with the bacterial growth inhibition caused by the bleach filtrates. It was evident that the use of chlorine containing chemicals during bleaching was the major cause of bacterial growth inhibition in the resulting waste waters. In addition, the reduction of the amount of chlorinated compounds would minimize also their teratogenic effect.
- Introduction of a washing step between X and D_1 stages during soda-aq bleaching might prevent problems such as the overloading of the plant waste water treatment system. The wash waters from the xylanase stage might be treated separately or even utilised for biotechnological applications. Alternatively, the xylanase-stage chemical oxygen demand (COD) can be sent to the recovery system of the mill where the organic matter will be utilized for heat generation and use in the mill.
- The reductions in the use of bleaching chemicals would not only minimize the impact of the pulp and paper waste waters on the environment, but also lead to cost savings for the industry. For instance, savings of 5.25 kg of ClO_2/t pulp and 1.4 kg NaOH/t pulp could be obtained as result of the enzyme bleaching. This can be translated into cost savings in excess of R2 million per annum.

Bio-utilisation of waste water for xylanase production with the fungi *Aspergillus oryzae* and *Aspergillus phoenicis*

- A comparison of xylanase activities obtained with *Aspergillus oryzae* NRRL 3485 showed that higher xylanase activities of up to 200 U/ml were obtained in batch cultures than in shake flasks (30 U/ml) cultures using the concentrated spent sulfite liquor (SSLc) as carbon feedstock at 20-fold dilution.

- The fed-batch cultivation of *A. oryzae* NRRL 3485 using SSLc as carbon substrate yielded activities of up to 200 U/ml, which was two-fold higher than activities obtained in batch cultures under similar conditions (40-fold dilution).
- The chemical composition of the SSL waste waters revealed that concentrating the waste water reduced the amount of acetic acid, thereby minimising the inhibitory effect the acid may have on microbial growth during utilisation of SSLc as carbon feedstock.
- The considerably high biomass concentrations obtained with SSLc as carbon feedstock could be attributed to the utilisation of additional carbon present in the SSLc, as is indicated by the total organic carbon (TOC) results.
- Xylanase production using SSLc as carbon feedstock indicated that the SSLc acted as inducer. Being a cheap carbon substrate, xylanase production costs would be greatly reduced and possibly enhance the economic viability of the biobleaching technology.
- Xylanase production using SSLc was favoured by a high culture pH of 7.5. The agitation rates between 400 to 800 rpm did not have any adverse effect on xylanase production. A better understanding of the limiting factor would enable even higher activities being achieved.
- The xylanases from both *A. oryzae* NRRL and *A. phoenicis* ATCC 13157 exhibited multiplicity. The *A. oryzae* xylanase showed unusual pH and temperature optima not generally exhibited by fungal xylanases. A higher level of pH stability was shown by the xylanase preparations from this fungus.
- The application of SSL-grown fungal xylanases in biobleaching resulted in a reduction in the use of active chlorine of up to 30 %. The xylanases increased the pulp brightness by up to 1.5 brightness points over the control. The application of these xylanase enzymes, produced with SSLc as carbon feedstock is highly significant.
- Xylanase production using SSLc as carbon feedstock indicated that the SSLc acted as carbon source and enzyme inducer. Overall, the xylanase yields in shake flasks obtained with both *A. oryzae* and *A. phoenicis* on SSL-based growth media were about 2-fold higher than those produced on xylan-based media. Furthermore, the xylanase activities induced in batch cultures on SSL and xylan were comparable.

- Since the carbon source in general constitutes 30-50% of the total enzyme production costs, the eventual replacement of xylan with SSL as inexpensive and abundant carbon substrate would result in significant cost reductions associated with xylanase production and enhance the economic viability of the biobleaching technology.

CONCLUSIONS

- It has been demonstrated that 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 use of enzymes can reduce the amount of chlorine-containing chemicals employed in bleaching. This impacts directly on the levels of toxic effects, chlorides and adsorbable organic halides of the waste waters.
- The use of polyoxometalates in pulp bleaching represents an alternative bleaching technology that can offer a significant reduction or complete elimination in the use of chlorine-containing bleach chemicals. However, further studies are required to demonstrate the reusability and economical viability and determine the environmental impact of this technology.
- The potential of pulp mill waste waters for biotechnical utilization has been demonstrated. The bio-utilization of these waste waters would alleviate problems associated with their discharge. Furthermore, the xylanase enzymes produced on the spent sulphite liquor could be successfully applied in biobleaching of pulp. This would further contribute to the overall reduction of the environmental impact of the bleach waste waters.

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

Dr G Offringa	Water Research Commission (Chairman)
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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	
MLSS	Mixed Liquor Suspended Solids	
H	Sodium Hypochlorite	
O	Oxygen	
P	Hydrogen Peroxide	
PCU	Platinum Cobalt Units	
POM	Polyoxometalates	
RS	Reducing Sugars	g/l
SS	Suspended Solids	g/l
SSL	Spent Sulfite Liquor	
TOC	Total Organic Carbon	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	

GLOSSARY OF SPESIALIZED TERMS

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

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

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

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

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

Xylanase: Carbohydrate hydrolyzing enzymes with high specificity towards xylan.

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

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

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

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

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

WRC PROJECT K/1367

BIO-REMEDIATION AND BIO-UTILIZATION OF PULPING AND BLEACHING WASTE WATERS

TABLE OF CONTENTS FOR FINAL REPORT TO THE WRC

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. 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 chemical cellulose. The end uses of chemical cellulose 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 chemical celluloseis 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 chemical cellulose. The efficiency of conversion of cellulose into the specific derivative is dependent upon the xylan and mannan content of chemical cellulose. 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 chemical celluloseproduction indicating that the cellulose pulp must be virtually lignin-free. Thus, the lignin and hemicellulose in chemical celluloseprocessing have to be degraded and removed substantially to ease the conversion of cellulose to its end-use product.

1.2. 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 contributes to their corrosiveness. The absence or reduction of chlorides allows the use of recovery-type processes with recycling wash waters. The colour is mainly due to the presence of solubilised lignin and its derivatives. Kraft bleach plant 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).

1.3. Biotechnology in the pulp and paper industry

The release of the bleach plant waste waters with high adsorbable organic halides (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 being 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 bioremediation and biobleaching technology to minimize the use of hazardous chlorine-based bleaching chemicals which would produce certain economical benefits as well as reduce the environmental impact of the pulp and paper industry.

- Development of a microbial fermentation technology of pulping waste waters to obtain high-value products such as enzymes to be utilized in a biobleaching process for environmental clean-up and upgrading the quality of pulp and paper products.

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

1. Literature survey on bioremediation and bio-utilization of pulp and paper waste waters
2. Waste water bioremediation

- POM-based waste water bio-remediation
- Xylanase-based waste water bioremediation

3. Waste water bio-utilization

- Xylanase production in batch and fed-batch cultures
- Application of xylanases in bleaching

CHAPTER 2
LITERATURE SURVEY

2.1. 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). Chloro-organics 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 treatment methods 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 studies indicate that certain physical/chemical method technologies 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.

2.2. Chemical composition of waste waters

The content of chlorinated substances in waste water is usually measured as total organically bound chlorine (TOCl) or adsorbable organic halides (AOX) (Eriksson 1993). Chloro-organic 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, toxic effect and colourisation (Martin et al. 1995; Jokela and Salkinoja-Salonen 1995).

2.2.1. 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 chloro-aromatics in high molecular weight material. Therefore there seem to be evidence both for adsorption as well as chemical decomposition as sources of chloro-aromatics 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.

2.2.2. 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).

2.3. Biobleaching-based waste water bioremediation

2.3.1. 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, microorganisms and biomimetic systems such as polyoxometalates (POM) which can selectively remove hemicelluloses and/or lignin from pulp without affecting the cellulose.

2.3.2. Bleaching with polyoxometalates

2.3.2.1. Structure and reaction mechanisms

Heteropolyacids (HPAs) are complex proton acids that incorporate polyoxometalate (POM) anions (heteropolyanions) having metal-oxygen octahedra as the basic structural units. The first characterized and the best known of these is the Keggin heteropolyanion typically represented by the formula $XM_{12}O_{40}^{x-8}$ where X is the central atom (Si^{4+}, P^{5+}, etc.), x is its oxidation state, and M is the metal ion (Mo^{6+} or W^{6+}). The M^{6+} ions can be substituted by many other metal ions, e.g., V^{5+}, Co^{2+}, Zn^{2+}, etc. The Keggin anion is composed of a central tetrahedron XO_4 surrounded by 12 edge- and corner-sharing metal-oxygen octahedra MO_6 groups (Kozhevnikov 1998). Each group is formed by three octahedra sharing edges and having a common oxygen atom which is also shared with the central tetrahedron XO_4 (Figure 2.1). Among a wide variety of HPAs, the Keggin's are the most stable and more easily available; these are the most important for catalysis. Multicomponent POM redox catalysts are considered as robust oxidation resistent inorganic metalloporphyrin analogs. The state of POMs in solution is generally complicated by a series of pH dependent equilibria, involving a variety of polyanions and mononuclear metallospecies.

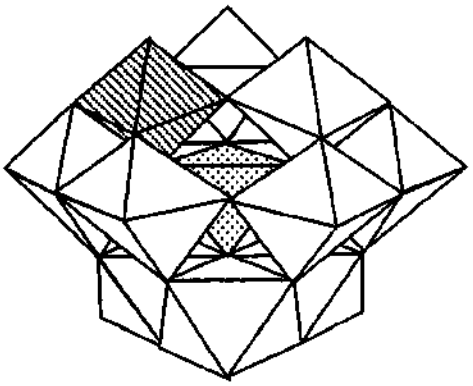

Figure 2.1. Structure of monosubstituted Keggin-type POM $[X^{n+}VW_{11}O_{40}]^{(8-n)-}$ where the central tetrahedron represents the XO_4 (X^{n+}= Al^{3+}, Si^{4+} or P^{5+}) unit, the shaded octahedron represents the VO_6 unit and the unshaded octahedra represents the WO_6 units

Generally reactions catalyzed by HPA-n proceed via a stepwise mechanism represented by the following equations:

$HPA\text{-}n + Red + mH^+ \rightarrow H_m(HPA\text{-}n) + Ox$

$H_m(HPA\text{-}n) + (m/4)O_2 \rightarrow HPA\text{-}n + (m/2)H_2O$

This mechanism includes stoichiometric nelectron oxidation of the substrate, Red, by HPA-n to form the product, Ox, followed by reoxidation of the reduced form of the oxidant ($H_m(HPA\text{-}n)=H_m(PMo_{12-n}{}^{6+}V_{n-m}{}^{5+}V_m{}^{4+})^{(3+n)-}$. Actually, it is the $V^{5+} \leftrightarrow V^{4+}$ transformation that is responsible for the redox properties of HPA-n. The reduction of HPA-n in solution is accompanied by protonation to maintain the charge of the polyanion. In aqueous solution, HPA-n are generally used in the pH range from 0.5 to 3.5, where they are relatively stable (Kozhevnikov 1998). The two reactions can be carried out in the same reactor (one-stage process) or separately as a combination of two stoichiometric reactions (two-stage process).

2.3.2.2. Delignification of wood and pulp

The HPA-5 catalyzed oxygen delignification was tested in the delignification of wood chips. However, delignification was limited to the external regions of the chips, because of diffusion limitations of the catalyst into the wood tissue (Evtuguin and Neto 2001). However, HPA-5 was tested in the oxygen delignification of eucalyptus sawdust in an ethanol-water medium and in aqueous solution. In the presence of HPA-5 (2 mM, pH 1.8), 76.5% delignification was achieved at 100°C, against only 15% to 24% in the absence of HPA-5 (Evtuguin and Neto 1997). The partly reduced form of HPA-5 was slightly more efficient than the oxidized form. The concentration of HPA-5 also influenced delignification. Maximum delignification was registered a 2 mM concentration. The decrease in the oxidizing ability at concentrations above 2-3 mM is probably a consequence of the increase in ionic strength in ethanol-water, due to addition of NaOH for pH adjustment. The wood species did not influence delignification efficiency of HPA-5 treatment. The spruce sawdust delignification occurred in a similar manner to that of eucalyptus sawdust. HPA/ O_2 bleaching of unbleached kraft pulp in ethanol-water at 3% consistency, 2 mM POM, 6 bar O_2 and 90°C for 2h reduced kappa number in excess of 60%, with pulp yield and viscosity losses of 4% and 25%, respectively (Evtuguin et al. 1998). The best temperature for the bleaching was found to be in the range 85-90°C. At higher temperatures, although delignification still increases, there is a dramatic drop in pulp viscosity (Evtuguin et al. 1998). Delignification was found to be independent of pulp consistency in the range 3-10%. Hence, variations in the pulp/ catalyst ratio do not effect the final bleaching results, suggesting that the catalyst regeneration stage is not the limiting step in oxidative delignification. Delignification was found to be independent of the oxygen pressure. The only requirement is to have enough oxygen to re-oxidize HPA-5. This requirement is fulfilled at oxygen pressures higher than 2 bar.

2.3.2.3.. Bleaching of pulp

Results with oxidation experiments of activated phenols using HPA-n suggest that POMs are capable of reducing wood lignin. Weinstock et al. (1998) suggested an oxygen based, waste water free POM delignfiication process (Figure 2.2). By separation of the aerobic

reoxidation of POM_{red} from anaerobic bleaching, oxygen can be utilized as a terminal oxidant while avoiding exposure of the pulp to non-specific oxygen radicals. The process includes four key unit operations: A. Bleaching, B. Pulp washing, C. Removal of wash water and undesired inorganic salts and D. POM catalyzed wet oxidation of dissolved organic material and simultaneous regeneration of the POM to its bleaching active form. The two key chemical steps (unit operations A and D), which represent the selective conversion of lignin and oxygen to carbon dioxide and water, are summarized in Figure 2.3. In the first step, a slurry of unbleached pulp in an aqueous solution of fully oxidized POM (POM_{ox}) is heated anaerobically. Here, the POM is converted to its reduced form (POM_{red}) as it removes electrons from the residual lignin. By separating reoxidation of POM_{red} from anaerobic bleaching, exposure of the pulp to non-specific oxygen radicals is avoided. After leaving the bleaching reactor, the pulp is washed (unit B). Washing experiments (99.9% washing efficiency) indicate that POMs such as α-$Na_5[SiVW_{11}O_{40}]$ are not adsorbed onto pulp fibers (Weinstock et al. 1998). Wash water could then be recycled by evaporation. After concentration, a small spent liquor stream may be diverted so that non-process elements, such as mineral salts of Fe, Mn and Ca carried in with the pulp, can be purged (unit C). A small portion of the POM stream may also be removed at unit C, or at an equally appropriate point, and re-refined at a rate dictated by its operational half-life. After delignification, the bulk of the POM solution is passed directly to unit D, POM reoxidation and wet oxidation of dissolved organic compounds. The much smaller POM stream derived from pulp washing, wash water concentration etc. will also be sent to unit D. In addition to the reduced POMs, the POM stream entering this reactor contains oxidized lignin fragments as well as polysaccharides, all of which enter the POM solution during delignification. POM_{red} is reoxidized by oxygen under conditions that convert the dissolved lignin and polysaccharide fragments to carbon dioxide and water.

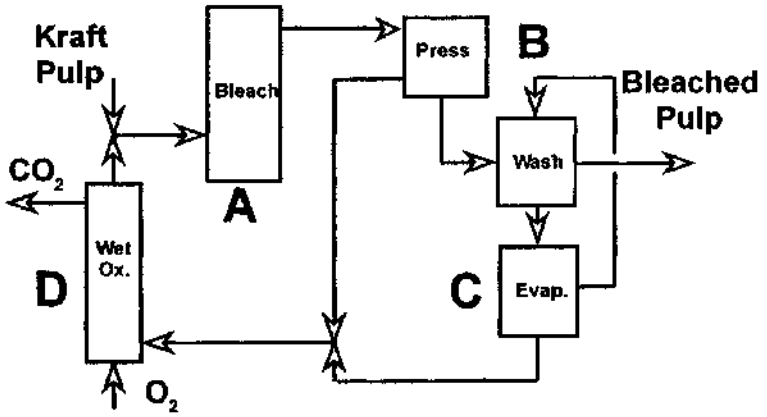

Figure 2.2. Closed mill scheme

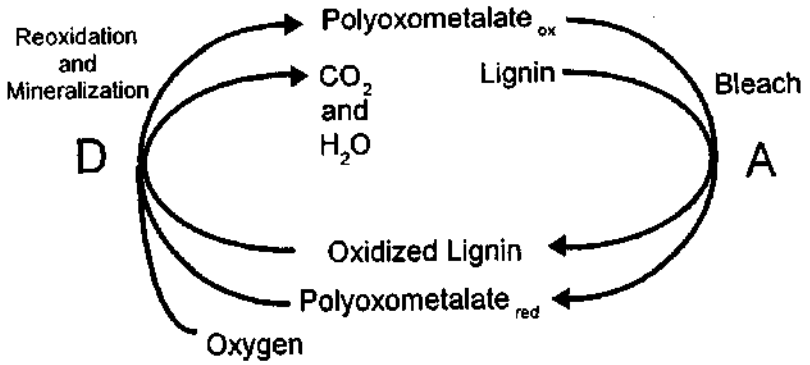

Figure 2.3. Net reactions of the POM process. Unit operation A denotes the delignification reactor under anaerobic conditions and unit operation D represents the wet-oxidation reactor

2.3.3. Bleaching with enzyme s

The application of xylanases in the pulp and paper industry is well documented (Viikari et al. 1994). Unbleached kraft pulps have a low brightness due to the brown coloured lignin. This is removed by the employment of successive bleaching stages. The

bleaching procedure, however, is chosen with respect to the pulp type so as to maintain the target brightness with retention of the strength properties (Buchert et al. 1994). Bleaching chemicals that are most reactive include elemental chlorine, ozone and peroxy acids. The benefits of using enzymes are dependent on the chemical bleaching sequence used as well as on the residual lignin content of the pulp. Xylanases have generally been used for the treatment of brown stock pulp or oxygen delignified pulp. Enzyme pre-treatment has been reported to result in a higher final brightness or lower bleaching chemical consumption. The strength properties of enzymatically treated pulps have been reported to be rather similar to those of reference pulps (Viikari et al. 1986; 1987). The treatment of kraft pulps with cellulose free xylanases increases pulp viscosity due to the partial hydrolysis of low DP xylan in the pulp (Paice et al. 1988; Clarke et al. 1990).

2.3.3.1. 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.

2.3.3.2. Biobleaching of sulfite pulps

Little attention has been paid to enzymatic prebleaching of sulphite pulps for chemical celluloseproduction in the past (Paice and Jurasek 1984). It was found that the enzymatic hydrolysis of xylan in chemical cellulose 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.

2.3.3.3. 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).

2.4. Bio-utilization of pulp and paper waste waters

The production of cellulose pulp using a calcium and magnesium-based sulphite pulping process results in the formation of a lignosulphonated black liquor referred to as spent sulphite liquor (SSL). The main components of SSL include 50 to 65 % lignosulphonates, 15 to 22 % total sugars and 2 to 5 % volatile acids such as acetic acid. The sugars found in the SSL include xylose, mannose, galactose, arabinose and glucose (Pretorius and Lempert, 1993; Streitet al., 1987). This makes the SSL a relatively concentrated waste with high BOD and COD levels and explains why the waste water has to be treated prior to disposal.

The presence of sugars in the SSL makes it an attractive source of cheap carbon for microbial growth. The SSL has been investigated and used as carbon feedstock for microbial biomass production. The production of other commercial products from SSL has also been reported. This includes various metabolites such as lactic acid, furfural, fumaric acid, propionic acid as well as ethanol (Kosaric et al. 1981; McKee and Quicke 1977; Taherzadeh et al. 2003). The productivity of a microbial process based on the SSL as carbon feedstock is limited by the relatively low assimilable carbonaceous concentration permitting only low levels of microbial products and severely hampering its effective commercial exploitation. The presence of inhibitors in the SSL also constitutes a problem for using this waste water as a substrate for microbial growth (Rychtera 1977; Khan and Miller 1983).

Fungal endo-ß-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. These enzymes are finding use as a pre-bleaching aid to chemical pulp bleaching and it has been shown that the application of these enzymes effected significant increases in brightness or reduced usage of active chlorine on kraft pulps (Paice et al. 1989; Bailey and Viikari 1993; Viikari et al. 1994). Recently, it was shown that these enzymes were also beneficial in

the biobleaching of sulphite pulps (Christov and Prior 1996; 1997; Christov et al. 1999). Xylanase preparations used in pulp bleaching have to be free from cellulolytic enzymes to avoid deterioration of strength properties and minimize yield loss (Bailey and Viikari 1993; Viikari et al. 1994).

There are few reports on xylanase production using pulp mill waste waters as carbon source. The use of spent sulphite liquor (SSL) for enzyme production, particularly xylanases, has not previously been reported. There are a number of factors that could negatively affect the production of xylanases using pulp mill waste waters. These include the presence of inhibitors, such as acetic acid and the degradation products of lignin. The inhibitory effect of the pulp mill waste waters may, however, be minimised by the use of various pretreatment methods. These methods include neutralisation, overliming, steam stripping, ion exchange and acclimatisation of organisms (Fein et al. 1984; Yu et al. 1987; Lawford and Rousseau 1992; 1998; Ranatunga et al. 2000; Nigam 2001). A number of reports showed that pretreatment of lignocellulosic material resulted in reduced toxic effect as a consequence of a reduction or total removal of acetic acid, phenolics, uronic acids, and extractives (Antal et al. 1991; Ingram et al. 1997; Ranatunga et al. 1997; 2000).

Besides the inhibitory factors of the SSL, there are other environmental factors that may adversely affect xylanase production and this would include culture pH, agitation rate as well as substrate concentration. It has been shown, for several fungi that cultivating the organism at an unfavourable pH-value results in increased yields of xylanase. Elevated pH-values favoured the production of xylanases by *T. laniginosus* (pH 7.5), *T. longibrachiatum* (pH 7.0), *T. reesei* (between pH 6.0 and 7.0). This effect of an increased xylanase production at elevated pH-values cannot, however, be generalised. Some strains, such as *A. fumigatus* and *A. awamori* were found to favour an acidic culture pH for increased xylanase production.

Thomas (1990) has described in detail the effect of shear stresses and mechanical forces on filamentous fungi. Included in the discussion are the change in morphology and

breakage of hyphae, together with the leakage of intracellular materials due to agitation. It has also recently been reported that the impeller speed which results in shear stress considerably affects xylanase production in the fungus *T. lanuginosus* (Parkarthor et al. 1993). In the case of *A. fumigatus*, cultivated in a stirred tank bioreactor, it was also reported that xylanase and cellulose production decreased at increased agitation rates and this was attributed to shear damage that occurred to the mycelium. However, by changing to an air-lift fermenter, the negative shear effect caused by the disc turbine agitator was avoided and the xylanase activity attained increased by more than 60 % (Wase et al. 1985). Optimum conditions are known to differ for different microorganisms, hence a manipulation of the conditions would enable the optimisation for xylanase production.

There are two major factors that must be considered for the efficient protein production; namely the production level per cell and the total cell density in the bioreactor. Fed-batch cultivation is a technique that allows for high cell density cultivations. The concentrations of nutrients in the bioreactor can be controlled and maintained at desired levels for the enhancement of protein production (Karlsson et al. 1999).

Studies conducted on bacterial and fungal xylanases have yielded most of the available information about the properties of xylanases. Microbial xylanases are single subunit proteins with molecular masses in the range of 8 to 145 kDa (Sunna and Antranikian 1997). Temperature optima for endoxylanases from bacterial and fungal sources vary between 40 and 60°C (Kulkarni et al. 1999). Xylanases from different organisms are usually stable over a wide pH range, between 3 and pH 10 and show optimum pH in the range of 4 to 7 (Kulkarni et al. 1999). Some fungal xylanases such as those from *Aspergillus kawachii* (Ito et al. 1992) and *Penicillium herue* (Funaguma et al. 1991) exhibit optimum pH towards the acidic side, between pH 2 to 6. Bacteria are generally known to produce two xylanases; high molecular weight acidic xylanase and low molecular weight basic xylanase. This type of relationship is, however, not observed in fungal xylanase preparations (Kulkarni et al. 1999).

2.5. Conclusions

- Biobleaching of pulp and paper with enzymes can offer some apparent benefits to the pulp and paper industry. These include the potential increase in pulp yield, higher brightness ceiling, modification of pulp and paper properties, increased pulp mill capacity.
- Biobleaching can be used as a means of waste water bioremediation by reducing the amounts of ble aching chemical used, especially chlorine containing chemicals. This would reduce the overall environmental impact of the industry.
- A viable 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, toxic effect and chemical load of the waste water.
- The reduction of the AOX and toxic effect of waste waters following waste water bioremediation can improve their bioutilization as substrates for enzyme production.
- The presence of sugars in the SSL makes it an attractive source of cheap carbon for microbial growth. The SSL has been investigated and used as carbon feedstock for microbial biomass production, metabolites such as lactic acid, furfural, fumaric acid, propionic acid as well as ethanol.
- There are few reports in literature on xylanase production using pulp mill waste waters as carbon source. The use of SSL for enzyme production, particularly xylanases, has not previously been reported.

CHAPTER 3

WASTE WATER BIO-REMEDIATION

3.1. Polyoxometalate-based waste water bio-remediation

3.1.1. Abstract

Unbleached hardwood soda-aq pulp and unbleached bagasse soda pulp were delignified using 7-molybdo-5-vanadophosphate (HPA-5). The HPA-5 pretreated pulps were subsequently bleached using DED (hardwood soda-aq pulp) and EH (bagasse soda pulp) sequences. Properties of the HPA-5 bleached pulps were compared to pulps bleached under standardized conditions. Savings of up to 50% ClO_2 during bleaching of soda-aq pulp, the replacement of the entire chlorine bleaching stage (100% chlorine savings) and 50% reduction in the use of hypochlorite during bleaching of bagasse pulp were attained without deterioration in the final pulp properties.

3.1.2. Introduction

Molecular oxygen is one of the leading candidates for chlorine replacement and subsequently reduced toxic effect of paper mill waste waters. Oxygen delignification is effective but its delignification effectiveness is limited to about 50% after which a severe loss of pulp strength occurs (Olm and Teder 1979). One approach to enhance the efficiency of oxygen bleaching is to use inorganic metal-oxygen cluster anions (polyoxometalates, POM) as catalysts. Two methods for use of POMs in pulp bleaching have been reported (Weinstock et al. 1997; Evtugin et al. 1998). According to the first one, pulp may be bleached with POM under anaerobic conditions and the regeneration of the POM occurs in a separate stage at temperatures higher than those applied in the bleaching stage (Weinstock et al. 1997). However, the high POM concentrations required (0.5M) and the need for a separate re-oxidation stage appear as drawbacks of this approach. Alternatively, the use of heteropolyanions (HPA) as catalysts in the oxygen delignification stage has been described (Evtugin et al. 1998). The aerobic lignin oxidation in the presence of POM allows regeneration of the catalyst in the same process

stage. The aim of this study was to determine the delignification efficiency of HPA-5 on unbleached hardwood soda-aq pulp and bagasse soda pulp and examine potential active chlorine savings due to the POM pretreatment.

3.1.3. Materials and methods

3.1.3.1. Pulps

Unbleached hardwood soda-aq pulp (Initial pulp properties: 31.2% brightness; 10.7 kappa number; 882 cm^3/g viscosity) and unbleached bagasse soda pulp (Initial pulp properties: 35.2% brightness, 7.8 kappa number, 999 cm^3/g viscosity) were washed until a neutral pH of the wash waters was attained and stored at 4°C until use.

3.1.3.2. Synthesis of polyoxometalates

The syntheses of 7-molybdo-5-vanadophosphate (HPA-5) was conducted according to a published procedure (Evtuguin et al. 1998).

3.1.3.3. Delignification of pulps with polyoxometalates

Unbleached hardwood soda-aq pulp was treated using 1.5 mM HPA-5 at 20% consistency and pH 1.9, 127°C and 8 bar O_2 for 45 min in ethanol/water (50%, v/v). Unbleached bagasse soda pulp was treated using 1mM HPA-5 at 10% consistency and pH 1.9, 140°C and 4.5 bar O_2 for 120 min in ethanol/ water (50%, v/v). The HPA-5 pretreated pulps were subsequently bleached with chemicals to reach certain target brightness levels as required for paper production.

3.1.3.4. Chemical bleaching of pulps

Two oxygen controls were prepared on soda-aq pulp. The acid oxygen control (O_{ac}) was run under the same conditions as the POM treatment but omitting the HPA-5 addition. The alkaline oxygen control (O_{alk}) was carried out under the same conditions as the O_{ac} with the exception that 3.5% NaOH was added instead of sulphuric acid. Following oxygen treatment, pulp was bleached using chlorine dioxide (D) and NaOH (E) in a DED sequence under standardized conditions (control brightness 83.3%). Unbleached bagasse soda pulp was bleached with chlorine (C), NaOH (E) and sodium hypochlorite (H) in a CEH sequence under standardized conditions (control brightness 76.4%).

3.1.3.5. Determination of pulp properties

Kappa number was determined according to Tappi Test Method T-236 (Technical Association of the Pulp and Paper Industry, Atlanta, GA). Viscosity was analysed according to the SCAN-CM 15:88 Method whereas the brightness determinations were performed on a brightness machine Technidyne Color Touch 2 Model ISO (New Albany, South Carolina, USA).

3.1.4. Results and discussion

3.1.4.1. Bleaching of hardwood soda-aq pulp with polyoxometalates

The HPA-5 treatment produced pulp with kappa number 5.2, 43.5% brightness and 486 cm^3/g viscosity. In terms of kappa number, HPA-5 was more efficient than alkaline oxygen (5.2 compared to 6.2). However, the latter produced pulp with higher brightness (45.8% compared to 43.5%) and viscosity (616 cm^3/g compared to 486 cm^3/g). The subsequent DED bleaching increased the brightness of HPA-5 pretreated pulp to 83.3%, 0.5 points higher than the alkaline oxygen control (Figure 3.1). The higher brightness of HPA-5 pretreated pulp could be translated into 10% ClO_2 savings compared to alkaline control.

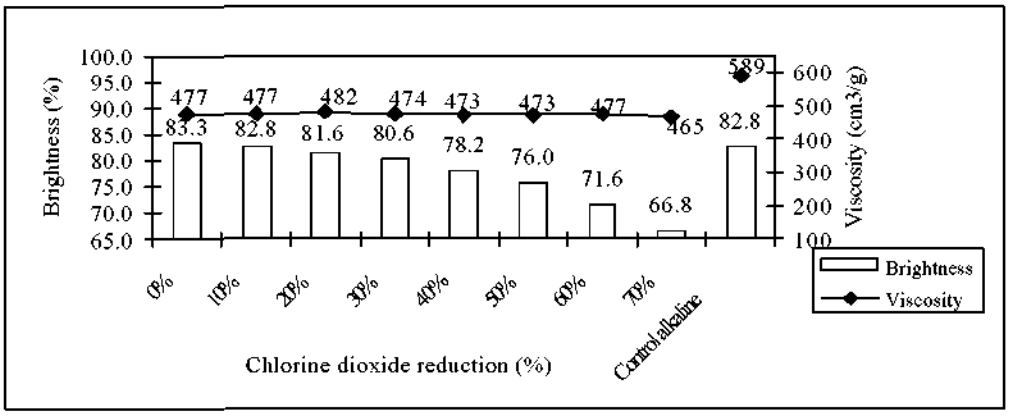

Figure 3.1. Impact of HPA-5 pretreatment (O_{HPA-5} DED) on brightness and viscosity of bleached pulp as compared to control (O_{alk}-DED)

3.1.4.2. Bleaching of bagasse soda pulp with polyoxometalates

HPA-5 treatment produced bagasse pulp with 40.7% brightness, kappa number 2.5 and viscosity of 353 cm^3/g (data not shown). The subsequent EH bleaching improved pulp brightness to 76.9% and reduced pulp viscosity to 243 cm^3/g. In comparison, the CEH control produced pulp with 76.4% brightness and 256 cm^3/g viscosity. It was possible to replace the entire chlorine stage with HPA-5 catalyzed oxygen treatment without negative influences on pulp brightness and viscosity (Figure 3.2). No additional reduction in H charge was possible without a brightness loss. By introducing peroxide at the extraction stage as POM-EpH it was possible to reduce the hypochlorite charge by up to 50% while retaining the pulp properties at the control levels (Figure 3.3). For example, the application of 1.38% act. Cl. (equal to 50% reduction in act. Cl) at the Hstage produced pulp with 78.4% brightness and 254 cm^3/g viscosity.

3.1.4.3. Recycling and recovery efficiency of polyoxometalates

The hardwood soda-aq pulp was treated in water rather than ethanol/ water (50%, v/v) due to the volatility of ethanol and the difficulty to determine the exact ethanol concentration after the treatment. The untreated pulp had a kappa number of 12.3 and 23.9% brightness. The O_{HPA} treatment reduced kappa number by 39.8% and improved pulp brightness by 3.7 points (Table 3.1). Re-using the same HPA-5 solution in two subsequent cycles produced similar kappa number reductions. However, the delignification efficiency declined during the fourth cycle (33.3% kappa number reduction). As expected, the O_{HPA} treatment in water had only a modest effect on pulp brightness.

Table 3.1. Recycling efficiency of HPA-5

Treatment cycles	Kappa no.	Kappa red. (%)	Brightness	Brightness change (points)
Untreated	12.3	-	23.9	-
Control O_{acid}	10.0	18.7	25.8	1.9
1x O_{HPA}	7.4	39.8	27.6	3.7
2x O_{HPA}	7.3	40.6	26.2	2.3
3x O_{HPA}	7.5	39.0	27.6	3.7
4x O_{HPA}	8.2	33.3	28.1	4.2

In washing experiments aimed to determine the recovery efficiency of POM, pulp pretreated with HPA-5 was filtered and then washed twice with distilled water. The concentration of Mo and V in the bleach liquor and combined wash waters was determined and compared to the initial amounts of these metals added with HPA-5. Only 63.3% of Mo and 55.3% of V were recovered suggesting that significant amounts of the heavy metals remained in the pulp.

3.1.5. Conclusions

- POM (HPA-5) pretreatment of hardwood soda-aq pulp enabled chlorine dioxide savings of 10% compared to alkaline oxygen treatment and 50% compared to the acid oxygen control while retaining brightness at the control level.

- POM (HPA-5) pretreatment of bagasse soda pulp could replace the entire chlorine bleaching step without loss in final pulp quality. In addition, up to 50% of the hypochlorite charge could be reduced without deterioration of bagasse pulp properties.

- The use of POM in pulp bleaching represents an alternative bleaching technology that can offer a significant reduction of the consumption of chlorine-containing bleach chemicals with reduced environmental impact. However, further studies are required to demonstrate the reusability and economical viability and determine the environmental impact of this technology.

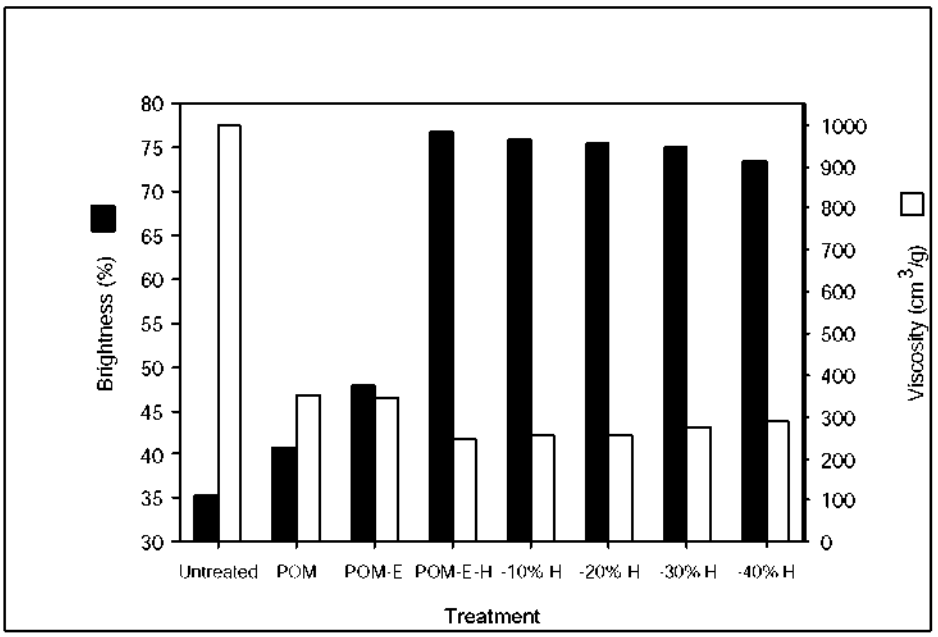

Figure 3.2. Impact of POM treatment of bagasse soda pulp at 140°C for 2 h on brightness and viscosity of bleached pulp (POM-EH)

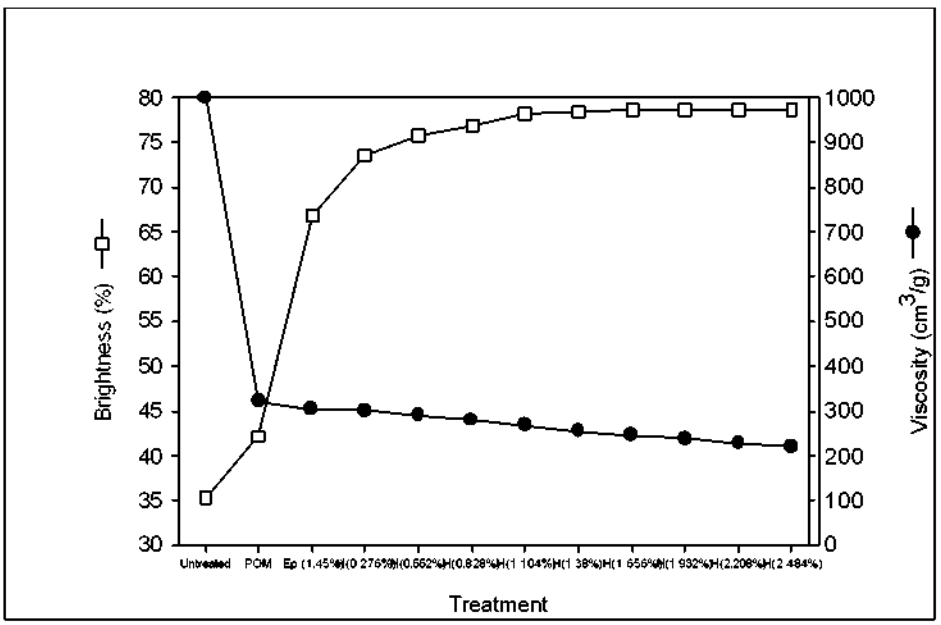

Figure 3.3. Impact of POM treatment of bagasse pulp at 140°C for 2 h on pulp properties of bleached pulp (POM-EpH)

3.2. Xylanase-based waste water bio-remediation

3.2.1. Abstract

The general use of chlorine-based bleaching chemicals and alkaline extraction in pulp and paper production leads to the production of waste waters with high chemical oxygen demand (COD) and absorbable organic halogen (AOX) values and/or high pH and colour. Reduction of the charges of these chemicals would have a beneficial effect on the concentration and composition of the waste waters produced. Enzymatic removal of hemicellulose and chromophore-based compounds from pulp renders the pulp lignin more accessible to bleaching chemicals resulting in increased pulp brightness or reduction of the chemical use. Prior to chemical bleaching, bagasse soda pulp and oxygen delignified soda-aq pulp were subjected to pre-treatment with xylanase (X) at a charge of $0.5 enzyme/t pulp. On bagasse pulp, it was determined that reduction of 32% in the hypochlorite (H) charge was possible without sacrificing the pulp brightness. Subsequently it was shown that the NaOH use during the alkaline extraction stage (E) can be reduced by 20% by lowering the extraction stage pH form 11.25 to 9.75 while keeping the brightness at the CEH (C, chlorine) control levels. On soda-aq pulp, it was found that the chorine dioxide (D) charge for both D_1 and D_2 stages could be reduced by 30% while a reduction of 20% in the NaOH charge during the E stage was possible. The inclusion of a washing step between the X and D_1 had no impact on brightness of the D_1ED_2-bleached pulp.

Filtrates obtained during the biobleaching of soda-aq pulp (X-DED) and bagasse pulp (X-CEH) with xylanase (X) were analysed. The effect on filtrate characteristics following the introduction of a washing step between the X and D_1 stages during biobleaching of soda-aq pulp was also investigated. In general, reductions in charges of chlorine containing bleaching chemicals, following application of xylanase, led to an overall improvement in filtrate quality. Various filtrate parameters were investigated to determine filtrate quality such as colour, pH, COD, AOX and toxic effect using the bacterial growth inhibition test. It was noted that there was a correlation between AOX levels and toxic effect. Filtrates from the xylanase pre-treatment steps did not repress bacterial growth, but indeed stimulated growth of the bacterial test strain. Chloroorganic

compounds present in selected samples were also assessed using thin layer chromatography (TLC). The presence of one chloroorganic compound in the extraction stage filtrate of soda-aq pulp could be identified as tetrachloroguaiacol. The results indicated that xylanase could be utilised in a pre-treatment step to lower the chlorine-containing chemical charges during bleaching and still obtain acceptable pulp characteristic. A further benefit would be the improvement in waste water quality. Washing between the X and D_1 stages resulted in filtrates having reduced COD, AOX and toxic effect levels which might be of assistance during the down stream processing of the waste water.

3.2.2. Introduction

The use of oxidative chlorine-based agents are ubiquitous in the South African pulp and paper industry. The waste waters resulting from bleaching with this class of compounds contain high levels of organic chlorides, have high COD values and often contain traces of unconsumed bleaching agents. Bleaching sequences often also contain alkaline extraction steps aimed at removing both organic chlorides produced by preceding bleaching steps and lignin liberated by the degradation of fibre structure. Waste waters from alkaline extraction have very high pH, COD and are, depending on the lignin content of the pulp, deeply coloured.

It is known that wood pulp contains mainly cellulose, hemi-cellulose and lignin. Hemi-celluloses are often called xylans due to a high content of the monosaccharide xylose. Enzymatic removal of xylan can be employed as auxiliary bleaching. Hemicellulose is only slightly coloured, its removal from pulp influences brightness only marginally. The hydrolysis of xylan however leaves the lignin contained in wood fibre more exposed to attack by bleaching chemicals in subsequent bleaching steps, improving lignin oxidation and extraction. Biobleaching can therefore be employed to improve brightness of pulp or can facilitate the reduction of bleaching chemical charges with the maintenance of brightness of the final product. The efficacy of biobleaching with xylanase in reduction of chemical use was examined for soda aq pulp, usually bleached in the sequence ODED, and bagasse pulp, bleached as CEH. Based both on mill requirements and bleaching

efficiency found in previous work, the xylanase treatment is carried out directly before the first chlorine based bleaching step i.e. D_1, for soda aq pulp, and C, for bagasse pulp.

Wastewaters from industries in general cause environmental problems and are increasingly coming under scrutiny from regulating authorities. In the pulp and paper industry, the employment of chlorinated chemicals as bleaching agents causes environmental concern, mainly because of the formation of chloro-organic substances during bleaching, measured as AOX. It has been demonstrated that application of xylanase as a pre-treatment step during pulp and paper production leads to a reduction in the bleaching charge necessary to achieve optimal pulp and paper brightness. This results in a lower AOX discharge and cost savings for industry. At the Sappi Biotechnology Laboratory, soda-aq pulp and bagasse pulp were treated with xylanase and bleaching executed with reduced bleaching chemical charges. Controls were also included in the study to compare the effect of reduction in bleaching chemicals charges on filtrate quality as obtained during various stages of the conventional bleaching process. Parameters such as colour, pH, bacterial growth, AOX and COD were determined. TLC was used to identify chloroorganic compounds in some of the filtrates. The effect on filtrate quality of having a washing step between X and D stages during soda-aq pulp bleaching was also assessed.

3.2.3. Materials and methods

3.2.3.1. Enzyme assay

The activity of xylanases used was determined in accordance of the method of Miller as adapted by Bailey et al (1992). A 1.8ml of a 1% suspension of birch wood xylan was incubated with 0.2ml of a suitable enzyme dilution (most commonly 0.02% and 0.001%). After incubation of exactly 5 minutes 1.5ml DNS solution was added and the samples heated to 100°C for 10 minutes. The samples were cooled to room temperature with water/ice and the absorbance at 540 nm determined. The instrument was zeroed with a un-incubated blank of identical composition to the sample to be analyzed. A standard curve of xylose concentration versus absorbance was prepared and the activity of the enzyme calculated.

3.2.3.2. Pulps

Bagasse soda and post O_2 soda aq pulp was obtained from the respective mills. The pulps were stored in plastic bags at 4°C until use.

3.2.3.3. Enzyme treatment of pulp

Enzyme treatment is carried out before the first chlorine based bleaching step for both types of pulp, i.e. chlorine dioxide (D_1) for soda aq and chlorine (C) for bagasse.

For all enzyme treatments, the pH of the pulp was pre-adjusted to the optimum pH of the enzyme. The pH adjustment was carried out by lowering the consistency of the pulp to less than 5% and by the addition of appropriate amounts of sodium hydroxide and/or sulphuric acid. The pulp was then filtered, fluffed and divided into the desired sample size. Enzyme charges were based on the cost of the enzyme involved. Enzymes were diluted with pH adjusted water and kept on ice before use. In all cases enzyme dilutions were used within 60 min of being prepared. Incubation was carried out in weighed plastic bags in a water bath temperature controlled to ± 1.5 °C of the desired temperature.

3.2.3.4. Chemical bleaching of pulp

Chemical bleaching was carried out at consistency, chemical charge and temperature in accordance with the conditions employed in the mill. Chemical reductions, when employed, were calculated as a percentage of the full charge used in the mill. The concentration of bleaching agents in stock solutions was determined on the day of use. Solutions of chlorine dioxide and chlorine was not used if older that two weeks. Care was taken to use solutions with similar concentrations. Reference bleaching of bagasse pulp in a CEH sequence without enzymes produced pulp with a brightness of 78.5% whereas the control brightness of soda aq pulp bleached in a DED sequence was 86.5%.

3.2.3.5. Analyses

Hand sheets for analysis was prepared in accordance with ISO Method 3886, with the exception that the sheets consisted of ca. 5g dry weight pulp instead of the 2.5 g

recommended in the method. Brightness was determined with a Color Touch 2 instrument from Technidyne Corporation (New Albany, South Carolina, USA).

3.2.3.6. Colour determinations

Samples for colour determinations were filtered through a 0.45 µm membrane filter before the pH was adjusted to pH 7.6. 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 to 500 colour units (PCU). One colour unit equals 1 mg/l platinum as chloroplatinate ion.

3.2.3.7. Analysis of chemical oxygen demand

COD was determined using the Hach spectrophotometer according to instructions set out in the manual for the instrument.

3.2.3.8. Chloride determinations

Chloride content of filtrates was assessed spectrophotometrically employing the procedures and reagents supplied by Hach. The method is based on following reactions: chloride in filtrates reacts with mercuric thiocyanate to form mercuric chloride and release thiocyanate ion which subsequently react with ferric ion to produce an orange coloured compound. The intensity of this orange colour, measured at 455 nm, is proportional to the chloride concentration in the sample.

3.2.3.9. Bacterial growth inhibition tests

Tests were performed to determine the effect of filtrate composition on bacterial growth response (Slabbert 1986; Slabbert 1988). *Pseudomonas putida* strain Berlin 33/2 (DSM, Teilsammlung, Bayreuth, Germany) was used as 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 $MgSO_4.7H2O$ and 0.25 g glucose per litre of deionised water, pH 7.15) in 50 ml medicine bottles. A culture of *P. putida* was grown overnight in minimal medium as static cultures in an incubator at 27°C. The culture was diluted with fresh minimal medium (to an absorption level of approximately 0.8) 30 min prior to the

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-fold concentrate of the minimal medium and used in 2.5 ml volumes for inoculation of 22.5 ml test samples. Samples were used at 25% of full strength (4-fold dilution) during the tests. Test samples were incubated at 27°C for 6 h after which growth was measured in terms of absorption. Absorption determinations were carried out spectrophotometrically at 600 nm. Solutions containing samples and medium only (without inoculate) were used to blank the spectrophotometer. Sterile deionised water was used for control tests. Results were expressed as percentage inhibition over control. Analytical values represented the mean of at least three determinations.

3.2.3.10. Solvent extraction

The pH of D_0E_0 and $XD_{30}E_{20}$ samples (50 ml) were adjusted to 2.0 and then treated with 50 ml of chloroform on an orbital shaker for 3 h. This procedure was repeated with fresh aliquots of chloroform and then the organic phases for each extract were combined. Extracts were subsequently treated with 100 ml of a 5% (w/v) $NaHCO_3$ solution. The organic phases were recovered and then extracted with 0.1 N NaOH for 3 h upon which the pH of each of the water phases was adjusted to pH 2.0. Thereafter another extraction of the water phases with chloroform was carried out for 3 h as described above. Finally, each organic phase was recovered, dried over anhydrous Na_2SO_4 and concentrated, before analyses using TLC were undertaken.

3.2.3.11. Thin layer chromatography procedures

Samples and known compounds (standards) were spotted on silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany) with micropipettes. Thereafter, each plate was developed in different organic solvents (methanol, dichloromethane; dichloromethane and hexane, etc.) Spots were detected under ultraviolet light. The R_f values for each sample were also determined. The R_f is defined as the distance moved by the centre of solute zone divided by the distance moved by the solvent front.

3.2.3.12. Determination of absorbable organic halides

The pH of waste water samples was adjusted to 1.8 with concentrated HNO_3 and each sample was frozen at -20°C in brown bottles before analysis of AOX was performed with an Euroglas AOX EC S 1000 analyser (Delft, The Netherlands) according to standard procedures (Odendahl et al. 1990).

3.2.4. Results and discussion

3.2.4.1. Effect of xylanase on reduction of chemical consumption during bleaching

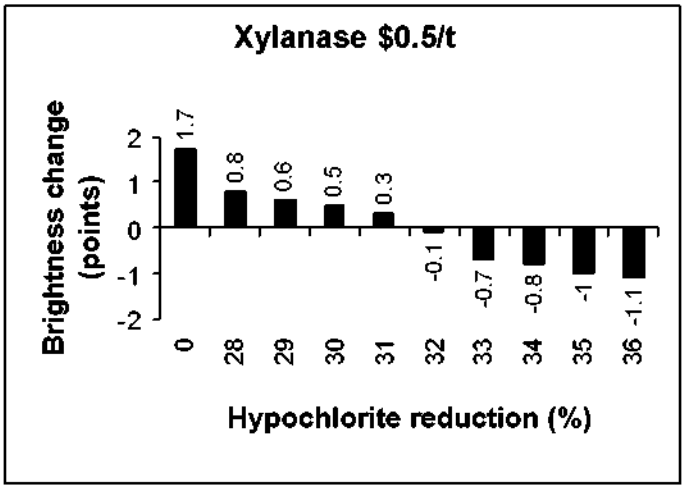

Figure 3.4. Impact of xylanase on hypochlorite reduction and brightness of bagasse pulp (X-CEH)

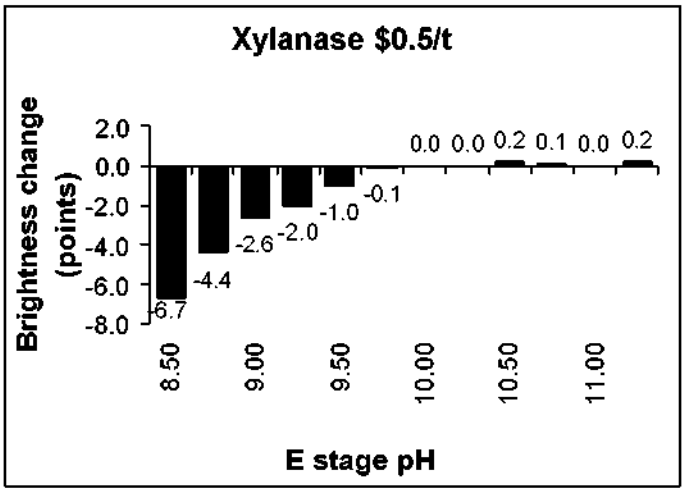

Figure 3.5. Impact of xylanase on pH reduction and brightness of bagasse pulp (X-CEH) at 32% hypochlorite reduction

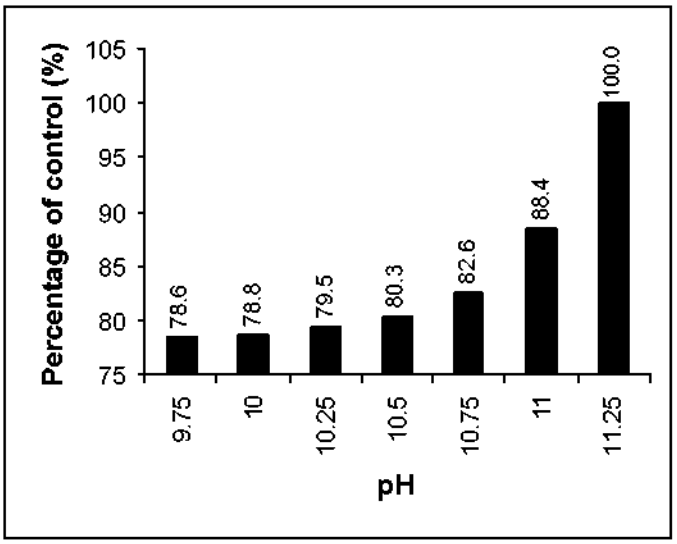

Figure 3.6. Percentage NaOH used to adjust 5 g bagasse pulp at 2.5% consistency from starting pH of 1.9

It was shown that biobleaching of bagasse pulp with \$0.5/t xylanase and 32% reduction in hypochlorite charge resulted in no significant brightness loss when bleaching bagasse (Figure 3.4). It should be noted that the biobleaching process becomes cost-effective at hypochlorite reductions higher than 20%. In addition, the E stage pH may be lowered to 9.75 without significant brightness loss (Figure 3.5). Reduction of E stage pH to the range 9.75 to 10.5 may represent up to 21.4% reduction in sodium hydroxide used (Figure 3.6).

On soda-aq pulp, reductions of 30% chlorine dioxide for both D_1 and D_2 as well as NaOH reduction of up to 20% with only marginal brightness loss was possible (Figure 3.7). The inclusion of a washing step between the X and the first chlorine dioxide step D_1 had an insignificant impact on brightness (Figure 3.8).

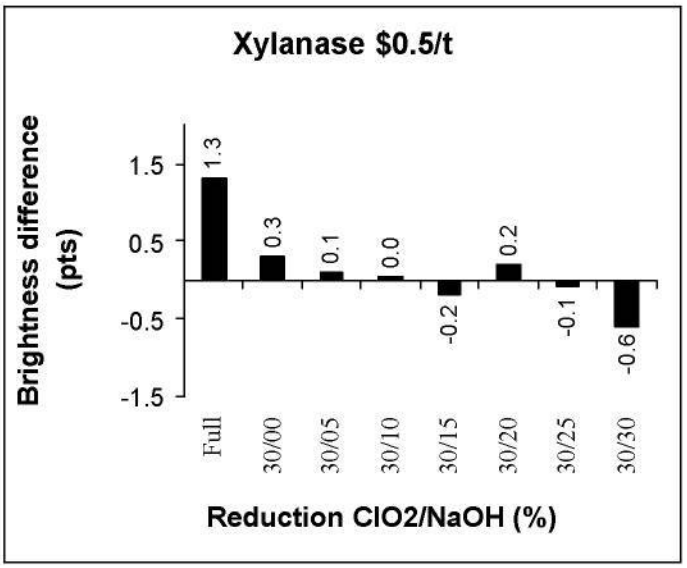

Figure 3.7. Impact of xylanase on chlorine dioxide and caustic reduction during biobleaching of soda aq pulp (O-X-DED)

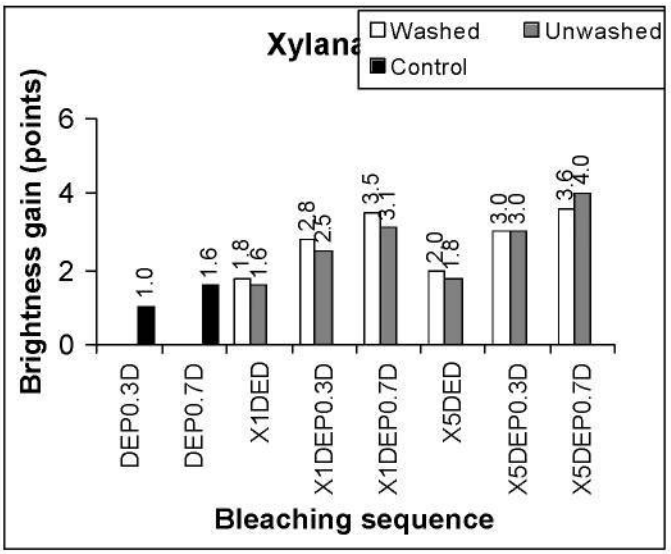

Figure 3.8. Impact of a washing step between X and D_1 on brightness during biobleaching of soda aq pulp (XwDEpD)

3.2.4.2. Effect of xylanase bleaching on waste water properties

The filtrate characteristics obtained following biobleaching of bagasse pulp are shown in Table 3.2.

Table 3.2. Impact of xylanase pre-treatment of bagasse pulp on the properties of bleach filtrates generated from a X-CEH bleaching

Sample	Colour (PCU)	pH	COD (mg/l)	Cl^- (g/l)	AOX (mg/l)	Change in bacterial growth (%)
X[a]	70	6.92	149	0.01	0.3	+3.6
C_0[b]	595	1.80	691	0.73	43.1	-7.7
X-C_0	385	1.84	531	0.74	34.8	-4.6
C_0E_0[c]	7100	7.30	5505	0.39	93.7	-7.1
X-C_0E_0	6800	7.60	4965	0.38	73.9	-4.8
$C_0E_0H_0$[d]	78	11.80	2100	34.7	57.8	-14.5
X-$C_0E_0H_0$	65	11.77	1879	33.5	52.6	-12.1
X-$C_0E_0H_{28}$	70	11.79	1763	30.5	42.9	-2.3
X-$C_0E_0H_{30}$	73	11.88	1781	25.0	39.2	-0.3
X-$C_0E_0H_{32}$	63	11.78	1436	24.0	37.3	0.0

[a]X: Xylanase stage (Cartazyme, Clariant, UK), [b]C: Chlorine stage, [c]E: Extraction stage, [d]H: Hypochlorite stage (Note that the values in subscripts indicate % reduction of charge over control)

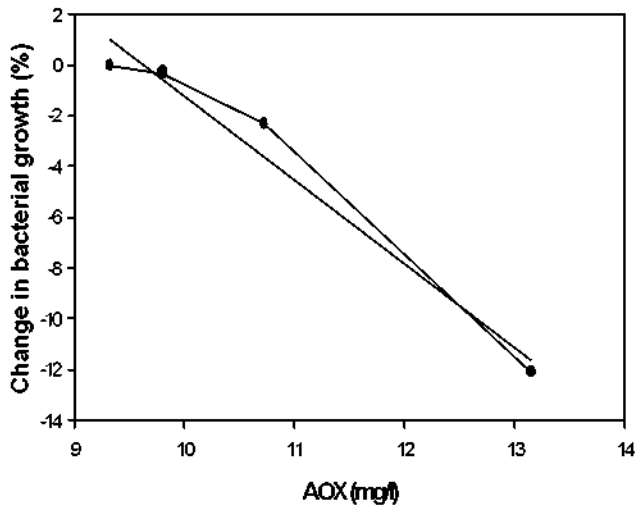

Figure 3.9. Change in bacterial growth vs AOX content of a hypochlorite filtrate from X-CEH bleaching of bagasse pulp

No bacterial growth inhibition was noted when the filtrate from the xylanase pre-treatment step was examined, furthermore the AOX and chloride levels were also very low (Table 3.2). However, following full bleaching of bagasse pulp, growth inhibition and relative high concentrations of AOX and chlorides were detected in the filtrates. The chloride concentrations in the hypochlorite filtrates were in particular very high. In general, the various parameters showed a decrease in value as the hypochlorite charges were reduced from full charge (0% reduction) to 68% (32% reduction). The bacterial growth inhibition caused by the hypochlorite filtrates was shown to be proportional to the AOX content of the filtrates (Figure 3.9).

The filtrate attributes obtained following biobleaching using soda-aq pulp are shown in Table 3.3. The filtrate quality also improved as chemical charges used during bleaching decreased. Overall, the AOX values correlated well with the bacterial growth inhibition caused by the bleach filtrates.

Table 3.3. Effect of xylanase pre-treatment on the filtrate quality obtained during biobleaching of soda-aq pulp as X-DED

Sample	pH	Colour (PCU)	COD (mg/l)	Chlorides (mg/l	AOX (mg/l)	Change in bacterial growth (%)
D_0	2.0	169	602	223	71.8	-31.3
X-D_{30}	1.6	78	397	143	55.3	-28.2
D_0E_0	12.8	103	637	43	2.97	-11.6
X-$D_{30}E_0$	12.0	150	340	32	2.55	-7.5
X-$D_{30}E_{20}$	11.8	155	336	30	1.57	-5.8
$D_0E_0D_0$	2.7	8	76	80	6.10	-28.2
X-$D_{30}E_0D_{30}$	2.7	0	72	53	4.94	-21.1
X-$D_{30}E_{20}D_{30}$	2.7	0	71	43	2.72	-17.2

Table 3.4 contains the R_f values obtained during TLC analysis of organic extracts and standard (plate 1). Because of similar R_f values it would appear that the D_0E_0 filtrate might contain tetrachloroguaiacol. The R_f values obtained using a mixture of dichloromethane and hexane as elluent in the ratio 1.7:1.3 (plate 2) are presented in Table 3.5.

The R_f values increased following TLC using the more polar mixture of dichloromethane and hexane combined in the ratio of 1.7:1.3. The results obtained with both TLC plates (Tables 3.4 and 3.5) lead to the suggestion that the D_0E_0 filtrate may contain tetrachloroguaiacol. Although three spots were obtained when the X-$D_{30}E_{20}$ filtrate was chromatographed on plate 1 (Table 3.4), only one spot was obtained when this sample was analysed on plate 2 (Table 3.5). The reason for this is unclear. Further work is necessary to confirm that tetrachloroguaiacol occurs in the D_0E_0 filtrate.

Table 3.4. R_f values of standards and organic extracts from the extraction stage during biobleaching of soda-aq pulp (TLC plate 1) using a mixture of dichloromethane and hexane as elluent in the ratio 1.3:1.7.

Sample name	R_f values (x100)
2-chlorosyrinaldehyde	1.90
D_0E_0	9.49; 18.99; 84.81
X-$D_{30}E_{20}$	11.39; 21.52; 93.04
Tetrachloroguaiacol	17.10
4,5-dichloroguaiacol	25.95
4-chlorocatechol	0.63
4,5,6-trichloroguaiacol	24.68
2,6-dichlorosyringaldehyde	0.63
3,4-dichlorophenol	30.57

Table 3.5. R_f values of organic extracts and known compounds obtained using a mixture of dichloromethane and hexane as elluents in the ratio of 1.7:1.3 (TLC plate 2)

Sample	R_f values (x100)
2-chlorosyrinaldehyde	4.38
D_0E_0	24.53; 54.72; 79.25
X-$D_{30}E_{20}$	25.08
Tetrachloroguaiacol	24.38
4,5-dichloroguaiacol	38.13

4-chlorocatechol	3.13
4,5,6-trichloroguaiacol	37.50
2,6-dichlorosyringaldehyde	3.75
3,4-dichlorophenol	39.06

The effects of adding a washing step between the D_1 and X stages on filtrate attributes following soda-aq bleaching are shown in Figures 3.10 to 3.12.

The introduction of washing between the X and D_1 stages of bleaching reduced the AOX, COD and toxic effect levels of filtrates. Xylanase treatment of pulp releases sugars and other substances that would end up in the plant waste water thereby increasing the COD levels in the plant waste water treatment. Since these treatment systems have been designed to perform at specific loading levels, increased COD concentrations can lead to overloading of these systems that might impair the treatment efficiency. Introducing a washing step might alleviate these problems. The wash waters from the xylanase stage might be treated separately or even utilised for biotechnological applications. Alternatively, the X-stage COD can be sent to the recovery system of the mill where the organic matter will be utilized for heat generation and use in the mill.

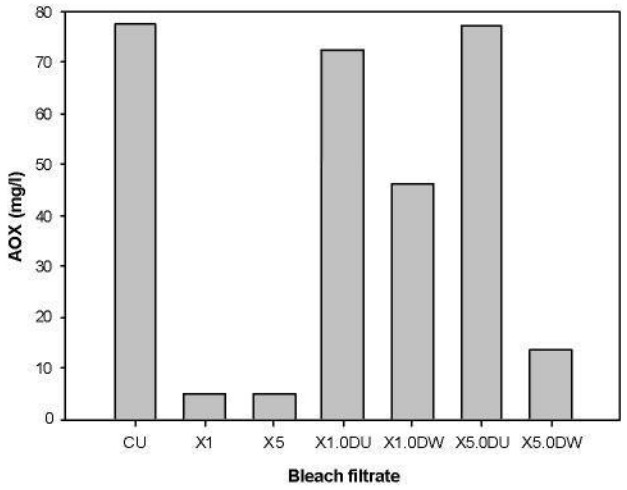

Figure 3.10. Filtrate AOX levels of filtrates obtained from X and D_1 stage (with or without inter-stage washing) during biobleaching of soda-aq pulp. Legend: CU, D_1

control without washing step; X1 and X5, xylanase applied at charges of 1$/t and 5$/t, respectively; X1.0DW and X5.0DW, xylanase pre-treatment followed by D_1-stage bleaching with a washing step between X and D_1 stages implemented; X1.0DU and X5.0DU xylanase pre-treatment followed by D_1 stage bleaching executed without a washing step between X and D_1 stages.

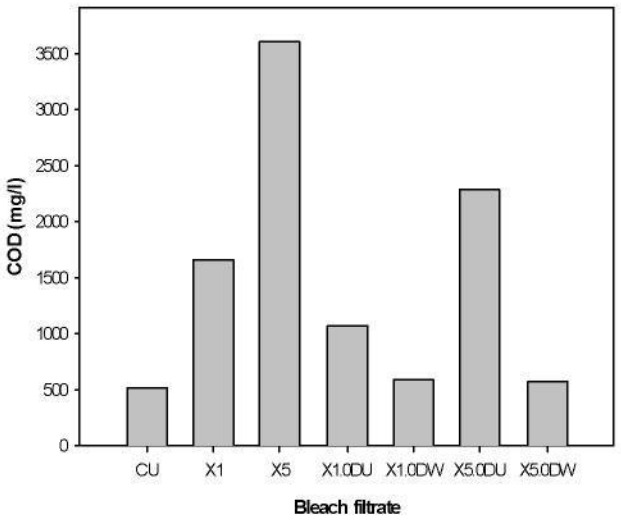

Figure 3.11. COD levels of filtrates obtained from X and D_1 stage (with or without inter-stage washing) during biobleaching of soda-aq pulp. Legend: CU, D_1 control without washing step; X1 and X5, xylanase applied at charges of 1$/t and 5$/t, respectively; X1.0DW and X5.0DW, xylanase pre-treatment followed by D_1-stage bleaching with a washing step between X and D_1 stages implemented; X1.0DU and X5.0DU xylanase pre-treatment followed by D_1 stage bleaching executed without a washing step between X and D_1 stages.

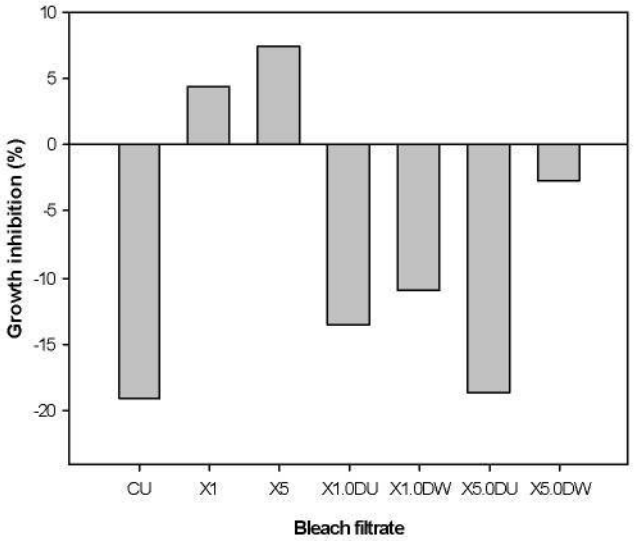

Figure 3.12. Toxic effect, as determined by the bacterial growth inhibition method, of filtrates obtained for X and D_1 stage (with or without inter-stage washing) during biobleaching of soda-aq pulp. Legend: CU, D_1 control without washing step; X1 and X5, xylanase applied at charges of 1$/t and 5$/t, respectively; X1.0DW and X5.0DW, xylanase pre-treatment followed by D_1-stage bleaching with a washing step between X and D_1 stages implemented; X1.0DU and X5.0DU xylanase pre-treatment followed by D_1 stage bleaching executed without a washing step between X and D_1 stages.

3.2.5. Conclusions

- Biobleaching with xylanase afforded reductions in sodium hypochlorite (32%) and sodium hydroxide (20%) when bleaching bagasse pulp in sequence X-CEH.
- Similar reductions of chlorine dioxide (30%) and sodium hydroxide (20%) were possible on soda-aq pulp during biobleaching in sequence O-X-D_1ED_2. Washing between X and D_1 steps had no impact on the final brightness of soda-aq pulp.
- Reduction in bleaching chemicals would not only reduce the impact of the pulp and paper waste waters on the environment, but also lead to cost saving for the industry. For instance, savings of 5.25 kg of ClO_2/t pulp and 1.4 kg NaOH/t pulp could be obtained as result of the enzyme bleaching. This can be translated into cost savings in excess of R2 million per annum.

- Overall improvements in filtrate quality and acceptable brightness levels could be achieved when xylanase pre-treatment was employed prior to bleaching. Because xylanase pre-treatment increased the brightness of pulp, lower concentrations of chlorine containing compound were required to reach acceptable pulp brightness. In addition, the reduction of the amount of chlorinated compounds would minimize also their teratogenic effect.
- No bacterial growth inhibition was noted when the filtrate from the xylanase pre-treatment step was examined, furthermore the AOX and chloride levels were also very low.
- The use of chlorine containing chemicals during bleaching was the major cause of bacterial growth inhibition. Overall, the AOX content correlated well with the bacterial growth inhibition caused by the bleach filtrates.
- Introduction of a washing step between X and D_1 stages during soda-aq bleaching might prevent problems such as the overloading of the plant waste water treatment system.
- The wash waters from the xylanase stage might be treated separately or even utilised for biotechnological applications. Alternatively, the X-stage COD can be sent to the recovery system of the mill where the organic matter will be utilized for heat generation and use in the mill.

CHAPTER 4
WASTE WATER BIO-UTILIZATION

4.1. Waste water utilization for xylanase production

4.1.1. Abstract

The spent sulfite liquor (SSL) waste water was characterised in terms of its chemical composition. Concentration of the SSL (SSLc) resulted in a significant increase in the total sugars in the waste water and a concomitant decrease in the acetic acid content of the SSL concentrate. HPLC analysis of sugars revealed that D-xylose was the main sugar component in the waste water. Cultivation of *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 using SSLc as carbon substrate showed that in addition to the sugars present in the SSLc, there was utilization of an additional carbon substrate in the SSLc-based medium. This finding was confirmed by the total organic carbon determination where up to 43 and 42 % of the total organic carbon utilised was from another carbon source. The highest xylanase activity of 322 U/ml was obtained with *Aspergillus foetidus* ATCC 14916 with xylan as carbon substrate. *Aspergillus oryzae* NRRL 3485 and *Aspergillus phoenicis* ATCC 13157 yielded activities of 164 and 146 U/ml, respectively, in the SSLc-based medium; these values were higher than those obtained on xylan and D-xylose with the same organism. The effect of SSLc pre-treatment by ultra filtration as well as overliming combined with heat was also investigated. Relatively high xylanase activities of up to 156 and 147 U/ml were obtained with *Aspergillus* oryzae NRRL 3485 and *Aspergillus phoenicis* ATCC 13157, respectively, in a medium based on the SSLc. Pretreatment of the SSLc by ultra filtration, overliming and heat resulted in even higher xylanase activities of up to 220 U/ml with *A. phoenicis*. These results indicated that the SSL concentrate, diluted ten-fold, constituted a suitable carbon feedstock for xylanase production by the *Aspergillus* species. Evaluation of culture pH resulted in considerably high xylanase activities of up to 290 and 199 U/ml with xylan and SSLc as carbon substrates, respectively at culture pH 7.5. The biomass concentrations, however, were lower than the biomass concentrations obtained under similar cultivation conditions at pH 6.0 or even at a lower cultivation pH of 4.0. No significant differences in xylanase

activities were observed at the different agitation rates, implying that the shear stress exerted at these agitation rates did not impact negatively on enzyme production. Preliminary fed-batch cultivations using SSLc as carbon substrate showed a two-fold increase in xylanase activities with possibilities of further increasing the activities considerably. Electrophoretic and zymogram analysis showed the presence of three xylanases having molecular weights of about 49, 34 and 30 kDa for *A. oryzae* where as *A. phoenicis* exhibited two xylanases having molecular weights of about 33 and 30 kDa. Crude xylanase preparations from the above *A. oryzae* and *A. phoenicis* strains grown in an SSLc-based medium exhibited optimal activities at pH 6.5 and 5.0 and at 65 and 55°C, respectively. The xylanolytic activity was stable at 50°C in a pH range of 4.5 to 10 for the *A. oryzae* strain. The crude xylanase preparations from *A. oryzae* NRRL 3485 grown with xylan and SSL as respective carbon substrates enhanced the brightness of hardwood pulp by 1.1 and 1.0 brightness points, respectively, whereas the xylanase of *A. phoenicis* ATCC 13157 enhanced pulp brightness by 1.3 and 1.5 points, respectively, compared to the control. Biobleaching of pulp with the xylanases from the *A. oryzae* and *A. phoenicis* strains grown with xylan and SSL as carbon substrates reduced the consumption of chlorine dioxide by 20 to 30 % without sacrificing brightness

4.1.2. Introduction

Spent sulfite liquor (SSL) results from the delignification of wood chips in an aqueous solution of acid bisulfites with an excess of SO_2, resulting in the solubilisation of lignin and leaving the wood cellulose largely undegraded. The main components of SSL include 50 to 65 % lignosulphonates, 15 to 22 % total sugars and 2 to 5 % volatile acids such as acetic acid (Pretorius and Lempert 1993).

There are few reports on xylanase production using pulp mill waste waters as carbon substrate. The use of SSL for enzyme production, particularly xylanases, has not previously been reported. SSL has been used as carbon feedstock for the production of yeast protein (McKee and Quicke 1977) as well as ethanol (Taherzadeh et al. 2003).

The present study evaluated the production of xylanases by fungi using SSL concentrated at a pulp mill as carbon feedstock. Conditions favouring increased levels of xylanase production were explored further in batch and fed-batch cultures. Xylanases were characterised and the efficacy of the crude xylanase preparations in the pre-bleaching of hardwood cellulose pulps was also determined.

4.1.3. Materials and methods

4.1.3.1. Characterization of spent sulfite liquor

The spent sulfite liquor waste waters investigated were the unconcentrated and concentrated liquors designated SSL and SSLc, respectively. The SSLc was concentrated at Sappi Saiccor, Umkomaas. The waste waters were characterised for their sugar composition using a Dionex 4000i HPLC (Dionex Corp., Sunnyvale, CA, USA) equipped with a pulse amperometric detector and gold electrode, using a 250 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. 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 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). Total polyphenols in the waste water and in the culture media were determined by the Prussian blue assay method (Graham, 1992). Total and suspended solids were determined by drying aliquots of the waste water and its sediment after centrifugation at 10 000 rpm for 10 min, respectively, to constant mass at 105°C.

4.1.3.2. Evaluation of fungal biomass using concentrated spent sulfite liquor as carbon feedstock

4.1.3.2.1. Fungal strains and cultivation conditions

Aspergillus oryzae NRRL 3485 and *Aspergillus phoenicis* ATCC 13157 were used. The SSLc, obtained in a concentrated form, was diluted ten-fold with distilled water and used

as carbon source . The following nutrients were also added (g/l): citric acid, 0.25; $(NH_4)_2SO_4$, 5; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 5; $CaCl_2.2H_2O$, 0.02; yeast extract, 10 and 1 ml of a trace element solution (Du Preez and van der Walt 1983). D-Xylose was used as the control carbon substrate at concentrations of 1, 3, 5, 8, and 10 g/l. All media were adjusted to pH 6.0 prior to autoclaving. The inoculum was prepared by inoculating each of 500 ml Erlenmeyer flasks containing 100 ml of the appropriate medium with a 5 ml of a fungal spore suspension (ca. 10^7 spores/ml) washed from a 5-day old Sabouraud dextrose agar plate culture with 10 ml of a 0.05 M KH_2PO_4 solution containing 0.1 % (v/v) Tween 80. These flasks were incubated at 210 rpm on a rotary shaker at 30°C for 48 h and 5 ml transferred to each of a second group of shake flasks containing the same medium. Biomass concentrations were determined over an extended incubation period of up to 100 h

4.1.3.2.2. Effect of pH on suspended solids in the concentrated spent sulfite liquor

The SSLc-based medium was adjusted to pH values of 2, 3, 4, 5, 6, 7, 8 and 9 using either a 3 M solution of KOH or H_2SO_4. Triplicate 20 ml aliquots of the pH adjusted samples were centrifuged, washed twice with equal volumes of distilled water and dried at 105°C to constant mass. Suspended solids concentrations were determined by weighing the precooled oven dried samples.

4.1.3.2.3. Determination of fungal biomass cultivated on concentrated spent sulfite liquor-based medium

Two different sets of experiments were set up to evaluate biomass production in the SSLc-based medium. The dry biomass concentration of triplicate 5 ml aliquots of the respective cultures were washed prior to gravimetric determination as above. A sterile control flask was also included which was sampled in parallel to the culture flasks. The control samples were adjusted to the pH of the culture samples at the time of sampling and these were treated in a similar manner as the culture samples. The control samples were used to correct for suspended solids in the cultivation medium at the time of sampling.

The second set of experiments involved sampling triplicate 5 ml aliquots of the respective cultures over time, centrifuging and washing the samples twice with an equal volume of a 0.05 M pH 4.0 acetate buffer and drying to constant mass at 105°C. Again, a sterile control flask was treated in a similar manner and used to correct for suspended solids in the culture medium.

4.1.3.2.4. Analytical methods

The sugar composition of the culture supernatants was determined using a Dionex 4000i HPLC (Dionex Corp., Sunnyvale, CA, USA) equipped with a pulse amperometric detector and gold electrode, using a 250 x 4 mm (I.D.) PA1 Carbopac column (Dionex Corp.) with inlet and oven temperatures of 30C and 1.8 mM NaOH as eluent at a flow rate of 1 ml/min. The chemical oxygen demand (COD) was determined by a standard spectrophotometric method (HACH Co. 1997) and total polyphenols were determined by the Prussian blue assay method (Graham 1992). The total organic carbon (TOC) of the SSLc-based media before and after cultivation was determined by the Institute of Groundwater Studies (University of the Free State, Bloemfontein).

4.1.3.3. Xylanase production with concentrated spent sulfite liquor as carbon substrate in shake flask cultures

4.1.3.3.1. Fungal strains

The fungi used in this study included six *Aspergillus* strains (*A. oryzae* NRRL 1808 and NRRL 3485, *A. niger* ATCC 10864, *A. foetidus* ATCC 14916, *A. phoenicis* ATCC 15555 and ATCC 13157) and *Gliocladium viride* CBS 658.70. The cultures were maintained on Sabouraud-dextrose agar slants (Biolab Diagnostics, Midrand, South Africa) and stored at 4°C with subculturing at 12-week intervals.

4.1.3.3.2. Media and cultivation conditions

The SSL was supplied in a concentrated form and had been concentrated approximately five-fold by a three-step evaporation process. The respective carbon sources used included the concentrated SSL (SSLc) diluted ten-fold with distilled water to give 11 to

13 g total sugars /l (depending on the SSLc batch), oat spelts xylan (10 g/l; Sigma Chemical Co., St. Louis, MO, USA) and D-xylose (10 g/l; Sigma). Other medium constituents included were (g/l): citric acid, 0.25; $(NH_4)_2SO_4$, 5; K_2HPO_4, 5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.02 and yeast extract, 10 as well as 1 ml of a trace element solution (du Preez and van der Walt 1983). These media were adjusted to pH 6.0 prior to autoclaving. The inoculum was prepared by inoculating each of 500 ml Erlenmeyer flasks containing 100 ml of the appropriate medium with 5 ml of a fungal spore suspension (ca. 10^7 spores/ml) washed from a 5-day old Sabouraud dextrose agar plate culture with 10 ml of a 0.05 M KH_2PO_4 solution containing 0.1 % Tween 80. These flasks were incubated at 210 rpm on a rotary shaker at 30°C for 48 h and 5 ml transferred to each of a second group of shake flasks containing the same medium. Xylanase activity and biomass concentrations were determined after a 5-day incubation period.

4.1.3.3.3. Analytical methods

The sugar composition of the SSLc and culture supernatants was determined using a Dionex 4000i HPLC (Dionex Corp., Sunnyvale, CA, USA) equipped with a pulse amperometric detector and gold electrode, using a 250 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. 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 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.

Cellulase activity in the culture supernatants was determined using the filter paper method with D-glucose as standard (Ghose 1987). Xylanase activity was determined using the dinitrosalicylic acid (DNS) assay method with birchwood xylan (Sigma) as substrate (Bailey et al. 1992). The fungal biomass concentration was determined gravimetrically after centrifugation of triplicate 5-ml aliquots of the respective cultures, washing twice with equal volumes of distilled water and drying at 105°C.

4.1.3.4. Pretreatment of spent sulfite liquor for xylanase production

4.1.3.4.1. Pretreatment of spent sulfite liquor

The waste waters investigated were the unconcentrated SSL and concentrated SSL (concentrated at Sappi Saiccor, Umkomaas, South Africa). These waste waters were subjected to two different pretreatment procedures, namely, by ultra filtration and by overliming combined with heating. The unconcentrated SSL and concentrated SSL (SSLc) waste waters were filtered through a cross-flow ultra filtration system equipped with a Prep/Scale-TFF (SD031) cartridge (Millipore, Millipore Corporation, Bedford, MA, USA) with an approximate molecular weight cut-off point of 30 000. The ultrafiltered SSL concentrate was designated as UFSSLc.

The overliming and heat pretreatment was conducted as described by Nigam (2001). The samples were boiled for 15 min and overlimed with $Ca(OH)_2$ up to pH 10 ± 0.1. The precipitates were removed by filtration and reacidified to pH 6.5 using 2 N H_2SO_4 followed by filtration. Samples subjected to this pretreatment procedure included the SSLc and UFSSLc which were designated OHSSLc and OHUFSSLc, respectively.

4.1.3.4.2. Microorganisms

The selected fungal strains were *Aspergillus oryzae* NRRL 3485 and *Aspergillus phoenicis* ATCC 13157. The cultures were maintained on Sabouraud-dextrose agar slants (Biolab, Biolab Dioagnostics, Midrand, South Africa) and stored at 4°C with subculturing at 12-week intervals.

4.1.3.4.3. Xylanase production

The above fungal strains were evaluated for xylanase production using xylan and SSLc as carbon substrates. In addition to the carbon substrate, the media also comprised (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 element solution.

These fungal strains were further evaluated for xylanase production using the SSL waste waters that had been subjected to the pre-treatment procedures as described above. All

media were adjusted to an initial pH value of 6.0 prior to autoclaving. Shake flask cultures were incubated on a rotary shaker at 30°C for 5 days during which aliquot samples were collected for xylanase assays.

4.1.3.4.4. Analytical procedures

The sugar composition of the SSLc and culture supernatants was determined using a Dionex 4000i HPLC (Dionex Corp., Sunnyvale, CA, USA) equipped with a pulse amperometric detector and gold electrode, using a 250 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.. The colour (true colour) of the unfiltered and ultra filtered waste water was determined using a standard spectrophotometric method described in the HACH DR/2000 spectrophotometer handbook (HACH Company, Loveland, Colorado, USA).

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 birch wood xylan (Sigma Chemical Co, St Louis, MO, USA) as substrate.

4.1.3.5. Xylanase production in batch cultures: Effect of culture pH

4.1.3.5.1. Cultivation conditions

The strain *Aspergillus oryzae* NRRL 3485 was evaluated for xylanase production under different cultivation pH values with concentrated SSL (SSLc; diluted twenty-fold with distilled water) as carbon source using a 15 l Biostat C STR (B. Braun Biotech International) with a working volume of 8.5 l. After *in situ* sterilisation of the bioreactor containing 8 l medium, the pH was adjusted to the desired cultivation pH and a 500 ml mycelial inoculum was added. The cultivation pH evaluated included pH 4.0, 5.0, 6.0, 7.0, 7.5 and 8.0. The dissolved oxygen in the medium was monitored with a polarographic pO_2 electrode (Mettler, Toledo, Halstead, UK) and maintained at or above 30 % of saturation by increasing the air flow rate, which was varied using an aeration rate from 0.03 to 8 l.min-1, and automatic adjustment of the stirrer speed between 400 and 800 $r.min^{-1}$. The pH was monitored and maintained at the pH set point by automatic

titration with 5 N KOH or 5 N H_2SO_4. The essence of diluting the SSLc 20-fold was to reduce the sugar concentration so that the biomass concentration obtained on the diluted SSLc would be comparable to biomass concentrations obtained with the xylan-based medium.

4.1.3.5.2. Cultivation medium

The bioreactor medium comprised (l^{-1}): 0.25 g citric acid, 10 g yeast extract, 5 g $(NH_4)_2SO_4$, 5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 1 ml trace element solution and 1 ml Dow corning 1520 Silicone antifoam. All cultivations were carried out at 30°C over 60 or 120 h during which xylanase activity assays, biomass, sugar and acetic acid determinations were conducted. The prolonged incubation times were used to generate sufficient data on the time-course of enzyme production. However, for the practical implementation of the process, significantly lower incubation periods would be required as xylanase activity peaks at the 40-60 h of incubation time.

4.1.3.5.3. Analyses

sugar composition of the culture supernatants was determined using a Dionex 4000i HPLC (Dionex Corp., Sunnyvale, CA, USA) equipped with a pulse amperometric detector and gold electrode, using a 250 x 4 mm (I.D.) PA1 Carbopac column (Dionex Corp.) with inlet and oven temperatures of 30C and 1.8 mM NaOH as eluent at a flow rate of 1 ml/min. 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 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.

Xylanase activity was determined using the dinitrosalicylic acid (DNS) assay method with birchwood xylan (Sigma) as substrate (Bailey et al. 1992). The fungal biomass concentration was determined gravimetrically after centrifugation of triplicate 5-ml aliquots of the respective cultures, washing twice with equal volumes of distilled water and drying at 105°C.

4.1.3.6. Xylanase production in batch cultures: Effect of agitation rate

4.1.3.6.1. Cultivation conditions

The strain *Aspergillus oryzae* NRRL 3485 was evaluated for xylanase production with concentrated SSL (diluted forty-fold with distilled water) as carbon source using a 15 l Biostat C STR (B. Braun Biotech International) with a working volume of 8.5 l. The parameter evaluated was the agitation rate and the stirrer speeds evaluated included 300, 400, 500, 600 and 800 rpm. The cultivation pH was monitored and maintained at pH 7.5 by automatic titration with 5 N KOH or 5 N H_2SO_4. The dissolved oxygen tension (DOT) of the medium was monitored with a polarographic pO_2 electrode (Mettler, Toledo, Halstead, UK) and maintained at or above 25 % of saturation by increasing the air flow rate, which was varied using an aeration rate from 0.05 to 8 l/min.

4.1.3.6.2. Cultivation medium

The bioreactor medium, in addition to the forty-fold diluted SSLc, comprised (/l): 0.25 g citric acid, 10 g yeast extract, 5 g $(NH_4)_2SO_4$, 5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 1 ml trace element solution and 1 ml Dow corning 1520 Silicone antifoam. All cultivations were carried out at 30°C over a three-day period during which xylanase activity assays and biomass determinations were conducted.

4.1.3.6.3. Analyses

Xylanase activity was determined using the dinitrosalicylic acid (DNS) assay method with birchwood xylan (Sigma) as substrate (Bailey et al. 1992). The fungal biomass concentration was determined gravimetrically after filtration of duplicate aliquot samples, washing twice with equal volumes of distilled water and drying at 105°C to constant mass.

4.1.3.7. Xylanase production in fed-batch cultures: Effect of xylose concentration

4.1.3.7.1. Cultivation conditions

The strain *Aspergillus oryzae* NRRL 3485 was evaluated for xylanase production with concentrated SSL (diluted forty-fold with distilled water) as carbon source using a 15 l Biostat C STR (B. Braun Biotech International). The initial medium volume used for the

cultivation was 5 l. The cultivation temperature was 30°C and the culture pH was monitored and maintained at pH 7.5 by automatic titration with 5 N KOH or 5 N H_2SO_4. The dissolved oxygen in the medium was monitored with a polarographic pO_2 electrode (Mettler, Toledo, Halstead, UK) and maintained at or above 25 % of saturation by increasing the air flow rate, which was varied using an aeration rate from 0.03 to 8 l.min-1, and automatic adjustment of the stirrer speed between 200 and 800 rpm. Feed addition was registered by placing the feed vessel on a balance. The feed period was started upon initial substrate depletion, indicated by the commencement of automatic acid addition to the medium for pH control. The feed was then added according to a predetermined constant feed flow of 120 g/l.

4.1.3.7.2. Cultivation medium

The batch medium, in addition to the forty-fold diluted SSLc, comprised (/l): 0.25 g citric acid, 10 g yeast extract, 5 g $(NH_4)_2SO_4$, 5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 1 ml trace element solution and 1 ml Dow corning 1520 Silicone antifoam. In the feed, however, the SSLc was diluted five-fold so as to have a substantial amount of sugars in the inflow to allow for a reasonable feed flow rate.

4.1.3.7.3. Analyses

Xylanase activity was determined using the dinitrosalicylic acid (DNS) assay method with birchwood xylan (Sigma) as substrate (Bailey et al. 1992).

4.1.3.8. Partial characterization of xylanases

4.1.3.8.1. Media and cultivation conditions

Aspergillus oryzae NRRL 3485 and *Aspergillus phoenicis* ATCC 13157 were grown on xylan and SSLc-based media for the production of xylanase. Other medium constituents added to the media included (g/l): citric acid, 0.25; $(NH_4)_2SO_4$, 5; K_2HPO_4, 5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.02 and yeast extract, 10 as well as 1 ml of a trace element solution (du Preez and van der Walt 1983). All media were adjusted to pH 6.0 prior to autoclaving. The inoculum was prepared by inoculating each of 500 ml Erlenmeyer flasks containing 100 ml of the appropriate medium with 5 ml of a fungal

spore suspension (ca. 10^7 spores/ml) washed from a 5-day old Sabouraud dextrose agar plate culture with 10 ml of a 0.05 M KH_2PO_4 solution containing 0.1 % Tween 80. These flasks were incubated at 210 rpm on a rotary shaker at 30°C for 48 h and 5 ml transferred to each of a second group of shake flasks containing the same medium which were then incubated on a rotary shaker at 30°C over a five-day period during which samples for xylanase activity determination were collected.

4.1.3.8.2. Effect of pH and temperature on activity of xylanases

Xylanase activity was determined using the dinitrosalicylic acid (DNS) assay method with birchwood xylan (Sigma) as substrate (Bailey et al. 1992). Different reaction buffers (0.05 M citrate for pH 4.0, 5.0, 5.5, 6.0; 0.1 M phosphate for pH 6.5, 7.0, 8.0; and 0.1 M carbonate-for pH 9.0, and 10.0) were used to assay for xylanase activity at 50°C to determine the effect of pH on xylanase activity. The temperature optimum was established by assaying for xylanase activity at different reaction temperatures (40 to 70°C) in 0.05 M citrate buffer (pH 6.0). To asses the pH stability of the xylanases, the enzyme was diluted in appropriate buffers of different pH (as mentioned above) and incubated at 50°C. Samples were removed at 30 min time intervals and the residual xylanase activity assayed at pH 6.0 and 50°C. The thermal stability of the xylanases was determined using 0.05 M citrate buffer (pH 6.0). The diluted enzyme was incubated at different temperatures and the residual activity measured at different time periods. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar (xylose equivalents) per minute of reaction time.

4.1.3.8.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Extracellular proteins were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using a 12.5 % polyacrylamide gel. Prior to loading, the protein samples were incubated at 37°C for 1 h. After electrophoresis, the protein bands were visualised by staining with 0.2 % Coomassie Brilliant Blue R250. SDS-PAGE molecular weight standards, broad range (BIO-RAD, Bio-Rad laboratories, 2000 Alfred Nobel Drive, Hercules CA 94547, USA) were used as marker.

4.1.3.8.4. Zymogram analysis

Zymogram samples were subjected to electrophoresis in a 12.5 % SDS-PAGE containing 0.1 % Remazol Brilliant Blue R-D-xylan (RBB xylan, Sigma). After electrophoresis the gel was washed twice for 1 h each in 0.02 M Tris-HCL buffer at pH 7.5 containing 0.7 % (w/v) Triton X-100 followed by a 20 min washing in the same buffer without Triton X-100. After washing the gel was incubated at 50°C in 0.05 M citrate buffer (pH 6.0) until clearing zones were observed. The zymogram was prepared by soaking the gel in 0.1 % Congo red solution for 15 min followed by destaining with 1 M NaCl. To visualise the xylanase active bands, the gel was soaked in 5 % acetic acid which contrasted the active bands against a dark blue background.

4.1.3.9. Evaluation of xylanases in bleaching of pulp

Oxygen-delignified soda-aq pulp was evaluated for biobleaching using xylanases produced by *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 with xylan and spent sulfite liquor concentrate (SSLc) as carbon substrates. The shake flask supernatants of the above strains served as crude xylanase preparations that were applied to the pulp. The xylanase of *A. phoenicis* was applied at an enzyme charge of 10 U/g dry pulp equivalent at pH 6.0 and 50°C, whereas pH 7.0 and 70°C was used in the case of the xylanase of *A. oryzae*. A pulp consistency of 10 % was maintained and the aliquots were incubated for 2 h. Pulp samples were chemically bleached after treatment with enzyme using the D_1ED_2 bleaching sequence (D_1 and D_2, chlorine dioxide, 2.63 and 1.315 % active chlorine, respectively, w/w; E, alkali extraction with 0.7 % NaOH, w/w). Subsequently, hand sheets were prepared and brightness measured in triplicate using a brightness meter (Technidyne Colour Touch 2 model ISO, Technidyne Corporation, New Albany, Indiana, USA).

4.1.4. Results and discussion

4.1.4.1. Characterization of spent sulfite liquor

The waste water had a high total sugar concentration with D-xylose constituting over 80 % of the total sugars in both the SSL and the concentrated SSL (SSLc). Other sugars present in smaller amounts included arabinose, galactose and glucose that collectively

amounted to less than 20 % of the total sugars (Table 4.1). Concentrating the SSL resulted in only a slight increase in the acetic acid content, indicating that a major portion of the initial acetic acid was lost through the evaporation process. As result of the concentration process, a 5.0-fold and 5.2-fold increase in the content of total sugars and total solids, respectively, was observed with a corresponding 5.5-fold increase of COD (Table 4.1). Due to the high total solids content, the SSLc was quite viscous. Total polyphenols also increased dramatically as a result of the SSL concentration, up to 139 g/l. The use of sulphuric acid during the cooking process renders the SSL a highly acidic waste water, as can be seen from the pH of both the SSL and SSLc.

Table 4.1. Composition and chemical properties of spent sulfite liquor[a]

Property	SSL	SSLc
Xylose (g/l)	23.6	119.0
Glucose g/l)	2.2	10.9
Arabinose (g/l)	0.9	4.2
Galactose (g/l)	2.4	11.1
Total sugars (g/l)	29.1	145.2
Acetic acid (g/l)	10.32	12.55
COD (mg/l)	2.0×10^5	1.1×10^6
Suspended solids (g/l)	10	18
Total solids (g/l)	145	753
Total polyphenols (g/l)	14	139
pH	2.7	3.3

[a] Mean values of duplicate determinations are shown
SSL Spent sulfite liquor
SSLc Spent sulfite liquor concentrate;
COD Chemical oxygen demand

4.1.4.2. Evaluation of fungal biomass

4.1.4.2.1. Biomass production with xylose as carbon substrate

Figures 4.1 and 4.2 show the biomass concentrations of *A. oryzae* and *A. phoenicis*, respectively, in a semi-defined medium containing yeast extract at different D-xylose

concentrations. Extrapolation of the regression lines to the Y-axes gives the values for the biomass yield coefficients on yeast extract. The values obtained by extrapolating the regression lines were 0.39 and 0.31 g/g yeast extract for *A. oryzae* and *A. phoenicis*, respectively. These correlate to the experimentally determined yield coefficients with yeast extract as carbon source, 0.38 and 0.34 g/g yeast extract for *A. oryzae* and *A. phoenicis*, respectively. These results indicate that the gravimetric biomass assay was fairly accurate despite the difficulty in obtaining homogeneous samples of a filamentous fungus in a liquid culture medium.

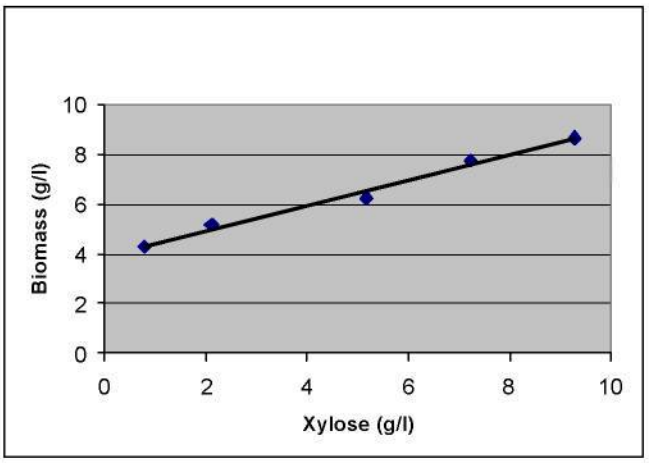

Figure 4.1. Biomass concentrations of *Aspergillus oryzae* NRRL 3485 as a function of xylose concentration

4.1.4.2.2. Effect of pH on suspended solids in the concentrated spent sulfite liquor

Results shown in Figure 4.3 indicate that the solubility of suspended solids in the SSLc were pH dependent. The solids were most soluble between pH values of 3.0 and 4.0, with higher concentrations of sediment solids found at lower and higher pH values. This finding suggested that during cultivation of the above fungal strains on SSLc in shake flasks, the change in pH of the medium could lead to an overestimation in biomass concentrations if not corrected for the change in the solubility of the suspended solids concomitant with the changing pH. Hence, two experiments were designed as described in the materials and methods in an attempt to clarify this aspect.

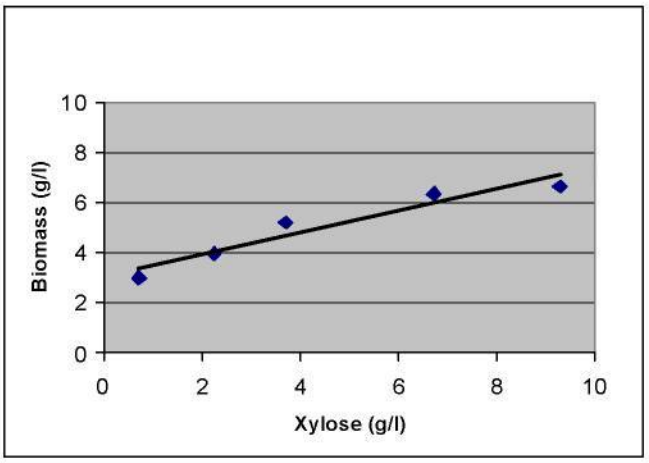

Figure 4.2. Biomass concentrations of *Aspergillus phoenicis* ATCC 13157 as a function of xylose concentration

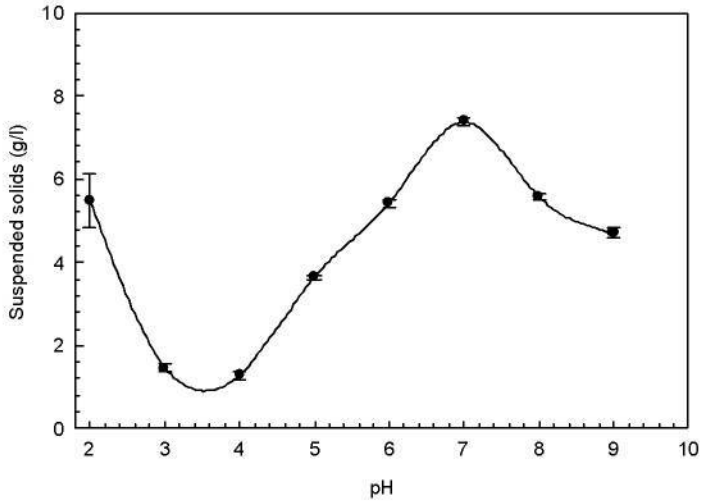

Figure 4.3. Effect of pH on the suspended solids content in the SSLc-based medium

4.1.4.2.3. Effect of pH adjustment on biomass concentration

The pH of the control medium was adjusted to the pH of the respective culture at time of sampling and the effect on biomass concentration was evaluated. Figure 4.4 shows the biomass concentration, pH and sugar utilization profiles obtained for the two strains. The pH profile of the *A. oryzae* culture increased from an initial value of pH 4.9 to a final value of pH 7.6, with a final biomass concentration of 23.6 g/l. The pH profile of the *A. phoenicis* culture, however, decreased from an initial value of pH 4.8 to a final value of

pH 3.4, with a final biomass concentration of 22.4 g/l. Figure 4.3 suggests that outside the range of pH 3.0 the concentration of suspended solids in the medium would increase. It follows, therefore, that adjusting the pH of the control medium to that of the culture would compensate for this increase in suspended solid concentration in the medium, thereby yielding a more accurate biomass value by subtracting the mass of the control. However, the biomass concentrations obtained using the above procedure did not differ significantly from previously obtained biomass values where this procedure was not applied, 22.6 and 22 g/l with *A. oryzae* and *A. phoenicis*, respectively. It was determined (data not shown) that the dry mass of the control only increases significantly after 33 h (indicative of an increase in the suspended solids in the medium). At this point, however, the biomass concentration in the culture medium is already at its maximum. A significant increase in the pH of the culture medium is also observed only after 33 h of cultivation, correlating to the increase in the dry mass of the control after this period. Taking this into consideration, the increase in suspended solids, as shown in Figure 4.3, only takes effect after the 33 h time period.

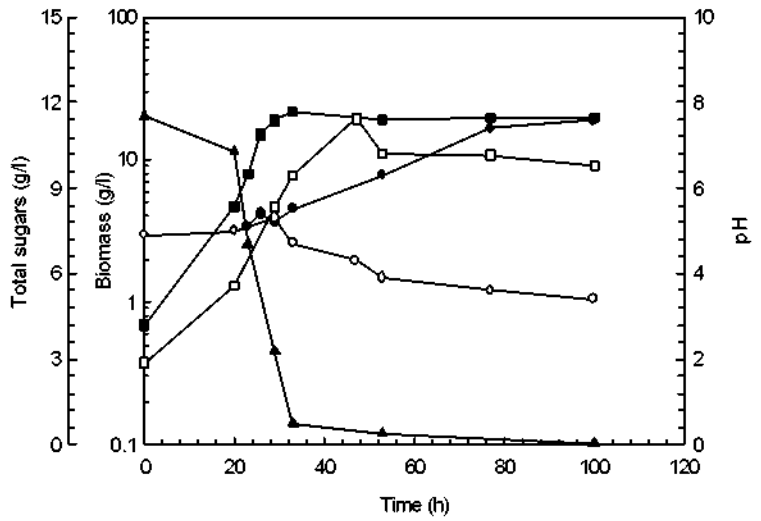

Figure 4.4. Cultivation profiles of *A. oryzae* NRRL 3485 (closed symbols) and *A. phoenicis* ATCC 13157 (open symbols) where the pH of the SSLc-based control medium was adjusted to that of the respective culture flask at time of sampling. Symbols: ¦ and ?, biomass concentration; ? and ?, pH; ? total sugars.

4.1.4.2.4. Effect of acetate buffer on biomass concentration

The rationale of washing the biomass with pH 4.0 acetate buffer followed the suspended solids profile shown in Figure 4.3. Washing the biomass with the buffer was expected to maintain the pH of the culture and control media at an average pH of 4.0 and thus increase the solubility of the suspended solids and minimize the adsorption of solids onto the biomass. Figure 4.5 shows that biomass concentrations of 22.3 and 24.5 g/l were obtained with *A. oryzae* and *A. phoenicis*, respectively. These values were comparable to those obtained in the study above where the pH of the control was adjusted to that of the culture at time of sampling. Therefore, washing the biomass with the pH 4.0 buffer had no significant influence on the values of biomass concentration.

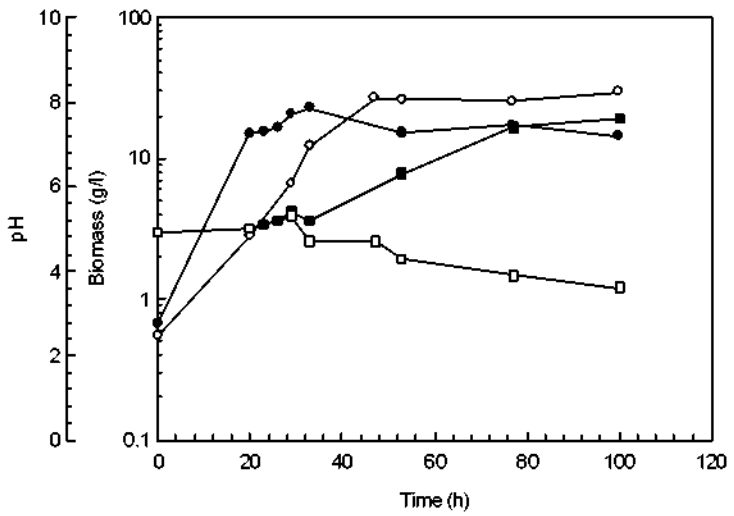

Figure 4.5. Cultivation profiles of *A. oryzae* NRRL 3485 (closed symbols) and *A. phoenicis* ATCC 13157 (open symbols) where samples from the control and culture flasks were washed with pH 4.0 acetate buffer at time of sampling. Symbols: ¦ and ?, biomass concentration; ? and ?, pH.

The above biomass concentrations translated into impossibly high biomass yield coefficients when calculated from the average amount of total sugars in the medium of 11.53 g/l. Taherzadeh et al (2003) investigated biomass production by *Rhizopus oryzae*

with SSL and with a semi-defined medium comprising the sugars D-xylose, galactose, glucose, mannose and arabinose as carbon sources. A biomass yield coefficient of 0.43 g/g sugar was obtained with the SSL, which was higher than the yield coefficient of 0.18 g/g sugar obtained with the semi-defined medium and was even higher (0.55 g/g sugar) when the organism was cultivated in a bioreactor with SSL as carbon source. These results follow a similar trend as to what was obtained in this study, although the yields obtained here were much higher. The biomass yields in terms of COD were also high, namely 0.95 (± 0.3) and 0.93 (± 0.4) g/g COD for *A. oryzae* and *A. phoenicis*, respectively. These yields were significantly higher than those obtained by Pretorius and Lempert (1993b) who reported a yield of 0.7 g/g COD for a strain of *A. fumigatus* with SSL as carbon substrate. To verify the high biomass concentrations, a protein assay method (Biurrete assay method) was attempted. This method was, however, not successful due to the dark colour of the cultivation medium.

4.1.4.2.5. Determination of total organic carbon

To investigate the utilization of additional carbon source present in the SSLc, a determination of total polyphenols as well as total organic carbon (TOC) was conducted. Results obtained for the determination of total polyphenols were not conclusive and this could be attributed to the waste water colour, as the procedure is a colorimetric procedure. On the other hand, TOC results, shown in Table 4.2, revealed that besides the sugars, there was utilization of other carbon sources in the SSLc-based medium, up to 4 g/l TOC, which was about 43 and 42 % of the total utilised TOC for *A. oryzae* and *A. phoenicis*, respectively. All the total sugars were completely utilised at the end of cultivation and a conversion of total sugars utilised into total organic carbon utilised showed that 57 and 58 % of the TOC was utilised by *A. oryzae* and *A. phoenicis*, respectively.

4.1.4.3. Xylanase production in shake flask cultures

The SSLc had a high total sugar concentration of up to 145.2 g/l with D-xylose constituting over 80 % of the total sugars (119 g/l). Other sugars present in smaller amounts included arabinose, galactose, mannose and glucose that collectively amounted

to less than 20 % of the total sugars. The acetic acid concentration of the waste water was 12.5 g/l. All of the strains exhibited negligible cellulase activity with xylan or SSLc as carbon feedstock. Xylan is known to be a good inducer of xylanase production (Leathers et al. 1986; Bahkali 1996; Siedenburg et al. 1998); accordingly, high xylanase activities were obtained on xylan as carbon substrate (Table 4.3). On xylan, the highest xylanase activity of 322 U/ml was obtained with *Aspergillus foetidus* ATCC 14916 (Table 4.3). The SSLc induced xylanase production in all of these fungal strains, with major strain-specific differences evident. Only strains *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 exhibited significantly higher xylanase activities on SSLc than on xylan (Table 4.3). D-xylose effected no or little induction of xylanase activity.

Table 4.2. Determination of total organic carbon (g/l) in the SSLc-based medium before and after cultivation of the *Aspergillus* strains

TOC_i	TOC_t	TOC_u	Total $carbon_i$ (sugars)	Total $carbon_t$ (sugars)	Additional $carbon_t$
Aspergillus oryzae NRRL 3485					
33.8	24.5	9.3	5.3	0	4.0
Aspergillus phoenicis ATCC 13157					
33.5	23.9	9.6	5.6	0	4.0

TOC_i	Initial total organic carbon in SSLc-based medium
TOC_t	Total organic carbon in SSLc-based medium after cultivation
TOC_u	Total organic carbon utilised during cultivation period
Total $carbon_i$	Initial total sugars in SSLc-based medium determined by HPLC converted to carbon equivalent
Total $carbon_t$	Concentration of total sugars determined by HPLC after cultivation converted to carbon equivalent
Additional carbon	Difference between total organic carbon utilised and total carbon sugars in SSLc-based medium

Figure 4.6 depicts typical xylanase production profiles of these two strains grown on xylan and SSLc as respective carbon sources. Total sugars in the SSLc-based medium were readily utilised and after 24 h of cultivation the total sugar concentration had decreased to below 0.2 g/l. The acetic acid was depleted after 72 and 48 h of cultivation

of *A. oryzae* and *A. phoenicis*, respectively. With *A. oryzae*, the pH of the media with xylan and SSLc as carbon substrates increased to final values of pH 8.3 and 7.8, respectively (Figure 4.6A). Growth of *A. phoenicis* resulted in the pH of the xylan-based medium decreasing to pH 3.8, whereas in the SSLc-based medium it increased to pH 6.2 (Figure 4.6B).

Table 4.3. Xylanase activities (U/ml)[a] produced by selected fungi on different carbon sources

Fungus	Carbon substrate		
	SSLc	Xylan	Xylose
Aspergillus oryzae NRRL 3485	164 (± 17.9)	96 (± 9.8)	27 ± 3.4
Aspergillus oryzae NRRL 1808	50 (± 10.2)	130 (± 19.0)	4 (± 1.2)
Aspergillus phoenicis ATCC 13157	146 (± 21)	77 (± 11.2)	21 (± 1.6)
Aspergillus phoenicis ATCC 15555	11 (± 1.2)	42 (± 6.3)	0
Aspergillus foetidus ATCC 14916	32 (± 10.6)	322 (± 33.1)	9 (± 2.0)
Aspergillus niger ATCC 10864	7 (± 1.6)	31 (± 12.6)	3 (± 0.9)
Gliocladium viride CBS 658.70	10 (± 3.0)	23 (± 5.2)	0

[a] Mean values and standard deviations (in brackets) of four experiments
SSLc Spent sulfite liquor concentrate, diluted ten-fold

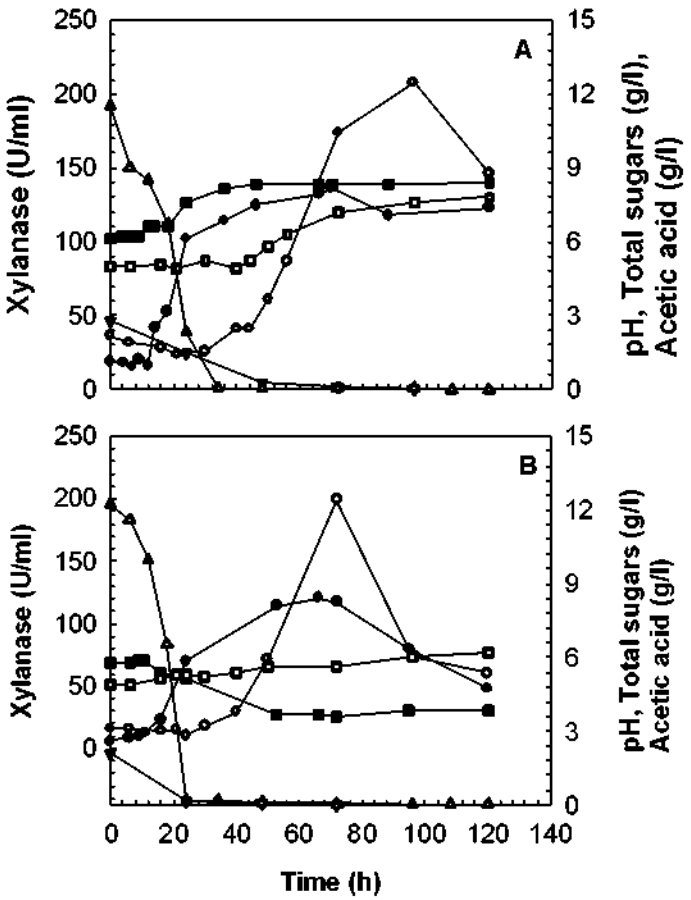

Figure 4.6. Typical cultivation profiles of *A. oryzae* NRRL 3485 (A) and *A. phoenicis* ATCC 13157 (B) with xylan (closed symbols) and SSLc (open symbols) as the respective carbon sources in shake flask cultures at 30°C. Symbols: ● and ○, xylanase activity; ■ and □, pH; △, total sugars; ▽, acetic acid.

The growth parameters of the above two strains are shown in Table 4.4. Both strains produced significantly higher xylanase activities with SSLc than with xylan as carbon substrate. However, from the values of the xylanase yield per g biomass ($Y_{p/x}$, Table 4.4), it is evident that xylan was a stronger inducer of xylanase production. Both strains gave higher maximum volumetric rates of xylanase production when grown in the SSLc medium than on xylan as carbon substrate, probably due to the higher biomass

concentrations reached in the SSLc-based medium.

Table 4.4. Growth parameters[a] of *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 in shake flask cultures with xylan and SSLc[b] as respective carbon substrates

Growth Parameter	*A. oryzae*		*A. phoenicis*	
	Xylan	SSLc	Xylan	SSLc
Xylanase (U ml^{-1})	96 (± 9.78)	164 (± 17.90)	77 (± 11.18)	146 (± 20.99)
Cellulase (FPUs[c])	0.1 (± 0.002)	0.5 (± 0.002)	0.2 (± 0.001)	0.3 (± 0.003)
Biomass (g l^{-1})	6.6 (± 0.74)	20.76 (± 2.51)	6.58 (± 0.73)	19.55 (± 1.76)
Q_p^{max} (U ml^{-1} h)	1.87 (± 0.22)	4.50 (± 0.69)	0.87 (± 0.25)	4.48 (± 0.52)
$Y_{p/x}$ (U g^{-1})	14 550 (± 980)	7 900 (± 560)	11 700 (± 1000)	7 470 (± 600)

[a] Mean values and standard deviations (in brackets) of four experiments
[b] Spent sulfite liquor concentrate, diluted ten-fold
[c] Filter paper units
Q_p^{max} Maximum volumetric rate of xylanase production, calculated from the maximum slope of the enzyme activity vs time curve
$Y_{p/x}$ Xylanase yield coefficient, U/g biomass

4.1.4.4. Effect of pretreatment of spent sulfite liquor

4.1.4.4.1. True colour of waste waters

Values for the true colour of the untreated and pretreated concentrated SSL waste waters are shown in Table 4.5. The UFSSLc had the lowest colour intensity indicating that ultra filtration of the waste water significantly decreased the waste water colour. The SSLc had the highest colour intensity, showing that the colour intensity of the waste water increases substantially during the concentration process.

Table 4.5. True colour of the various SSL waste waters (untreated and pretreated). Mean values of triplicate determinations are shown

Waste water	True colour (PCU)
SSL	5.3×10^4
SSLc	1.8×10^5
UFSSLc	3.5×10^4

PCU Platinum Colour Unit
SSL unconcentrated SSL waste water
SSLc concentrated SSL waste water
UFSSLc concentrated ultra filtered SSL waste water

4.1.4.4.2. Ultrafiltration

The SSLc as well as the UFSSLc (ultra filtered SSLc) were evaluated for xylanase production using *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157. The results showed that *A. oryzae* and *A. phoenicis* yielded comparable xylanase activities on the SSLc and UFSSLc of up to 156 and 171 U/ml, respectively for *A. oryzae* and 158 and 157 U/ml, respectively for the *A. phoenicis* strain (Figure 4.7). Significantly higher xylanase activities were obtained with the SSLc and UFSSLc-based media than with xylan as carbon substrate. Overall, the xylanase yields in shake flasks obtained with both *A. oryzae* and *A. phoenicis* on SSL-based growth media were about 2-fold higher than those on produced on xylan-based media. This indicates that the SSL has the capacity to induce xylanase production at high levels which provides a solid base for a potential scale-up and use in the pulp and paper industry.

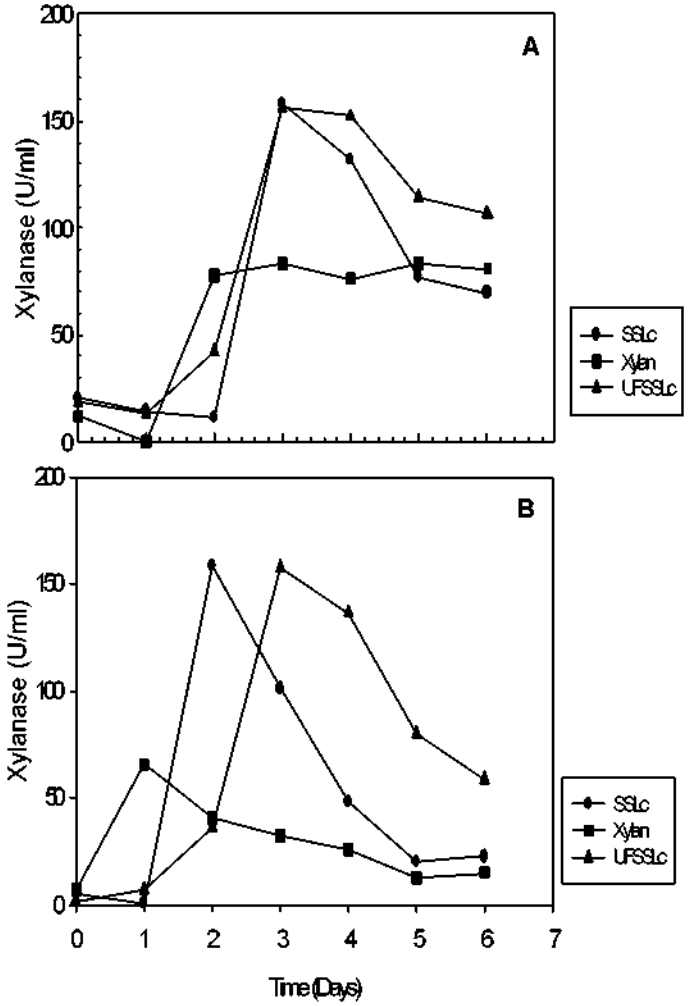

Figure 4.7. Xylanase production profile of *A. oryzae* NRRL 3485 (A) and *A. phoenicis* ATCC 13157 with xylan, SSLc and ultra filtered SSLc (UFSSLc) as carbon substrates in shake flask cultures incubated at 30°C for 6 days.

4.1.4.4.3. Overliming and heat treatment

The *A. oryzae* strain yielded comparable xylanase activities with SSLc and OHSSLc (overliming combined with heat treatment of SSLc) as carbon substrates, up to 156 and 167 U/ml, respectively. Significantly lower activities were, however, obtained with the OHUFSSLc (ultra filtration followed by overliming combined with heat treatment of SSLc; 94 U/ml), as carbon source (Figure 4.8).

The *A. phoenicis* strain yielded considerably higher xylanase activities on the overlimed and heated-treated waste water-based media (Figure 4.8) than the *A. oryzae* strain. The SSLc fraction that was overlimed combined with heat treatment (OHSSLc) yielded highest xylanase activities of up to 220 U/ml, significantly higher than activities obtained with the SSLc fraction (158 U/ml). The results in Figure 4.8 indicated that overliming and heat-treating the SSLc had a significant effect on xylanase production by the *A. phoenicis* strain but not with the *A. oryzae* strain.

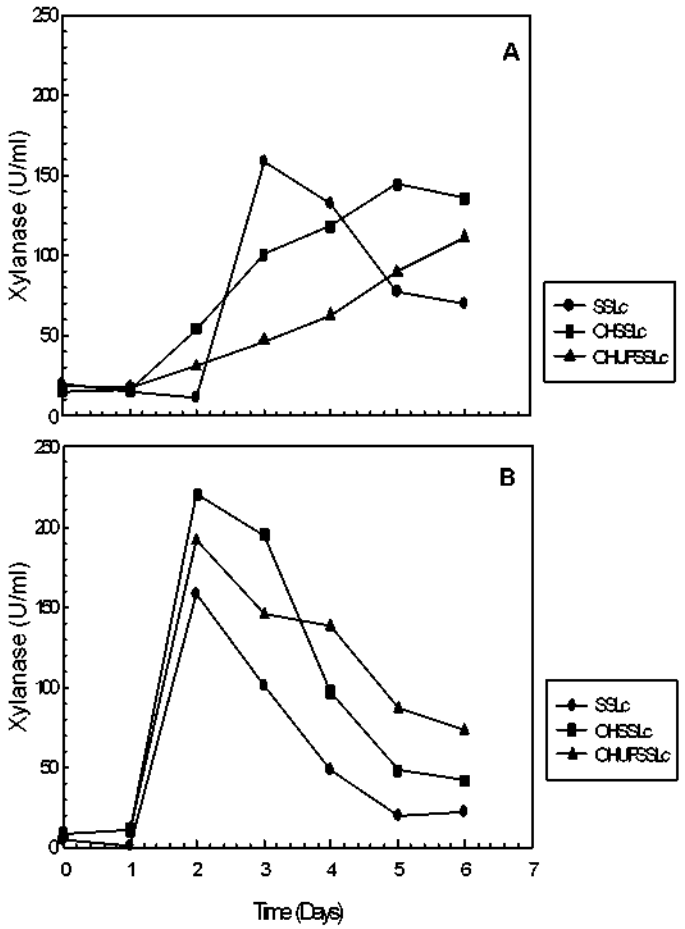

Figure 4.8. Xylanase production profile of *A. oryzae* NRRL 3485 (A) and *A. phoenicis* ATCC 13157 with xylan, SSLc and ultra filtered SSLc (UFSSLc) as carbon substrates in shake flask cultures incubated at 30°C for 6 days

The increase in xylanase production as a result of waste water pretreatment correlates well to other reports where an increase in certain target products (ethanol, 2,3-butanediol) occurred after the pretreatment of hemicellulosic hydrolysates (Frazer & McCaskey 1987; Olsson & Hahn-Hägerdal 1996; Ranatunga et al. 1997; Nigam 2001). These results 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.

4.1.4.5. Xylanase production in batch cultures: Effect of culture pH

4.1.4.5.1. Xylanase production with xylan as carbon substrate

The *A. oryzae* strain was cultivated at pH 6.0 and 7.5 with xylan as carbon substrate. Comparable xylanase activities of up to 289 and 286 U/ml were obtained at these cultivation pH values (Figure 4.9). These activities are significantly higher than activities obtained in shake flask cultivations with xylan as carbon substrate (77 U/ml). A higher biomass concentration was obtained at cultivation pH 6.0 than at pH 7.5. The xylanase yield per gram biomass was, however, significantly higher at pH 7.5 than at pH 6.0 (Figure 4.9).

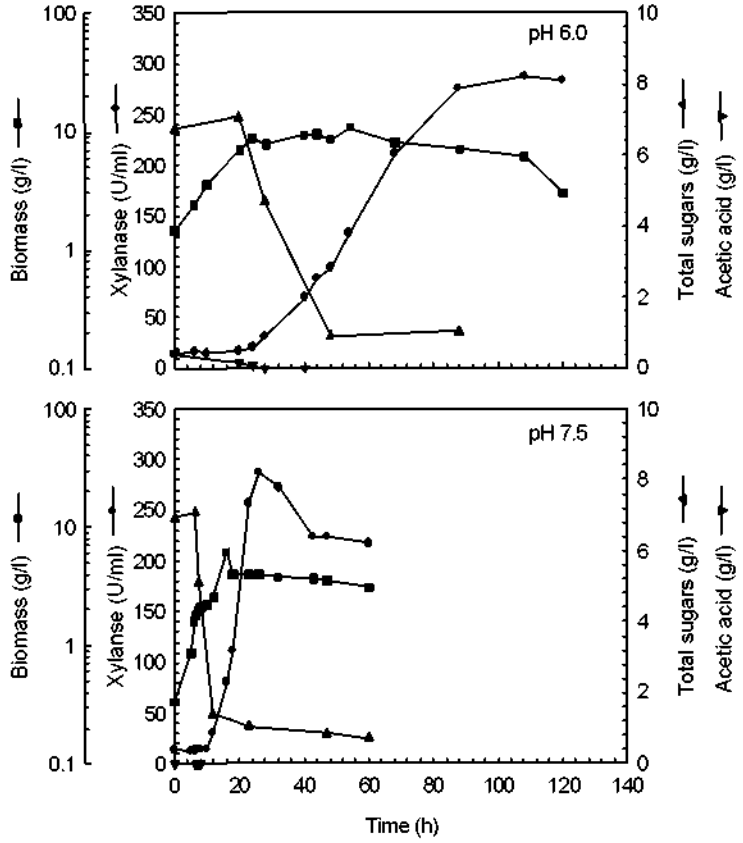

Figure 4.9. Typical cultivation profiles of *A. oryzae* NRRL 3485 with xylan as carbon source in bioreactor cultures at 30°C and cultivation pH 6.0 and 7.5.

4.1.4.5.2. Xylanase production with concentrated spent sulfite liquor as carbon substrate

Highest xylanase activities of up to 199 U/ml were obtained at cultivation pH 7.5 with SSLc as carbon source. Cultivation of *A. oryzae* at pH 4.0 yielded the lowest xylanase activity of 69 U/ml (Figure 4.10). Although high xylanase activities of up to 156 U/ml were obtained in shake flask cultivations with SSLc as carbon source, the cultivation pH of 7.5 yielded significantly higher activities. Xylanase activities obtained with SSLc as carbon substrate in bioreactor cultivations were, however, lower than activities obtained with xylan as carbon substrate. In shake flask cultures, it was observed that higher activities were obtained with SSLc than with xylan as respective carbon substrate. The biomass concentration was observed to decrease as the cultivation pH was increased, as

was the case when xylan was used as carbon source. There was a prolonged lag phase at cultivation pH 4.0 which ended as the acetic acid concentration in the medium reduced to minimal levels. The effect of weak acids such as acetic acid on microbial growth is well documented. It is the undissociated acid that is toxic due to the fact that it freely diffuses across the plasma membrane; therefore, the inhibitory effect of acetic acid is pH-dependent (Brown and Booth 1991; Axe and Bailey 1995; Nigam 2001). The cultivation profile for pH 4.0 with SSLc as carbon source suggests that the acetic acid present in the SSLc exerted a major effect on growth, despite its relatively low concentration in the culture medium (SSL diluted twenty-fold) and the low cultivation pH.

The growth parameters for *A. oryzae* NRRL 3485 across the different cultivation pH values are summarised in Figure 4.11. The maximum volumetric rate of xylanase production, xylanase activities, xylanase yield as well as the maximum specific growth rates were observed to increase as the cultivation pH increased from pH 4.0 to pH 7.5. Above this pH, these parameters decreased significantly (Figure 4.11). The biomass concentration on the other hand was highest at cultivation pH 4.0, up to 11 g/l, but decreased to 5.7 g/l at cultivation pH 7.5 and decreased even further to 4.9 at cultivation pH 8.0. This could be due to the fact that some fungi thrive at lower cultivation pH values, but this may, however, not be their optimal pH for xylanase production.

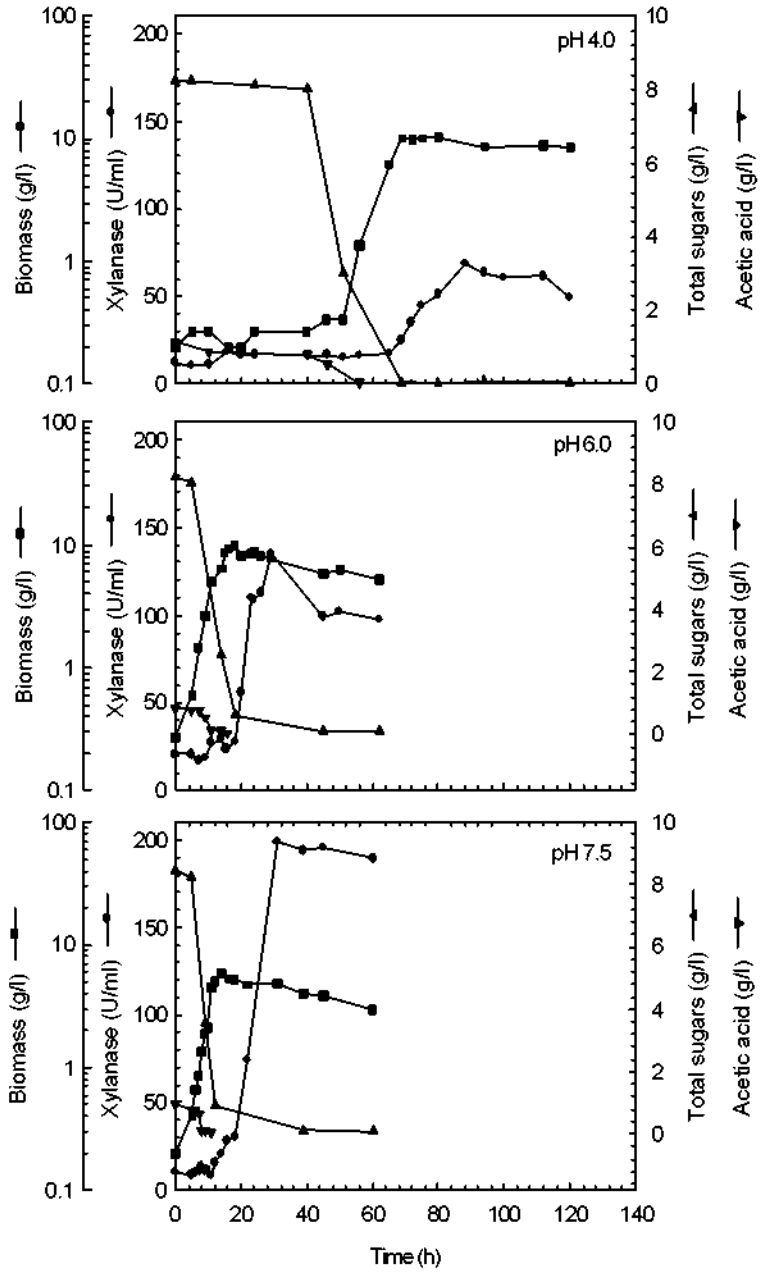

Figure 4.10. Typical cultivation profiles of *A. oryzae* NRRL 3485 with SSLc as carbon source in bioreactor cultures at 30°C and cultivation pH 4.0, 6.0 and 7.5.

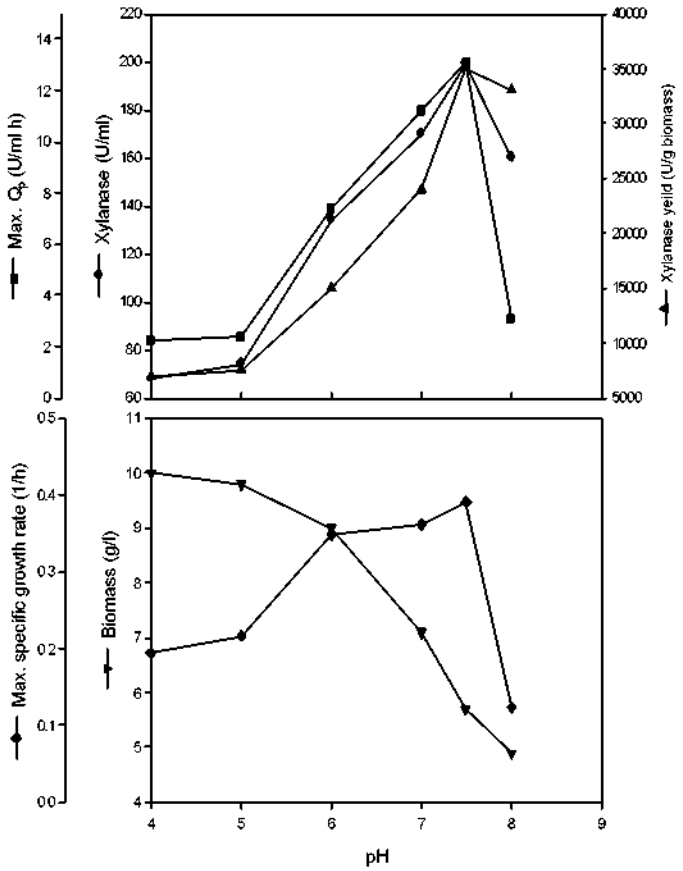

Figure 4.11. Growth parameter profiles of *A. oryzae* NRRL 3485 with SSLc as carbon source in bioreactor cultures at 30°C and cultivation pH 4.0, 5.0, 6.0, 7.0, 7.5 and 8.0.

4.1.4.6. Xylanase production in batch cultures: Effect of agitation rate

Typical cultivation profiles for xylanase production, biomass concentration, stirrer speed as well as dissolved oxygen tension are shown in Figures 4.12 and 4.13. There was no significant difference in xylanase activities at the different stirrer speeds evaluated (Figures 4.12 and 4.13). The SSLc was diluted forty-fold and not twenty-fold as was previously diluted during the cultivation pH study so as to reduce the amount of biomass formed during the cultivation. A higher biomass would make it difficult to maintain the DOT at or above 25 %, hence the higher dilution of the SSLc. The cultivation profile obtained at 300 rpm has been omitted. As a result of the low stirrer speed (300 rpm) and

the increase in biomass concentration, the DOT could not be maintained at or above 25 %, even at maximal aeration rate.

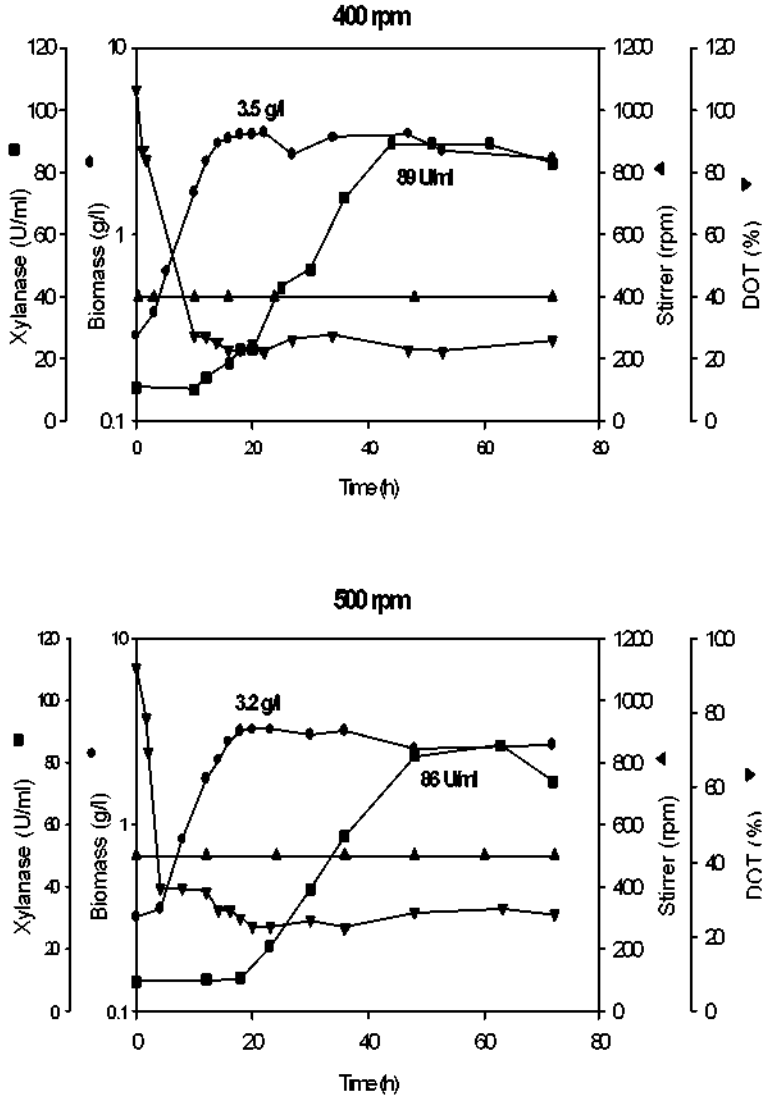

Figure 4.12. Typical cultivation profiles of *A. oryzae* NRRL 3485 at agitation rates of 400 and 500 rpm with SSLc as carbon source in bioreactor cultures at 30°C and cultivation pH of 7.5.

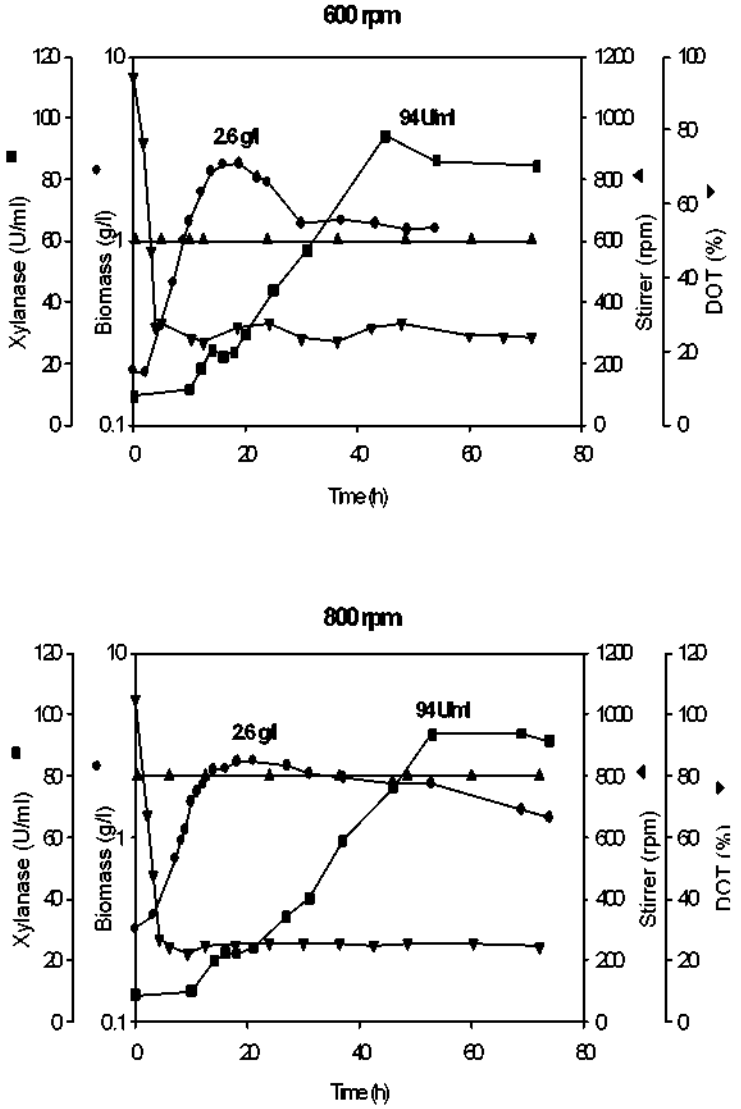

Figure 4.13. Typical cultivation profiles of *A. oryzae* NRRL 3485 at agitation rates of 600 and 800 rpm with SSLc as carbon source in bioreactor cultures at 30°C and cultivation pH of 7.5.

The growth characteristics of *A. oryzae* NRRL 3485 at different agitation rates are summarised in Table 4.6. Xylanase activities of between 86 to 94 U/ml were obtained throughout all the stirrer speeds evaluated. Slightly higher biomass concentrations were obtained with the 400 and 500 rpm agitation rates but lower maximum growth rates were

obtained at these agitation rates. The xylanase yield per gram biomass was higher for the 600 and 800 rpm agitation rates.

Table 4.6. Growth parameters for *A. oryzae* NRRL 3485 in bioreactor cultivations[a] with SSLc[b] as carbon substrate

Parameter	Stirrer Speed (rpm)			
	400	500	600	800
Xylanase (U/ml)	89 ± 12	86 ± 14	94 ± 9	94 ± 13
Biomass (g/l)	3.5 ± 0.1	3.2 ± 0.1	2.6 ± 0.2	2.6 ± 0.1
μ_{max} (h^{-1})	0.204	0.200	0.230	0.265
Q_p^{max} ($U.ml\ h^{-1}$)	2.72	2.43	2.58	2.31
$Y_{p/x}$ ($U.g^{-1}$)	25 428	26 875	36 154	36 154

[a] Cultivation pH maintained at pH 7.5, DOT at = 25 % and temperature at 30°C
[b] Spent sulfite liquor concentrate diluted 40-fold with distilled water
μ_{max} Maximum specific growth rate
Q^P_{max} Maximum volumetric rate of xylanase production, calculated from the maximum slope of the enzyme activity vs time curve
$Y_{p/x}$ Xylanase yield coefficient, $U.g^{-1}$ biomass

Shear stress is known to cause morphological as well as physiological changes to some filamentous fungi (Tanaka et al. 1975). A number of studies have shown some fungi to have increased xylanase production at relatively low agitation rates, as low as 200 rpm or less (Hoq et al. 1994; Purkarthofer et al. 1993b). Higher agitation rates result in increased oxygen transfer rates but also results in increased hyphal branching. Our results, however, do not show an increase or decrease in xylanase activities at the different agitation rates. This could imply that the shear stress imposed on the organism at these rates is not that severe as to affect xylanase production.

4.1.4.7. Xylanase production in fed-batch cultures

Fed-batch cultivations in an SSLc medium were carried out for the production of xylanase and the profile is shown in Figure 4.14. By using the constant feed strategy, xylanase activities increased almost two fold from 89 U/ml in batch cultures using a forty-fold diluted SSLc to a maximum of 210 U/ml. The feed was started at the 14 h

period and from the xylanase profile (Figure 4.14) xylanase production began soon after, peaking after 46 h of cultivation.

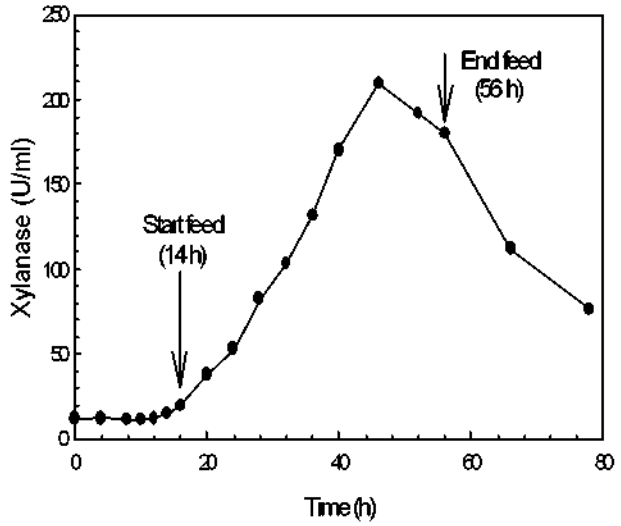

Figure 4.14. Xylanase activities of *A. oryzae* NRRL 3485 in fed-batch cultures with SSLc as carbon substrate

4.1.4.8. Xylanase characterization

4.1.4.8.1. Physico-chemical properties

The use of high temperatures during the pulping and bleaching processes dictates the use of xylanases with a high pH and temperature tolerance (Viikari et al. 1994). The optimum pH of the *A. oryzae* and *A. phoenicis* xylanases under the standard assay conditions was 6.5 and 5.0, respectively (Figure 4.15.

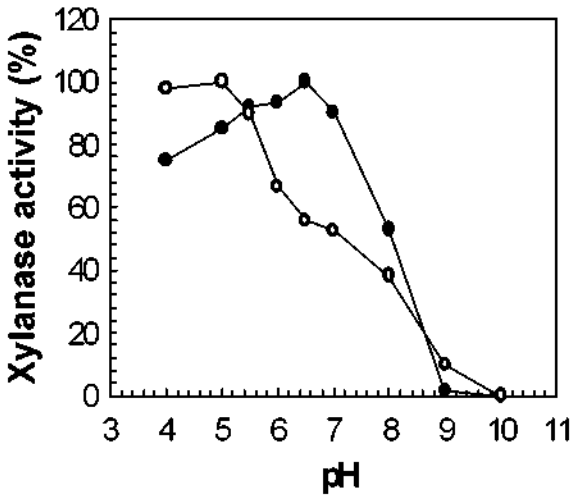

Figure 4.15. Xylanase activity of *A. oryzae* NRRL 3485 (closed symbols) and *A. phoenicis* ATCC 13157 (open symbols) grown on spent sulfite liquor concentrate as carbon source as a function of the assay pH. Mean values of triplicate determinations are shown.

The optimal temperatures of the *A. oryzae* and *A. phoenicis* xylanases was 65 and 50°C (Figure 4.16). The pH and temperature optimum exhibited by *A. phoenicis* was typical of fungal xylanases, whereas the *A. oryzae* xylanase exhibited unusually high pH and temperature optima thereby rendering this enzyme of potential industrial importance.

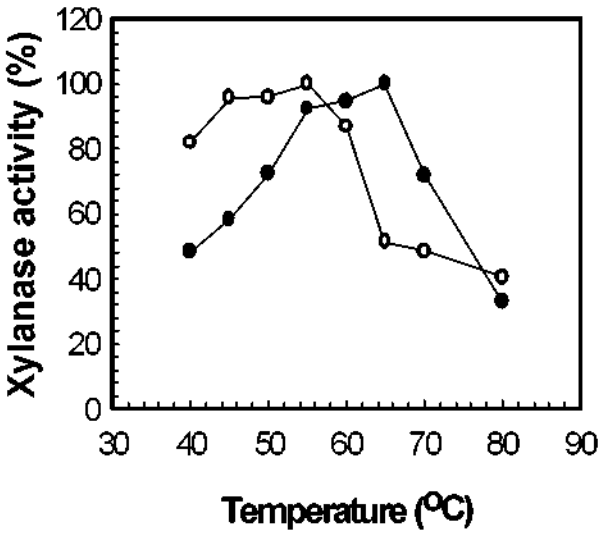

Figure 4.16. Xylanase activity of *A. oryzae* NRRL 3485 (closed symbols) and *A. phoenicis* ATCC 13157 (open symbols) grown on spent sulfite liquor concentrate as carbon source as a function of the assay temperature. Mean values of triplicate determinations are shown.

Xylanases from *A. oryzae* showed better pH stability than the *A. phoenicis* xylanases, retaining up to 90 % of activity in a wide range of pH from 4.5 to 10 whereas nearly 20 % activity was lost at pH 8.5 by *A. phoenicis* xylanases which exhibited stability between pH 4.0 and 8.0, retaining up to 90 % activity (Figure 4.17).

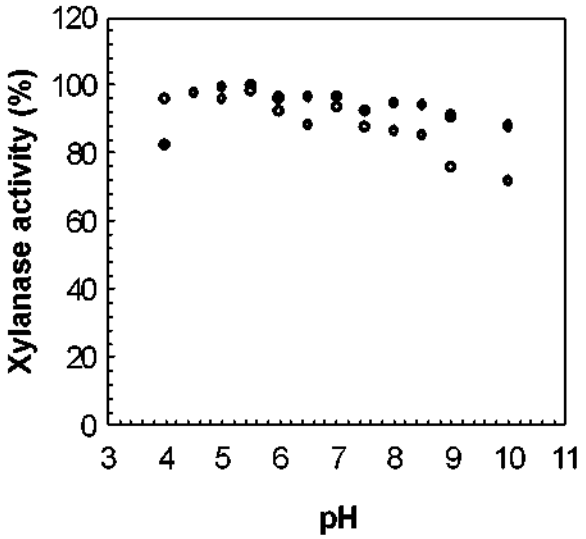

Figure 4.17. Xylanase activity of *A. oryzae* NRRL 3485 (closed symbols) and *A. phoenicis* ATCC 13157 (open symbols) grown on spent sulfite liquor concentrate as carbon source as a function of the pH stability. Mean values of triplicate determinations are shown.

Xylanases from both the above strains were not stable beyond 50°C with the *A. oryzae* xylanases losing almost 50 % of its activity whereas the *A. phoenicis* xylanases lost up to 70 % activity at 60°C after 5 min incubation (Figure 4.18). At 60°C, xylanases from both strains rapidly lost activity with denaturation half-life of 5 and 2 min for *A. oryzae* and *A. phoenicis* xylanases, respectively.

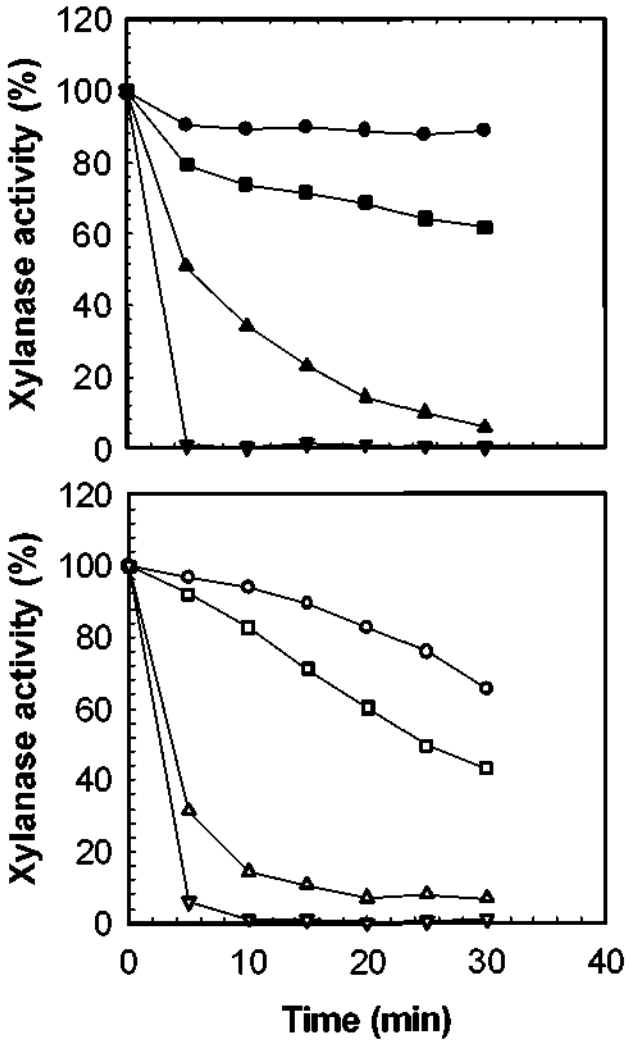

Figure 4.18. Xylanase activity of *A. oryzae* NRRL 3485 (closed symbols) and *A. phoenicis* ATCC 13157 (open symbols) grown on spent sulfite liquor concentrate as carbon source as a function of temperature stability. Symbols: ● and ○, 40°C; ■ and □, 50°C; △ and ? , 60°C; ▽ and ? , 70°C. Mean values of triplicate determinations are shown.

4.1.4.8.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymograms

The zymogram analysis confirmed that xylan as well as SSLc acted as inducing substrates for xylanase production. Multiple xylanases were shown to be present in all the crude enzyme preparations. *Aspergillus oryzae* and *A. phoenicis* revealed three and two xylanase bands, respectively (Figure 4.19). By comparing the zymogram activity bands to the SDS-PAGE stained bands and marker proteins with each other, the molecular weights of the *A. oryzae* xylanases were 49, 34 and 30 kDa whilst the *A. phoenicis* xylanases were 32 and 29 kDa (Figure 4.19).

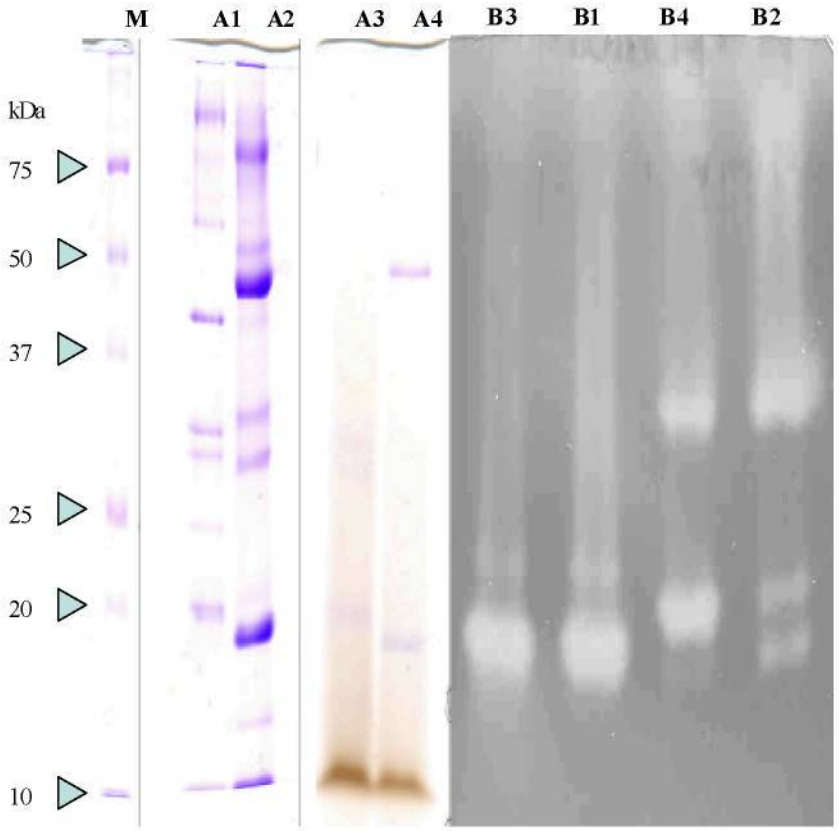

Figure 4.19. SDS-PAGE (lanes A) and zymogram (lanes B) analysis of xylanase preparations from *A. oryzae* NRRL 3485 (lanes A2, A4, B2, B4) and *A. phoenicis* ATCC 13157 (lanes A1, A3, B1, B3) with xylan (lanes A1, A2, B1, B2) and SSL (lanes A3, A4, B3, B4) as carbon substrates.

Most xylanolytic fungi produce at least two or more xylanases (Wong et al. 1998). This has also been reported for some *Aspergillus sp* (Bailey et al. 1991; Anthony et al. 2003). It has been suggested that the production of multiple xylanases may reflect the need to produce xylanases with different specificities which would be able to act on either highly substituted, unsubstituted or differentially soluble substrates (Ryan et al. 2003).

4.1.4.9. Application of xylanases in beaching of pulp

Using the full chlorine dioxide charge, a brightness gain was achieved with both xylanases (Figure 4.20). However, the crude xylanase of *A. phoenicis* grown in the SSLc-based medium produced the highest gain of 1.5 brightness points over the control. Alternatively, a 30% reduction in the chlorine dioxide charge could be attained without sacrificing brightness. The application of the xylanases of *A. oryzae* and *A. phoenicis* grown on xylan resulted in gains of 1.1 and 1.3 brightness points, respectively, over the control (Figure 4.20). On the overall, the *A. phoenicis* xylanase was more efficient in pulp bleaching than the *A. oryzae* xylanase. Furthermore, the xylanase of *A. phoenicis* grown in the SSLc-based medium had a greater bleaching potential than the xylanase obtained with xylan as carbon substrate. It was demonstrated that the application of these xylanases at a charge of 10 U/g pulp could reduce the usage of chlorine dioxide up to 30% without compromising pulp brightness.

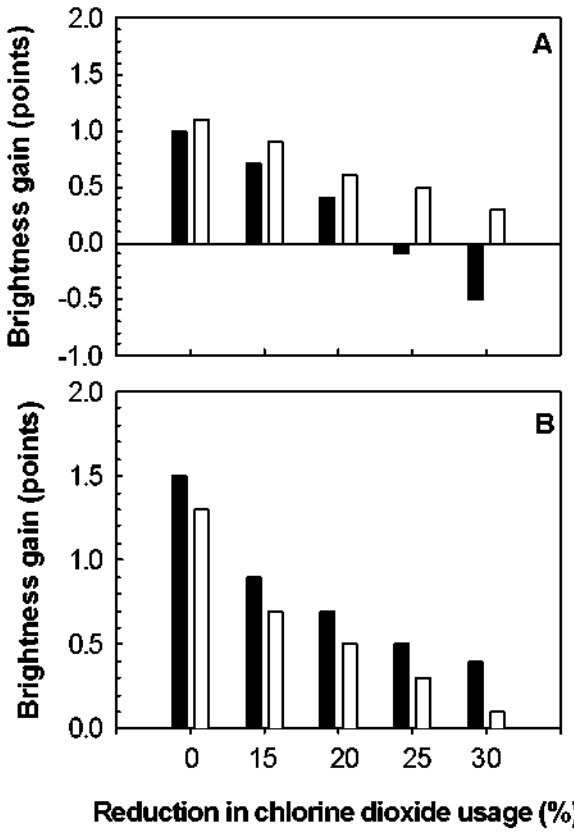

Figure 4.20. Impact of biobleaching oxygen-delignified soda aq pulp with xylanases of *A. oryzae* NRRL 3485 (A) and *A. phoenicis* ATCC 13157 (B) grown in the SSL concentrate (solid bars) and on xylan (open bars) on chlorine dioxide consumption.

4.1.5. Conclusions

- A comparison of xylanase activities obtained with *Aspergillus oryzae* NRRL 3485 showed that higher xylanase activities of up to 200 U/ml were obtained in batch cultures than in shake flasks (30 U/ml) cultures using the SSLc as carbon feedstock at 20-fold dilution.
- The fed-batch cultivation of *Aspergillus oryzae* NRRL 3485 using SSLc as carbon substrate yielded activities of up to 200 U/ml, which was two-fold higher than activities obtained in batch cultures under similar conditions (40-fold dilution).
- The chemical composition of the SSL waste waters revealed that concentrating the waste water reduced the amount of acetic acid, thereby minimising the inhibitory

effect the acid may have on microbial growth during utilization of SSLc as carbon feedstock.

- The considerably high biomass concentrations obtained with SSLc as carbon feedstock could be attributed to the utilization of additional carbon present in the SSLc, as is indicated by the TOC results.
- Xylanase production using SSLc was favoured by a high culture pH of 7.5. The agitation rates between 400 to 800 rpm did not have any adverse effect on xylanase production. Fed-batch trials revealed a two-fold increase in xylanase activities. A better understanding of the limiting factor would enable even higher activities being achieved.
- The xylanases from both *A. oryzae* NRRL and *A. phoenicis* ATCC 13157 exhibited multiplicity. The *A. oryzae* xylanase showed unusual pH and temperature optima not generally exhibited by fungal xylanases. A higher level of pH stability was shown by the xylanase preparations from this fungus.
- The application of SSL-grown fungal xylanases in biobleaching resulted in a reduction in the use of active chlorine of up to 30 %. The xylanases increased the pulp brightness by up to 1.5 brightness points over the control. The application of these xylanase enzymes, produced with SSLc as carbon feedstock, is highly significant.
- Xylanase production using SSLc as carbon feedstock indicated that the SSLc acted as inducer. Overall, the xylanase yields in shake flasks obtained with both *A. oryzae* and *A. phoenicis* on SSL-based growth media were about 2-fold higher than those produced on xylan-based media. Furthermore, the xylanase activities induced in batch cultures on SSL and xylan were comparable.
- Since the carbon source in general constitutes 30-50% of the total enzyme production costs, the eventual replacement of xylan with SSL as inexpensive and abundant carbon substrate would result in significant cost reductions associated with xylanase production and enhance the economic viability of the biobleaching technology.

CHAPTER 5
GENERAL CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

- It has been demonstrated that the implementation of the new bleaching technologies (enzyme- and polyoxometalate-based bleaching) in the pulp and paper industry could improve the existing technology of pulp and paper manufacture. The research findings of this work provide evidence for the potential of xylanases as a means of improving pulp brightness and rendering the waste waters more environmentally friendly.

- The potential of pulp mill waste waters (spent sulfite liquor) for the production of high-value products such as enzymes has been demonstrated. The bio-utilization of these waste waters would alleviate problems associated with their discharge and make use of the produced enzymes in pulp bleaching. This would further contribute to the overall reduction of the environmental impact of the bleach waste waters.

5.2. Recommendations

- The use of polyoxometalates in pulp bleaching represents an alternative bleaching technology that can offer a significant reduction or complete elimination in the use of chlorine-containing bleach chemicals such as chlorine and sodium hypochlorite. However, further studies are required to demonstrate the reusability and economical viability and determine the environmental impact of this technology.

- Biobleaching with xylanase can reduce the amount of chlorine-containing chemicals used in bleaching by 30% (chlorine dioxide and hypochlorite) with about 20% reduction in the use of sodium hydroxide. This impacts directly on the levels of toxic effects, chlorides and absorbable organic halogen of the waste waters contributing to the overall reduction of the environmental impact of the mill. Furthermore, the chemical reductions can be translated into cost savings, alternatively, pulp brightness

can be increased. It is therefore recommended that the potential of xylanase is evaluated on a large scale to verify the lab generated results.

- The use of the spent sulfite liquor as an inexpensive and abundant carbon source for xylanase production would improve the overall economics of the biobleaching technology. Further work in this direction can continue with optimization of xylanase production in fed-batch and continuous cultures using genetically modified organisms that can produce high levels of xylanase and other enzymes required for bleach boosting.

- In light of the above, it is recommended that a thorough techno-economical assessment of the enzyme fermentation technology is carried out that could lead to a pilot plant scale study to be undertaken as a follow-up WRC-funded project to verify the lab generated results and bridge up the eventual industrial implementation of this promising bio technology.

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ANNEXTURE

PRODUCTS EMANATING FROM THE PROJECT

1. Book chapters

1. Bissoon, S., Singh, S. and Christov, L. (2002) Evaluation of the bleach-enhancing effect of xylanases on bagasse pulp. In: *Biotechnology in the Pulp and Paper Industry* (Ed. by Viikari, L. and Lantto, R.), Progress in Biotechnology, Elsevier, Amsterdam, pp 247-254. ISBN 0-444-51078-8

2. Szendefy, J., Szakács, G. and Christopher, L. (2003) In situ solid-state fermentation and utilization of xylanase in pulp bleaching. In: *Application of Enzymes to Lignocellulosics* (S Mansfield & JN Saddler, Eds.), American Chemical Society, Washington, DC, 2003, pp. 255-284.

3. Szendefy, J., Szakács, G. and Christopher, L. (2004) Biobleaching efficiency of *Aspergillus oryzae* xylanases produced by solid substrate fermentation. In: *Lignocellulose biodegradation* (B Saha, Ed.), American Chemical Society, Washington, DC (in press).

4. Christopher, L. (2004) Application of microbial xylanases in the pulp and paper industry. In: *Concise Encyclopedia of Bioresource Technology* (A Pandey, Ed.). The Haworth Press, Inc., Binghamton, NY (in press).

2. Peer-reviewed publications

1. 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.

2. Van Driessel, B. and Christov, L. (2002) Adsorption of colour from a bleach plant waste water using biomass and cell wall fractions from *Rhizomucor pusillus*. *J. Chem. Technol. Biotechnol.* 77: 155-158.

3. Bissoon, S., Singh, S. and Christov, L. (2003) Synergistic interactions of purified xylanases on bagasse pulp hemicellulose. *Tappsa J.* 2:28-30.

4. Christov, L. and Van Driessel, B (2003) Waste water bioremediation in the pulp and paper industry. *Ind. J. Biotechnol.* 2: 444-450.

5. Christopher, L., Bissoon, S., Singh, S., Szendefy, S. and Szakacs, G. (2004) Bleach-enhancing abilities of *Thermomyces lanuginosus* xylanases produced by solid-state fermentation. *Process Biochem.* (accepted).

6. Van Driessel, B. and Christopher, L. Mechanisms prevalent during bioremediation of wastewaters from the pulp and paper industry. *Crit. Rev. Biotechnol* (in press).

3. Presentations.

1. Christov, L., Bissoon, S. and Singh, S. (2002) Effects of purified xylanolytic enzymes of *Aspergillus niger* on isolated and fiber-bound hemicellulose of bagasse pulp. *ACS National Meeting 1st International Symposium on Xylans, Mannans and Other Hemicelluloses: Biology, Chemistry and Technology*, April 7-11, 2002, Orlando, FL, USA.

2. Chipeta, Z.A., du Preez, J.C. and Christov, L. (2002) Xylanase production by *Aspergillus oryzae* NRRL 3485 and *Aspergillus phoenicis* ATCC 13157 on pulp mill waste waters. *12th Biennial Congress of the SASM*, April 2-5, 2002, Bloemfontein, South Africa.

3. Kandioller, G. and Christov, L. (2002) Efficiency of *Trametes versicolor* laccase in combination with various mediators in pulp delignification and bleaching. *12th Biennial Congress of the SASM*, April 2-5, 2002, Bloemfontein, South Africa.

4. Chipeta, Z.A., du Preez, J.C. and Christov, L. (2002) Use of industrial waste water for xylanase production by two *Aspergillus* strains. *Tappsa 2002 Pulp and Paper Week "Adding Value in a Global Industry"*, October 7-11, 2002, Durban, South Africa.

5. Kandioller, G. and Christov, L. (2002) Catalyst potential of laccase-mediator systems and transition metal polyoxometalates in oxygen bleaching of pulp. *Tappsa 2002 Pulp and Paper Week "Adding Value in a Global Industry"*, October 7-11, 2002, Durban, South Africa.

6. Bissoon, S., Singh, S. and Christov, L. (2002) Synergistic interactions of purified xylanases on bagasse pulp hemicellulose. *Tappsa 2002 Pulp and Paper Week "Adding Value in a Global Industry"*, Durban, South Africa, October 7-11, 2002.

7. Christov, L, Chipeta, Z.A., Szakacs, G. and du Preez, J.C. (2002) Use of industrial waste waters for enzyme production. *43rd Annual Conference of the Association of Microbiologists of India (AMI)*, Hisar, India, December 11-13, 2002.

8. Szendefy, J., Szakacs, G. and Christov, L. (2003). Potential of *Aspergillus oryzae* xylanase produced by solid substrate fermentation for biobleaching of pulp. *225th ACS National Meeting, Symposium on Advances in Biodegradation and Biotransformation of Lignocellulosics*, March 23-27, 2003, New Orleans, LA, USA.

9. Chipeta, Z.A, du Preez, J.C., Szakacs, G. and Christov, L. (2003) Enzyme production by two *Aspergillus* strains using spent sulfite liquor. *12th International*

Symposium on Wood and Pulping Chemistry (ISWPC), June 8-12, 2003, Madison, USA.

10. Kandioller, G. and Christov, L. (2003) Hardwood soda-aq pulp bleaching with molybdovanadophosphoric acids. *12th International Symposium on Wood and Pulping Chemistry (ISWPC)*, June 8-12, 2003, Madison, USA.

11. Du Preez, J.C, Chipeta, Z.A., Christov, L. and Szakacs, G. (2003) Xylanase production by *Aspergillus* strains on sulphite waste liquor as carbon feedstock. *FEMS 2003 – First Congress of European Microbiologists*, June 29-July 3 2003, Ljubljana, Slovenia.

12. Du Preez, J.C, Chipeta, Z.A., Christov, L. and Szakacs, G. (2003) Evaluation of sulphite waste liquor as carbon feedstock for xylanase production by natural and recombinant strains of *Aspergillus*. *11th European Congress on Biotechnology*, August 24-29, 2003, Basel, Switzerland.

13. Kandioller, G. and Christopher, L. (2004) Delignification efficiency of *Trametes versicolor* laccase in combination with various mediators. *9th International Conference on Biotechnology in the Pulp and Paper Industry (ICBPPI)*, October 10-14, 2004, Durban, South Africa.

14. Szakacs, G., Szendefy, J. and Christopher, L (2004) Biobleaching efficiency of thermostable fungal xylanases produced by solid state fermentation. *9th International Conference on Biotechnology in the Pulp and Paper Industry (ICBPPI)*, October 10-14, 2004, Durban, South Africa.

15. Chipeta, Z.A, du Preez, J.C. and Christopher, L. (2004) Production, characterization and application of *Aspergillus oryzae* xylanase using sulphite waste liquor as carbon feedstock. *9th International Conference on Biotechnology in*

the Pulp and Paper Industry (ICBPPI), October 10-14, 2004, Durban, South Africa.

4. Reports

1. Annual reports to the NRF (THRIP)

5. Degrees

This project funded by the Water Research Commission has enabled:

1. Mr Z Chipeta to complete part of PhD his thesis (University of the Free State) on "Comparative evaluation of xylanase producing fungal strains".
2. Mr G Kandioller to complete part of his thesis and obtain a PhD degree (2004) from the Technical University of Vienna (Title of thesis: Laccase-mediator systems and phosphomolybdovanadates as potential catalysts for improved oxygen delignification of pulps)