EVALUATION OF THE BLEACH-ENHANCING EFFECTS OF XYLANASES ON BAGASSE-SODA PULP

Sadhvir Bissoon

Submitted in fulfilment of the requirements for the Degree of Doctor of Technology, Department of Biological Sciences, Faculty of Science, M L Sultan Technikon, Durban, South Africa

Supervisor	:	Prof S. Singh
Co- supervisor	:	Prof L. Christov
Date submitted	:	January 2002

DECLARATION

I hereby declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Technology to the M L Sultan Technikon, Durban, South Africa. It has not been submitted before for any degree or examination to any other institute

S. Bissoon

January, 2002

ACKNOWLEDGEMENTS

The author gratefully acknowledges the following people:

Prof S. Singh, Department of Biological Sciences, M L Sultan Technikon, for his expert supervision, invaluable discussions, and extensive motivation,

Prof L. Christov, for his knowledge and wisdom, and his critical input and guidance during the course of this project,

Miss N. Singh for her endless patience, support, time and continued interest in this study,

Staff and students in the Department of Biological Sciences, M L Sultan Technikon, for their interest and support,

Mr T. Govender, Sappi (Stanger) for his invaluable assistance and cooperation

Sappi Management Services, National Research Foundation (NRF) and M L Sultan Technikon, for funding this reseach.

Technicians at Sappi Biotechnology laboratory (UOVS) for their excellent technical supervision and advice,

Staff and students in the Department of Microbiology and Biochemistry, UOVS, for their expert advice and assistance with certain aspects of the study

His family for their support, patience and encouragement,

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AOX:	adsorbable organic halogen
BSA:	bovine serum albumin
C:	chlorine
D:	chlorine dioxide
DP:	degree of polymerization
E:	NaOH extraction
EC:	elemental chlorine
ECF:	elemental chlorine-free
H:	hypochlorite
HPLC:	high performance liquid chromatography
kDa:	kilodalton
k.f:	kappa factor
kV:	kilovolt
LDC's	lignin-derived compounds
O:	oxygen
M.W:	molecular weight
N.D:	not determined
pI:	isoelectric point
PBS:	phosphate buffered saline
qPa:	peracetic acid with chelant
SDS-PAGE:	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM:	scanning electron microscopy
SF:	submerged fermentation
SSF:	solid substrate/state fermentation
TCF:	total chlorine-free
TEM:	transmission electron microscopy
X:	xylanase

LIST OF PUBLICATIONS

Journal publications

i) Madlala, A. M., **S, Bissoon**, S. Singh, L. Christov. 2001. Xylanase-induced reduction of chlorine dioxide consumption during elemental chlorine-free bleaching of different pulp types. *Biotechnology Letters*, 23:345-351.

ii) **Bissoon, S.**, L. Christov, S. Singh. 2002. Bleach boosting effects of purified xylanase from *Thermomyces lanuginosus* SSBP on bagasse pulp. *Process Biochemistry*, **37**:567-572.

iii) **Bissoon, S.**, V. Bandu, L. Christov, S. Singh. 2001. Immunolocalization of a purified *Thermomyces lanuginosus* xylanases during hydrolysis of bagasse pulp. Proceedings of Microscopy Society of South Africa 2001, ISBN 0-620-28339-4, pp. 49.

Book chapters

Bissoon, S., S. Singh, L. Christov. 2002. Evaluation of the bleach-enhancing effect of xylanases on bagasse pulp. In: Biotechnology in the Pulp and Paper Industry. Viikari, L. and R. Lantto, (eds.), Progress in Biotechnology, Elsevier Scinece (in press).

SUMMARY

A novel thermophilic fungus, *Thermomyces lanuginosus* SSBP, isolated locally produced a high level of extracellular xylanase (1095 U/ml) under shake-flask conditions using a medium of coarse corn cobs as a carbon source, and yeast extract as a nitrogen. Application of this enzyme to bagasse pulp in a pretreatment process increased the brightness by 1.34 points over the control at 65°C and pH 6.0. Spectrophotometric analyses of the enzyme filtrates revealed the release of chromophoric material, lignin derived compounds (LDC's) and reducing sugars (RS) with corresponding increase in brightness and decrease in kappa number of bagasse pulp. Evaluation of this xylanase in elemental chlorine-free (ECF) bleaching produced ClO_2 savings of 20% on bagasse pulp. In comparison commercial xylanases Ecopulp TX-200C and Xylanase P reduced the ClO_2 consumption by 20 and 33%, respectively. TCF bleaching with these enzymes was less efficient than ECF bleaching. Negligible difference in the physical properties of xylanase pretreated and control bagasse pulp samples were detected. In CEH bleaching of bagasse pulp Cartazyme 9704-E produced the highest brightness gain of 2.8 points over the control. This enzyme at a charge of 5 U/g pulp had the ability to reduce the chlorine consumption by 25% and proved to be the most efficient xylanase in CEH bleaching of bagasse pulp. Xylanase produced by *T. lanuginosus* and other thermophilic fungi under SSF conditions indicated a vast difference in enzyme titres. Strain F 980 was the most efficient producer of xylanase (6037 U/g bagasse pulp), however, bleaching of bagasse pulp with SSF samples revealed that strain ATCC 36350 displayed the most effective bleach enhancing ability. This strain reduced the Cl_2 consumption by 30% in CEH bleaching at SSF/raw pulp ratio of 1:50. Purified xylanase from *T. lanuginosus, A. pullulans, and T. viride* pretreatment on alkali-extracted hemicellulose and commercial birchwood xylan produced varying degrees of RS release. *A. pullulans* xylanase was the superior enzyme on xylan substrates as well as on bagasse pulp. The xylanases when treated in combination on these substrates produced the highest concentration of RS, with the commercial xylan being more susceptible to xylanase hydrolysis. The combined xylanase treatment of bagasse pulp displayed synergistic effects in CEH bleaching producing brightness gains of 2.2 points and reduction in Cl_2 consumption of 30%. SEM of bagasse pulp pretreated with *T. lanuginosus* xylanase showed a great degree of surface modification of the pulp with evidence of a noticeable "tearing-off" effect. TEM showed penetration of xylanase through the primary and secondary cell walls with a homogenous distribution of the enzyme. This demonstrates the ability of *T. lanuginosus* xylanase to penetrate bagasse pulp fibres with efficient access to xylan located in the inner layer of the cells, which could subsequently enhance chemical bleaching. Current data indicated the bleach enhancing effect of xylanases on bagasse pulp with concomitant reductions in chemical consumption during ECF and chlorine bleaching, and significant brightness gains in TCF bleaching. This project has demonstrated the important bleach enhancing potential of xylanases in bleaching of bagasse pulp and warrants investigation on a larger scale.

CHAPTER ONE: INTRODUCTION

The globalization of the pulp and paper industry is a relatively new phenomenon that has been a harbinger of change and opportunities. Today's paper industry employs advanced chemical and mechanical-based technologies to improve high-quality consumer products that are in worldwide demand and support the lifestyles of the new global economy. And yet, from these successes arise many of the current and future difficulties of the industry. Various pulp industry leaders have stated that the capital requirements of manufacturing paper products are too high and are limiting creativity and the entrepreneurial spirit of the industry. Coupled with these challenges, the emergence of low-cost fibre resources has contributed to further pressures on the pulp and paper industry to significantly reduce its manufacturing costs through a major redesign of its core manufacturing technologies. Within these difficulties are disguised but unparalleled opportunities for researchers to efficaciously develop new biotechnology-based processes for this industry. These new technologies must reduce the capital cost of pulp production, be readily implemented in today's mill, and provide exceptional return for the resources invested if they are to be commercially feasible. Basically the business of pulp and paper has provided research and development a challenge for change and biotechnology is destined to address this problem (Ragauskas, 2001).

Biotechnology in the pulp and paper industry has received increasing attention during the past fifteen years because of its commercial potential in many fields, which includes removal of bark, shives, pitch, slime control retting of flax fibres, and biopulping.

Currently, bleaching of pulp types uses high concentrations amounts of chlorine and chlorine chemicals, viz: chorine dioxide and hypoclorite. By products from using these chemicals are chlorinated organic substances, some of which are toxic, mutagenic, persistent, bioaccumulating and cause numerous harmful disturbances in biological systems (Bajpai and Bajpai, 1997). The options open to pulp mills considering a change to chlorine-free bleaching are oxygen delignification, extended cooking, and substitution of chlorine dioxide for chlorine, hydrogen peroxide and ozone. These alternatives involve process modifications and/or additional capital investment to modify the operating plant set-up. This has therefore provided an opportunity for the application of enzymes in the pulp and paper industry.

Until a few years ago the use of enzymes in the pulp and paper industry was not considered technically or economically feasible. The enzymes were not readily available, except for limited use in the modification of starch for paper coatings. However, research by various groups in several commercial companies has lead to the development of enzymes that offer significant benefits for the industry. Currently, the most important application of enzymes is in the prebleaching of kraft pulp. Enzymes provide a very simple and cost effective way to reduce the use of chlorine, chlorine compounds, and other bleaching chemicals. Enzymes also offer a simple approach that allows for a higher brightness ceiling to be achieved. Xylanases have been found to be most effective for this purpose and this technology is now in use at several mills worldwide. This technology has been successfully transferred to full industrial scale in just a few years (Bajpai, 1999). The virtue of enzyme technology lies in its potential to supply more

specific reactions, to provide less environmentally deleterious processes, to save energy, and to be used where non-biological chemistry is impractical (Eriksson, 1991).

Within a short period of time (1988-1991), xylanase prebleaching technology has become one of the solutions considered by the pulp and paper industry to give an innovative, environmentally and economically acceptable answer to the pressures exerted on chlorine bleaching by regulatory authorities in Western countries and by more demanding, environmentally minded consumers. New, much lower limits of adsorbable organo-halogen (AOX) levels in pulp mill effluents and the fast development of totally chlorine-free (TCF) and low AOX pulp markets led to quick responses from the industry in Canada and Northern Europe. Presently, more than 20 Scandinavian and North American mills are bleaching on a full scale with xylanases (Lavielle, 1992; Bajpai and Bajpai, 1992, Viikari *et al.*, 1994).

South Africa is a major international producer of pulp and paper products. Of the five major pulp and paper companies in South Africa, the South African Pulp and Paper Industry (Sappi) supplies half the countries requirements and is the largest papermaker on the African continent (Christov and Prior, 1998). Among the many agricultural fibres used for pulp manufacuture, sugar cane bagasse stands out because it appears to become a major raw material for the pulp and paper industry. Bagasse is readily available and easily accessible in many counties of the world, and it is especially abundant in some of the wood-poor countries. Sappi Fine Papers (Stanger) produces pulp from bagasse fibres under the soda process, which is then bleached in a CEH bleaching sequence. The

bleaching process utilizes elemental chlorine at a concentration of 49 kg/ton of air-dried pulp thereby contributing to effluents containing chlorinated organic compounds. The problems encountered with the use of chlorine are: corrosion of storage tanks, high losses in pulp yield and extensive emissions of chlorine in the bleach plant effluent. During recent years, substantially large sums of money have been expended to improved effluent treatment methods but with no significant contributions to improving the quality of the effluent. The current effluent treatment method at this mill is the use of flocculant K 300, which is aluminium based together with a flocculant aider AP 161 in aerated lagoons. The characteristics of the effluent and stipulated concentration of the residual chemicals are as follows: Supended solids: < 70 ppm, COD: < 390 ppm, O_2 absorption: < 90 ppm, Na: < 275 ppm and conductivity: < 220 ppm. The concentration of Cl_2 used in the bleaching process is approximately 49 kg/ton of air-dried pulp. The mill has been keeping within these guidelines, however, on average these values do exceed the normal limits. In this regard a biotechnological approach to the bleaching process has been suggested in view of improving the bleaching process with subsequent improvement to the effluent.

Unlike conventional wood species, bagasse has several inherent drawbacks, the most prominent of them being that it suffers rapid discolouration during storage, resulting in pulp with a low initial brightness (Prasad *et al.*, 1996). The low initial brightness of bagasse pulp has created a harsh bleaching sequence utilizing extensive quantities of Cl_2. In response to environmental concerns and stringent emission standards, modifications of the production process at the pulping and bleaching stages will be implemented in the

near future. Most of the published studies on enzyme treatment of pulps have focussed on either hardwood or softwood (Shah *et al.*, 1999). To the best of our knowledge there are limited published reports on xylanase pretreatment of bagasse pulp. Preliminary studies indicated that the application of xylanase to bagasse pulp improved the brightness and reduced kappa number significantly with concomitant release of reducing sugars and chromophoric material (Madlala *et al.*, 2001).

The objectives of this study were to:

(i) optimize xylanase pretreatment conditions on bagasse pulp;

(ii) determine the effect of xylanase pretreatment on the brightness, kappa number, removal of chromophoric material and reducing sugars present in bagasse pulp;

(iii) Assess the effectiveness of xylanase pretreatment when incorporated into an elemental chlorine-free (ECF) and total chlorine-free (TCF) bleach sequence;

(iv) Evaluate the effect of xylanases on an equal cost basis in CEH bleaching sequence

(v) Evaluate xylanase production levels by thermophilic fungi under SSF conditions and subsequent CEH bleaching of bagasse pulp

(vi) Examine the effect of purified xylanases on fibre-bound and isolated hemicellulose of bagasse pulp

(vii) Immunolocalize the purified *Thermomyces lanuginosus* xylanase on bagasse pulp to visualize accessibility of the enzyme to the hemicelluloses and examine surface modification of the fibres.

An overview of international research on the use of enzymes in biobleaching, and future prospects/developments in this area are discussed. Data obtained from the above investigations will provide relevant information on establishing the feasibility of incorporating an enzyme process for bleaching of bagasse pulp.

CHAPTER TWO: LITERATURE REVIEW

2.1 THERMOPHILIC FUNGI

Among the eukaryotic organisms, only a few species of fungi have the ability to thrive at temperatures between 45 to 55°C. Such fungi comprise thermophilic and thermotolerant forms, which are arbitrarily distinguished on the basis of their minimum and maximum temperature of growth (Cooney and Emerson, 1964). Thermophilic fungi are the chief components of the microflora that develops in heaped masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter wherein the warm, humid, and aerobic environments provides the basic conditions for their development (Maheshwari *et al.*, 2000). The extracellular enzymes of thermophilic fungi are appreciably thermostable. Currently, enzymes from thermophiles are being favoured, with prospects in biotechnology, and one of the most widely studied fungi of thermophilic origin is the *Thermomyces lanuginosus* strain.

2.1.1 *Thermomyces lanuginosus*

Thermophilic hyphomycetes and their distribution in diverse habitats are now well documented (Cooney and Emerson, 1968). The worldwide distribution *of T. lanuginosus* is a result of the common occurrence of self heating masses of organic debris. The fungus has been isolated in the British Isles, Denmark, Italy, USA, Canada, Nigeria, Ghana, South Africa, India, Indonesia, Brazil and Japan (Emerson, 1968). Colonies of *T. lanuginosus* are fast growing, reaching 2.5 to over 5 cm on various media at 45-50°C in 2

days. Colonies appear white and felty at first, less than 1 mm high, but soon turn grey or greenish grey, beginning at the centre of the colony. Gradually the colony turns purplish brown, at this time, the agar substratum stains are deep pink or wine colour, due to diffusible substances secreted by the colony. Mature colonies appear dull dark brown to black (Cooney and Emerson, 1968).

2.2 BAGASSE

Sugar cane, *Saccharum officnarum*, is grown in most of the tropical and subtropical countries for its high sucrose content. Following the processing of the cane in the sugar mill through a series of pressers and difussers to remove the sugar juice, the fibrous bagasse residue is normally burned in the sugar mill boilers. In this way, the sugar mill is completely self sufficient in steam and power. Nevertheless, bagasse in many areas of the world has greater economic value if it is processed into pulp, paper, paperboard and/or reconstituted panelboard. In fact, bagasse appears to satisfy the requirements for a successful papermaking raw material better than any other crop fibre (Atchison, 1993).

Despite these favourable factors, however, and despite research and developmental efforts for more than one hundred years, progress has been very slow toward the successful commercial utilization of bagasse. The slow progress is the result of three principle factors:

i) Although fibres of bagasse are fine, strong, flexible and suitable for the manufacture of many grades of paper, about 30 % of the weight of the bagasse consists of

pith or parenchyma cells and about 5 % of dense epidermal material. Neither of these components is fibrous in nature and, when left in the pulp, they have a deleterious effect on its quality.

ii) In the early efforts to utilize bagasse, no satisfactory storage method was developed and huge losses were suffered from deterioration between sugar cane grinding seasons.

iii) In addition to these problems, the greatest single obstacle to rapid progress was the use of bagasse by people who lacked understanding of its properties and limitations.

This had led to research and development institutions and large industrial companies to concentrate their efforts on overcoming the technical obstacles, which had held back the use of bagasse. These efforts resulted in greatly improved methods of storing, preparing, depithing, handling, and pulping bagasse, which drastically improved the economics of its use. Furthermore, the quality of the pulp was vastly improved (Atchison, 1993). The chemical composition of bagasse pulp is essential in determining the type of pulping and bleaching process to be undertaken. Bagasse pulp differs from the typical hardwood and softwood pulp in the concentration of hemicelluloses, lignin and cellulose. In order to pursue a biotechnological approach to the pulp and paper industry, the chemical characteristics and composition of the raw material have to be investigated and researched to provide maximum benefits achieved by the technology.

2.3 XYLANS

Xylan is the major component of the plant cell wall and the most abundant renewable hemicellulose (Whistler and Richards, 1970). Xylans are structurally of mixed composition, thus the enzymatic degradation of the substrate to its monomer, xylose, is a complex process involving a battery of enzymes (Biely, 1985; Zimmerman, 1992). Endo-xylanase and β-xylosidase have the most important activities among the xylanolytic enzymes involved in xylan hydrolysis, while the side chain cleaving enzymes like α-arabinofuranosidase, α-glucuronidase, and acetylxylan esterase play important roles in the removal of side substituents of heteroxylans. Xylanases are the most studied enzymes among hemicellulases since their substrate xylan constitutes the largest portion of hemicelluloses in plants. The interest in xylanases has increased greatly in the last decade due to their potential biotechnological applications, especially in the paper industry (Viikari *et al.*, 1994; Yang *et al.*, 1992; Daneault *et al.*, 1994).

2.3.1 Structure and occurrence of xylan

Hemicelluloses are noncellulosic polysaccharides that are found in plant tissues (Woodward, 1984). In the cell walls of plants, xylan is the most common hemicellulose polysaccharide, representing more than 30% of the dry weight (Joseleau *et al.*, 1992). Xylans are comprised of 1,4-linked β-D-xylopyranosyl residues (Whistler and Richards, 1970). Most xylans occur as heteropolysaccharides, containing different substituent groups in their backbone chain and in the side chain (Biely, 1985; Puls and Poutanen,

1989). The common substituents found on the backbone of xylan are acetyl, arabinosyl, and glucuronosyl residues (Whistler and Richards, 1970). Homoxylans, on the other hand, consist exclusively of xylosyl residues. This type of xylan is not widespread in nature and has been isolated from esparto grass (Chandra *et al.*, 1950), tobacco stalks (Eda *et al.*, 1976), and guar seed husk (Montgomery *et al.*, 1956).

2.4 HARDWOOD HEMICELLULOSE

Xylan is the major polyoses component in hardwoods and in woody parts of dicotyledonous annual fibre crops. The xylan of hardwoods is comprised of *0*-acetyl-4-*0*-methylglucuronoxylan (Fig. 2.1). The sugar cane bagasse xylan is similar to that found in hardwoods, with acetic and glucuronic acid side linkages attached to the main xylan chain. Hardwood xylan consists of linear chains of about 200 D-xylopyranose units, connected by β-(1-4) glycosidic bonds (Timell, 1964). About one in every 10 xylose residues contains a β-(1-2) – linked 4-*0*-methyl-D-glucuronic acid residue, at least 7 out of 10 xylose residues are esterified with an *0*-acetyl group at C-2 or C-3 (Lindberg *et al.*, 1973). The presence of these acetyl groups is responsible for the partial solubility of xylan in water (Whistler and Richards, 1970).

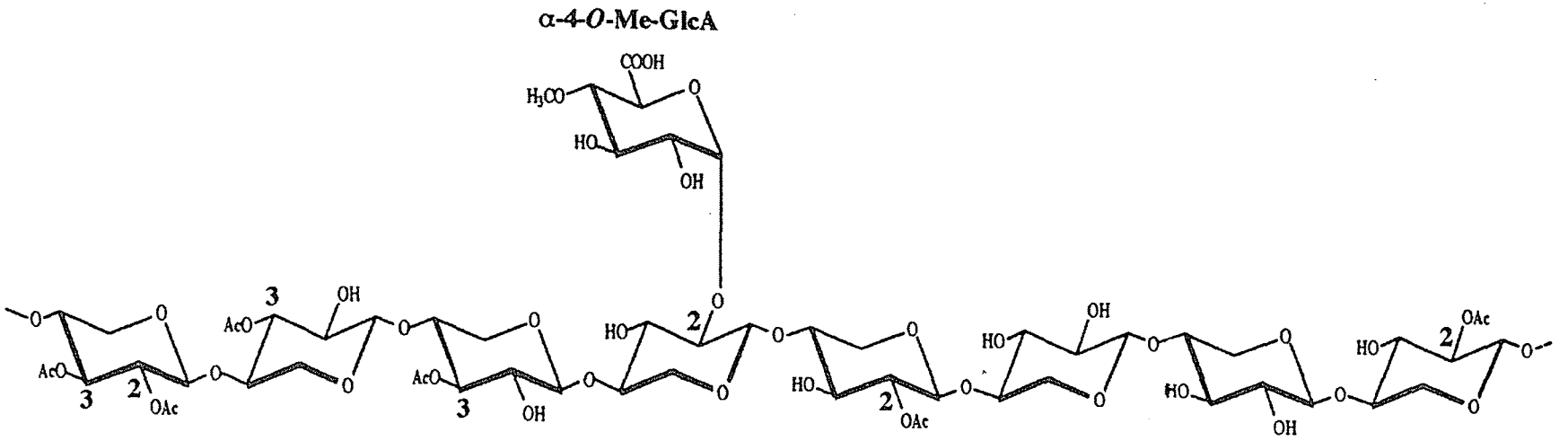


Fig. 2.1 Structure of hardwood xylan (*O*-acetyl-4-*O*-methylglucuronoxylan (Sunna and Antranikian, 1997)

2.5 SOFTWOOD HEMICELLULOSE

Softwoods are composed of arabino-4-*O*-methylglucoronoxylans (Fig. 2.2). They have a higher 4-0-methylglucuronic acid content than do hardwood xylans. The 4-0-methylglucuronic acid residues are attached to the C-2 position. Softwood xylans are not acetylated, and instead of acetyl groups they have α-L-arabinofuranosidase units linked by α-1,3-glycosidic bonds to the C-3 position of the xylose (Puls and Schuseil, 1993). The arabinosyl substituents occur on almost 12% of the xylosyl residues (Wong *et al.*, 1988). The ratio of β-D-xylopyranose, 4-0-methyl-α-D-glucuronic acid, and L-arabinofuranose is 100:20:13 (Puls and Schuseil, 1993). Softwood xylans are shorter than hardwood xylans, with a degree of polymerization (DP) between 70 and 130. They are also less branched (Zimbo and Timell, 1967).

2.6 THE XYLANOLYTIC ENZYME SYSTEM

Due to the heterogeneity of xylan, its hydrolysis requires the action of a complex enzyme system (Fig. 2.3). This is usually composed of β-1.4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, and phenolic acid esterases. All these enzymes act cooperatively to convert xylan to its constituent sugar. The presence of such multifunctional xylanolytic enzymes systems is quite widespread among bacteria and fungi (Wong *et al.*, 1988; Coughlan and Hazlewood, 1993).

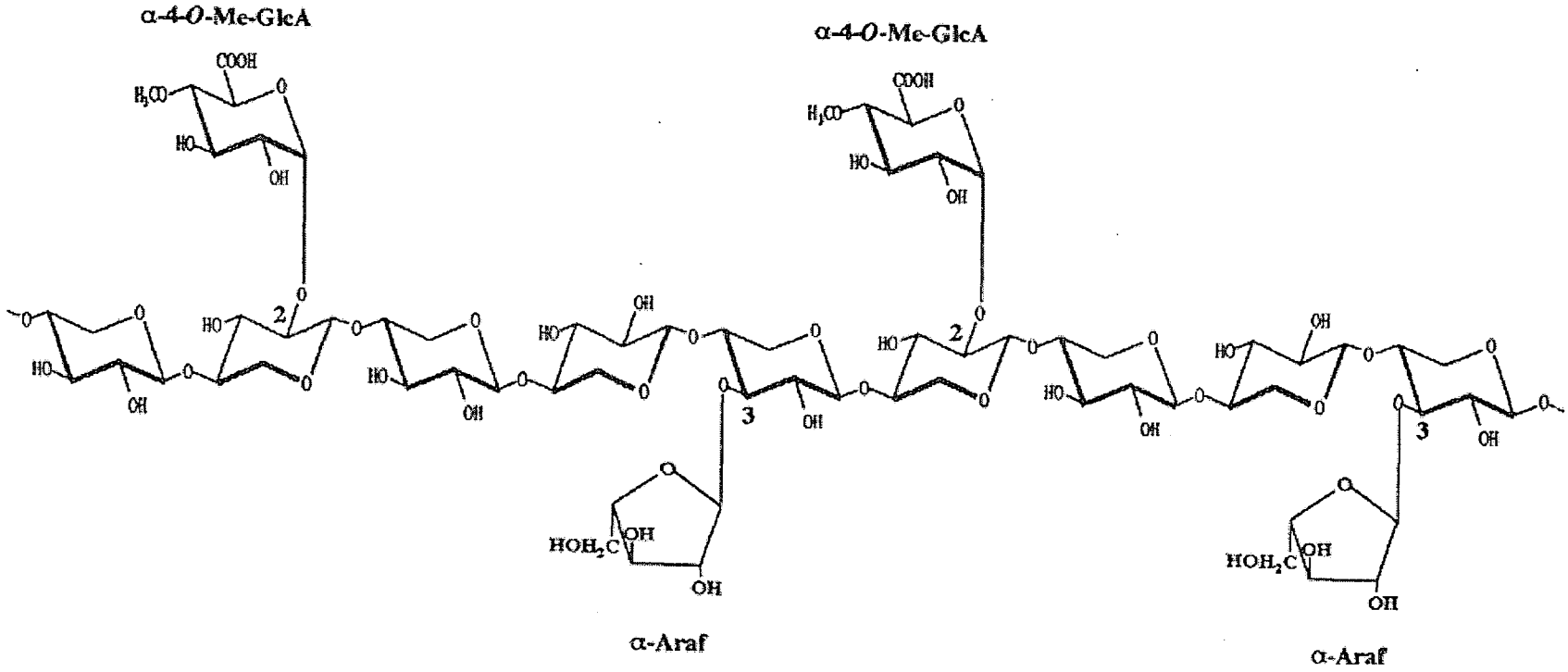


Fig. 2.2 Structure of softwood xylan (arabino -4-*O*-methylglucuronoxylan (Sunna and Antranikian, 1997)

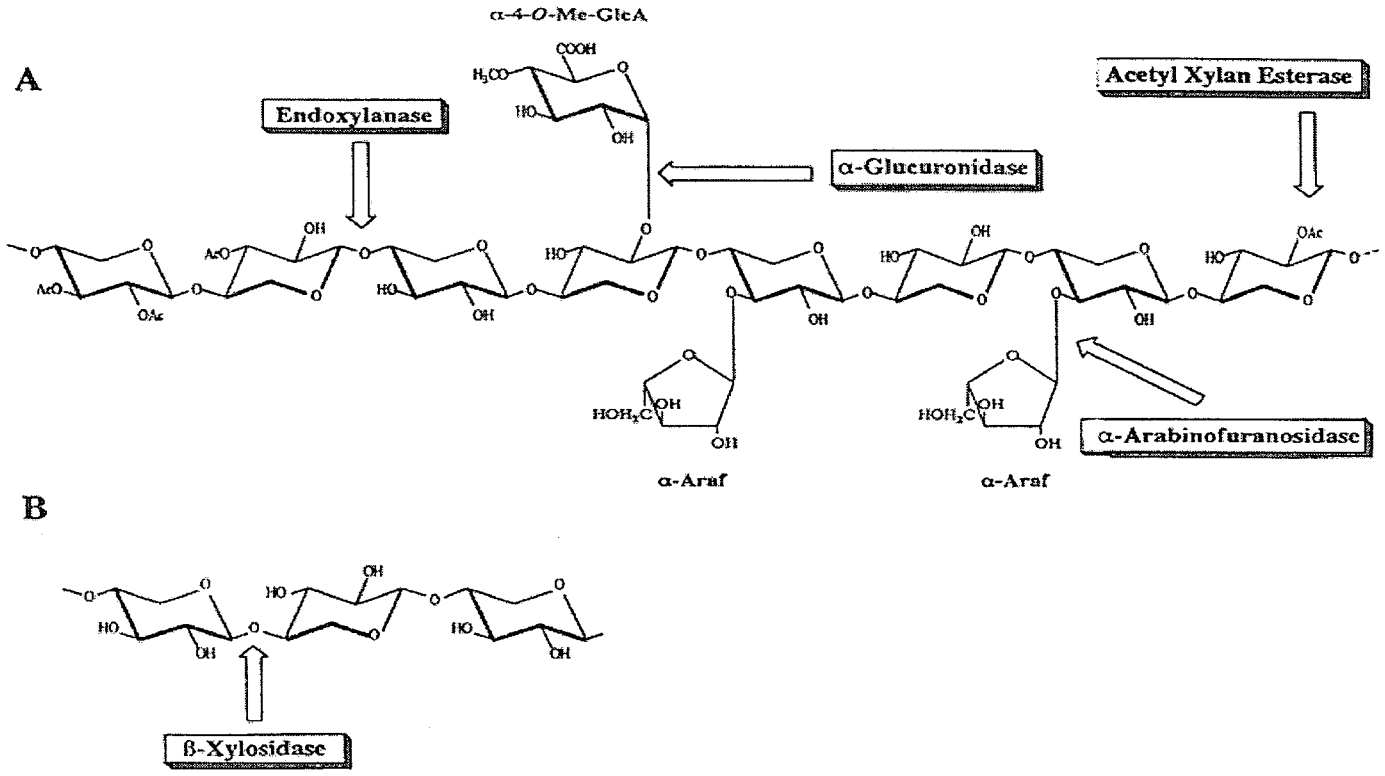


Fig. 2.3 The xylanolytic enzymes involved in the degradation of xylan (A). Hydrolysis of xylooligosaccahride by β-xylosidase (B)
(Sunna and Antranikian, 1997)

Heteroxylans contain different substituent groups in the backbone and side chain. Thus, the degradation of such a complex polysaccharide may involve synergistic action between the different components of the xylanolytic enzyme system (Kleupful *et al.*, 1992).

2.7 PRODUCTION OF XYLANASES

There has been an increasing interest in the use of fungi for xylanase production because of their ability to produce high levels of xylanolytic enzymes (Maheshwari and Kamalam, 1985). Several criteria essential for choosing a microorganism to produce xylanases. In addition to giving the desired biobleaching effect, the resulting enzyme preparation must be prepared in sufficiently high quantity, and the xylanase technology must be compatable with the technology of the pulp mill. Also, it is essential that the enzyme preparation be completely free of cellulase activity as this will have serious economic implications in terms of cellulose loss, degraded pulp quality, and increased effluent treatment cost. Non-cellulolytic preparations have been produced by genetic engineering, selective inactivation, or bulk scale purification (Barnoud *et al.*, 1986; Pederson *et al.*, 1992). The strains reported to be used for commercial production of xylanases include *Trichoderma reesei*, *Thermomyces lanuginosus*, *Aureobasidium pullulans*, and *Streptomyces lividens* (Bajpai and Bajpai, 1996; Jager *et al.*, 1992; Senior *et al.*, 1992).

Xylanolytic acitivity has been reported in a wide variety of different genera and species of fungi under mesophilic growth conditions (Gilkes *et al.*, 1991). These include *A. niger, A.*

fumigatus, T. reesei, T. viride, Penicillium capsulatum, P. stipidis, and *A. pullulans*, and a report by Elisashvili (1993) describes thermophilic fungi degrading xylan which include *H. lanuginosa, Thermoascus aurantiacus, Sporotrichum thermophile* and *Talaromyces byssochlamydoides*. Xylanases from thermophilic fungi have been produced under submerged, static or solid state fermentation (SSF) conditions (Table 2.1), among which the first type is most frequently employed.

Xylanases with half lives from a few minutes up to 90 minutes at 80°C have been produced by *Thermoascus aurantiacus*, *Bacillus stearothermophilus*, *Caldocellum saccharolyticum*, *Clostridium stercorarium* and *Thermomonospora* spp. (Zamost *et al.*, 1991; Luthi *et al.*, 1990). A newly identified *T. lanuginosus* strain MED 4B1 was recently characterized by Haarhoff *et al.* (1998) and growth temperatures indicated that the organism was inhibited at temperatures lower than 37°C and higher than 60°C, classifying it as a thermophile. Similarly, *T. lanuginosus* SSBP has been characterized as possessing an optimum growth temperature of 50°C (Singh *et al.*, 2000a). The highest production level of β-xylanase fom *T. lanuginosus* SSBP was reported to be 60 000 nkat/ml (Singh *et al.*, 2000a). β-xylanase production levels of 36 200 nkat/ml (Purkarthofer *et al.*, 1993); 41 630 nkat/ml (Hoq *et al.*, 1994); 24 000 nkat/ml (Gomes *et al.*, 1993a) 31 670 nkat/ml (Alam *et al.*, 1994), and 47 340 nkat/ml (Bennett *et al.*, 1998) were reported for other *T. lanuginosus* strains.

Table. 2.1 Production characteristics of xylanases from thermophilic moulds

Organism	Substrate	Temperature	pH	Max Prod U/ml	Time (days)	Mode of cultivation	Reference
Allerscheria terrestris	Cellulose	45	6.5	266	LF	3	Grajek, 1987
Aspergillus fumigatus	Wheat bran	45	-	10	SSF	3	Kitpreechavanich, 1984
Humicola lanuginosa	Wheat bran	45	-	2050	SSF	4	Kitpreechavanich, 1984
Malbranchea pulchella	Xylan	42	6.5	18.4	LF	4	Matsuo *et al.*, 1977
Melanocarpus albomyces	Sugar cane bagasse or Xylose	40	6.0	22.5	LF	3-4	Maheshwari and Kamalam, 1985
Mucor pusillus	Cellulose	45	6.5	2	LF	3	Grajek, 1987
Sporotrichum thermophile	Wheat straw or wheat bran	45	-	28.5 15.0	LF	3	Margaritis, 1983
Sporotrichum sp.	Wheat straw	45	5.0	-	-	3	Dubey and Johri, 1987
Talaromyces byssochlamydoides	Xylan	50	-	-	SSF	3	Yoshioka *et al.*, 1981
Talaromyces emersonii	-	45	4.5	-	SSF& LF		Tuohy *et al.*, 1994
Thermoascus aurantiacus	Sugarbeet	45	6.5	9.67	SSF	6	Grajek, 1987
Thermoascus aurantiacus	Xylan	45-50	4.0	-	LF	4	Gomes *et al.*, 1994
Thermoascus aurantiacus	Xylan	45	-	-	LF	10	Tan *et al.*, 1987
Thermomyces lanuginosus	Corn cob	-	-	-		7	
Thermomyces lanuginosus	Xylan followed by barley husk	50	6.0 6.5	9168 9502 (nKat/ml)	LF	5-6	Gomes *et al.*, 1993a; Gomes *et al.*, 1993b
Thielavia terrestris	1 % Solkafloc BW 200	48	4.0	18.8	LF	1.4	Merchant *et al.*, 1988
Torula thermophila	Cellulose	45	6.5	87	LF	3	Grajek, 1987

In practical process conditions, some properties of the enzymes, such as substrate specificity and the pH and temperature optima, are of utmost importance. Enzymes with high pH and temperature optima have been isolated and tested for improving the bleachability of the pulps (Davis *et al.*, 1992; Jager *et al.*, 1992). Similar to the fungi xylanolytic degradation also extends across bacterial gene lines involving both Gram positive and Gram negative staining aerobic and anaerobic microbes (Uffen, 1997). The use of microorganisms at temperatures above 50°C and in alkaline conditions is especially desirable for pulp treatment in the paper industry. For this purpose, hyperthermophilic eubacteria have been isolated that grow anaerobically at temperatures above 80°C. Several alkali-tolerant strains of *Bacillus* species have been used for production of xylanases with pH optima around 9 (Bajpai, 1997). The most thermostable xylanases, with a half-life of 90 min at 95°C, have been produced by *Thermotoga* strain (Simpson *et al.*, 1991). The pH range has been increased to about 8 to 9 and the temperature range to 75°C in some commercial preparations (Bajpai, 1997).

Multiple xylanases have also been purified from culture filtrates of *A. niger* (Wong *et al.*, 1988), *A. oryzae* (Bailey *et al.*, 1991), *A. kawachii* (Ito *et al.*, 1992), *A. awamori* (Kormelink *et al.*, 1993) and other fungi, as well as bacteria (Wong *et al.*, 1988; Viikari *et al.*, 1993). By production of multiple xylanases the microorganisms may maximize the utilization of xylans with different structures (Buchert *et al.*, 1994). The synthesis of multiple xylanases as distinct gene products by one microorganism was revealed on the basis of their different physicochemical properties, such as molecular mass and isoelectric point, rather than on the basis of different catalytic properties. A careful comparison of xylanases by Wong *et al.*

(1988) indicated that these enzymes can be divided into two groups, one consisting of high molecular mass enzymes with low pI values, and the other consisting of low molecular mass enzymes with high pI values. Acidic high molecular mass xylanases (> 30kDa) were found to belong to one family, assigned as glycanase family 10 (Henrissat and Bairoch, 1993; formerly family 11), and basic low-molecular mass xylanases, were found to belong to another family, designated as glycanase family 11 (formerly family 11). In spite of limited knowledge on the differences in catalytic properties of xylanases in the two families, it is certain that the enzymes belonging to family 10 exhibit greater catalytic versatility or lower substrate specificity than enzymes of family 11. A common feature of both enzyme families is their endocharacter demonstrating viscometrically and the double displacement mechanism of the hydrolysis of glycosidic bond, which means that both types of enzymes are retaining glycanases (Gebler *et al.*, 1992).

2.7.1 Solid substrate/state fermentation (SSF)

Solid-substrate/state fermentation (SSF) is the growth of microorganisms on moist substrates in the absence of free-flowing water. In solid substrate fermentation (SSF), enzyme productivity levels are strongly affected by the water activity (a_w) of the substrate. Production of xylanases by *T. aurantiacus* under solid-state fermentation was enhanced by optimization of the type of carbon and nitrogen source (Kalogeris *et al.*, 1998).

However, SSF has been found to prove more economical on account of the following:

i) Better mycelium and substrate contact results in greater bioconversion of the substrate.

ii) Wood shavings and/or loosely ground agro-residues can be used in contrast to finely powdered substrate required for liquid state culture.

iii) The organism colonizing the solid state is forced to produce greater amount of specific enzymes and this, therefore results in smaller levels of other extracellular proteins, on account of limited moisture. This makes the purification of the desired protein relatively simpler (Grajek, 1987b).

Submerged fermentation was found to be more suitable for xylanase production by *H. lanuginosa* and *Sporotricum thermophile*, while *Thermoascus aurantiacus* produced higher enzyme titres by solid-state fermentation (Grajek, 1987b). *Melanocarpus albomyces* IIS-68 also produced higher titres in solid-state fermentation than in submerged fermentation (Jain, 1995). Furthermore, the authors found a distinct difference in temperature and pH optima depending upon the method of cultivation of the fungus employed. The use of abundantly available and cost effective agricultural residues, such as wheat bran, corn cobs, rice bran, rice husk, and other similar substrates, to achieve higher xylanase yields using SSF allows reduction of the overall manufacturing cost of biobleached paper. Several workers have also showed a high yield of xylanase at various moisture levels in SSF studies. In SSF using wheat bran and eucalyptus kraft pulp as the primary solid substrates, *Streptomyces* sp. QG-11-3 (Beg *et al.*, 2000) produced maximum xylanase yield at substrate-to-moisture ratio of 1:2:5 and 1:3, respectively. However, on increasing or decreasing the moisture level, the xylanase

yield marginally decreased. In contrast, a lower solid substrate-to-moisture level of 1:1 has been reported for maximum xylanase production by *Bacillus* sp. A-009 (Gessesse and Mamo, 1999). An improvement in xylanase production by fungal mixed culture (*Trichoderma reesei* LM-UC4 E 1, *Aspergillus niger* ATCC 10864, and *A. phoenicis* QM 329) using SSF has also been reported (Gutierrez-Correa and Tengerdy, 1998). A higher xylanase yield using SSF compared with submerged fermentation using wheat straw and sugar cane bagasse has been reported from thermophilic *Melanocarpus albomces* IIS-68 (Jain, 1995).

2.8 BLEACHING OF PULP

There are a number of processes, and hybrid processes, used for the pulping of lignocellulosic materials to obtain fibres for making paper and board products (Smook, 1992). At present chemical pulping of wood commonly applies the kraft process, which uses sulfide in alkali to remove lignin and yield a particularly strong pulp. However, this process leaves behind small amounts of modified lignin that are mainly responsible for the dark colour of the pulp (Hartler and Norrstrom, 1969; Schwartz *et al.*, 1940; Pigman and Csellak, 1948). The removal of this residual lignin is traditionally achieved using a chlorine-based bleaching sequence, where chlorine gas (C) is used during prebleaching to chlorinate lignin for subsequent extraction (E) under alkaline conditions, and chlorine dioxide (D) is used to oxidize the remaining residual lignin to brighten the pulp (Reeve, 1989). A final pulp brightness of 90% ISO is sought for many fine paper products.

Since reports of the toxic nature of certain organochlorine compounds (Kringstad and Lindstrom, 1984), there have been movements to minimize their generation during pulp bleaching by substituting chlorine gas with chlorine dioxide or by eliminating the use of chlorine containing compounds (Pyke and Mckenzie, 1996). Bleaching without chlorine gas has been termed "elemental chlorine free" (ECF), while that without chlorine containing chemicals as been termed "totally chlorine-free" (TCF) (Johnson, 1994). The former has been dependant on the use of expensive chlorine dioxide while the latter has been dependant on oxygen based reagents such as oxygen (O), ozone (Z) and peroxide (P). Although the use of oxygen reagents avoids the need to handle corrosive chlorine based reagents, it is presently less selective and efficient so that the target brightness for TCF bleaching tends to be slightly lower, 75-85% ISO (Kukkonen and Reilama, 1993). The modification of process conditions and the use of additives are two strategies being tested to improve both TCF and ECF bleaching processes. Enzymes have been recognized as a biotechnological alternative that warrants consideration along with other chemical alternatives (Forber, 1992a, b).

2.9 BRIEF HISTORY OF XYLANASE-AIDED BLEACHING

2.9.1 Discovery and laboratory research

The first report on the possibility that xylanase (X) can enhance the bleaching of kraft pulp was presented in Stokholm in 1986, at the Third International Conference on Biotechnology in the Pulp and Paper Industry (Viikari *et al.*, 1986). The pretreatment of

pulp with crude hemicellulases, prepared from a fungus and an actinomycete, was found to improve peroxide delignification of birch and pine kraft pulp, reducing the amounts of lignin remaining in the pulp as measured by kappa number. (DC)E delignification (using chorine dioxide and chlorine with subsequent alkaline extraction) was also enhanced, permitting reduction in the amount of chlorine use in the prebleaching stage. Subsequent work using partially purified enzymes suggested that xylanase, and not other hemicellulases or debranching enzymes, is mainly responsible for the observed increase in pulp delignification as well as in pulp brightness (Kantelinen *et al.*, 1988). Although more recent work has shown that mannanase also contributes to the bleaching of softwood pulps (Suurnakki *et al.*, 1994; Allison *et al.*, 1996; Gubitz *et al.*, 1996), the ability of xylanase to enhance the bleaching of kraft pulp has been verified using genetically cloned xylanases that have no other known enzymatic activity (Paice *et al.*, 1988; Senior and Hamilton, 1993). Laboratory studies by many other workers have now confirmed the occurrence of xylanase aided bleaching (Viikari *et al.*, 1994).

2.9.2 Mill trials and current status

Since the first mill trial in 1988, there have been more than 75 trials in Canada and Europe (Jurasek and Paice, 1992). Most of these trials used chlorine based bleaching sequences (Koponen, 1991; Lavielle *et al.*, 1992; Pedersen *et al.*, 1992; Turner *et al.*, 1992; Dunlop-Jones and Gronberg, 1995), although some TCF trials were also reported (Gronberg *et al.*, 1995; Lundgren *et al.*, 1994). Only a few of these trials were reported in detail and the results tended to be encouraging in terms of both chemical savings and

reduction of organochlorine in the effluent. The enzyme can be conveniently applied before the storage tower between the kraft digester and the bleach plant, where a residence time 1-2 hours has been found to be sufficient.

Xylanase treatment has been shown to reduce the requirement of chlorine for bleaching while still achieving a high brightness and good pulp properties as shown in Table 2.2, (Bajpai *et al.*, 1994). Results from laboratory studies and mill trials show about 35 to 41 % reduction in active chlorine at the chlorination stage hardwoods and 10 to 20 % for softwoods, whereas savings in total active chlorine were found to be 20 to 25 % for hardwoods and 10 to 15 % for softwoods (Buchert *et al.,* 1992a; Clark *et al.*, 1991; Viikari *et al.*, 1986, 1987, 1994). A recent report by Jain *et al.* (2001) showed xylanase mediated reduction in chlorine from 42 kg/ton pulp to 36 kg/ton pulp in the case of kraft wood and from 51 kg/ton pulp to 42 kg/ton pulp in the case of kraft bagasse pulp indicating a net saving of 14 and 18 % respectively, when bleaching was performed to achieve brightness levels of 83 to 85 % ISO. Examination of the strength properties under optimized xylanase pretreatment conditions on kraft bagasse pulp indicated an improvement in burst and tensile strength by 20 %. The AOX, COD and BOD of the bleach effluent after the CE stage of the enzyme treated pulps was subsequently reduced by 20 % with kraft wood pulp and 25 % with kraft bagasse pulp.

Similarly, the bleaching of ethanol/water-bagasse pulps by using xylanase from *T. lanuginosus* IOC-4145 and Catazyme (Sandoz) indicated a decrease in kappa number with a corresponding increase in viscosity (Goncalves *et al.*, 2001). These two enzymes

demonstrated the same bleaching efficiency with a decrease in kappa number of 66 % after 8 h of xylanase treatment followed by alkaline extraction. The effect of xylanase bleaching of non-woody (*Saccharum spontanem*) pulp was also recently evaluated by Jahan *et al.* (2001). They reported a 2 % brightness increase in DED bleaching with improved hand sheet properties in comparison to the control. A report by Madlala *et al.* (2001) had shown an increase of 2.0 brightness points produced by *T. lanuginosus* xylanase on bagasse soda pulp, which correlated to 2.5-3.0 kg/ton saving of ClO_2 during DED bleaching. These reports indicate the effective introduction of xylanases into ECF bleaching processes.

Table 2.2 Effect of xylanase treatment on the increase in brightness improvement of eucalyptus kraft pulp in a conventional bleaching sequence (Bajpai and Bajpai, 1994)

Enzyme	Bleaching sequence	Brightness (% ISO) Control enzyme		Viscosity (mPa.s)
Cartazyme	CEHH	80.4	84.2	3.8
HS-10	CEH	78.1	83.0	4.9
Novozyme 473	CEHH	80.6	83.6	3.0
VAI xylanase	CEHH	80.4	82.5	2.1

In elementary chlorine-free bleaching sequences, the use of enzymes increases the productivity of the bleaching plant when the production capacity of ClO_2 is a limiting factor. This is often the case when the utilization of chlorine gas has been abandoned (Table 2.3).

Table 2.3 Effect of xylanase treatment on conventional bleaching for various *k* factors and chlorine dioxide substitution (Senior and Hamilton, 1992)

	Control		Treated	
ClO_2 substitution (%)	*k* factor	Final brightness (% ISO)	*k* factor	Final brightness (% ISO)
10	0.200	90.0	0.05	90.0
	0.233	90.2	0.10	90.8
	0.266	90.3	0.15	91.8
40	0.150	89.2	0.05	90.0
	0.173	90.0	0.10	90.8
	0.200	91.0	0.15	91.8
70	0.200	90.0	0.05	88.7
	0.233	90.6	0.10	90.0
	0.250	90.9	0.15	90.3
100	0.150	83.5	0.10	87.4
	0.173	85.3	0.15	87.4
	0.200	87.0		

Evaluation of xylanases on nonwoody fibre material in TCF bleaching has received enormous attention over the recent years. In totally chlorine-free bleaching sequences, the addition of enzymes increases the final brightness value, which is a key parameter in marketing of the chlorine-free pulps. In addition, savings in the TCF bleaching are important with respect to both costs and strength properties of the pulp. Raugauskas *et al.* (1994) examined the effect of xylanase pretreatment on bleaching efficiency for a variety of non-chlorine bleaching agents. It was found that xylanase pretreatment of softwood kraft pulps can enhance the bleaching efficiency of non-chlorine based bleaching agents. Optimal biobleaching results were obtained with ozone, which exhibited enhanced bleaching selectivity and brightness gains. Xylanase petreatment also improved brightness and delignification of peracetic acid bleached pulp (Table 2.4)

Table 2.4 Effect of xylanase treatment on bleaching efficiency of peracetic acid and ozone (Bajpai and Bajpai, 1997)

Bleaching treatment	Kappa number	Brightness (%)	Viscosity (m Pa s)
Brownstock	22.3	24.0	14.2
Xylanase-treated brownstock	21.7	26.8	15.1
X(PAA-2% charge) E	7.2	51.4	7.6
(PAA-2% charge)E	8.8	49.4	78
X(PAA-3.9% charge)E	5.4	51.4	7.4
(PAA-3.9% charge)E	6.3	48.7	7.8
X(PAA-4.4% charge)E	5.8	51.2	8.9
(PAA-4.4% charge)E	6.2	49.8	8.5
Bleached with 1.2% ozone/Ph 2.5			
Xylanase treated	9.9	-	-
Control	11.6	-	-
Followed by caustic extraction:			
Xylanase treated	6.3	10.3	48
Control	7.6	9.4	44
Bleached with 0.6% ozone/pH 2.5			
Xylanase treated	15.2	-	-
Control	15.8	-	-
Followed by caustic extraction:			
Xylanase treated	11.2	12.1	38
Control	12.5	11.4	36
Bleached with 0.6% ozone pH 5.0			
Xylanase treated	15.0	-	-
Control	16.1	-	-
Followed by caustic extraction:			
Xylanase treated	12.2	12.2	36
Control	13.9	17.7	32

The impact of xylanases on kenaf AQ pulp, a nonwoody material was clearly demonstrated in a TCF bleaching sequence using oxygen, hydrogen peroxide and ozone de la Rosa *et al.* (2001a). This study revealed increases in brightness of 1 point with xylanase pretreated pulp in comparison to the controls. The enzyme application displayed its influence in the kappa number and the brightness of the pulp, however no conclusions could be drawn for the viscosity of the pulp. The application of xylanases (Xyl A and Xyl B) from *Thermotoga martima* in TCF bleaching of straw has also been reported (Hernadi *et al.*, 2001). These two xylanases have demonstrated high temperature optimum of 90°C, and brightness increases of 7 and 4 %, respectively in comparison to the control. The xylanases from this organism therefore display favourable characteristic with respect temperature stability, which is highly attractive in the pulp and paper industry.

There have been reports of routine usage of xylanase at six kraft mills in Canada (Tolan *et al.*, 1995; Jurasek and Paice, 1992) and several mills in Europe (Jurasek and Paice, 1992; Fastén, 1993; Grant, 1996). The motivation for using xylanase has been the increased production from mills limited in chlorine dioxide generation, the marketing advantage of pulp that was produced by a more "environmentally friendly" process, the cost savings associated with lower chemical usage, and the minimal process modification required (Grant, 1996; Lavielle, 1993). Another attractive advantage of implementing xylanase prebleaching has been the low capital investment required, estimated to be between US$ 10 000 to 100 000 for most mills (Capps, 1995; Farrell *et al.*, 1996a). The target cost for enzyme application has been estimated at US$ 3.50 per ton in 1995 (Farrell

et al., 1996a). Further improvements desired for xylanase technology include, enzymes that are effective at higher pH's, cost less, or have greater and more consistent effects on pulp brightness (Tolan *et al.*, 1995).

2.10 DIFFERENT RESPONSES OF XYLANASES IN PULP BLEACHING

There are many variables in a pulp bleaching sequence, ranging from differences in the feedstock to those in the combinations of chemicals. Each variable could alter the effects observed with xylanase treatment, thus providing alternative views to the mechanisms of xylanase aided bleaching. The difficulties encountered when elucidating the mechanisms with which xylanase enhances pulp bleaching include, differences associated with wood species, pulping processes, bleaching agents and enzyme preparations (Wong *et al.*, 1997a).

2.10.1 Wood species

Compared to softwood kraft pulp, hardwood kraft pulp contains more xylan, more carboxylic acid groups and surface charge (Laine *et al.*, 1994; Buchert *et al.*, 1997) and smaller xylan macromolecules (Eremeeva *et al.*, 1995; Wong *et al.*, 1996). Relatively more lignin-xylan complexes, where lignin and xylan are covalently linked, seem to occur in hardwood pulps while more lignin-cellulose complexes occur in softwood pulps (Karlsson and Westermark, 1996). There is a tendency for hardwood kraft pulp to show

higher benefits with regard to chlorine savings (Munk *et al.*, 1993; Hamilton *et al.*, 1996) during xylanase-aided bleaching. Some variation in response has also been reported among hardwood (Farrell *et al.*, 1996a) and softwood (Suurnakki *et al.*, 1996; Wong *et al.*, 1996) species, as well as among feedstocks from different plantations of Eucalyptus globulus (Nelson *et al.*, 1995). These differences may be partly attributed to differences in xylan solubilization from pulp, which may in term be related to the amounts of accessible xylan present, the ion composition (Buchert *et al.*, 1995a) and buffering capacity that could moderate enzyme-fibre interactions, or the wood's response to kraft pulping.

2.10.2 Pulping processes

Xylanase-aided bleaching appears most effective on pulp produced by a conventional batch process where there is substantially more redeposited xylan (Kantelinen *et al.*, 1993). Although the effects of the enzyme may be smaller, these effects have been observed in continuous cooked pulps such as those produced by MCC (modified continuous cook) (Suurnakki *et al.*, 1994; Allison *et al.*, 1996; Suurnakki *et al.*, 1996) and EMCC (extended MCC) (Suurnakki *et al.*, 1994), and in low kappa pulps such as those produced by extended cooking (Allison *et al.*, 1995), superbatch (Yang *et al.*, 1994), kraft-anthraquinone (Saake *et al.*, 1995) and prehydrolysed kraft (Chang and Farrell, 1996) processes. Pulps from other pulping processes have also been reported to be responsive to xylanase treatment: alkaline sulphite-anthraquinone-methanol (ASAM) and pre-hydrolysed ASAM pulps that are produced using processes designed to preserve

carbohydrates (Saake *et al.*, 1995), acid sulfite pulp that was alkaline extracted or oxygen delignified before bleaching (Christov and Prior, 1994; 1996a, b), organosolv pulp (Leite *et al.*, 1995; Gliese *et al.*, 1996), soda pulp (Orgill and Pichler, 1992), and even themomechanical pulp (Grant, 1996; Gronberg *et al.*, 1995). More work is however needed to confirm these findings. In particular, xylanase treatment did not significantly enhance the bleaching of acid sulfite pulps in other studies (Buchert *et al.*, 1995a; Frühauf *et al.*, 1996), and such results are expected due to the high degradation of xylan in its pulping process (Buchert *et al.*, 1995a). Furthermore the mechanisms with which xylanase enhances the brightening of non-kraft pulp types may be quite different from those occurring in kraft pulp.

2.10.2.1 Dependence on kappa number and brightness

Xylan content of pulps produced by different kraft conditions tended to decrease with lower kappa numbers (Johnson, 1994). There was however no clear relationships between the response of pulps at different kappa numbers to xylanase-aided bleaching. Brighter pulps that have been partially bleached with oxygen (Suurnakki *et al.*, 1996; Allison *et al.*, 1995, 1996) or peroxide (Wong *et al.*, 1997b; Nelson *et al.*, 1995) remained responsive to xylanase treatment. When the oxygen delignified pulps were compared with extensively cooked kraft pulps having the same kappa number, their bleachability after xylanase treatment appeared greater (Suurnakki *et al.*, 1994). Indeed, Alison *et al.* (1995) reported that oxygen delignified pulps derived from radiata pine become more responsive with lower kappa numbers while kraft pulps become less

responsive. It would seem that the delignification process is more important than the kappa number of the pulp.

2.10.2.2 Dependence on pulp washing

Immediately after pulping, washing can remove as much as 30% of the bulk lignin found in the secondary walls, with minimal effect on the lignin present in cell corners (Choi *et al.*, 1976). The leaching of lignin is increased with increasing temperature (Hagström-Näsi *et al.*, 1987; Smith *et al.*, 1993), increasing pH (Li *et al.*, 1996), increasing pH beating (Abe, 1987), and decreasing ionic strength (Hagström-Näsi *et al.*, 1987; Favis *et al.*, 1983). Lignin leaching seems associated with the leaching of carbohydrates, particularly xylan (Li *et al.*, 1996), and with an increase in the water retention value of the pulp (Wilcox and Goring, 1990). The decrease of lignin content as a result of pulp washing can be measured as a drop in kappa number (Allison *et al.*, 1993a,b), by as much as 4 units (Allison and Clark, 1994). Its occurrence means that a proper control is required before any enhancement in pulp bleaching can be reliably attributed to the action of xylanase. Furthermore Tolan and Canovas (1992) suggested that pulp washing may remove enzyme inhibitors occurring in the black liquor or help stabilize enzymes at high temperatures, although Farrell *et al.* (1996b) have indicated that it has minor impact on enzyme performance. Inconsistent results have also been reported on the effects of washing the pulp after enzyme treatment. In contrast to findings by Pedersen *et al.* (1992), Senior *et al.* (1992), as well as Tolan and Canovas (1992) reported that the

washing of enzyme treated pulp is not necessary for the subsequent enhancement of pulp bleaching.

2.10.3 Bleaching agents

Xylanase-aided bleaching has been tested in numerous chlorine based, ECF and TCF bleachng sequences (Viikari *et al.*, 1994; Farrell *et al.*, 1996a; Daneault *et al.*, 1994), including those that use recently advocated chlorine-free reagents such as ozone (Ragauskas *et al.*, 1994; Pham *et al.*, 1995), dimethyldi-oxirane (Nelson *et al.*, 1995), iso-urea peroxide (Hamilton *et al.*, 1996), peroxide activated with nitrilamine (Nelson *et al.*, 1995), oxone, performic acid (Ragauskas *et al.*, 1994), and peroxymonosulfate (Allison *et al.*, 1996). There have been suggestions that the enzyme stage is more effective before acidic oxidation stages with chlorine, ozone or peracetic acid than before alkaline oxidation stages using oxygen or peroxide (Allison *et al.*, 1993b). Bleaching sequences that yield higher final brightness are also thought to benefit less from enzyme treatment (Vicuña *et al.*, 1995; Leduc *et al.*, 1995). However, although the magnitude of the brightness gain becomes smaller with higher pulp brightness, there remains a large potential for chemical savings (Senior and Hamilton, 1993; Dunlop-Jones and Gronberg, 1995). A description of the functions, advantages and disadvantages of bleaching chemicals is summarized in Table 2.5.

Table 2.5 Functions, advantages and disadvantages of bleaching chemicals (Singh, 1993)

Oxidant	Function	Pulp type[a]	Advantages	Disadvantages
Chlorine	Oxidize and chlorinate lignin	C	Effective, economical deligninfication, good particle removal	Organochlorine formation; highly corrosive
Hypochlorite	Oxidize, decolourize and solubilize lignin	C	Easy to make and use; Low cost	Can cause loss of pulp strength; chlroform formation
Chlorine dioxide	1) Oxidize, solubilize and decolourize lignin 2) In small amounts with Cl_2, protects against cellulose degradation	C	Achieves high brightness without loss of pulp strength; good particle bleaching	Must be made on-site; cost; some organochlorine formation; highly corrosive
Oxygen	Oxidize and solubilize lignin	C	Low chemical cost; provides chloride-free effluent for recovery	Requires significant capital equipment when used in large amounts; potential loss of pulp strength
Hydrogen peroxide	Oxidize and decolourize lignin	C/M	Easy to use; low capital cost	High chemical cost; poor particle bleaching; can cause loss of pulp strength
Ozone	Oxidize, decolourize and solubilize lignin	C	Effective, provides chloride-free effluent for recovery	Must be made on-site; cost; poor particle blaeching and pulp strength
Reductant hydrosulfite	Reduce and decolourize lignin in high-yield pulps	M	Easy to use; low capital cost Effcetive and econimical	Limited effectiveness; cost
Alkali: sodium hydroxide	Hydrolyse chlorolignin and solubilize lignin	C		Darkens pulp
Chelants EDTA or DTPA	Remove metal ions	C	Improves peroxide selectivity and efficiency	Cost

2.10.3.1 Oxygen delignification

Extensive use of oxygen will be favoured to obtain high pulp brightness when less chlorine or chlorine containing agents are used (Collodette *et al.*, 1994; McDonough, 1995). Oxygen delignification has been considered as an extension of pulping because the filtrate is sent back to the chemical recovery cycle of the kraft process, and as bleaching stage because it brightens the pulp substantially. It is considered as bleaching stage here because fibres rather than wood chips are being delignified. Besides enhancing subsequent bleaching of oxygen delignified pulps, the xylanase stage has been reported to directly yield a small drop in kappa number (Nelson *et al.*, 1995). Reports have also indicated that xylanase treatment can be applied successfully before oxygen delignification (Roncero *et al.*, 1996; Vidal *et al.*, 1997).

2.10.3.2 Chlorine dioxide bleaching

The effectiveness of xylanase pretreatment appears dependant on the degree of substitution of elemental chlorine by chlorine dioxide, with the response of hardwood pulps being better at low substitution (du Manoir *et al.*, 1991). The response of certain softwood pulps was found to be best at 100% substitution (Senior and Hamilton, 1993) but not in pulp derived from radiata pine (Allison *et al.*, 1993b). There can be substantial savings in chlorine dioxide (15-35%) when xylanase is used to achieve target brightness desired from an ECF sequence (Senior *et al.*, 1992; Lahtinen *et al.*, 1993; Vicuña *et al.*, 1995), and this has been confirmed in mill trials (Pedersen *et al.*, 1992; Jean *et al.*, 1994).

The brightness gains achieved with low levels of DED bleaching (≤80% ISO, gain ≥ 5% ISO) are generally much higher than those achieved with high levels (90% ISO, gain ≤ 2% ISO). There are also results that suggest the existence of a higher brightness ceiling for enzyme treated pulps (Munk *et al.*, 1993; Hamilton *et al.*, 1996). Other results indicate that the chemical savings can be obtained at either the prebleaching or the brightening stages of the bleach sequence (Gibson, 1992; Aumont, 1996).

2.10.3.3 Peroxide bleaching

Similar to oxygen, peroxide has been found to be useful in ECF and TCF bleaching sequences, being used both as an alkaline bleaching stage or as a reinforcement for alkaline extraction. As high as 4% ISO brightness gain (Tolan, 1992; Suurnakki *et al.*, 1996) and 30% saving in peroxide (Dunlop-Jones and Gronberg, 1995) have been reported after xylanase treatment. A chelation stage (Q) is important for peroxide bleaching because it removes metal ions that cause decompostion of peroxide. Since chelation is applied at a pH and temperature stability for the activity of many commercial xylanases, the two treatments have been successfully combined into one stage (Allison *et al.*, 1996) although it has been suggested that certain xylanases are inhibited by chelating agents (Gubitz *et al.*, 1996).

The brightness gains after low levels of peroxide bleaching are generally similar to those after high levels, and there are claims that the xylanase treatment raises the brightness ceiling of peroxide bleaching (Tolan, 1992; Pham *et al.*, 1995). The systematic study of

the QPP bleaching of hardwood and softwood krat pulps also indicate that enzyme stage is equally effective at the beginning or end of the bleaching sequence (Wong *et al.*, 1997c). This study showed that the lignin solubilized and that which became extractable after xylanase pretreatment was smaller in size, but larger in quantity, than the respective lignin fractions after xylanase post treatment (de Jong *et al.*, 1996). These results therefore suggests that xylanase attacks a target that is not very amenable to peroxide bleaching and corroborate an early report that the location of the enzyme stage does not seem to be very important in TCF bleaching sequences (Ledoux *et al.*, 1993).

2.10.4 Enzyme preparations

2.10.4.1 Purified xylanases

Xylanases are known to differ in their structure as well as in their activity under a variety of pH and temperature conditions. Several reports have revealed that xylanases can vary in their ability to enhance pulp bleaching (da Silva *et al.*, 1994; Elegir *et al.*, 1996). An interesting physicochemical property of fungal and bacterial xylanases seems to be the strong relationship between their molecular weight (MW) and isoelectric points (pI). In Table 2.6, the physicochemical properties of purified xylanases are presented. Almost 70 % of the acidic endoxylanases have molecular weight values above 30,000. However, it should be mentioned that there are several exceptions to this general pattern and endoxylanases with low pI and low MW values have been reported, and vice versa (Sunna and Antranikian, 1997).

Table 2.6 Physico-chemical properties of purified endoxylanases (Sunna and Antranikian, 1997)

Organism	Growth temperature(°C)	Optimal temperature (°C)	Optimal pH	pI	MW	Km (mg/ml)	Vmax (U/mg)
Aspergillus awamori CMI 142717	24	55	5.5-6.0	5.7-6.7	39.0	1.00	1.00×10^4
Aspergillus kawachii	30	60	5.5	6.7	35.0	N.D	N.D
Aureobasidium pullulans Y-2311-1	28	54	4.8	9.4	25.0	7.20	2.65×10^3
Humicola grisea var. *thermoidea*	40	70	5.5	N.D	25.5	3.30	2.29×10^2
Trichoderma reesei Rut C30	30	N.D	5.0-5.5	9.0	20.0	3.80	1.41×10^3
Thermomyces lanuginosus SSBP	50	70	6.5	3.9	23.6	3.26	6.3×10^3
Bacillus sp. NIM 59	48-50	55-60	7.0	9.2	22.0	N.D	N.D
Bacillus Stearothermophilus T-6	60	75	6.5	7.0	43.0	1.63	2.88×10^2
Streptomyces sp. T7	50	60	4.5-5.5	7.8	21.8	10.00	7.60×10^3
Streptomyces thermoviolaceus OPC-520	50	60	7.0	8.0	33.0	N.D	N.D
Thermotoga maritime SB8	80	105	5.4	5.6	40.0	0.29	4.76×10^3

Most characterized endoxylanases are optimally active at temperature ranges between 45 and 75°C, and only a small number of purified bacterial and fungal endoxylanases show maximal activities at temperatures above 80°C, with xylanases from the genus *Thermotoga* being one of them (Sunna and Antranikian, 1997).

The use of purified xylanases for pulp bleaching have to date been done with enzymes belonging to family 11, one of two structural families of xylanase distinguished using amino acid sequences (Henrissat and Bairoch, 1993). The *T. lanuginosus* purified xylanase clearly demonstrated an increase of 4.5 brightness points on bagasse pulp in DED bleaching, corresponding to 18 % chlorine dioxide savings in the bleach sequenece (Bissoon *et al.*, 2002). Evaluation of the strength properties of the pulp after full bleaching revealed negligible differences in comparison to the control. Two Family 11 xylanases, both isolated from the fungus *Trichoderma reesei*, have been examined extensively. Their optimum pH for activity on soluble xylan differed from that for activity on pulp. One enzyme was found more effective in solubilizing sugars from pulp and in enhancing pulp bleaching, and more tolerant to the removal of metal ions from pulp (Buchert and Viikari, 1995). Part of these differences may be attributed to charge interactions between pulp and enzyme (Buchert *et al.*, 1993). With these considerations, it is clear that enzyme assays which use soluble xylan as the substrate are not very useful for evaluating the effectiveness of xylanase in enhancing pulp bleaching. Even the use of dyed pulp to measure xylanase activity on insoluble substrates (Freiermuth *et al.*, 1994) may not be adequate for this purpose.

2.10.4.2 Commercial xylanases

Commercial enzymes vary with respect to the activities and optimum conditions of their constituent xylanases. In addition they may also differ in the formulation used for their stabilization, in the amounts of contaminating cellulase (Tolan, 1995) and other enzymatic activities, and perhaps more importantly in the cost of their production and subsequent cost to mills. It is therefore of no surprise that some differences among commercial enzymes have been reported in their effectiveness for enhancing pulp bleaching (Nelson *et al.*, 1995; Leduc *et al.*, 1995; Bajpai *et al.*, 1994; Clark and Allison, 1996). This variation among enzyme preparations also seems to depend on the type of pulp (Wong *et al.*, 1996; Allison *et al.*, 1993a) as well as the bleaching sequence (Leduc *et al.*, 1995). A list of industrial suppliers of commercial xylanases used for prebleaching is represented in Table 2.7. Cost benefits during mill applications should be the ultimate comparison of commercial enzymes because there is no other simple basis for standardization. Moreover reports in the literature seldom associate the identity of commercial enzymes with their corresponding data, or characterize their enzymatic and non-enzymatic components.

Table 2.7 Industrial suppliers of commercial xylanases (Bajpai, 1999)

Supplier	Product trade name
Clariant, U. K.	Cartazyme HS 10
Clariant, U. K.	Cartazyme HT
Clariant, U. K.	Cartazyme SR 10
Clariant, U. K.	Cartazyme P S 10
Clariant, U. K.	Cartazyme 9407 E
Clariant, U. K.	Cartazyme NS 10
Clariant, U. K.	Cartazyme MP
Genencor, Finland/Ciba Geigy Switzerland	Irgazyme 40-4X/ Albazyme 40-4X
Genencor, Finland/Ciba Geigy Switzerland	Irgazyme-10A/ Albazyme 10A
Voest Alpine, Austria	VAI- Xylanase
Novo Nordisk, Denmark	Pulpzyme HA
Novo Nordisk, Denmark	Pulpzyme HB
Novo Nordisk, Denmark	Pulpzyme HC
Rohn Enzyme OY, Finland	Ecopulp X-100
Rohn Enzyme OY, Finland	Ecopulp X-200
Rohn Enzyme OY, Finland	Ecopulp X-200/4
Rohn Enzyme OY, Finland	Ecopulp TX-100
Rohn Enzyme OY, Finland	Ecopulp TX-200
Rohn Enzyme OY, Finland	Ecopulp M
Solvay Interox, USA	Optipulp L-8000
Thomas Swan Co., UK	Ecozyme
Iogen Corp., Canada	GS-35
Iogen Corp., Canada	Hs-70

2.10.4.3 Enzyme dosage

In general the effects that xylanase has on pulp bleaching increase with enzyme dosage until a threshold is reached (Brown *et al.*, 1994; Skerker *et al.*, 1991; Buchert *et al.*, 1992b; Clark and Allison 1996). This threshold depends on the type of pulp (Allison *et al.*, 1993a), the chemical loadings in the bleaching sequence (Brown *et al.*, 1994), and no doubt on whether the conditions of the enzyme stage favour enzyme activity. Generally the optimal enzyme dose lies within the range of 2-5 IU of enzyme per gram of dry pulp (Bajpai, 1999).

2.10.4.4 Other enzyme applications

Although initial results were inconclusive because of a concomitant solubilization of xylan macromolecules (Clark *et al.*, 1991), more recent work indicates that certain mannanases can enhance the bleaching of softwood kraft pulp (Yu *et al.*, 1998; Cuevas *et al.*, 1996; Farrell *et al.*, 1996a), but not hardwood kraft pulp were glucomannans occur in very small amounts. Although there seems to be a great range in the effectiveness of different mannanases (Allison *et al.*, 1996; Clark *et al.*, 1991), certain mannanases appear to be more effective on pulp with lower kappa numbers and the increase in pulp brightness seem associated with very small decreases in kappa number (Suurnakki *et al.*, 1996). After oxygen delignification, the effects of mannanse seems particularly strong in superbatch pulp (Suurnakki *et al.*, 1994) but weak on MCC pulp (Allison *et al.*, 1996). Additive effects between certain mannanases and xylanases have been reported in

peroxide bleaching (Cuevas *et al.*, 1996). In contrast, α-arabinosidase that removes arabinosyl branches from xylan was reported to interfere with xylanase aided bleaching despite its inherent ability to slightly enhance pulp bleaching (Luonteri *et al.*, 1996). No consistent role has so far been demonstrated for other xylan-degrading enzymes (Kantelinen *et al.*, 1988). There is also no conclusive evidence indicating that endoglucanases and cellobiohydrolases, that attack cellulose only, can enhance pulp delignification (Buchert *et al.*, 1994).

Early work failed to show any enhancement of pulp bleaching using oxidative enzymes from ligninolytic fungi (Viikari *et al.*, 1987; Kantelinen *et al.*, 1993). Indeed these enzymes appeared to darken the pulp samples. Research has indicated that peroxide bleaching is enhanced when laccase (Kantelinen *et al.*, 1993), lignin peroxidase (Niku-Paavola *et al.*, 1994) or manganese peroxidase (Harazono *et al.*, 1996) was used after xylanase treatment. The difficulties with obtaining positive results appear to be the relatively specific conditions required for pulp delignification by ligninolytic enzymes. The breakthroughs in this area in the past few years (Kondo *et al.*, 1994; Paice *et al.*, 1995), particularly concerning the use of mediators with laccase (Call and Mücke, 1994), suggest that the cooperation between xylanase and ligninolytic enzymes needs to be reconsidered.

2.11 PROPOSED MECHANISMS OF XYLANASE-AIDED BLEACHING

A number of hypotheses have been proposed to explain the effect that xylanase pretreatment has on subsequent bleaching of kraft pulp (Buchert *et al.*, 1994; Viikari *et al.*, 1994). The early hypothesis that xylanase attacks redeposited xylan was readily accepted because it is highly plausible that redeposited xylan entraps residual lignin and thus interferes with chemical bleaching. Irrespective of their physical distribution across the fibre wall, xylan substrates may be in one of the four forms which are xylan derived chromophores, lignin-xylan complexes, physical entrapment of lignin and fibre swelling (Wong and Saddler, 1992) as shown in Fig. 2.4 .

2.11.1 Xylan-derived chromophores

Xylose and xylan macromolecules are known to be modified during alkaline cooking to produce coloured (de Jong *et al.*, 1997) and partly aromatic (Gellerstedt and Li, 1995) structures. Xylan derived chromophores may therefore occur in kraft pulp and their removal may be more effectively achieved using xylanase than bleaching chemicals. Recent work has already found that methyl-glucuronic acid substituents on xylan are converted to hexenuronic acid (Teleman *et al.*, 1996), a modified component that is thought to contribute to kappa number and brightness reversion in kraft pulp (Buchert *et al.*, 1997).

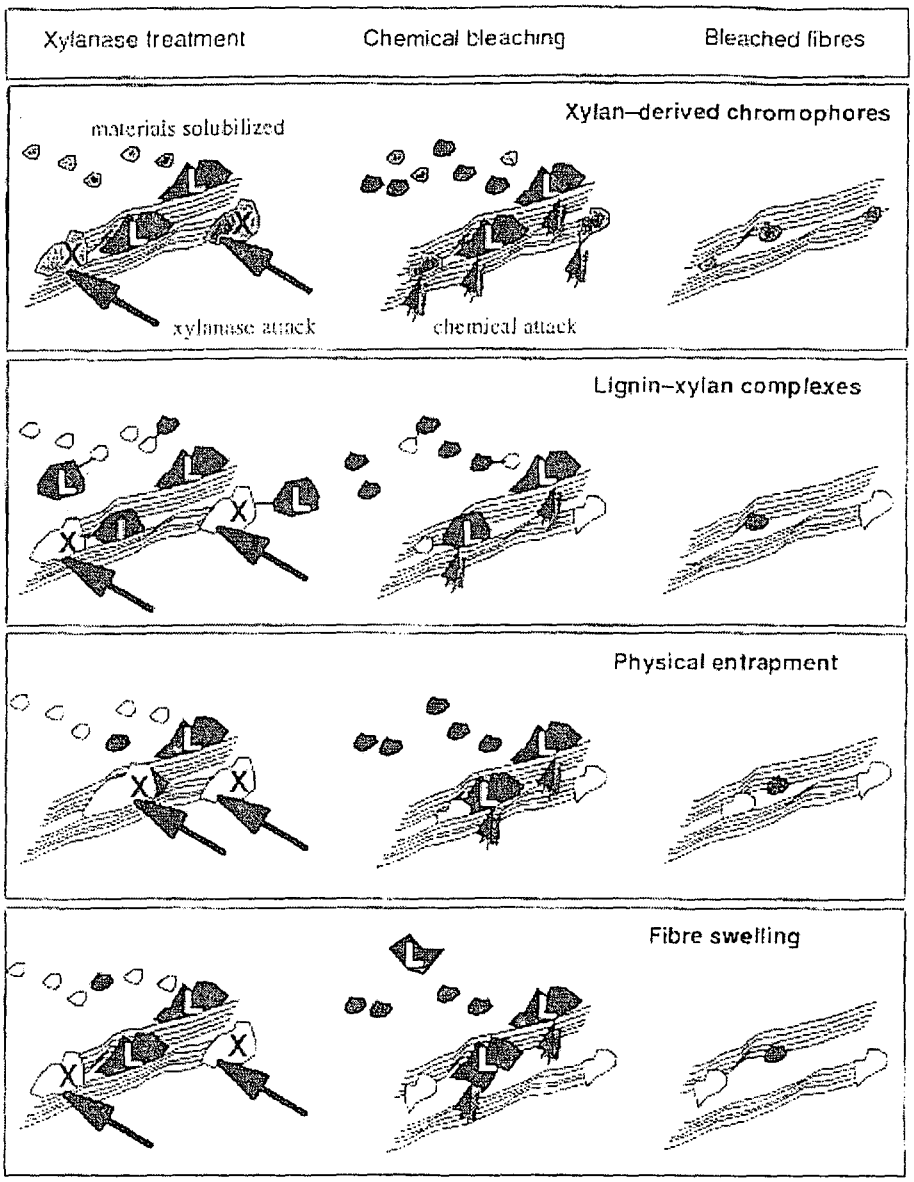


Fig. 2.4 Proposed mechanism of xylanase-aided bleaching (Wong *et al.*, 1997)

2.11.2 Lignin-xylan complexes

There is some evidence that lignin-carbohydrate linkages occurring in kraft pulp (Yamasaki *et al.*, 1981; Wong *et al.*, 1996) restrict the removal of residual lignin (Yamasaki *et al.*, 1981). Xylanase cleavage of the carbohydrate portion of lignin-xylan complexes could facilitate subsequent chemical delignification by releasing the lignin component (Yang and Eriksson, 1992) or by reducing the overall size of macromolecules containing residual lignin.

2.11.3 Physical entrapment of lignin

Deposits of xylan may physically entrap residual lignin on fibre surfaces (Kantelinen *et al.*, 1993). Not only would its removal facilitate the diffusion of residual lignin out of the fibre matrix, it may also enhance the accessibility of this lignin to bleaching chemicals. A similar hypotheisis has arisen from past work on the topochemistry of kraft pulping, in which xylan *in situ* was thought to hinder the delignification process (Kerr and Goring, 1975). In this scenario, the removal of xylan would leave behind pores having sizes similar to those of the macromolecules removed.

2.11.4 Fibre swelling

Adsorptive interactions between cellulose and xylan may contribute to the integrity of pulp fibres (Roberts *et al.*, 1990). The removal of xylan accessible to xylanase may disrupt fibre structure to a degree that facilitates subsequent removal of the residual lignin (Clark *et al.*, 1991). In this scenario, fibre swelling generates pores that are longer than the xylan macromolecules removed. A portion of the residual lignin in kraft pulp is already known to be larger than 20 000 to 40 000 molar mass (Wong *et al.*, 1996), the typical size of xylanase enzymes (Coughlan and Hazlewood, 1993; Viikari *et al.*, 1993). There is also evidence that during the course of the kraft cook, there is a slight increase in fibre swelling at the beginning and a subsequent decline below the original fibre dimensions (Stone and Scallan, 1968).

2.12 MATERIALS SOLUBILIZED FROM PULP AFTER ENZYME TREATMENT

2.12.1 Carbohydrate and lignin

Only a portion of the xylan in lignocellulosic substrates and kraft pulps is accessible to hydrolysis by xylanase (Buchert *et al.*, 1992a; Viikari *et al.*, 1994). The inaccessibility of the remaining xylan has often been attributed to the location of xylan within the pulp fibre matrix. For xylanase-aided bleaching, as little as 1% of hardwood kraft pulp (Pham *et al.*, 1995) and 0.4-2% of softwood kraft pulp (Saake *et al.*, 1995; Suurnaki *et al.*, 1996)

needs to be solubilized, representing less than 16% of the residual xylan. The sugars solubilization of carbohydrate during xylanase treatment are predominant composed of xylan derived residues (Buchert *et al.*, 1996; Allison *et al.*, 1996). The arabinose:xylose ratio in the solubilized oligosaccharides may differ from that in the pulp, suggesting that a certain class of pulp xylan is being attacked (Suurnaki *et al.*, 1994). The profile of these oligosaccharides is dependant on the enzyme (Elegir *et al.*, 1996) or the pH (Elegir *et al.*, 1995) used in the treatment. The solubilization of carbohydrate during xylanase treatment is associated with the solubilization of apparent lignin that absorbs in the UV range from 230-280 nm (Wong *et al.*, 1997b). Infrared spectroscopy of the filtrates from enzymic hydrolysis also suggested the presence of lignin (Pham *et al.*, 1995)

2.12.2 Chromophores

Extensive work has been done on examining the solubilization of colour by measuring absorbance around 460 nm because the wavelengths near 457 nm are used for the determination of pulp brightness (Patel *et al.*, 1993, Garg *et al.*,1996). In other attempts to measure chromophores, filtrate absorbance was monitored in the UV range at 237, 254 and 350 nm (Garg *et al.*,1996, Shoham *et al.*, 1993). Two reports suggested that there is a direct correlation between the solubilization of chromophores absorbing at 237 nm (Garg *et al.*, 1996) and 465 nm (Dunlop *et al.*, 1996) and the brightness achieved after bleaching with chlorine based reagents.

2.13 PROPERTIES OF XYLANASE TREATED PULPS

2.13.1 Optical properties after xylanase treatment

Since xylanase is not expected to act directly on residual lignin, little attention has been paid to the direct effects that this enzyme has on brightness, light absorption coefficient, light scattering coefficient and opacity. Evidence for direct brightening of hardwood brown stock can nevertheless be found in literature, increasing brightness immediately after enzyme treatment by 1.4 % ISO (de Jong *et al.*, 1996; Wong *et al.*,1997). There have also been several reports that xylanase directly brightens oxygen delignified (Wong *et al.*, 1996; Yang *et al.*, 1994) and peroxide bleached (Wong *et al.*, 1997c), as well as hypochlorite bleached wheat straw pulp (Chen *et al.*, 1996) and bagasse pulp (Madlala *et al.*, 2001). However, direct brightening of softwood brownstock remains contentious, with positive (Yang and Eriksson, 1992) and negative (de Jong *et al.*, 1996) reports. For these pulps of low brightness, an increases in brightness might not be detected with a small drop in kappa number (Nelson *et al.*, 1995) because the direct brightening relationship between the absorption coefficient and brightness becomes less prominent with increasing lower brightness (Robinson, 1975).

2.13.2 Optical properties after bleaching

There have been several reports that xylanase pretreatment increases the brightness ceiling of pulp under certain conditions (Pham *et al.*, 1995; Suurnakki *et al.*, 1996), meaning that xylanase treated pulp can attain a brightness that cannot be reached solely

by increasing chemical loading. These claims have not been fully accepted because the extent of chemical bleaching was not examined thoroughly, and because the use of various chemical combinations or additional chemical stages could also increase the brightness ceiling. However, the reports indicating that xylanase can directly brighten peroxide bleached pulps (Wong *et al.*, 1997c), suggests that this matter should be examined more closely, particularly in chlorine bleached pulps. Xylanase treated pulp has also been reported to be less prone colour reversion (Bajpai *et al.*, 1994) This has been associated with a decrease in the amount of charge groups on fibre surfaces (Buchert *et al.*, 1995b), which may in turn be associated with the acidic substituents in xylan.

2.13.3 Porosity

When the wall thickness of kraft fibres was measured after xylanase treatment, no indication of fibre swelling was found (Kibblewhite and Clark, 1996). Using nitrogen porosimetry, however, a decrease in the mean pore radius of kraft pulp was found after treatment with crude xylanase (Mora *et al.*, 1986). The reliability of both of these techniques can be questioned because drying the pulp was required before measurements. Porosity determination under aqueous conditions would seem more appropriate because these conditions are present during pulp processing. To date conflicting results have been obtained when the water retention of pulp was measured after xylanase treatment. If there is an increase in fibre porosity, it remains unclear whether these pores are larger than the xylan macromolecules removed during enzyme treatment (Wong *et al.*, 1997b)

2.13.4 Other physical properties

The viscosity of pulp may increase with xylanase treatment because xylan has a molar mass that is generally lower than that of cellulose, so that its removal leaves behind cellulose that has a higher viscosity (Paice *et al.*, 1988; Senior *et al.*, 1992). Therefore the conventional relationship, where higher pulp strength is observed with higher pulp viscosity, might not hold for xylanase treated pulp. Moreover, after bleaching to the same brightness, changes to pulp strength are only indirectly related to the action of xylanase the lower chemical loadings required can reduce damage to pulp strength and modify refining properties. After bleaching with the same chemical loadings, most reports claim little change to papermaking properties while some indicate an improvement to the tear index (Chauvet *et al.*, 1987; Roncero *et al.*, 1996). A difficulty in evaluating the effects of xylanase is the possible presence of contaminating cellulolytic activity in the enzyme preparation, which can have detrimental effects on the pulp (Tolan, 1995).

2.13.5 Alkaline extractability

After xylanase treatment, there is an increase in the amount of xylan that can be extracted in alkali (Saake *et al.*, 1995) and DMSO (Allison *et al.*, 1995). There is also an increase in the extractable lignin as detected in the extract by absorbance at 280 nm (Suurnakki *et al.*, 1994; de Jong *et al.*, 1996), as well as in the pulp by a drop in the kappa number (Allison and Clark, 1994). The enzyme seems to improve lignin extractability more in

hardwood kraft pulp than in softwood and oxygen delignified softwood kraft pulps (Wong *et al.*, 1997b). The increase in lignin extractability is an excellent explanation for the ability of xylanase to enhance pulp bleaching. However the xylanase treatment of peroxide bleached pulp can increase pulp brightness while the increase in lignin extractability is relatively small. (Wong *et al.*, 1997c). These recent results suggest that although xylanase can increase lignin extractability in unbleached pulp, this phenomenon might not be necessary for xylanase enhancement of peroxide bleaching.

2.14 OTHER INDUSTRIAL APPLICATIONS OF HEMICELLULASES

Applications of hemicellulases have also been found in the food and feed industry. Most of these applications don't require the utilization of highly purified enzymes. In fact, some applications are enhanced by the presence of other enzyme activities (Wong and Saddler, 1992). It has been suggested that, in the food industry, hemicellulases may be used along with pectinases and cellulases for the maceration of fruit and vegetable materials (Biely, 1985), and for clarification of juices and wines (Zeikus *et al.*, 1991). The use of hemicellulases has also been suggested for the extraction of coffee (Godfrey, 1983) and starch (Biely, 1993), plant oils (Godfrey, 1983) and starch (Biely, 1993), and for the modification of textile (McClearly, 1986) and staling (Reichelt, 1983) properties of baked products. The use of xylanases to modify the properties of dough and its resulting baked products have also been reported (Maat *et al.*, 1992). There are reports on the use of xylanases as a supplement to improve the nutritional value of rye, so as to promote the use of this grain in chick feed (Godfrey, 1983).

2.15 SCOPE OF THE PRESENT STUDY

The forestry industry makes a significant contribution to the South African economy. Reducing the environmental impact of mill effluents has been a major concern for the pulp and paper industry in recent years. Kraft pulps have traditionally been bleached using chlorine and chlorine-containing chemicals, releasing effluents that contain organochlorine. Chlorine-free bleaching represents an alternate for eliminating undesirable chlorinated compounds. Xylanase enzymes have been found to be most effective for this purpose.

A *Thermomyces lanuginosus* strain was isolated from soil produced high levels of xylanases on various carbon sources (Singh *et al.*, 2000a). It is imperative, not only from an academic viewpoint that xylanase application in a laboratory scale bleach process be investigated. Xylanases have great potential in industrial application and could provide an alternative to chemical bleaching, with reduced chloroorganic compounds in process waters and improved pulp properties. Application of xylanases in mill trials have proven to reduce the consumption of chlorine and chlorine-containing bleaching agents. Sappi Fine Papers (Stanger, South Africa) has been experiencing extensive problems with effluent treatment, high losses in pulp yield and the corrosive nature of harsh chemicals utilized in the bleach process. Hence the objective of this research was to examine the bleach boosting effect of xylanases on bagasse soda pulp with the aim of improving pulp properties and reducing chemical consumption during elemental chlorine (EC), ECF, TCF bleach sequences. In addition to submerged fermentation an alternate xylanase

production method (SSF) was evaluated with subsequent chemical bleaching conducted on bagasse pulp. Morphological studies together with immunolocalization of *T. lanuginosus* xylanase on bagasse pulp was performed to determine the extent of enzyme accessibility on the pulp. These analyses together with the evaluation of the bleach boosting potential of the xylanases provided in depth knowledge on the potential application of xylanases in the bleaching of bagasse pulp.

CHAPTER THREE: OPTIMIZATION OF *Thermomyces lanuginosus* XYLANASE PRETREATMENT CONDITIONS ON BAGASSE SODA PULP

3.1 ABSTRACT

A locally isolated thermophilic fungus, *Thermomyces lanuginosus* SSBP produced a high level of extracellular β-xylanase (1095 U/ml) under shake-flask conditions using a medium of coarse corn cobs as a carbon source, and yeast extract as a nitrogen source. The enzyme filtrates were analysed for LDC's, chromophoric material and RS, while kappa number and brightness changes were evaluated on the pretreated bagasse soda pulp. There was a gradual increase in the release of LDC'S, chromophoric material and reducing sugars with increasing temperature up to 65°C. The enzyme was most effective at pH 6.0 and 65°C, producing a direct brightening gain of 1.34 points over the control. The kappa number was reduced by 0.6 points at pH 4.0, 5.0 and 6.0, and 0.4 points at pH 7.0 immediately after xylanase treatment, indicating removal of lignin from the pulp. Extraction with NaOH after xylanase treatment improved the brightness by 3.8 points, demonstrating the removal of material in the pulp contributing to the low initial brightness. Bleaching of the enzyme treated pulp with H_2O_2 increased the to levels of 60 %, with brightness gain of 3.1 points over the control.

3.2 INTRODUCTION

In modern biotechnology a wide range of enzymes are industrially exploited, not for total degradation, but for overall modification of structures in textile, feed and forest industries (Juracek and Paice, 1992). Until recently, in the pulp and paper industry, the use of enzymes was not considered technically or economically feasible and suitable enzymes were not readily available, except for limited use in the modification of starch for paper coatings. However, research by scientific institutions and enzyme producers has led to the development of enzymes that offer significant benefits for the industry (Bajpai, 1999).

With the current trends to employ "clean technologies" xylanases have come into focus for their potential application in reducing the consumption of chlorine based chemicals required for bleaching of pulp (Bajpai and Bajpai, 1997). The use of xylanases as bleach boosters is a recent application of biotechnology to the pulp and paper industry. The positive results obtained with these enzymes, which significantly increase fibre bleachability (Pham *et al.*, 1995; Roncero *et al.*, 1996), seem to be a consequence of the enzymatic removal of xylan from the fibre surface, which facilitates chemical bleaching of lignin derived substances (Roncero *et al.*, 2000).

With xylanase pretreatment of pulps, mills have reported savings of 8 to 15 % of the total amount of chemicals across the bleach plant (Bajpai and Bajpai, 1997). Decrease in adsorbable organic halogens (AOX) levels of 12 to 25 % in the effluent, increased tear strength and an increase in pulp throughput have also been reported as benefits achieved

with xylanase pretreatment. (Shah *et al.*, 2000). These positive effects have generated global interest for the industrial application of xylanases in pulp bleaching.

Various explanations have been proposed with regards to the mechanism of xylanase pretreatment on pulp. It may degrade the xylan in the fibre pores, thus enhancing the flow of bleaching chemicals into the fibre. Alternatively, it may break down the lignin-carbohydrate bonds, improving the extractability of soluble lignin (Viikari *et al.*, 1996; Li *et al.*, 1996). With the cost of enzyme production decreasing appreciably, the implementation of this technology has become a commercial reality and a large number of mills are already using xylanases on a regular basis (Viikari *et al.*, 1994; Tolan *et al.*, 1996).

Information about the nature of the chromophores responsible for the colour in kraft pulps is limited. Researchers have assumed that colour is attributable to residual lignin, however, there is increasing evidence that modified carbohydrates contribute to kappa number, and perhaps pulp colour (Wong *et al.*, 1997). Controversy exists concerning the relative merits of different enzyme preparations for treating kraft pulps. Paice *et al.*, (1992) have found that xylanases are more effective than mannanases in enhancing the bleaching of both hardwood and softwood pulps, despite the fact that xylans constitute only a small fraction of hemicellulose in softwoods. Moreover, the efficacy of enzyme treatment was associated with a drop in the average DP rather than with the solubilization of xylan (Paice *et al.*, 1992). A significant role for xylan-derived chromophores would ensure that xylanase has a unique role in pulp bleaching.

The South African Pulp and Paper has recognized the implementation of biotechnology to existing bleaching processes on account of environmental problems associated with chemical bleaching. In view of the foregoing, the aim of this study was to optimize pretreatment conditions of xylanase from *T. lanuginosus* on bagasse pulp. It is well documented that xylanases release lignin derived compounds, chromophoric material and reducing sugars with consequential decreases in kappa number and increases in brightness of pulp types. In this regard the enzyme pretreated pulps and filtrates were analysed and evaluated at various conditions and the optimized conditions were based on the criteria mentioned above.

3.3 MATERIALS AND METHODS

3.3.1 Strain and maintenance

T. lanuginosus (designated as SSBP) was isolated from soil at the University of Durban-Westville, identified by the council for Scientific and Industrial Research, and deposited in the Industrial Biotechnology MIRCEN Culture Collection, Bloemfontein (Accession number PRI 0226). The strain was grown at 50°C on potato dextrose agar and stored either at room temperature or at 4°C and subcultured every 3-4 weeks

3.3.2 Xylanase production

An agar block (1 cm^2) of an actively growing 5 day old culture of *T. lanuginosus* strain SSBP was used to inoculate the growth medium (100 ml) in 300 ml Erlenmeyer flasks. The medium comprised of 3.14 g coarse corn cobs (carbon source), 3.02 g yeast extract and 0.5 g KH_2PO_4 per 100 ml distilled water, adjusted to pH 6.5 and autoclaved for 15 min at 121°C. Inoculated flasks were continuously shaken at 150 rpm on a rotary shaker at 50°C for 7 days. All experiments were run in triplicate. After the incubation period the growth medium was filtered and centrifuged at 13 000g for 5 min. The supernatant liquid was used as the crude enzyme for pretreatment of bagasse pulp.

3.3.3 Xylanase assay

Xylanase was assayed according to Bailey *et al.* (1992) by incubating the diluted crude extract (100 µl) in sodium citrate buffer (pH 6.5) at a temperature of 50°C for 5 min using a substrate solution of 1% birchwood xylan (Roth 7500, Karlsruhe, Germany). One unit of enzyme activity is defined as the amount of enzyme that catalyses the release of one µmole of xylose equivalent per minute under the assay conditions. All assays were conducted in triplicate from triplicate independent samples.

3.3.4 Pulp type

Bagasse unbleached soda pulp was obtained from SAPPI Fine Papers (Stanger). The pulp had an initial solid consistency of 12%, kappa number of 8.76 and brightness of 39.3%. This brown stock was thoroughly washed with water until a neutral pH in the wash waters was obtained thereafter a final wash with distilled water. The excess water was filtered with the moisture content of the washed pulp being 84 % (w/w). The pulp was then stored in sealed plastic bags at 4°C and until use.

3.3.5 Xylanase pretreatment of bagasse pulp

3.3.5.1 Temperature optimization

Washed unbleached bagasse pulp equivalent to 10 g of dry weight was placed in plastic bags to which the xylanase diluted in distilled water (pH 6.5) to attain a concentration of 20 U/g pulp was added. The total weight of the reaction mixture was brought to 100 g by the addition of distilled water (pH 6.5), to a final pulp consistency of 10 %. The bags were incubated at temperatures from 50°C to 75°C at increments of 5°C. Controls were carried

out in a similar manner using inactivated xylanase (boiled). The pulp was mixed with kneading at 30 min intervals for 3h, after which pulp was filtered through filter paper (Whatman No 1) using a buchner funnel. The filtrate and pulp were retained for analysis as described below.

3.3.5.2 pH optimization

The enzyme pretreatment was carried out as described in section 3.3.5.1 at pH values from 3 to 11. Pretreatment was carried out homogenizing the pulp in distilled water followed by incubation at the optimum temperature determined above.

3.3.6 Analyses of pulp filtrates

The enzyme-mediated release of lignin-derived compounds (LDC'S) and chromophoric material form pulp was monitored in filtrates by measuring the absorbance at 280 nm and 465 nm, respectively (Wong *et al.*, 1997c). The filtrates were adjusted to pH 7.0 prior to the measurement. The amount of reducing sugars (RS) released from pulp was determined spectrophotometrically at 540 nm according to the DNS method (Miller, 1959).

3.3.7 Extraction with NaOH and H_2O_2 bleaching

Enzyme pretreated bagasse pulp (5g d/w) was placed in plastic bags and treated with 5% NaOH for 1hr at 70°C with a final pulp consistency of 10%. A further 5 g of enzyme pretreated pulp was bleached with 1.5% H_2O_2 (30%), 0.7% NaOH (10%), and 0.5% $MgSO_4$. $7H_2O$ (10%), for 3h at 70°C with a consistency of 10%. After incubation, the

pulp was filtered and the filtrate was retained for analyses as described above. The pulp was washed several times with distilled water and thereafter handsheets were prepared and analysed for brightness.

3.3.8 Analyses of pulp properties

After enzyme treatments, pulp samples were analyzed for kappa number using the TAPPI test method T 236 cm-85, and brightness was measured with the Color Touch 2 brightness machine (Technidyne Corp, New Albany, Indiana, USA)

3.4 RESULTS

3.4.1 Production of xylanase by *T. lanuginosus* SSBP

T. lanuginosus SSBP produced 950 U/ml of β-xylanase in a medium containing coarse corn cobs, yeast extract and KH_2PO_4 under shake-flask cultures after an incubation period of 7 days. The enzyme solution had a total protein content of 8.5 mg/ml with a specific activity of 112 U/ml.

3.4.2 Xylanase-mediated release of chromophores and lignin derived compounds (LDC's)

The enzyme filtrate was analyzed for the presence of chromophores (A_{465} nm) and LDC's, (A_{280nm}). There was a gradual increase in the amount of chromophoric material detected in the filtrate with increasing temperature up to 65°C (Fig. 3.1). The absorbance at 465 nm varied between 0.23 and 0.30 at 50 and 65°C, representing a 35 % increase in chromphore release at the higher temperature. On the other hand temperature variance did not appear to have a significant effect on the release of LDC's (Fig. 3.2). There was only a 4 % increase in LDC's from 50 to 75°C. However, the absorbance values of the filtrates containing the active enzyme were significantly higher than that with the inactive enzyme.

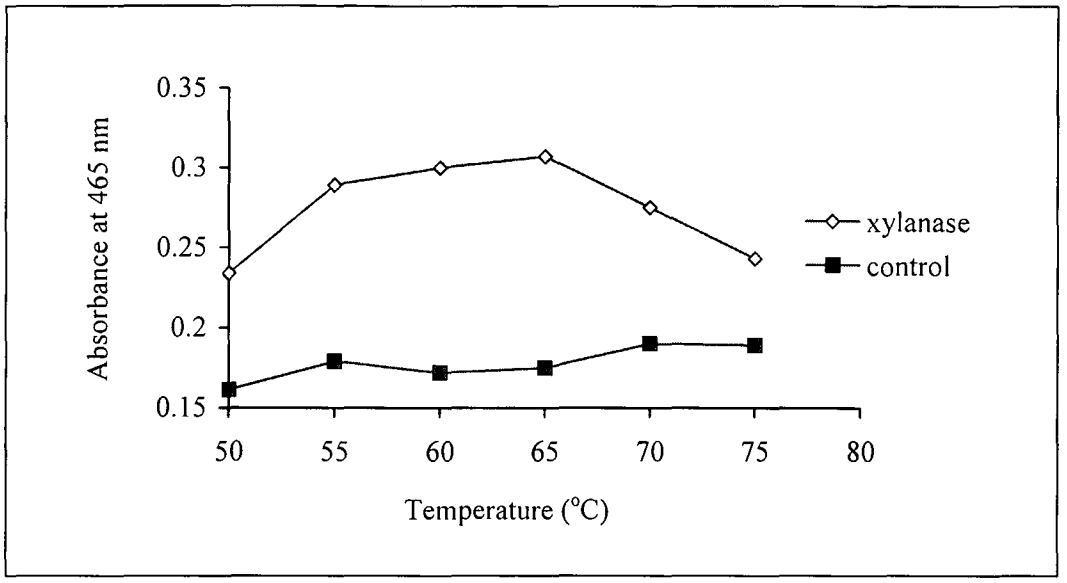


Fig. 3.1 Influence of temperature on the release of chromophoric compounds ($A_{465\ nm}$) from bagasse pulp pretreated with *T. lanuginosus* crude xylanase at pH 6.5

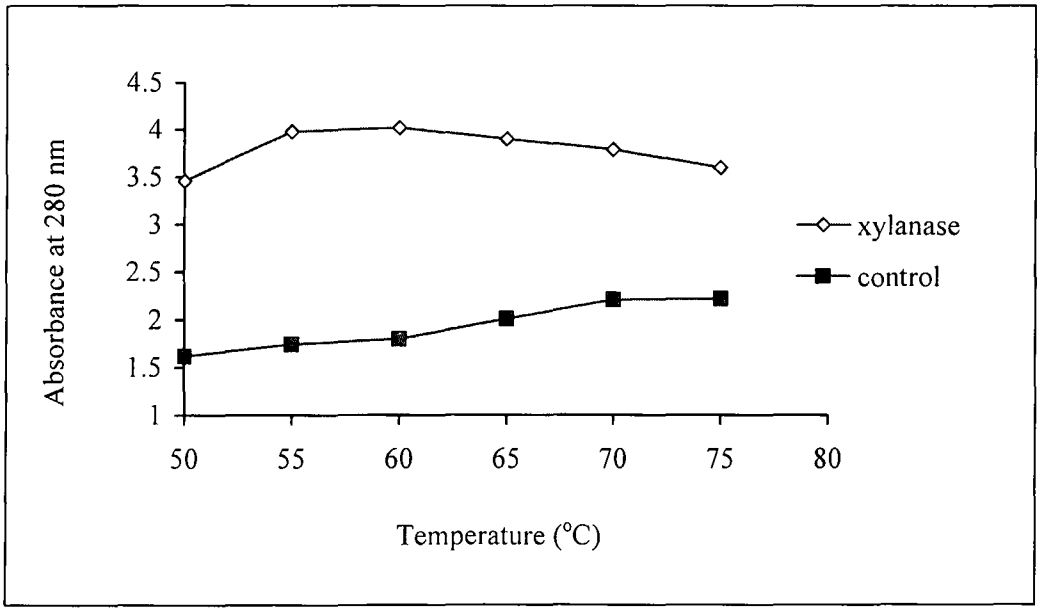


Fig. 3.2 Influence of temperature on the release of LDC's ($A_{280\ nm}$) from bagasse pulp treated with *T. lanuginosus* crude xylanase at pH 6.5

3.4.3 Xylanase-mediated release of reducing sugars

There was an increase in reducing sugar concentration with increasing temperature, between 50 and 60°C, however, this concentration decreased at temperatures beyond 60°C (Fig. 3.3). A peak concentration of 0.31 mg/g reducing sugar was obtained at 60°C, and RS concentration at 65°C was 0.3 mg/g, while 0.175 mg/g was detected at 75°C. There was a slight increase in RS content of the control samples with increasing temperature but at values incomparable to that achieved with the active enzyme.

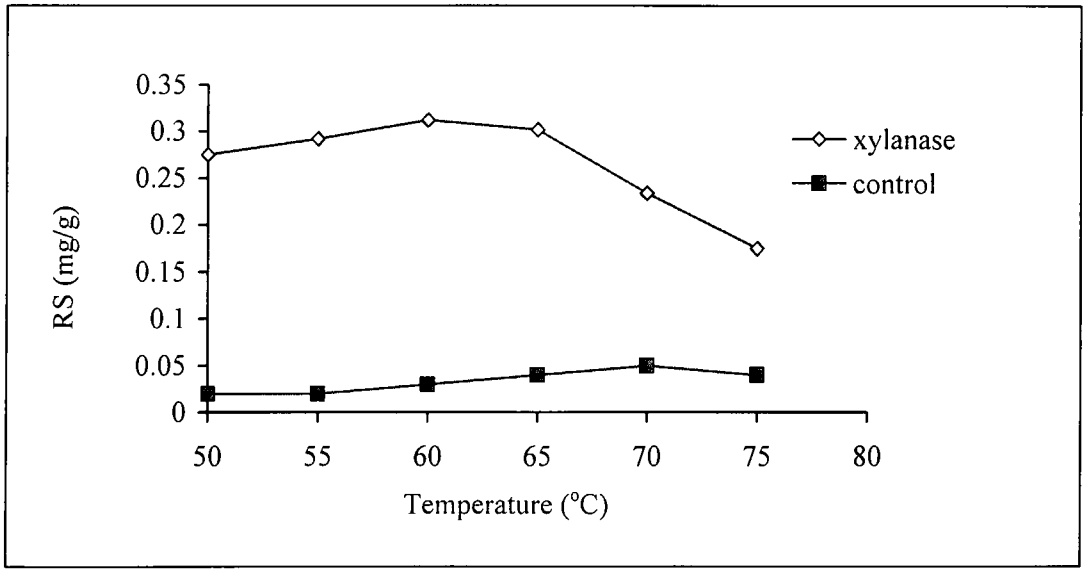


Fig. 3.3 Influence of temperature on the release of RS from bagasse pulp treated with *T. lanuginosus* crude xylanase at pH 6.5

3.4.4 Effect of xylanase pretreatment on the brightness of bagasse pulp

The brightness of bagasse pulp immediately after xylanase pretreatment improved at all the temperatures under investigation in comparison to the control. The brightness increase at 50, 55 and 60°C was 1.6, 1.3 and 1.37 points, respectively (Fig. 3.4). Treatment with the enzyme above 60°C produced brightness increases lower than that obtained between 50 to 60°C, with a brightness gain of 0.97 points achieved at 65°C. It therefore appears that enzyme pretreatment at temperatures between 50 to 60°C are equally effective in improving the brightness of the pulp.

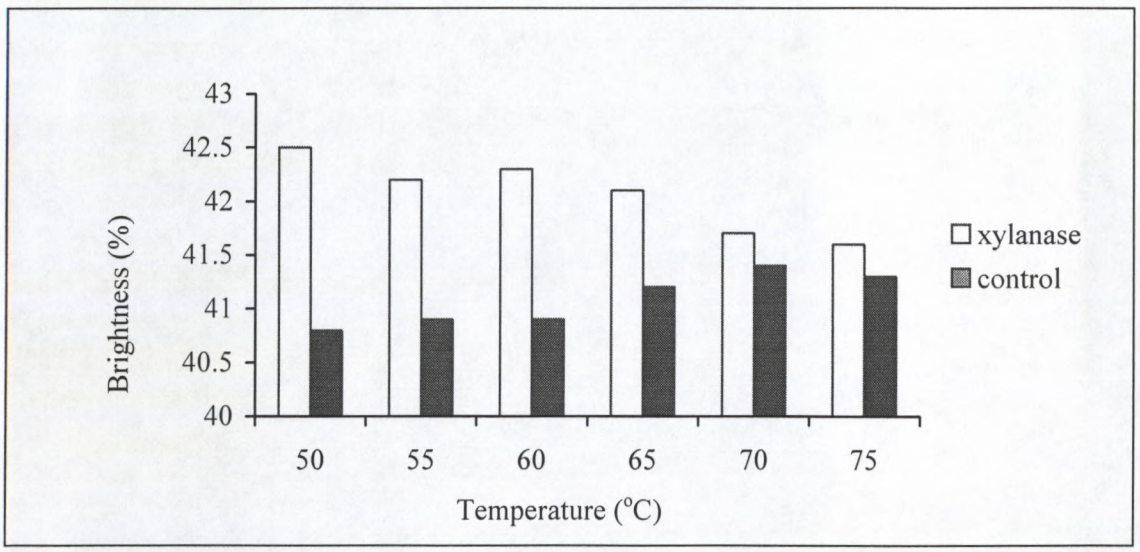


Fig. 3.4 Influence of temperature on the brightness of bagasse pulp treated with *T. lanuginosus* crude xylanase at pH 6.5

3.4.5 Effect of xylanase pretreatment on the kappa number of bagasse pulp

Xylanase pretreatment had minimal effect on the kappa number of bagasse pulp. The maximum reduction in kappa number obtained with the enzyme was 0.65 points, achieved at 55°C (Fig. 3.5). However, similar variations of approximately 5% were detected at temperatures between 50 to 65°C. At higher temperatures enzyme mediated kappa reduction was lower with values of 0.39 and 0.42 obtained at 70 and 75°C.

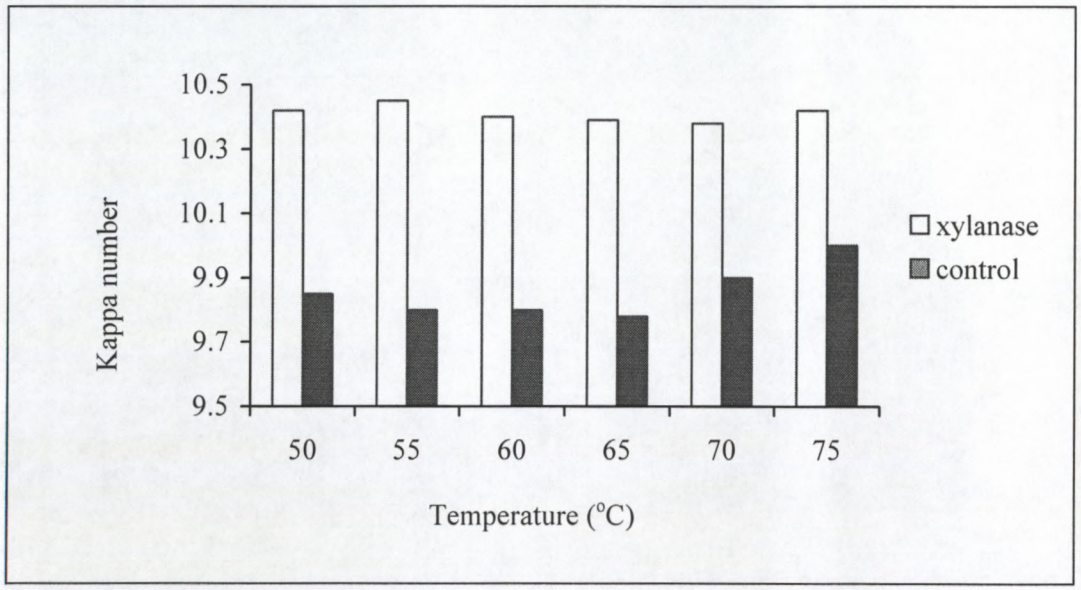


Fig. 3.5 Influence of temperature on the kappa number of bagasse pulp treated with *T. lanuginosus* crude xylanase at pH 6.5

3.4.6 Evaluation of xylanase pretreatment of bagasse pulp at varying pH values

3.4.6.1 Xylanase-mediated release of chromophoric material from bagasse pulp

From the data obtained the optimum temperature for xylanase pretreatment on bagasse pulp was 60°C. This temperature was used for optimizing the pH pretreatment conditions, and the pH varied between pH 3 and 11. The enzyme-mediated release of chromoporic material was constant between pH 5 to 8, with maximum chromophoric material released between pH 6-7 (Fig. 3.6). Treatment at pH values below 5 and above 8 seemed to be relatively ineffective in releasing colour material from the pulp.

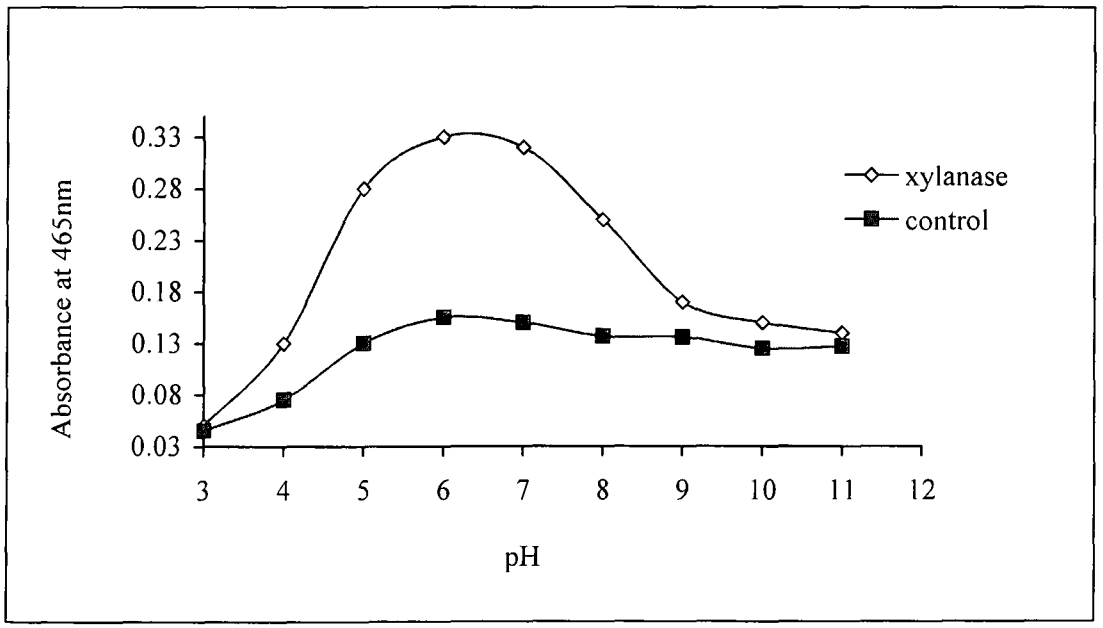


Fig. 3.6 Influence of pH on the release of chromophoric compounds from bagasse pulp treated with *T. lanuginosus* crude xylanase at 60°C

3.4.6.2 Xylanase-mediated release of LDC'S from bagase pulp

The enzyme-mediated release of LDC's was constant over a broad pH range between pH 5 and 8 (Fig. 3.7). The optimum pH range for the release of LDC's was between pH 5 and 7, with a maximum absorbance detected at pH 6.0. At pH values below 5 and above 9, the amount of LDC's present in the filtrate decreased significantly. Control samples displayed a constant release of LDC'S between pH 4 and 11, which was 46 % less than that achieved with the crude enzyme.

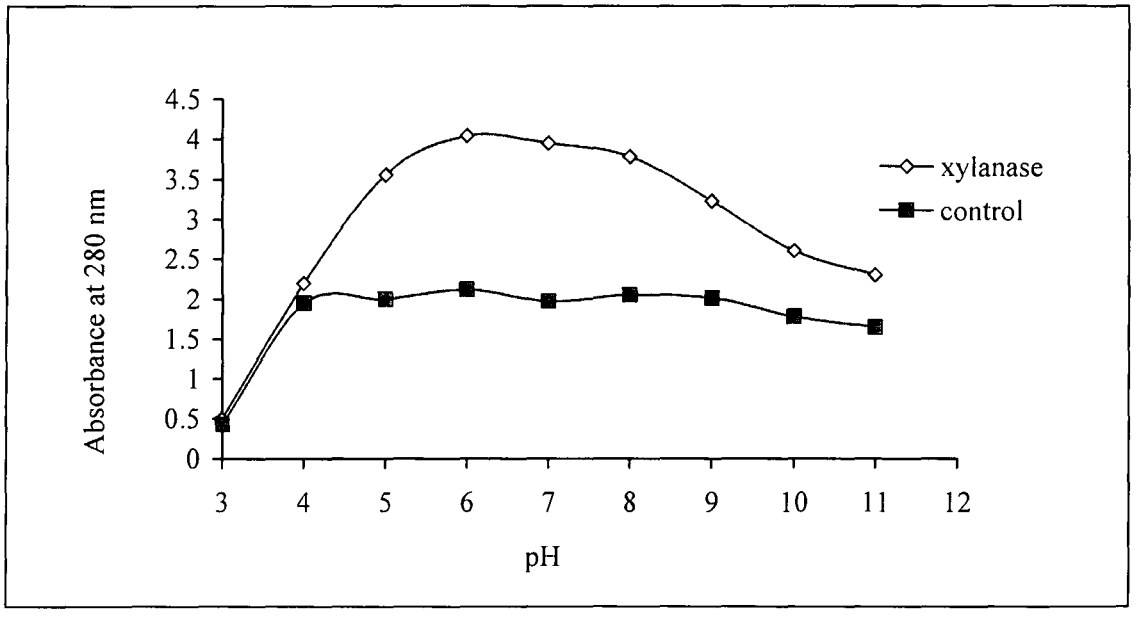


Fig. 3.7 Influence of pH on the release of lignin derived compounds from bagasse pulp treated with *T. lanuginosus* crude xylanase at 60°C

3.4.6.3 Xylanase-mediated release of RS from bagasse pulp

The enzyme was most effective between pH 5 and 7. At pH values higher than 7 and below 5, the efficiency of the enzyme in releasing RS decreased (Fig. 3.8). There was a 33 % drop in reducing sugars at pH 4 in comparison to pH 5, similarly, the reducing sugars detected at pH 8 was 16 % lower than at pH 7.0. The maximum concentration of reducing sugars present in the filtrate was 0.32 mg/g pulp at pH 6.0. At pH 3, 10 and 11 the enzyme appeared to be ineffective in releasing RS from the pulp. The control samples had a constant concentration of reducing sugars at all pH values investigated, with values significantly lower than the active xylanase treated pulp.

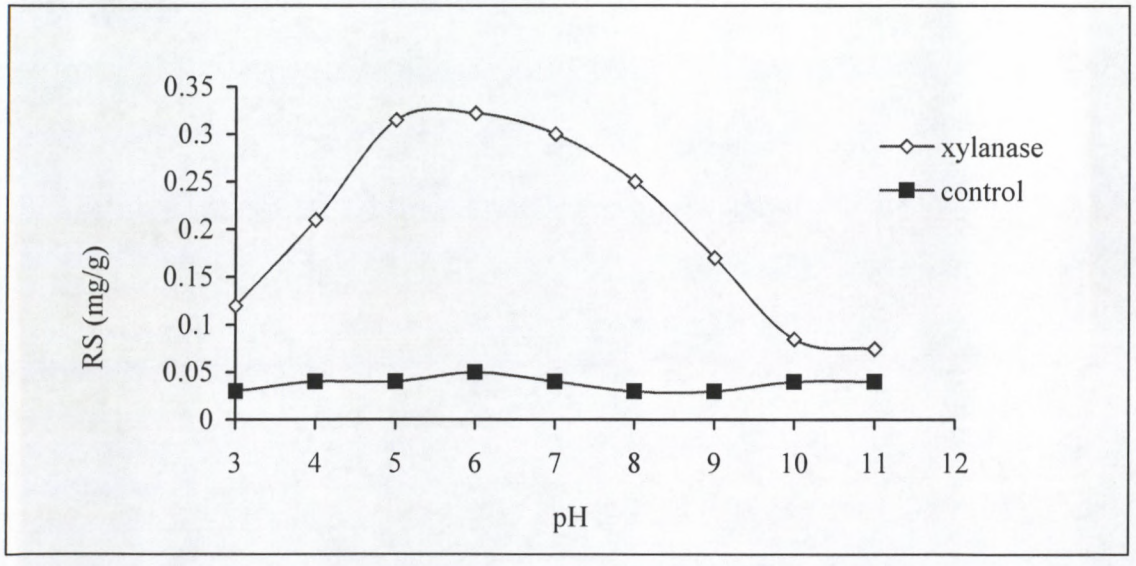


Fig. 3.8 Effect of pH on the release of RS from bagasse pulp treated with *T. lanuginosus* crude xylanase at 60°C

3.4.6.4 Effect of xylanase pretreatment on the brightness of bagasse pulp

The enzyme was most effective at pH 6.0, improving the brightness by 1.3 brightness points to 42.2 % (Fig. 3.9). Similar brightness gains of 1.05 and 1.06 points were achieved at pH 5 and 7, respectively. These results correlate positively with the reducing sugar released between pH 5 and 7. At pH 10 and 11 only minimal increases in brightness were observed indicating enzyme instability at higher pH values. Pretreatment at pH 3 and 4 also proved to be less effective in improving the brightness of the pulp.

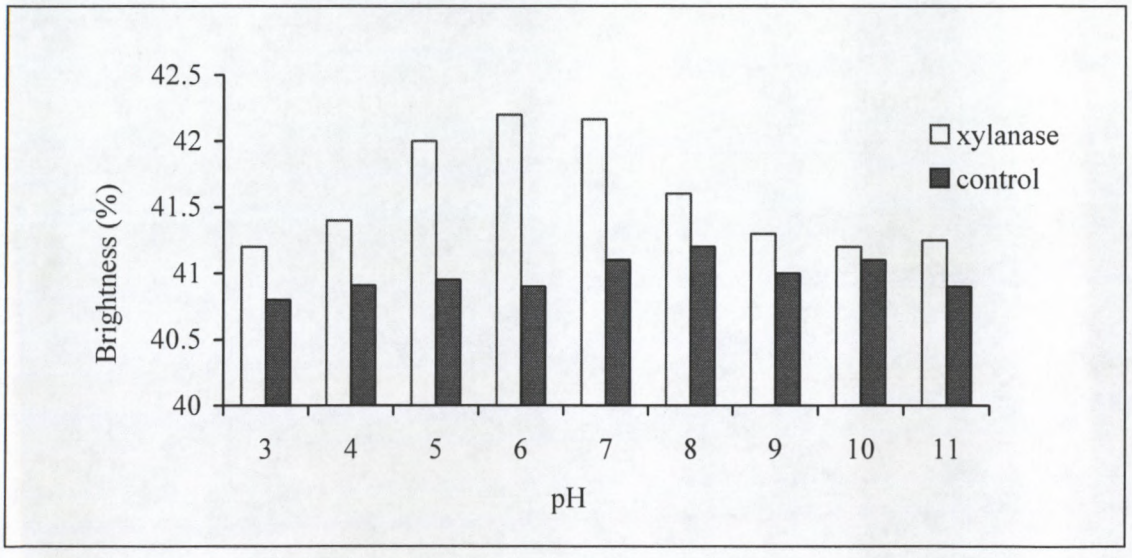


Fig. 3.9 Influence of pH on brightness of bagasse pulp treated with *T. lanuginosus* crude xylanase at 60°C

3.4.6.5 Effect of xylanase pretreatment on kappa number of bagasse pulp

Xylanase pretreatment showed similar kappa number reductions between pH 4 to 8. The enzyme reduced the kappa number by 0.6 points, at pH 4, 5 and 6, and 0.4 points at pH 7.0 (Fig. 3.10). At pH 3 the kappa number was reduced by 0.15 points, and at pH 9 to 11 the reduction in kappa number was 0.2 points, illustrating minimal effect of the enzyme in kappa number reduction of bagasse pulp at these respective pH values.

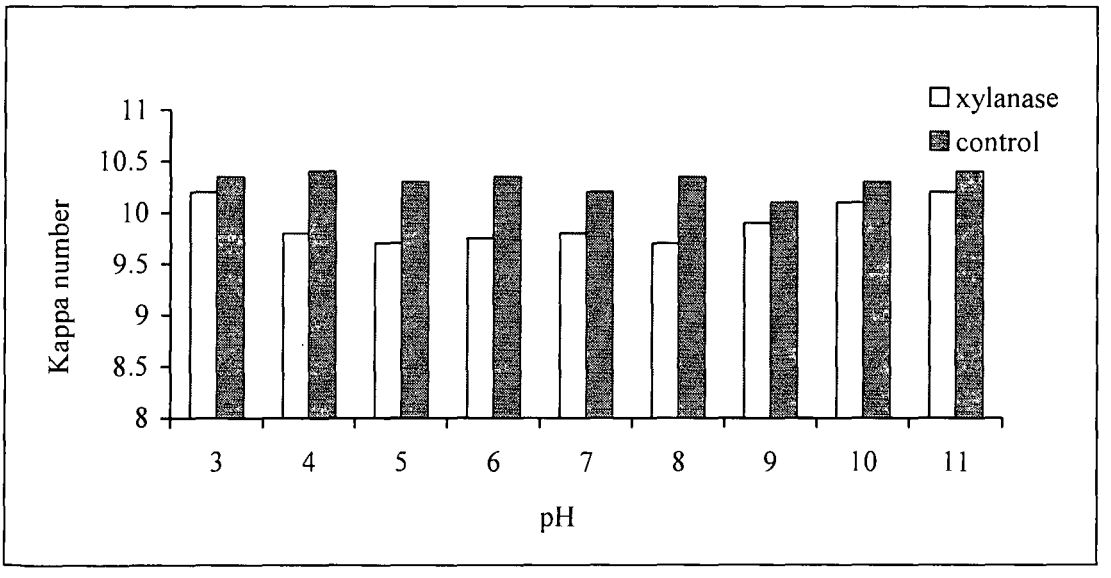


Fig. 3.10 Influence of pH on kappa number of bagasse pulp treated with *T. lanuginosus* crude xylanase at 60°C

3.4.7 Effect of pH and temperature on the release of LDC'S, chromophoric material and reducing sugars from bagasse pretreated with *T. lanuginosus* xylanase

In order to clearly define the optimum conditions for xylanase pretreatment of bagasse pulp, pH and temperature conditions were further analyzed in a narrow range of 5 to 7 and 55 to 70°C, respectively. In terms of RS release a temperature and pH range of 60 to 65°C and 55 to 6.0 was the most effective (Table 3.1). Analysis of the LDC's indicated a pH optimum of 6.0 and a optimum temperature range between 60-65°C (Table 3.2). The chromophoric material released also showed a similar profile as the LCD's (Table 3.3). Evaluation of the effect of the enzyme on the brightness showed conclusively that the pH and temperature producing the highest brightness gain was 6.0 and 65°C, respectively (Table 3.4). A brightness gain of 1.34 points was achieved during these conditions.

Table 3.1 Influence of temperature and pH on the release of RS from bagasse pulp treated with *T. lanuginosus* crude xylanase

pH	Temperature (°C)			
	55	60	65	70
5.5	0.322	0.326	0.337	0.307
6.0	0.311	0.328	0.335	0.278
6.5	0.298	0.302	0.315	0.254
7.0	0.273	0.277	0.296	0.231

Xylanase treatment conditions:Consistency: 10%; Time: 3h

Table 3.2 Influence of temperature and pH on the release of LDC's ($A_{280\ nm}$) from bagasse pulp treated with *T. lanuginosus* crude xylanase

pH	Temperature (°C)			
	55	60	65	70
5.5	3.996	4.132	4.167	4.022
6.0	4.103	4.186	4.196	3.925
6.5	4.178	3.982	3.921	3.841
7.0	3.847	3.643	3.514	3.687

Xylanase treatment conditions:Consistency: 10%; Time: 3h

Table 3.3 Influence of temperature and pH on the release of chromophoric compounds ($A_{465\ nm}$) from bagasse pulp treated with *T. lanuginosus* crude xylanase

pH	Temperature (°C)			
	55	60	65	70
5.5	0.301	0.302	0.321	0.287
6.0	0.314	0.342	0.344	0.256
6.5	0.311	0.307	0.305	0.243
7.0	0.294	0.276	0.240	0.223

Xylanase treatment conditions:Consistency: 10%; Time: 3h

Table 3.4 Influence of temperature and pH on the brightness of bagasse pulp treated with *T. lanuginosus* crude xylanase

pH	Temperature (°C)			
	55	60	65	70
5.5	1.25	1.10	1.25	0.70
6.0	1.20	1.20	1.34	0.75
6.5	1.10	1.05	1.20	0.50
7.0	0.90	1.06	0.96	0.30

Xylanase treatment conditions:Consistency: 10%; Time: 3h

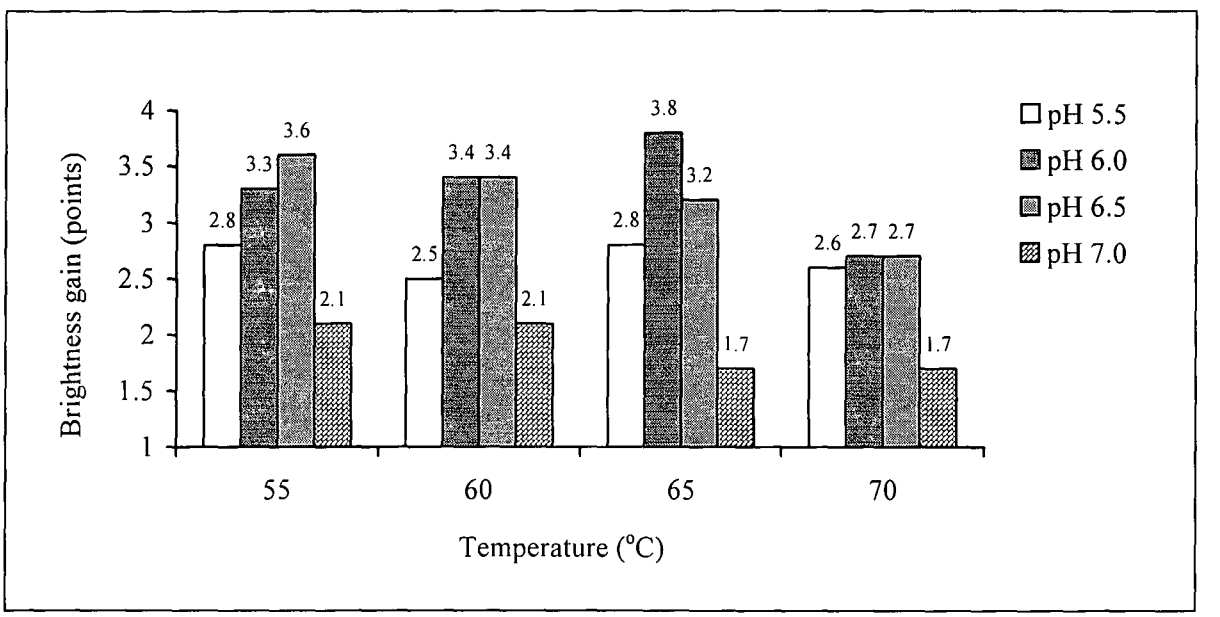
3.4.8 Evaluation of *T. lanuginosus* xylanase in chemical bleaching

Bagasse soda pulp pretreated with *T. lanuginosus* xylanase was extracted with NaOH and bleached with with H_2O_2, to evaluate the effects of the enzyme in subsequent chemical treatments. After NaOH extraction the brightness improved significantly in comparison to the control. The highest brightness gain of 3.8 points was achieved with xylanase pretreatment conditions of 65°C at pH 6.0 (Table 3.5, Fig. 3.11). Pretreatment at 55 and 70°C at all pH values did not produce brightness gains comparable to that achieved at pH 6.0 and 65°C. Bleaching of pulp with H_2O_2 improved the brightness to levels of 60 %, with brightness gain of 3.1 points achieved at pH 6.0 and 65°C (Table 3.6, Fig 3.12). The bleaching results indicate that the optimum pH and temperature for *T. lanuginosus* xylanase on bagasse pulp is 6.0 and 65°C, respectively.

Table 3.5 Influence of pH and temperature on the brightness of bagasse pulp treated with *T. lanuginosus* crude xylanase followed by NaOH extraction

pH	Temperature (°C)			
	55	60	65	70
5.5	51.02	50.63	50.40	50.00
6.0	51.21	51.55	51.70	50.75
6.5	51.43	51.56	51.40	50.54
7.0	50.21	50.25	50.07	49.64

Xylanase treatment conditions: Consistency: 10%; Charge: 20 U/g; Time: 3h
NaOH extraction conditions: Consistency: 10%; Temperature: 70°C; Charge: 5%; Time: 1h



Xylanase treatment conditions: Consistency: 10%; Charge: 20 U/g; Time: 3h
NaOH extraction conditions: Consistency: 10%; Temp: 70°C; Charge: 5%; Time: 1h

Fig. 3.11 Influence of pH and temperature on the brightness of bagasse pulp treated with *T. lanuginosus* crude xylanase followed by NaOH extraction

Table 3.6 Influence of pH and temperature on the brightness of bagasse pulp treated with *T. lanuginosus* crude xylanase followed by H_2O_2 bleaching

pH	Temperature (%)			
	55	60	65	70
5.5	60.21	60.54	60.34	60.21
6.0	61.32	61.55	61.64	60.23
6.5	61.44	60.94	61.49	59.98
7.0	59.42	59.52	59.78	59.32

Xylanase treatment conditions: Consistency: 10%; Charge: 20 U/g;Time: 3h
H_2O_2 bleaching conditions: Consistency: 10%; Temperature: 70°C; H_2O_2 (1.5%); NaOH (0.7%); MgSO4 $7H_2O$ (0.5%); Time: 3h

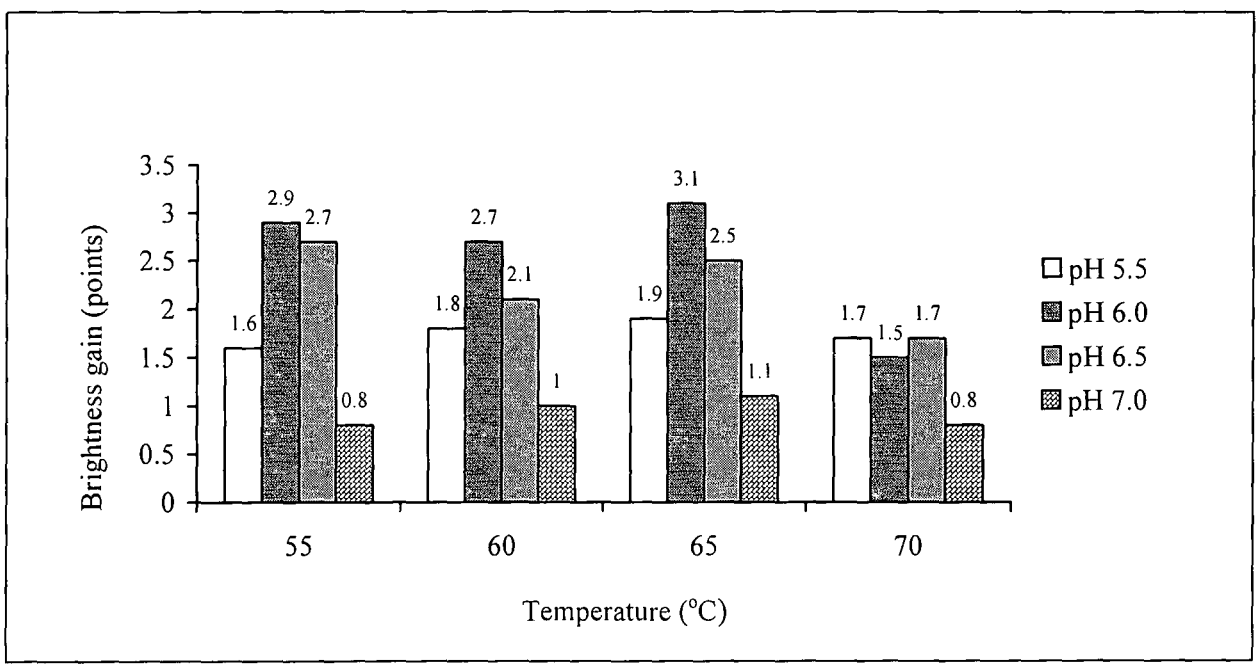


Fig. 3.12 Influence of pH and temperature on the brightness of bagasse pulp treated with *T. lanuginosus* crude xylanase followed by H_2O_2 bleaching

3.5 DISCUSSION

Thermomyces lanuginosus SSBP is a thermophilic hyphomycete, producing a range of thermostable extracellular enzymes which include β-xylanases, α-galactosidase, α-L-arabinofuranosidase, esterases and β-mannanase (Singh *et al.*, 2000a). In the present study this organism produced up to 950 U/ml of xylanase activity in shake-flask cultures at 50°C after a 7-day incubation period. However, xylanase activity of 3 600 U/ml was previously reported for this strain (Singh *et al.*, 2000a). A report by Bennet *et al.* (1998) indicated a production level of 2840 U/ml by *T. lanuginosus* ATCC 46882 when grown on corn cobs. According to Gomes *et al.* (1993) various factors including the surface area, pore size, maturity and geographical origin of the corn cobs affect the production levels of xylanase. Nevertheless, *T. lanuginosus* SSBP produced the highest xylanase activity among the *Thermomyces* strains investigated to date (Singh *et al.*, 2000a).

T. lanuginosus SSBP has been reported to produce xylanase which is very active at high alkaline pH values (5.5-9.5) and has an optimum temperature of 70°C (Singh *et al.*, 2000b). These two characteristics allow the enzyme to be extremely stable since xylanases with high thermostability and pH stability are of particular interest for enzymatic hydrolysis of xylan in pulp bleaching. *T. lanuginosus* SSBP xylanase has similar properties to many of the commercial xylanases currently available to the pulp and paper industry. Primalco Ltd has genetically modified a *Trichoderma* strain to produce a hemicellulose enzyme that tolerates higher temperature and alkalinity. Ecopulp TX-100 can operate at 85°C and at a pH of 4.5 to 8.5, well within the 60 to 90°C

and pH 4 to 6 present in most bleaching plants (Jeffries and Davis, 1998). A xylanase from the thermohyperphilic bacterium *Thermotoga maritima* has been demonstrated to be stable at 92°C, for a longer period of time than is required for the enzyme pretreatment stage (Bragger *et al.*, 1989). Although genetic engineering is quite promising in modifying several xylanase producing strains, their application in the pulp and paper industry has not been fully exploited. However, current research on xylanase applications has revolved around natural organisms producing highly thermostable and alkaline tolerant xylanases. This study evaluated the effect of *T. lanuginosus* xylanase on bagasse soda with the aim of optimizing pretreatment conditions.

Analysis of the bleach filtrate at wavelengths of 280 nm and 465 nm revealed significant differences between the active xylanase the control (heat inactivated xylanase) samples at all the temperatures under investigation. The release of chromophoric compounds beyond 65°C decreased drastically. It appeared that the enzyme-mediated release of these compounds were inhibited due to the instability of the enzyme at higher temperatures on bagasse soda pulp. Spectrophotometric analysis of the filtrate could not be used to determine the optimum temperature for xylanase pretreatment since, the enzyme pretreatment at all the temperatures under study released similar quantities of ultraviolet absorbing material.

Contrary to this, the reducing sugar content in the filtrate did reflect differences with pretreatment carried out at varying temperature. There was an increase in the release of reducing sugar from 50°C to 60°C, thereafter a steady decrease was detected when

pretreatment was carried out beyond 60°C, with a 44 % reduction in reducing sugars at 75°C in comparison to that obtained at 60°C. This pattern was similar to that of the chromophore release, indicating a correlation between the release of chromophoric material xylan from the pulp. Besides the chromophores derived from lignin, evidence has implicated carbohydrates (Ziobro, 1990; de Jong *et al.*, 1997), extractives (Hartler and Norrstrom, 1969) and tannins (Bard, 1941) as contributors to kraft colour. Although lignin derived chromophores are still thought to be the dominant contributor (Pigman and Csellak, 1948) the importance of aromatic and highly conjugated structures in lignin is uncertain (Furman and Lonsky, 1988). It is therefore important in developing bleaching strategies that not only target lignin derived chromophores but other compounds contributing to the dark colour of unbleached pulp.

There were negligible differences in brightness increases with xylanases pretreatment of bagasse pulp between 50 and 60°C. The enzyme at this stage appeared to be optimally active on bagasse pulp at 60°C, producing the highest brightness gain of 1.37 points after pretreatment, while a brightness gain of 0.3 points was achieved at 70°C. This decrease in brightness correlated well with the decrease in reducing sugars released at 70°C indicating instability of the enzyme. Although one study suggested that there is a correlation between the amount of sugars solubilized from conventional kraft pulp and the final brightness achieved by elemental chlorine-free bleaching (Allison *et al.*, 1995), other studies have found no relationship (Hoffmann, 1991) and no correlation between solubilized sugars and the direct brightening of pulp before bleaching (Wong *et al.*, 1996).

The xylanase had minimal effect on the kappa number of bagasse pulp, with similar reductions detected at 50 to 60°C. Direct delignification of pulp by xylanase has often been overlooked because the enzyme is not expected to act directly on residual lignin. There is however, a small but measurable drop in the kappa number of kraft pulp immediately after xylanase treatment (Nelson *et al.*, 1995; Gellerstedt and Li, 1995; Li *et al.*, 1996) and further decreases could be achieved using additional xylanase treatment (Munk *et al.*, 1993). Other analytical methods are required to verify the occurrence of direct delignification because the specificity of kappa number determination of lignin has been questioned (Gellerstedt and Li, 1995; Buchert *et al.*, 1995). Besides being inaccurate for samples with small quantities of lignin, the measure of acid insoluble lignin also requires careful consideration because adsorbed proteins may also register (Gellerstedt and Li, 1995). However, it should be noted that adsorbed xylanase is quickly removed with subsequent chemical bleaching (Bernier *et al.*,1994).

The enzyme filtrate analyses revealed that the enzyme removed the optimal amount of lignin material (A_{280} nm) between pH 5 to 8, while the release of chromophoric material was optimal at pH 5 to 7. Xylanase treatment performed at pH values less than 4 and above 8 were not considered favourable since considerably less amounts of UV and visible light absorbing material were released. The reducing sugar analyses indicated that the enzyme was effective in removing most of the accessible hemicelluloses in the pulp at pH 5 to 7. A drop in reducing sugar was prevalent in the filtrate at pH values below 5 and above 7. Similarly, brightness levels showed maximum increases between pH 5 to 7, while kappa number reductions were stable between pH 4 to 8, illustrating no direct

correlation between kappa reductions and brightness increases. This verifies the assumption that other compounds apart from lignin contributes to the low brightness of bagasse pulp.

In order to verify these results pretreatments were performed between pH 5 and 7 at increments of 0.5 pH points at 55 to 70°C, followed by extraction with NaOH and bleaching with H_2O_2. Examination of the enzyme filtrate, and pulp after treatment at the above conditions showed optimum conditions for xylanase pretreatment as pH 6.0 and 65°C. This was confirmed by chemical treatment, which was performed following xylanase pretreatment. The enzyme rendered the bagasse fibres more amenable to the extraction of the residual lignin by NaOH with brightness gains of 3.8 points over the control. Bleaching with a one step H_2O_2 stage improved the brightness by 3.1 points at pH 6.0 and 65°C over the control, to final brightness levels of 61 %.

One of the impediments to elucidating the mechanisms of xylanase aided bleaching is the lack of convenient methods for distinguishing xylan-derived from lignin-derived chromophores. This difficulty was well illustrated by a recent report concerning the hexenuronic acid substituents that are formed on xylan under alkaline conditions (Buchert *et al.*, 1995). This modified carbohydrate, carrying one double bond, seems to contribute to the kappa number of low kappa pulps and to colour reversion in kraft pulp. Since hexenuronic acid persists under alkaline peroxide, it would be present for removal by xylanase at the beginning and at the end of such a bleaching sequence, thereby leading to minor brightness gains and kappa drops in both cases. Xylanase is more effective than

peroxide for the removal of certain xylan derived chromophores (Wong *et al.*, 1997b). Recent results challenge plausible hypotheses that were proposed earlier for the role of lignin xylan linkages and lignin entrapment. The challenge provides an excellent opportunity for researchers to reconsider the process determinants, while more thorough analyses are required to determine whether a common target substrate is responsible for the apparently different phenomena that are observed under various process conditions. A major role for xylan-derived chromophores would ensure that xylanase has a unique role in pulp bleaching.

3.6 CONCLUSIONS

i) The current data indicated the efficient application of xylanase from *T. lanuginosus* SSBP on bagasse pulp with optimum conditions achieved at pH 6.0 and 65°C.

ii) The xylanase from *T. lanuginosus* was effective in releasing LDC'S, chromophoric material and RS from bagasse soda pulp.

iii) The enzyme had minimal effect on the kappa number of the pulp, but exhibited direct brightening abilities.

iv) This xylanase demonstrated significant increase in brightness of 3.8 and 3.1 points after NaOH and H_2O_2 bleaching, respectively.

The evaluation of the bleach enhancing abilities of *T. lanuginosus* xylanase as well as commercial xylanases during chemical bleaching is presented in chapter four.

CHAPTER FOUR: EVALUATION OF THE BLEACH ENHANCING EFFECT OF XYLANASES ON BAGASSE SODA PULP IN AN ELEMENTAL CHLORINE-FREE (ECF) AND TOTAL CHLORINE-FREE (TCF) BLEACH SEQUENCE

4.1 ABSTRACT

In this study crude xylanase from *Thermomyces lanuginosus* SSBP and three commercial xylanases (Xylanase P, Ecopulp TX-200C and Cartazyme NS-10) were evaluated in both ECF (DED) and TCF (X-OqPaP) bleaching to enhance the bleachability of bagasse pulp. The enzyme pretreatment was carried out at charges of 1, 5 and 10 U/g dry weight pulp under optimized conditions. The final brightness achieved with 10 U of xylanase P, Ecopulp TX-200C, *T. lanuginosus* xylanase and Cartazyme NS-10 at the highest chlorine charge (D_1: 6.57%; D_2: 3.285) applied was 85.4%, 85.1%, 84.8% and 84.7%, respectively. In all instances, however the biobleaching effect induced due to xylanase pretreatment was dependant on both the enzyme and chlorine dioxide charges used. Xylanase P was able to reduce the consumption of chlorine dioxide by 33% while, *T. lanuginosus* xylanase, Ecopulp TX 200C and Cartazyme NS 10 were equally effective producing chlorine dioxide savings of 20 %. Mechanically pretreatment of the pulp prior to the ECF biobleaching slightly increased final brightness, indicating "activation" of the pulp to facilitate enzyme and chemical bleaching. At similar brightness levels, the TCF biobleaching was less efficient than the ECF DED biobleaching. Negligible differences in the physical properties of the xylanase treated and control bagasse pulp samples were detected.

4.2 INTRODUCTION

Growing concerns on environmental issues have prompted governments to develop stringent regulations against polluting processes. The use of chlorine and chlorine chemicals during the bleaching process results in the formation of chlorinated organic substances, some of which are toxic, mutagenic and non-biodegradable (Bajpai and Bajpai, 1997).

Xylanases hydrolyze the xylans redeposited on pulp fibers during kraft pulping. This is thought to facilitate the diffusion of lignin fragments degraded and solubilized during subsequent bleaching (Kantelinen *et al.*, 1993). It has been already demonstrated that xylanases can enhance the bleaching of kraft pulps on a large scale without major capital investments (Viikari *et al.*, 1993). Xylanases can provide a cost effective way to reduce the use of chlorine-containing compounds and other bleaching chemicals. There are reports of 8 to 15% savings of the total amount of chemicals utilized in the bleaching process and a decrease in the adsorbable organic halogen levels of 12 to 25% in the bleach effluent (Bajpai and Bajpai, 1997). Increased tear strength and pulp throughput have also been reported (Shah *et al.*, 2000).

Several commercially available xylanase preparations, most of which are active at slightly acidic or neutral pH, have been investigated in pulp bleaching. Pulpzyme HA (Novo Nordisk, Denmark), produced by *Trichoderma reesei,* was the first commercial xylanase to be used in biobleaching. It achieved a 20% kappa number decrease of oxygen-delignified birch kraft pulp (Zamost *et al.*, 1991). Cartazyme (Clariant) also improved the brightness of kraft pulps (Garg *et al.*, 1998). There is a tendency for

hardwood kraft pulp to show higher benefits with regard to chlorine savings (Hamilton *et al.*, 1996) during xylanase bleaching in comparison to softwood pulps. These differences may be partly attributed to differences in xylan solubilization from pulp, which may in tern be related to the amounts of accessible xylan present, the ion composition (Buchert *et al.*, 1995) and buffering capacity that could moderate enzyme-fibre interactions, or the wood's response to kraft pulping.

The effectiveness of xylanases have been well documented for wood pulps, however, very little attention has been given to the application of xylanases to non-woody materials such as bagasse pulp. A recent report by Jain *et al.*, 2001 indicated a xylanase mediated reduction of Cl of 9 kg/ton pulp of kraft bagasse pulp, which corresponds to 18% savings in chemicals when bleaching was performed to achieve brightness levels of 83 to 85% ISO. The strength properties of the enzyme treated pulp sheets were increased by 20% while the BOD, COD and AOX levels of the bleach effluent was reduced by 25%. This study verified the significant biobleaching potential of xylanases on non-woody fibre material.

We have demonstrated that crude xylanase preparations form *T. lanuginosus* SSBP in the form of culture supernatants can be used to remove chromphores and xylan from pulp types and therefore improve the brightness of pulp (Madlala *et al.*, 2001). The successful application of purified xylanase from *T. lanuginosus* SSBP on bagasse pulp has also been demonstrated by Bissoon *et al.* (2002). However, the incorporation of xylanases in ECF and TCF bleaching processes is of great significance in demonstrating reductions in chemical consumption induced by these hydrolytic enzymes. The aim of this study was to evaluate and compare the bleaching potential

of *T. lanuginosus* SSBP xylanase and three commercial xylanase preparations in ECF and TCF bleaching of bagasse soda pulp.

4.3 MATERIALS AND METHODS

4.3.1 Pulp

Unbleached bagasse soda pulp was prepared as described in section 3.3.4.

4.3.2 Enzymes and enzyme assays

T. lanuginosus xylanase and three commercial enzymes, Xylanase P (Iogen Corp., Canada), Ecopulp TX-200C (Röhm Enzyme, Finland) and Cartazyme NS-10 (Clariant, UK), were evaluated in bleaching of bagasse pulp. All four xylanases belonged to family 11 of glycosyl hydrolase, of which three commercial xylanases were produced from genetically modified *Trichoderma reesei* (Xylanase P and Ecopulp TX-200C) and *Bacillus* strains (Cartazyme NS-10). The xylanase activities were determined according to the method of Bailey *et al* (1992) as described in section 3.3.3.

4.3.3 Optimization of xylanase pretreatment conditions on bagasse pulp

Washed unbleached bagasse pulp equivalent to 5 g of dry weight was placed in plastic bags to which the xylanases diluted in distilled H_2O of varying pH were added. The enzyme equivalent to 5 U/gram dry weight pulp was added to the experiment bag and thoroughly mixed. The bags were incubated at varying temperatures from 50°C to 75°C as described in section 3.3.5. The optimum conditions for the use of each enzyme preparation (Table 4.1) were determined based on the release of R.S from the pulp (data not shown).

Table 4.1 Optimized conditions used for xylanase pretreatment on bagasse soda pulp

Enzyme	Temperature (°C)	Time (h)	Consistency(%)	pH
T. lanuginosus xylanase	65	2	10	6.0
Xylanase P	60	2	10	5.0
Cartazyme NS-10	60	2	10	8.0
Ecopulp TX-200 C	65	2	10	7.0

Washed unbleached bagasse pulp was treated with various xylanases at charges of 1, 5 and 10 U/g under the optimum pH and temperature conditions for each enzyme at a 10 % pulp consistency for 2h. Boiled enzyme (inactive) was used as control. After the incubation period, the pulp was filtered and washed. Pulp and enzyme filtrates were retained for further analyses.

4.3.4 Mechanical treatment of pulp

Prior to the application of each xylanase at a charge of 1 U/g, washed unbleached bagasse pulp was pretreated mechanically using a pulp homogenizer (Heidolph, Germany). Pretreatment was carried out at 26 000 rpm, room temperature and 1.5% pulp consistency for 15 min.

4.3.5 Analyses of pulp filtrates

The enzyme mediated release of ultraviolet light (UV) absorbing and chromophoric material as well as reducing sugars from pulp was monitored in filtrates as described in section 3.3.6.

4.3.6 Chemical bleaching of bagasse pulp

Xylanase pretreated pulp was subjected to ECF bleaching under conditions described in Table 4.2. Chlorine dioxide charges were based on kappa factors (kf) of 0.3, 0.4, 0.5, 0.6 and 0.75. The conditions for TCF bleaching are shown in Table 4.3.

4.3.7 Pulp properties

The kappa number and physical properties of bagasse pulp were determined as described in section 3.3.8.

Table 4.2 Conditions used for ECF bleaching of bagasse pulp

Bleaching step	Charge (%)	Temperature (°C)	Time (h)	Consistency (%)	pH
Chlorine dioxide (D_1)	kappa no x kf	70	1	10	3.0
Alkali extraction (E)	2.5% NaOH	70	2	10	-
Chlorine dioxide (D_2)	0.5 x D_1	70	3	10	4.0

Table 4.3 Conditions used for TCF bleaching of bagasse pulp

Bleaching step	Charge	Temperature (°C)	Time (h)	Consistency (%)	pH
Oxygen (O)	1.2% NaOH; 400 kPa O_2	95	1	10	>10.0
Chelated peracetic acid (qPa)	0.3% Pa; 0.3% H_2O_2	75	2	10	8.5
Hydrogen peroxide (P)	3% H_2O_2; 1.8% NaOH	75	3	10	11.0

4.4 RESULTS

4.4.1 Analyses of enzyme filtrates

Xylanase P was most effective in releasing RS, UV and visible light absorbing material from bagasse pulp while Ecopulp TX-200C, *T. lanuginosus* xylanase and Cartazyme NS-10 followed in decreasing order of efficiency (Table 4.4). The amount of UV absorbing material detected in the filtrates was significantly greater than that of the visible light absorbing material. The concentration of material released from pulp increased with increasing enzyme charges. Xylanase P at a charge of 10 U/g was able to release 62% and 69% more UV and visible light absorbing material, respectively, and 2.14 fold more RS than the control.

Table 4.4 Analyses of enzyme filtrates following xylanase pretreatment of bagasse pulp

Enzyme	Charge (U/g)	$A_{280\ nm}$	A_{465nm}	RS (mg/g)
T. lanuginosus xylanase	1	3.90	0.18	0.32
	5	4.16	0.19	0.37
	10	4.29	0.21	0.43
Xylanase P	1	4.35	0.20	0.34
	5	4.50	0.21	0.42
	10	4.64	0.24	0.59
Ecopulp TX-200C	1	4.20	0.19	0.30
	5	4.37	0.20	0.37
	10	4.50	0.22	0.45
Cartazyme NS-10	1	3.96	0.18	0.30
	5	4.21	0.20	0.36
	10	4.31	0.22	0.40
Control	0	2.86	0.15	0.19

4.4.2 Impact of xylanase pretreatment on kappa number and brightness of bagasse pulp

Xylanase pretreatment at a charge of 1 U/g had no effect on the kappa number of bagasse pulp with the exception of xylanase P (Table 4.5). An increase in the enzyme charge to 10 U/g produced a minimal decrease in kappa number with reductions of 4 %, 6%, 7 % and 12% achieved with *T. lanuginosus* xylanase, Cartazyme NS-10, Ecopulp TX-200C and Xylanase P, respectively. Of the four enzymes under study, Xylanase P and Ecopulp TX-200C produced directly brightening effects under all enzyme charges investigated. At 10 U/g, Xylanase P was the most effective enzyme increasing the brightness by 2.2 brightness points while Cartazyme NS-10 and Ecopulp TX-200C and *T. lanuginosus* xylanase produced brightness increases of 1.4, 1.8 and 1.6 points over control, respectively.

Table 4.5 Impact of xylanase pretreatment on kappa number and brightness of bagasse pulp

Enzyme	Charge (U/g)	Kappa no reduction (%)	Brightness increase (points)
T. lanuginosus xylanase	1	0.0	0.0
	5	0.0	0.5
	10	4.0	1.6
Xylanase P	1	5.0	0.6
	5	8.0	1.3
	10	12	2.2
Ecopulp TX-200C	1	0.0	0.4
	5	5.0	1.0
	10	7.0	1.8
Cartazyme NS-10	1	0.0	0.0
	5	0.0	0.6
	10	6.0	1.4

4.4.3 ECF biobleaching of bagasse pulp

Xylanase P was the most efficient enzyme to enhance bleachability of bagasse pulp. The biobleaching effect induced by this enzyme was between 0.3 and 1.6 points brightness gain over control at a kf of 0.75 (Fig. 4.1) and between 4.9 and 8.2 points when pulp was bleached at a kf of 0.3 (Fig. 4.2). Ecopulp TX 200-C, Cartazyme NS 10 and *T. lanuginosus* xylanase at a charge of 10 U/g and kf of 0.75, improved the brightness by 1.2, 0.9 and 1 point, respectively. At the kf factor of 0.3 the brightness gain produced by Ecopulp TX-200C, Cartazyme NS 10 and *T. lanuginosus* xylanase was 4.9%, 31.0% and 31.2% less than that achieved with Xylanase P. Brightness of the bleached pulp progressively increased with the increase of the enzyme charges.

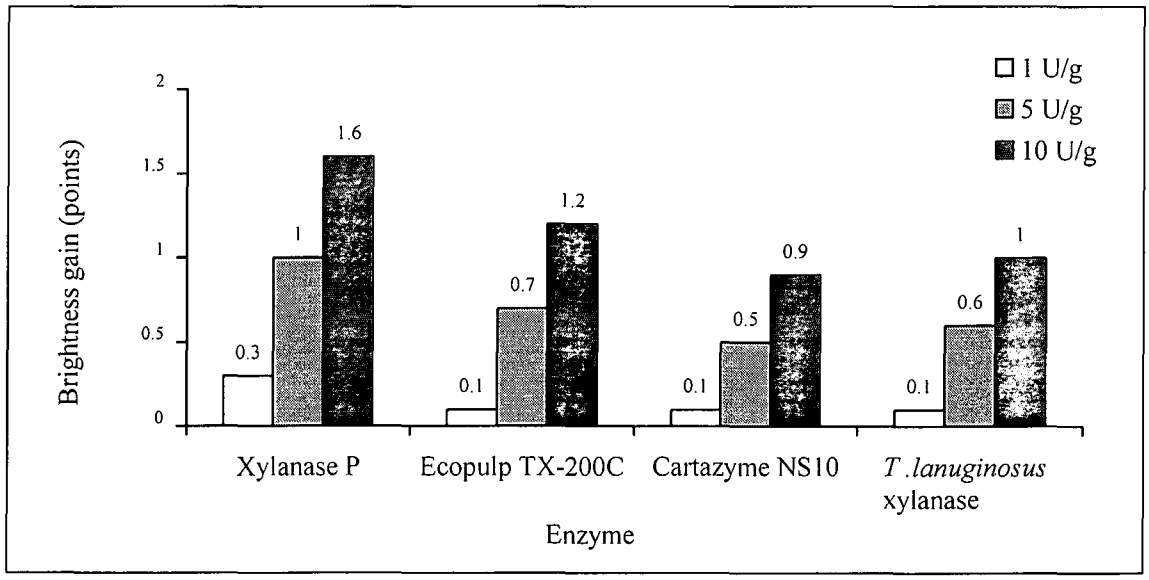


Fig. 4.1 Influence of Xylanase P, Ecopulp TX-200C, Cartazyme NS 10 and *T. lanuginosus* xylanase on the brightness of bagasse pulp in X-DED bleaching at kf of 0.75

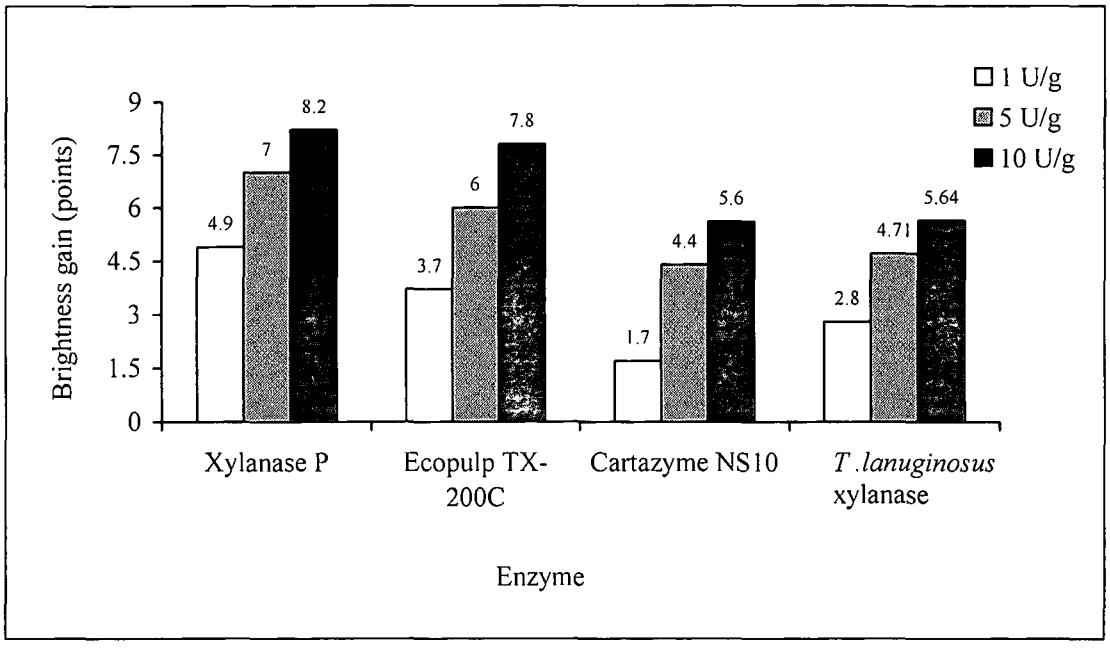


Fig. 4.2 Influence of Xylanase P, Ecopulp TX-200C, Cartazyme NS 10 and *T. lanuginosus* xylanase on the brightness of bagasse pulp in X-DED bleaching at kf of 0.3

Using Xylanase P, the bagasse pulp could be bleached with a kf of 0.5 to the same brightness value of 83.8 % as the control bleached with a kf of 0.75 (Fig. 4.3). This represents a reduction in the chlorine dioxide consumption of 33.3 %. The brightness gain induced by *T. lanuginosus* xylanase, Ecopulp TX 200C and Cartazyme NS 10, on the other hand, could be translated into chlorine dioxide savings of 20 %.

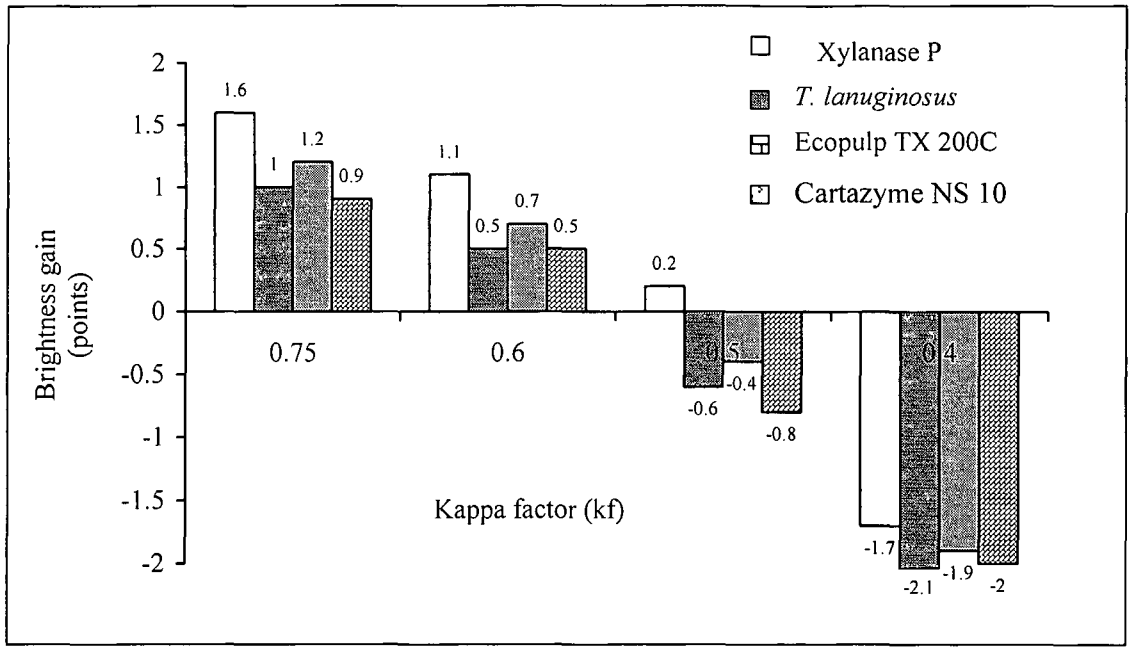


Fig. 4.3. Reduction of chlorine dioxide consumption following X-DED bleaching using *T. lanuginosus* xylanase, Ecopulp TX 200C and Cartazyme NS 10 at a charge of 10 U/g

Mechanical pretreatment of pulp prior to xylanase treatment (1 U/g) rendered the pulp more susceptible to bleaching in comparison to pulp that was not pretreated mechanically. The brightness increase induced by Xylanase P, Ecopulp TX-200C, Cartazyme NS 10 and *T. lanuginosus* xylanase on the mechanically pretreated pulp was 0.3, 0.4, 0.2 and 0.3, respectively (Fig. 4.4).

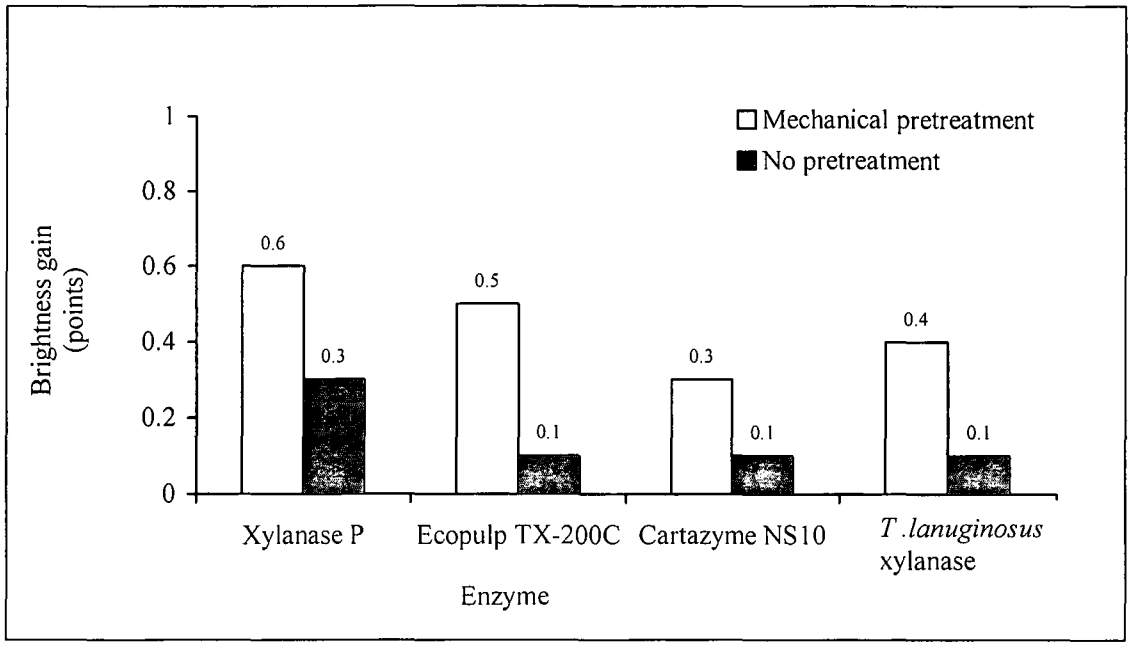


Fig. 4.4 Influence of xylanase treatment (1 U/g) on mechanical pretreated bagasse pulp in XDED bleaching at a kf of 0.75

4.4.4 TCF biobleaching of bagasse pulp

With the application of Xylanase P, the brightness of bagasse pulp was improved by up to 2.3 points (Fig. 4.5) to a final brightness of up to 71.9%. For comparison, using Xylanase P in the ECF bleaching, a brightness increase of 8.2 points was gained to produce a final brightness of 76.1% (Fig. 4.2). *T. lanuginosus* xylanase, Ecopulp TX-200C and Cartazyme NS-10, when used at 10 U/g, brightened pulp by 2.0, 2.2 and 1.9 points, respectively (Fig. 4.5). At a charge of 1 U/g Xylanase P, Ecopulp TX-200C, Cartazyme NS 10 and *T. lanuginosus* xylanase improved the brightness by 1.4, 1.5, 1.2 and 1.2 points respectively.

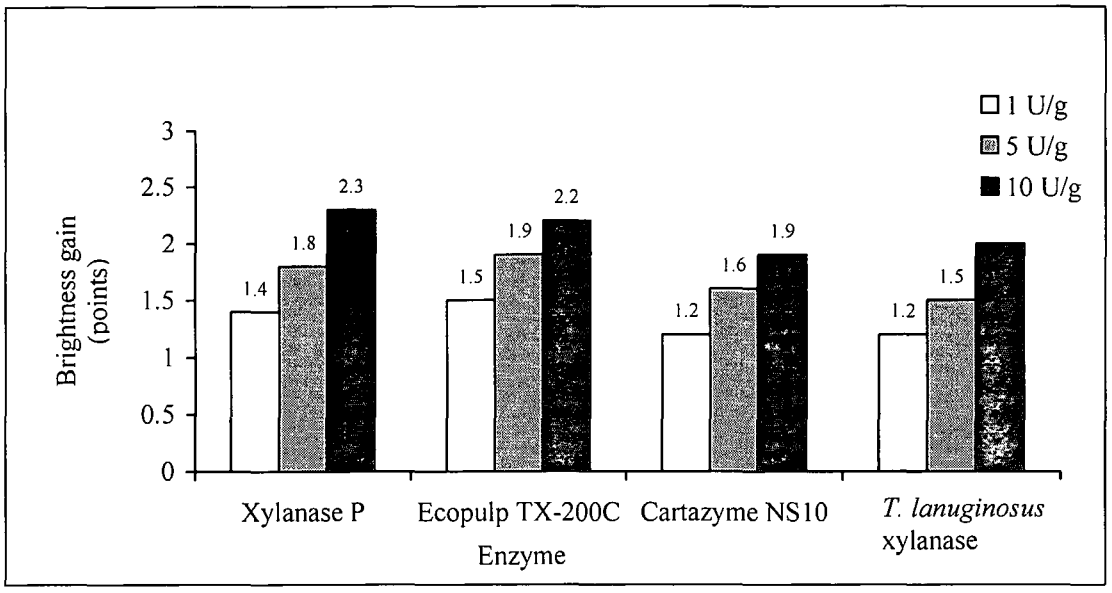


Fig. 4.5 Influence of Xylanase P, Ecopulp TX-200C, Cartazyme NS 10 and *T. lanuginosus* xylanase in TCF (X-OqPaP) bleaching of bagasse pulp

4.4.5 Physical properties

Analysis of the physical properties of bagasse pulp indicated minimal changes of the enzyme treated pulp compared to the control (Table 4.6). Xylanase P and Ecopulp TX-200C, however, did increase the tensile strength of the paper with a corresponding increase in the amount of energy required to rupture the test strip. The percentage stretch of the paper strips was marginally lower with the enzyme treated samples. The stretchability values obtained with Cartazyme NS-10 were lower than the three xylanases under study. The viscosity of the enzyme treated pulp was decreased minimally. However, there were no significant changes in the physical properties of bagasse pulp with the incorporation of xylanases into the bleach sequence.

Table 4.6 Effect of xylanase pretreatment on the physical properties of bagasse pulp

*Enzyme	Tensile strength(N/15mm)	Stretch(%)	TEA(J/m^2)	Viscosity(mPa·s)
Control	13.76	2.72	13.83	48.4
Xylanase P	14.12	2.55	14.56	46.9
Ecopulp TX-200C	13.87	2.58	14.03	47.2
Cartazyme NS-10	13.56	2.31	13.65	47.3
T. lanuginosus xylanase	13.34	2.35	13.54	46.5

*Enzyme charge: 10 U/g, D_1: 6.57% ClO_2,E: 0.7% NaOH, D_2: 3.285% ClO_2

4.5 DISCUSSION

The bleach boosting effects of xylanases on kraft pulps (Kantelinen *et al.*, 1992; Hortling *et al.*, 1994; Viikari *et al.*, 1994), and dissolving pulps (Christov and Prior, 1996; Bucchert *et al.*, 1995; Scott *et al.*, 1998) are well documented. However, xylanase pretreatment of non-woody material such as cereal straw (Sabharwal and Young, 1996), ramie fibres (Zheng *et al.*, 2000) wheat straw (Jimenez *et al.*, 1999) and bagasse pulp (Kulkarni and Rao, 1996; Shah *et al.*, 1999) has only recently received attention.

In view of producing a pulp of high brightness with minor impact to the environment the evaluation of xylanases in ECF and TCF bleaching processes have been investigated. Xylanase P, when compared to Ecopulp TX-200C, *T. lanuginosus* xylanase and Cartazyme NS-10 at equal enzyme doses as U/g, was the most efficient bleach-enhancing enzyme on bagasse pulp in both ECF (DED) and TCF (OqPaP) bleaching. In DED bleaching (kf of 0.75), a brightness gain of 1.6 points over control could be attained using Xylanase P. Overall, Ecopulp TX-200C *T. lanuginosus* xylanase and Cartazyme NS-10 followed Xylanase P in order of decreasing biobleaching efficiency.

Xylanases do not directly attack the lignin, the use of xylanases is an indirect bleaching method. This could explain the insignificant reductions in kappa number immediately after enzyme treatment with all the xylanases in this study. Previously, kappa reduction has been attributed to lignin removal, however, recent studies indicate that hexeneuronic acid (Hex A), accounts for a significant fraction of the

oxidizable components in kraft pulps (Gellerstedt, 1996). A report by Jeffries and Davis (1998) showed that there is an excellent correlation between xylanase activity and Hex A release, but the kappa number did not correlate directly with either of these factors. These findings indicate that other materials released during enzyme treatment also contribute to kappa reduction. It therefore, emphasizes the importance of assessing the properties of various xylanases from different sources, since the reduction of kappa number depends on the origin and processing of the pulp type, access of xylanase to the xylan substrate, and the nature of the enzymes themselves (Jeffries and Davis, 1998).

Xylanase P was able to reduce the use of chlorine dioxide by 33% as compared to a 20% reduction of the chlorine dioxide consumption achieved with the other three enzymes. These results compare favourably with laboratory trials conducted by Allison *et al.* (1993) with a thermophilic xylanase, Cartazyme HT, on kraft pulp in the conventional (DED) bleach sequence, in which brightness improvements of 3.5 points was correlated to reduced chlorine dioxide consumption of 19 to 24 %. Similar chemical savings were achieved a highly thermostable and alkalotolerant xylanase (90°C, pH 10.0) from *Thermotoga maritima* pretreated on hardwood kraft pulp in DEDED bleaching. The xylanase reduced the chlorine dioxide consumption by 25 % at a target brightness of 86.7 % ISO (Shah *et al.*, 2000).

In this study it was noticed that the biobleaching effect induced by xylanase pretreatment depended on both the enzyme and chlorine dioxide charges used. The use of high kappa factors were required for a satisfactory bleaching which could be due to a low initial brightness and a relatively low brightness ceiling of bagasse pulp.

The xylan content of pulps play a significant role in xylanase induced bleaching. This was confirmed by Wong *et al.* (2001) in which Irgazyme 40-X4 was tested on pine kraft pulps. For the low and high xylan pulps, the results indicated that the response of the former to xylanase-aided bleaching was smaller than that of the latter. The low xylan pulp showed ClO_2 savings of 6 to 7 % for achieving 85 % ISO brightness with xylanase pretreatment, while the high xylan pulp showed savings of 11 %. The efficient bleach boosting capabilities of the xylanases even at high kappa factors could be attributed to the high xylose content of approximately 20% present in bagasse pulp.

Mechanical pretreatment of the pulp prior to ECF biobleaching did produce an increase in final brightness, indicating "activation" of pulp to enzyme and chemical bleaching. Further studies on optimization of the parameters during mechanical pretreatment have to be performed to confirm the results obtained thus far. However, it appears that mechanical pretreatment could play an important role in improving the accessibility of the hemicelluloses in the pulp to xylanases.

Analysis of the physical properties of bagasse pulp indicated minimal changes of the enzyme treated pulp compared to the control. Xylanase P and Ecopulp TX-200C however, did increase the tensile strength of the paper with a corresponding increase in the amount of energy required to rupture the test strip. The percentage stretch of the paper strips was slightly lower with the enzyme treated sample with stretchability values obtained with Cartazyme NS 10 lower than the other xylanases under study. A recent report by Jain *et al.* (2001) showed an improvement in burst and tensile strength by 20 % on xylanase pretreated kraft bagasse pulp. Findings by Prasad *et al.* (1996) revealed a slight decrease in strength properties of xylanase pretreated bagasse

pulp. Contrary to this, Beg *et al.* (2000) reported a 63 and 8 % increase in tensile strength and burst factor, respectively with the application of a thermostable xylanase from Streptomyces sp. QG-11-3 on eucalyptus kraft pulp. This concludes that the effects of xylanases on the physical properties of the paper depends on the pulp type investigated.

The lower viscosity of the enzyme treated pulps could be attributed to the presence of low levels of cellulase activity. This could also be attributed to activities of other hemicelllases present in the enzyme suspension since the xylanases under study are not purified. A report by Kulkarni *et al.* (1996) on the treatment of crude xylanase on bagasse pulp indicated that the viscosity of the enzyme treated samples were unaltered. However, Goncalves *et al.* (2001) indicated an increase in viscosity of ethanol/water bagasse pulp using xylanase from *Thermomyces lanuginosus* IOC-4145 and Cartazyme (Sandox). In the present study xylanase pretreatment displayed minimal changes on the physical properties of bagasse soda pulp after final bleaching.

At similar brightness levels, the TCF biobleaching in sequence X-OqPaP appeared as less efficient than the ECF biobleaching in terms of biobleaching effect. The final brightness obtained with the control samples was on average 69.5%. However, Xylanase P, *T. lanuginosus* xylanase, Ecopulp TX 200C and Cartazyme NS 10 improved the brightness by 2.35, 2.0, 2.2 and 1.85 points, respectively. These results corroborate with reports by Nelson *et al.* (1995) that different xylanase preparations can vary in their ability to enhance peroxide bleaching of pulps. The potential of hydrogen peroxide as a bleaching agent has been the subject of several recent studies (Jimenez *et al.*, 1999; de la Rosa *et al.*, 2001 and Hernadi *et al.*,2001). A recent report

by Roncero *et al.* (2000) showed that the application of Pulpzyme HHC to eucalyptus kraft pulp in a TCF bleach sequence (X-OZRP) improved lignin removal with better preservation of carbohydrates, giving rise to pulps with higher brightness. Xylanase treatment enhanced brightness by 3 points and without xylanase pretreatment, it was necessary to increase ozone consumption by 37 % to obtain the same final results. Similarly, Kulkarni and Rao (1996) reported a 2.5 % increase in brightness on bagasse pulp when bleached with an alkaliphilic thermophilic *Bacillus* sp. NCIM xylanase in a TCF (X-PHH) sequence. The impact of xylanases on kenaf AQ pulp, a nonwoody material was also clearly demonstrated in a TCF bleaching sequence using oxygen, hydrogen peroxide and ozone (de la Rosa *et al.*, 2001). Similarly, xylanase treatment of straw pulp by *Thermotoga maritima* improved the brightness by 7% in TCF bleaching (Hernadi *et al.*, 2001). These reports on xylanase treatment of non-woody material as well as our present study on bagasse soda pulp signifies the potential interest of implementing enzyme application in TCF bleaching.

The leading motivations for the use of xylanases in the pulp and paper industry are increasing pulp throughput and obtaining a marketing advantage. Cost savings, pulp quality, and environmental benefit are cited in decreasing order (Tolan *et al.*, 1995). The results obtained indicate that xylanase pretreatment has a great potential in improving the bleachability of bagasse pulp especially in an ECF bleach sequence. The use of xylanase could result in improved bleachability of bagasse pulp. Alternatively, a decrease in chemical consumptions of ClO_2 could be obtained to attain a target brightness while still maintaining the desired physical properties.

4.6 CONCLUSIONS

i) Comparison of enzyme efficiency in this work was based on equal activity unit enzyme charges on pulp (U/g) rather than equal enzyme costs therefore conclusions drawn herein do not represent a commercial evaluation of enzyme products or enzyme manufacturers.

ii) Xylanase P was the most efficient enzyme in ECF and TCF bleaching followed by Ecopulp TX-200C, Cartazyme NS 10 and *T. lanuginosus* xylanase in decreasing order of efficiency.

iii) In DED bleaching Xylanase P reduced the ClO_2 consumption by 33%, while Ecopulp TX-200C, Cartazyme NS 10 and *T. lanuginosus* xylanase produced reductions of 20%.

iv) Mechanically pretreated pulp produced an enhanced biobleaching effect.

v) Xylanase-mediated changes to the physical properties of bagasse pulp were negligible.

The evaluation of the bleachboosting efects of xylanases in CEH bleaching of bagasse pulp is presented in chapter five

CHAPTER FIVE: COMPARISON OF THE BLEACH ENHANCING EFFECT OF XYLANASES ON BAGASSE PULP IN CEH BLEACHING

5.1 ABSTRACT

The bleach boosting effect of Xylanase P, Ecopulp TX 200C, Cartazyme NS 10, Cartazyme 9704-E, Pulpzyme HC and *T. lanuginosus* SSBP xylanase was investigated on bagasse-soda pulp in the CEH bleach sequence. These xylanases were evaluated on a cost rather than charge basis. The enzymes showed different degrees of bleachability on the pulp resulting in differences in chlorine saving during the bleaching process. Xylanase P (10 U/g) and Cartazyme 9704-E (5 U/g) were the most efficient xylanases reducing the chlorine consumption between 20 to 25 % in CEH bleaching. Cartazyme 9704-E produced the greatest gain of 2.8 brightness points over the control, followed by Xylanase P, which improved the brightness by 2.6 points. In terms of economical efficiency, Cartazyme 9704-E proved to be the best xylanase on bagasse soda pulp. Xylanase P, Ecopulp TX 200C, Pulpzyme HC, *T. lanuginosus* SSBP and Cartazyme NS-10 followed in decreasing order of efficiency. Cartazyme 9704-E (3.75 U/g) had the ability to reduce the hypochlorite consumption by 33.33 % while still maintaining a higher brightness than the control sample.

5.2 INTRODUCTION

Enzyme treatments of pulps to specifically remove hemicellulose is a well demonstrated technology for investigating and potentially modulating paper properties (Roberts *et al.*, 1990), but particularly for boosting the subsequent chemical bleaching treatment. The dark brown colour of unbleached pulp is attributed to lignin and its reaction products, and certain degraded polysaccharides (Viikari *et al.*, 1993). These are usually removed by multistage chlorine-based bleaching processes which increase the brightness of the pulps to marketable grades but produce highly coloured toxic and recalcitrant phenols and dioxins in the waste bleach waters. Chlorine is the cheapest and most versatile bleaching agent for pulp but at increasing environmental cost (Eriksson, 1991). In addition, a high concentration of chlorides in the effluents contribute to their corrosiveness (Rydholm. 1965).

In the search to produce pulp with non-polluting chemicals, more efficient pulping methods, which reduce the amount of residual lignin, and alternative bleaching methods are being developed (Kovasin and Tikka, 1992). Among the new bleaching technologies, enzymes have already shown their potential (Koponen, 1991; Lavielle *et al.*, 1992). The use of enzymes in the bleaching process provides an alternative to alleviate the heavy chemical loads during the bleaching process and development of environmentally friendly processes.

The residual lignin covalently bound to the carbohydrates acts as a physical barrier to the entry of bleaching chemicals into the fibers and its partial removal by the application of

xylanases, which can also remove chromophores (Patel *et al.*, 1993), is the basis of the enzyme bleach boosting effect. Enzymes should be effective in their application in all bleach sequences including chlorine, elemental chlorine free (ECF) and total chlorine free (TCF) bleaching. It has been reported that savings could be gained either in the prebleaching stage, as chlorine gas, or in the final bleaching stage, as chlorine dioxide (Viikari *et al.*, 1986, 1987).

Xylanases have been tested on different types of wood and non-wood plants using diverse bleaching sequences for mechanical, semi-chemical, and chemical pulps at both the laboratory and pilot plant level. Some of these processes have already been implemented on an industrial scale in several countries (Jiménez *et al.*, 1999). Xylanases have shown enormous potential in the pretreatment of bagasse pulp with subsequent improvements in brightness, reductions in kappa number and chemical consumption. (Madlala *et al.*, 2001).

We have demonstrated in the previous chapter that xylanases are effective in improving the brightness ceiling of bagasse soda pulp in ECF and TCF bleaching processes. For environmental reasons, the possibility of minimizing the consumption of chlorine gas in bleaching was of particular interest. This chapter presents data on the bleach boosting efficiency of xylanases on bagasse soda pulp based on equal price evaluation as well as determining reductions in chlorine consumption in the chlorine based CEH bleach sequence.

5.3 MATERIALS AND METHODS

5.3.1 Pulp

Two bagasse soda pulp batches were used in the bleaching process. Batch 1 was thoroughly washed with distilled water and the kappa number and brightness was 9.42 and 39.70 %, respectively. The second batch of pulp was not washed and the kappa number and brightness was 8.89 and 42.00 %, respectively.

5.3.2 Xylanase source

Six xylanase preparations were investigated in this study. The commercial xylanase sources are as follows: Xylanase P (Iogen Corp, Canada); Pulpzyme HC (Novozyme); Ecopulp TX-200C (Röhm, Europe); Cartazyme NS 10 and 9704 E (Clariant). *T. lanuginosus* xylanase was produced in shake-flask cultures as described in section 3.3.2.

5.3.3 Xylanase Assay

Xylanase assay was performed according to Bailey *et al.* (1992) as described in section 3.3.3.

5.3.4 Treatment of bagasse soda pulp (Batch 1)

5.3.4.1 Xylanase pretreatment of bagasse soda pulp

The pretreatment conditions for the xylanases on bagasse soda pulp are described in Table 5.1. The xylanases applied to bagasse soda pulp were based on equivalent costs rather than equal charges.

5.3.5 Chemical bleaching of bagasse pulp

Conditions for CEH (C: chlorine; E: NaOH hydroxide; H: sodium hypochlorite) bleaching of bagasse pulp are described in Table 5.2. The Cl_2 charges were subsequently reduced to evaluate the bleach boosting capabilities of the xylanases.

Table 5.1 Conditions for xylanase pretreatment of bagasse soda pulp

Enzyme	Activity (U/ml)	Charge (U/g)	pH	Temperature (°C)	Time (min)	Consistency (%)
T. lanuginosus SSBP xylanase	1095	5, 10	6.0	65	120	10
Xylanase P	84 620	5, 10	5.0	60	120	10
Pulpzyme HC	15 310	2, 4	7.0, 8.0	60	120	10
Ecopulp TX-200C	14 390	3, 6	6.5, 7.0	65	120	10
Cartazyme NS-10	11 436	2, 4	7.0	65	120	10
Cartazyme 9740 E	23 713	2.5, 5	6.0, 6.5, 7.0	70	120	10

Table 5.2 CEH bleaching conditions for bagasse soda pulp

Treatment	Charge (%)	Time (min)	Temperature (°C)	Consistency (%)
C	3.5 active Cl	45	45	3.4
E	4.5 NaOH	90	70	12
H	1.5 active Cl	135	70	12

5.3.6 Treatment of bagasse soda pulp (Batch 2)

5.3.6.1 Optimization of chlorine charges during CEH bleaching

The second batch of bagasse pulp was used to optimize the Cl_2 charges to achieve a target brightness of 80%. The charges under investigation were between 3.25 and 5 % active Cl_2.

5.3.6.2 Xylanase treatment of bagasse soda pulp

Only Cartazyme 9740 E was evaluated on batch 2 bagasse soda pulp. The enzyme pretreatment was carried out as described in section 5.3.4.1 with a few modifications as described in Table 5.3.

Table 5.3 Pretreatment conditions of Cartazyme 9740-E on bagasse soda pulp

Enzyme	Charge (U/g)	pH	Time (min)	Temperature (°C)	Consistency (%)
Cartazyme 9740-E	1.25, 2.5, 3.75	6.0, 6.5, 7.0	90	60	10

5.3.7 Chemical bleaching conditions

The CEH bleaching conditions for batch 2 was performed as described in section 5.3.5. However, the Cl_2 charge used was 4.75 % active Cl_2. The enzyme pretreatment was evaluated at reduced Cl_2 charges (15 %, 20 % and 25 % reduction) as well as reduced H charges (16.67 % and 33.33 % reduction) in the CEH sequence.

5.3.8 Pulp properties

The brightness of bagasse pulp was determined as described in section 3.3.8

5.4 RESULTS

5.4.1 Effect of xylanases in biobleaching of bagasse soda pulp

The xylanases under investigation had significantly different activities ranging from 1095 U/ml for *T. lanuginosus* SSBP xylanase to 84 620 U/ml for Xylanase P. These enzymes showed different degrees of enhancing the bleachability of bagasse soda pulp. The approximate final brightness achieved with the control samples at a Cl_2 charge of 3.5 % was between 75 to 76 %. Xylanase P improved the brightness over the control by 2.3 and 3.5 brightness points to 77.8 and 78.2 % with 5 and 10 U/g respectively (Table 5.4 and Fig 5.1). This increase in brightness produced savings of 20 and 25 % Cl_2 with the application of 5 and 10 U/g, respectively, while still maintaining brightness levels similar to the control. Any further reductions in Cl_2 did produce brightness values lower than the control samples.

Table 5.4 Effect of Xylanase P pretreatment at pH 5.0 and 60°C on the final brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Charge (U/g)	pH	Temperature (°C)	Brightness at reduced Cl_2 charges (%)					
			0	-15	-20	-25	-30	-35
5	5.0	60	77.8	76.8	75.6	74.8	73.0	70.9
10	5.0	60	78.2	77.0	76.4	75.4	73.1	71.5
Control	5.0	60	75.5	-	-	-	-	-

Brightness of unbleached pulp: 39.7%; Kappa number of unbleached pulp: 9.4

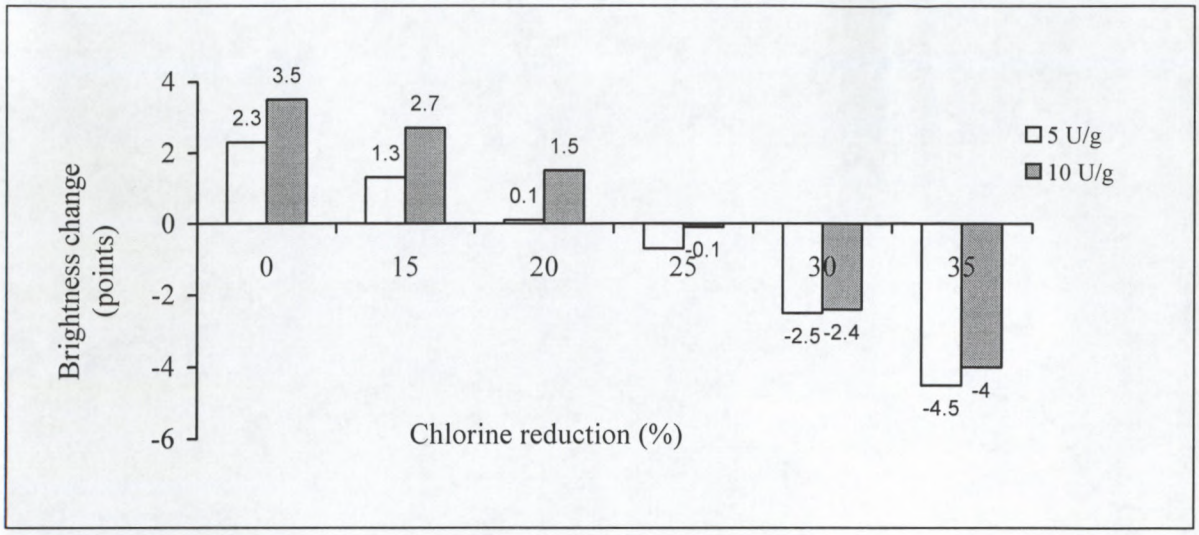


Fig 5.1 Evaluation of the bleach enhancing effect of Xylanase P pretreatment at pH 5.0 and 60°C on the brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Cartazyme NS-10 and *T. lanuginosus* SSBP xylanases were less efficient in improving the final brightness of bagasse pulp in the X-CEH bleach sequence (Table 5.5 and 5.6). At charges of 2 and 4 U/g, Cartazyme NS-10 improved the brightness by 0.6 and 1.5 brightness points, respectively, at full chlorine charges (Table 4.5). The enzyme at a charge of 2 U/g failed to reduce chlorine consumption, but still maintained the target brightness of the control. However, at a charge of 4 U/g, Cartazyme NS-10 reduced the Cl_2 consumption by 15 % and still maintained brightness values higher than the control. *T. lanuginosus* SSBP xylanase also produced a saving of 15 % at a charge of 10 U/g (Table 5.6). The absolute brightness achieved with *T. lanuginosus* SSBP xylanase with a charge of 10 U/g at Cl_2 reductions of 15 % was 76.7 %, which was 0.4 brightness points higher than the control.

Table 5.5 Effect of Cartazyme NS-10 pretreated at pH 7.0 and 65°C on final brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Charge (U/g)	pH	Temperature (°C)	Brightness at reduced Cl_2 charges (%)					
			0	-15	-20	-25	-30	-35
2.0	7.0	65	76.5	75.6	74.3	73.3	71.5	70.5
4.0	7.0	65	77.4	76.6	75.3	74.2	73.1	71.2
Control	7.0	65	75.9	-	-	-	-	-

Table 5.6 Effect of *T. lanuginosus* SSBP xylanase pretreated at pH 6.5 and 65°C on final brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Charge (U/g)	pH	Temperature (°C)	Brightness at reduced Cl_2 charges (%)					
			0	-15	-20	-25	-30	-35
5	6.5	65	77.2	76.0	74.1	71.3	70.5	70.0
10	6.5	65	77.9	76.7	75.5	72.7	71.9	71.4
Control	6.5	65	76.3	-	-	-	-	-

Pulpzyme HC was evaluated at pH 7.0 and 8.0. At pH 8.0, the enzyme appeared to be less efficient in improving the final brightness than ph 7.0 (Table 5.7 and Fig 5.2). On the other hand, enzyme treatment at pH 7.0 did produce Cl_2 savings of 15 % at a charge of 4 U/g, achieving a final brightness of 76.7%, which was 0.4 points higher than the control. The Cl_2 savings and final brightness obtained with Pulpzyme HC was comparable to results obtained with Cartazyme NS-10 and *T. lanuginosus* SSBP xylanase.

Table 5.7 Effect of Pulpzyme HC pretreated at 60°C and varying pH (7 to 8) on the final brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Charge (U/g)	pH	Temperature (°C)	Brightness at reduced Cl_2 charges (%)					
			0	-15	-20	-25	-30	-35
2.0	7.0	60	77.1	75.5	74.3	73.1	71.7	70.8
4.0	7.0	60	78.0	76.7	75.1	74.2	72.1	71.1
2.0	8.0	60	76.2	74.7	73.5	72.8	71.5	70.3
4.0	8.0	60	77.1	75.3	74.3	73.1	71.0	70.4
Control	7.0	60	76.3	-	-	-	-	-
Control	8.0	60	76.1	-	-	-	-	-

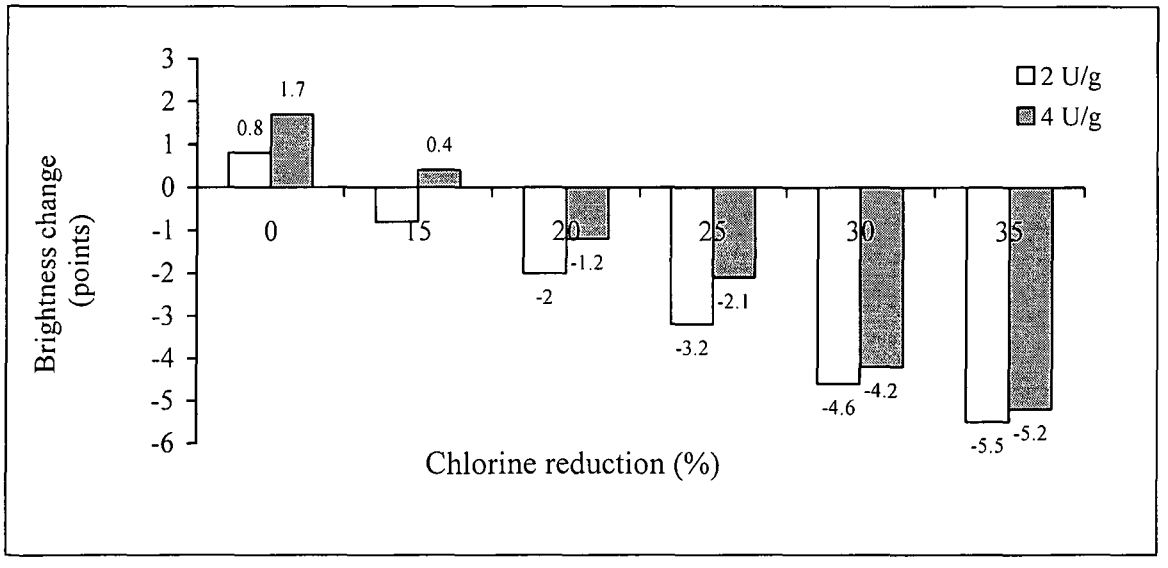


Fig. 5.2 Evaluation of the bleach enhancing effect of Pulpzyme HC pretreatment at pH 7.0 and 60°C on the brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Evaluation of Ecopulp TX-200C on bagasse soda pulp was carried out at pH 6.5 and 7.0. The enzyme induced similar effects to that of Cartazyme NS 10, *T. lanuginosus* SSBP xylanase and Pulpzyme HC. Ecopulp TX 200C exhibited greater bleach enhancing capabilities at pH 6.5 than pH 7.0 with 1.3 and 1.7 brightness points increase over the control at full Cl_2 charge and enzyme charges of 3 and 6 U/g, respectively (Table 5.8 and Fig. 5.3). The enzyme was also efficient in reducing the Cl_2 consumption by 15 % at a charge of 6 U/g.

Table 5.8 Effect of Ecopulp TX 200C pretreated at varying pH (6.5 to 7.0) at 65°C on final brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Charge	pH	Temperature	Brightness at reduced Cl_2 charges (%)					
(U/g)		(°C)	0	-15	-20	-25	-30	-35
3.0 U	6.5	65	77.6	76.0	75.2	74.0	72.3	70.6
6.0 U	6.5	65	78.5	76.6	76.1	75.0	73.3	71.2
3.0 U	7.0	65	77.0	75.3	74.4	72.4	71.4	70.4
6.0 U.	7.0	65	77.4	75.5	74.8	73.3	71.3	70.4
Control	6.5	65	76.3	-	-	-	-	-
Control	7.0	65	75.9	-	-	-	-	-

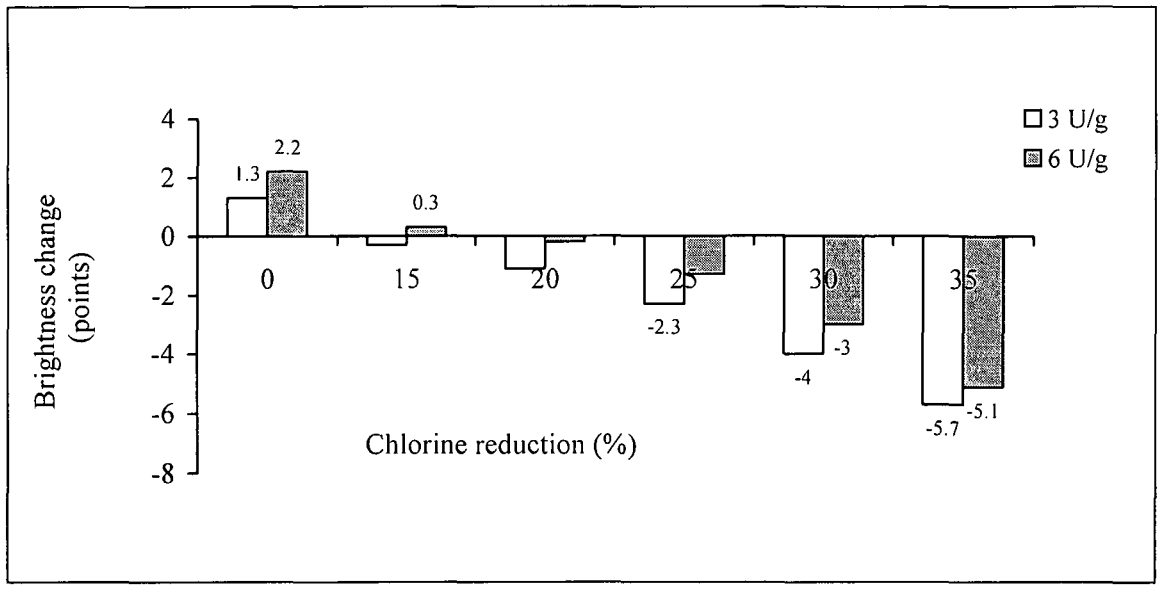


Fig 5.3 Evaluation of the bleach enhancing effect of Ecopulp TX-200C pretreatment at pH 6.5 and 65°C on brightness of bagasse pulp at reduced Cl_2 charges in X-CEH bleaching

Cartazyme 9704-E pretreatment was performed at pH 6.0, 6.5 and 7.0 at charges of 2.5 and 5 U/g. Pretreatment at the respective pH's did reduce the chlorine consumption by 15 %, with 5 U/g producing higher brightness values than the control. (Table 5.9, Fig 5.4, 5.5, 5.6). At pH 6.0, Cartazyme 9704-E improved the brightness by 0.3 and 1 point at charges of 2.5 and 5 U/g, respectively, with a reduced Cl_2 charge of 15 %. Similarly, at pH 6.5 and 7.0, the enzyme improved the brightness by 0.7 and 1.5; and 0.9 and 1.4 at charges of 2.5 and 5 U/g, respectively. At a charge of 5 U/g, Cartazyme 9704-E was able to reduce the Cl_2 consumption by 20 % when pretreatment was carried out at pH 6.0, 6.5 and 7.0. It appears that pH 6.5 is the optimum on bagasse pulp inducing a reduction of 25 % Cl_2 while still maintaining brightness at the control level.

Table 5.9 Effect of Cartazyme 9704-E pretreated at varying pH (6.0 to 7.0) and 70°C on the final brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Charge (U/g)	pH	Temperature (°C)	Brightness at reduced Cl_2 charges (%)					
			0	-15	-20	-25	-30	-35
2.5	6.0	70	78.0	76.1	75.3	73.9	72.9	71.5
5.0	6.0	70	78.4	76.8	76.2	74.9	73.9	72.1
2.5	6.5	70	77.8	75.6	73.9	72.8	71.7	70.4
5.0	6.5	70	78.0	76.4	76.0	74.9	73.5	72.7
2.5	7.0	70	76.7	76.3	75.2	74.1	72.4	70.9
5.0	7.0	70	77.2	76.8	75.8	74.1	72.9	71.2
Control	6.0	70	75.8	-	-	-	-	-
Control	6.5	70	74.9	-	-	-	-	-
Control	7.0	70	75.4	-	-	-	-	-

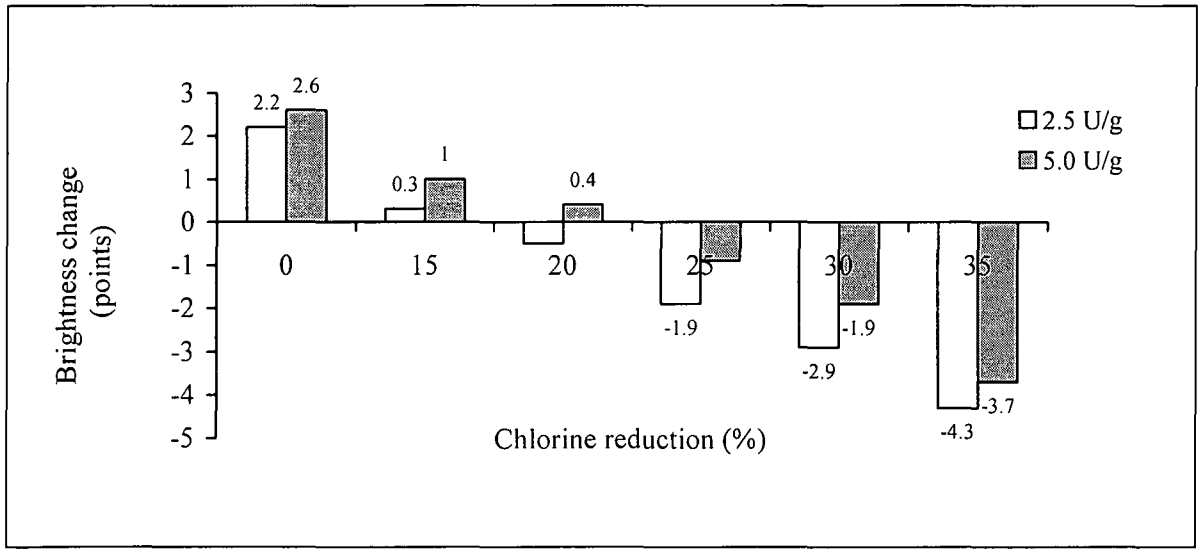


Fig 5.4 Evaluation of the bleach enhancing effect of Cartzyme 9704-E pretreatment at pH 6.0 and 70°C on brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

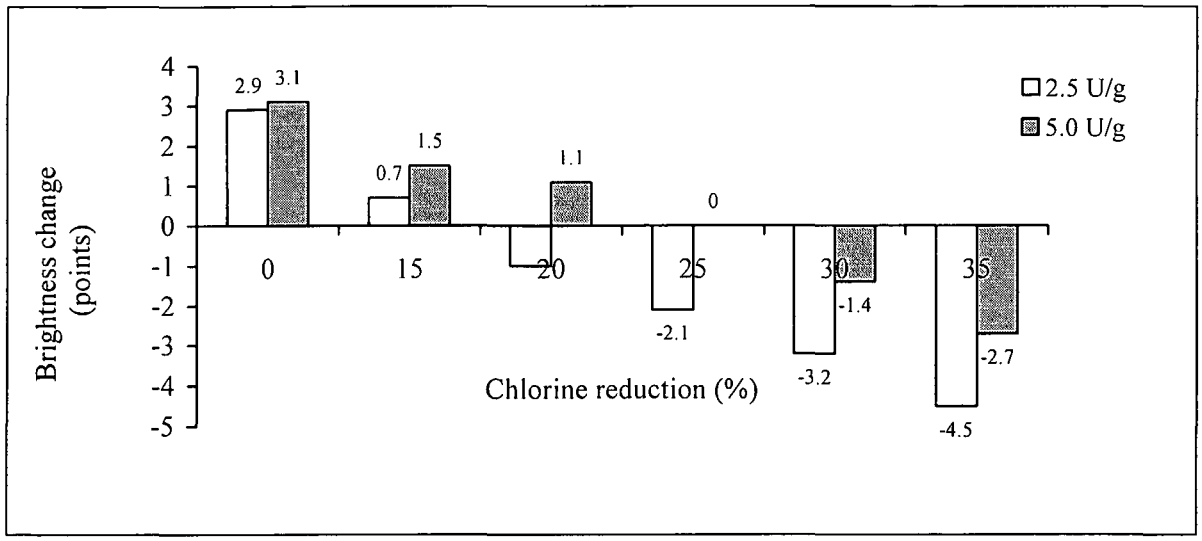


Fig. 5.5 Evaluation of the bleach enhancing effect of Cartazyme 9704-E pretreatment at pH 6.5 and 70°C on brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

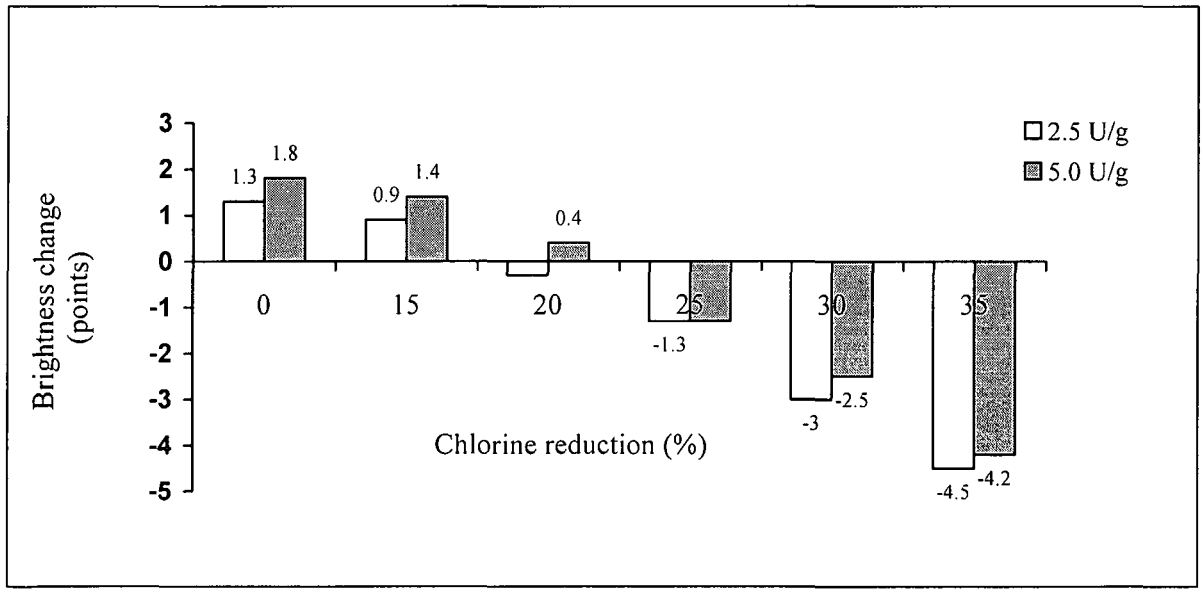


Fig 5.6 Evaluation of the bleach enhancing effect of Cartazyme 9704-E pretreatment at pH 7.0 and 70°C on brightness of bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

A comparison of the efficiency of the xylanases on bagasse pulp in terms of enzyme costs indicated that Cartazyme 9704-E and Xylanase P were most effective in reducing the Cl_2 consumption during X-CEH bleaching (Table 5.10). The other xylanases under investigation did not produce significant brightness increases resulting in Cl_2 savings of up to 15 % in the bleach sequence.

Table 5.10 Potential benefits from biobleaching of bagasse pulp (X-CEH) with xylanases used at equal enzyme cost

Enzyme	Charge (U/g)	Conditions			Benefit	
		pH	Temperature (°C)	Time (h)	Brightness gain (points)	Savings Cl_2 (%)
Cartazyme 9704E	2.5 U/g	6.0	70	2	2.3	15-20
Cartazyme 9704E	5.0 U/g	6.0	70	2	2.8	20
Cartazyme 9704E	2.5 U/g	6.5	70	2	2.6	15-20
Cartazyme 9704E	5.0 U/g	6.5	70	2	2.7	20-25
Xylanase P	5.0 U/g	5.0	60	2	2.1	20
Xylanase P	10 U/g	5.0	60	2	2.6	20-25
Ecopulp TX-200C	3.0 U/g	6.5	65	2	1.2	10-15
Ecopulp TX-200C	6.0 U/g	6.5	65	2	2.1	15-20

5.4.2 Optimization of Cl_2 charges in the bleaching of unwashed bagasse soda pulp in CEH bleaching

The chlorine charges applied to bagasse soda pulp were between 3.25 and 5 % active chlorine in the CEH bleach sequence (Table 5.11). An increase in the Cl_2 charges produced corresponding increases in the final brightness of the pulp. At a charge of 5 % Cl_2 the final brightness achieved was 81.31 %. A target brightness of 80 % was required and the Cl_2 charge of 4.75 % produced an absolute brightness of 79.87 %. This charge was used in the subsequent bleaching experiments.

Table 5.11 Brightness of bagasse soda pulp after CEH bleaching at various Cl_2 charges

Cl_2 charge (%)	Brightness (%)	Mean Brightness (%)
5.00	81.27	81.31
5.00	81.35	
4.85	80.65	80.53
4.85	80.41	
4.75	79.94	79.87
4.75	79.79	
4.60	79.26	79.15
4.60	79.04	
4.50	78.45	78.55
4.50	78.64	
4.00	77.30	77.11
4.00	76.92	
3.85	76.58	76.79
3.85	76.99	
3.75	76.86	76.55
3.75	76.24	
3.60	76.02	76.00
3.60	75.98	
3.50	75.66	75.80
3.50	75.94	
3.25	74.36	74.23
3.25	74.10	

5.4.3 Evaluation of the bleach enhancing effect of Cartazyme 9704-E on unwashed bagasse soda pulp

The unwashed bagasse soda pulp was pretreated with Cartazyme 9704-E at various charges (1.25, 2.5 and 3.75 U/g) and pH 6.0, 6.5, and 7.0. The optimized Cl_2 charge of 4.75 % was used in the X-CEH bleaching sequence. Pretreatment at pH 6.0 improved the brightness by 0.3 and 0.4 points with enzyme charges of 2.5 and 3.75 U/g, respectively (Table 5.12; Fig. 5.7). The absolute brightness achieved with the enzyme at pH 6.0 with charges of 2.5 and 3.75 U/g was 80.9 and 81 %, respectively. These charges reduced the Cl_2 consumption by 25 % and still maintained a higher brightness than the control samples.

Table 5.12. Effect of Cartazyme 9704-E pretreated at pH 6.0 and 70°C on the final brightness of unwashed bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Enzyme charge (U/g)	Brightness at reduced Cl_2 charges (%)			
	0	-15	-20	-25
Control	80.6	ND	ND	ND
1.25	81.7	80.9	80.5	80.5
2.50	81.8	81.5	81.0	80.9
3.75	82.2	81.7	81.0	81.0

ND: not determined

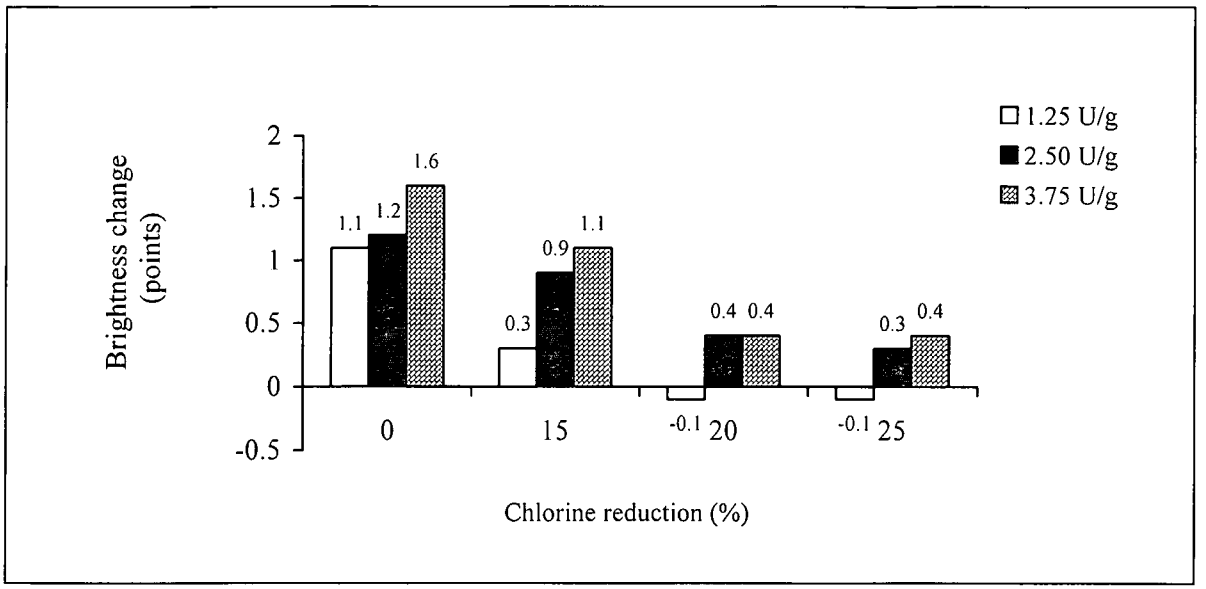


Fig. 5.7 Evaluation of the bleach enhancing effect of Cartazyme 9704-E pretreatment at pH 6.0 and 70°C on brightness of unwashed bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching.

Pretreatment of bagasse soda pulp with Cartazyme 9704-E at pH 6.5 and 7.0 produced similar increases in brightness and Cl_2 saving with all charges under investigation (Tables 5.13 and 5.14 and Fig 5.8 and 5. 9). At pH 6.5 and a charge of 3.75 U/g, the enzyme reduced the Cl_2 consumption by 25 % while still achieving a brightness of 81.3 %, which was 1 point higher than the control (Table 5.13, Fig 5.8). Even at a charge of 1.25 U/g, the enzyme could produce Cl_2 saving of 25 %, maintaining the brightness of the control. Pretreatment carried out at pH 7.0 produced absolute brightness values of 80.7, 81.1 and 81.3 % with 1.25, 2.5 and 3.75 U/g, respectively in comparison to the 80.35 % achieved with the control (Table 5.14, Fig 5.9).

Table 5.13 Effect of Cartazyme 9704-E pretreatment at pH 6.5 and 70°C on the final brightness of unwashed bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Enzyme charge	Brightness at reduced Cl_2 charges (%)			
(U/g)	0	-15	-20	-25
control	80.3	ND	ND	ND
1.25	81.6	81.2	80.8	80.2
2.50	82.0	81.3	81.2	80.8
3.75	82.4	81.3	81.1	81.3

ND: not determined

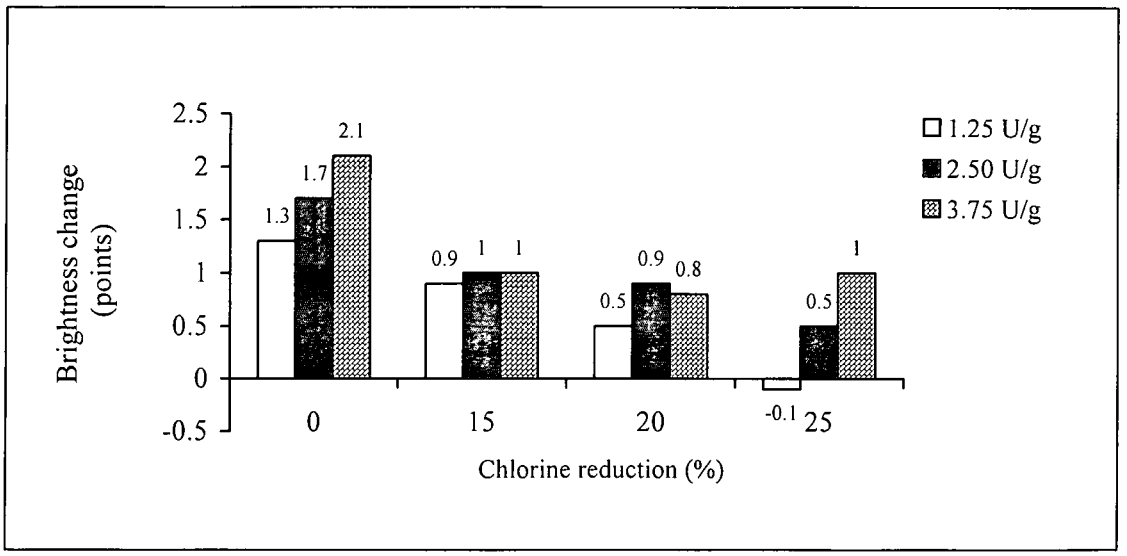


Fig. 5.8 Evaluation of the bleach enhancing effect of Cartazyme 9704-E pretreated at pH 6.5 and 70°C on brightness of unwashed bagasse soda pulp at reduced Cl_2 charge in X-CEH bleaching

Table 5.14 Effect of Cartazyme 9704-E pretreatment at pH 7.0 and 70°C on brightness of unwashed bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

Enzyme charge (U/g)	Brightness at reduced Cl_2 charges (%)			
	0	-15	-20	-25
Control	80.35	ND	ND	ND
1.25	81.6	81.3	81.2	80.7
2.50	81.9	81.3	81.5	81.1
3.75	81.9	81.8	81.6	81.3

ND: not determined

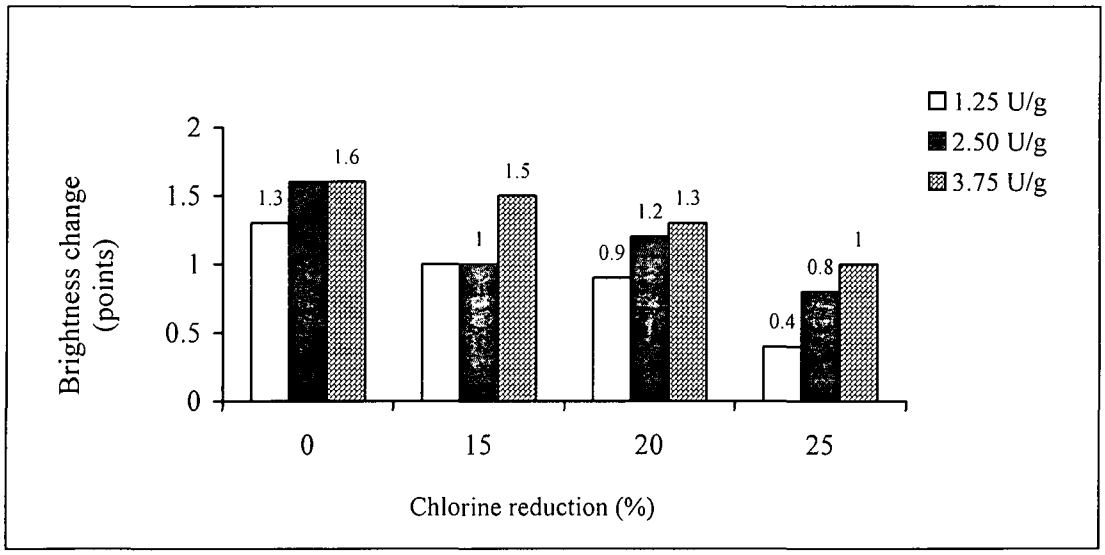


Fig. 5.9 Evaluation of the bleach-enhancing effect of Cartazyme 9704-E pretreatment at pH 7.0 and 70°C on brightness of unwashed bagasse soda pulp at reduced Cl_2 charges in X-CEH bleaching

5.4.4 Bleach enhancing ability Cartazyme 9704-E on unwashed bagasse pulp at reduced hypochlorite charges

Unwashed bagasse soda pulp was pretreated with Cartazyme 9704-E at pH 6.5 followed by bleaching with full chlorine charge (4.75 %) at reduced hypochlorite (H) charges. The enzyme used at all three charges was able to reduce the H dose by 16.67 % (Table 5.15, Fig 5.10). The absolute brightness achieved with Cartazyme 9704-E at a reduced H charge of 16 .67 % was 80.7, 80.6 and 80.7 % with 1.25, 2.5 and 3.75 U/g, respectively. Application of 3.75 U/g, reduced the H charge by 33.33 % while still maintaining a brightness higher than the control of 80.3%.

Table 5.15 Effect of Cartazyme 9704-E pretreated at pH 6.5 and 70°C on brightness of unwashed bagasse soda pulp at reduced hypochlorite charges in X-CEH bleaching

Enzyme charge (U/g)	Brightness at reduced H charges (%)		
	0%	-16.67%	-33.33%
Control	80.3	ND	ND
1.25	81.6	80.7	79.6
2.5	82.0	80.6	79.8
3.75	82.4	80.7	80.4

Control of unincubated pulp: 78.76%; ND: not determined

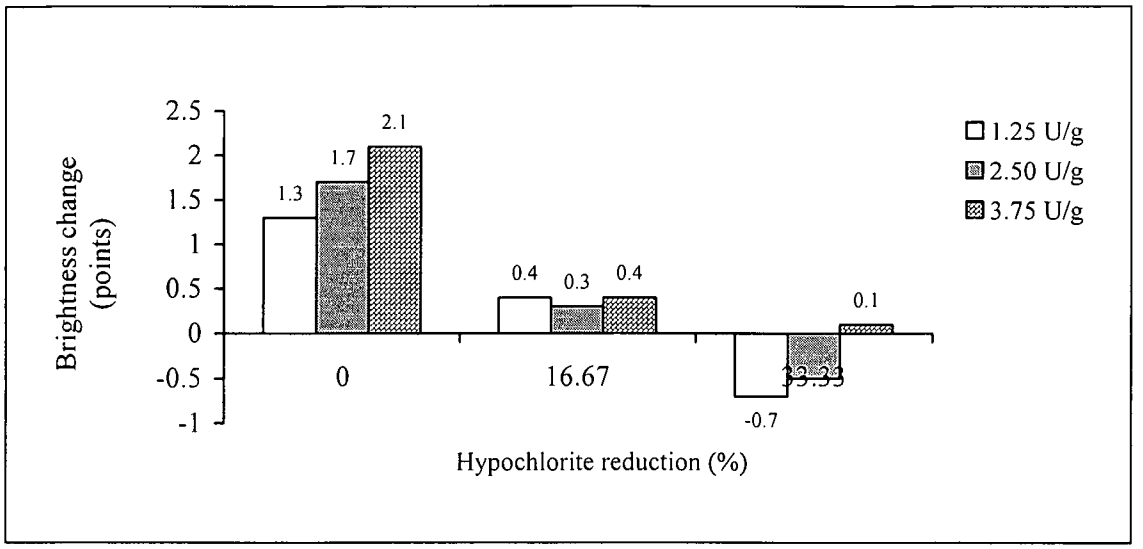


Fig. 5.10 Evaluation of the bleach enhancing effect of Cartazyme 9704-E pretreatment at pH 6.5 and 70°C on the brightness of unwashed bagasse soda pulp at reduced hypochlorite charges in X-CEH bleaching

5.5 DISCUSSION

Historically, chlorine has been the cheapest and the best agent for bleaching of chemical pulps. However, the needs of the pulp and paper industry have changed, and the economic superiority of chlorine has been eroded. The discovery of certain dioxins that seem to be generated in the bleach plant effluent has forced pulp and paper companies into seeking alternatives to chlorine (Ragauskas *et al.*,1994).

Although chlorine is highly versatile, corrosion of storage tanks and extensive emissions of chlorinated organic compounds in the bleach plant effluent can occur. The use of xylanolytic enzymes in pulp bleaching has been considered as one of the most important new biotechnological applications of these enzymes (Viikari *et al.*, 1994). To our knowledge, there are no reports on the evaluation of xylanases on non-woody materials such as bagasse pulp in a CEH bleach sequence and this is the first report thereof.

The bleach boosting potential of Xylanase P, Ecopulp TX 200C, Cartazyme NS 10, Cartazyme 9704-E, Pulpzyme HC and *T. lanuginosus* SSBP xylanase was investigated on bagasse-soda pulp in CEH bleaching. These xylanases were investigated on an equal cost rather than on equal charge basis. The xylanases showed different degrees of bleachability on the pulp with significant differences in chlorine saving during the bleaching process. Pretreatment of batch 1 bagasse soda pulp with xylanases revealed that Xylanase P (10 U/g) and Cartazyme 9704-E (5 U/g) were most efficient, reducing the chlorine consumption between 20 to 25 %. The final brightness after CEH bleaching was approximately 76 % and all the xylanases under investigation produced a higher final

brightness than the controls with full Cl_2 charges. Cartazyme 9704-E produced the greatest gain of 2.8 brightness points over the control, followed by Xylanase P, which improved the brightness by 2.6 points. Ecopulp TX 200C, Pulpzyme HC, *T. lanuginosus* SSBP and Cartazyme NS-10 followed in decreasing order of efficiency. Ecopulp TX 200C achieved a reduction of 20 % Cl_2 while Pulpzyme HC, *T. lanuginosus* SSBP xylanase and Cartazyme NS-10 reduced by Cl_2 charge by 15 %.

Previous reports by Bajpai *et al.* (1994) revealed that pretreatment of eucalyptus kraft pulp with commercial xylanases, (Pulpzyme HA, Novozyme 473 and VAI xylanase), in the CEH bleaching sequence resulted in a 31 % reduction in chlorine consumption at the chlorination stage. In the same study, it was also shown that pretreatment with Cartazyme HS-10 reduced the chlorine consumption by 31%, and brightness increased by 2.5 points. At a constant chemical dose, the final brightness of the pulp was increased by 4.9, 3.0, and 2.1 points with Cartazyme HS-10, Novozyme 473 and VAI xylanase, respectively, in the CEH sequence. This report is comparable to the present X-CEH bleaching of bagasse pulp with respect to Cl_2 saving.

Crude xylanase preparations from two fungal strains, TNPL 193 and TNPL 293, displayed an improvement in the bleachability of bagasse mechanical pulp with improvements in brightness of 2 to 3 points following peroxide bleaching (Prasad *et al.*, 1996). Similarly Kulkarni *et al.* (1996) reported an increase in brightness of 2.5 points by a xylanase (10 U/g) from an alkaliphilic thermophilic *Bacillus* sp. NCIM 59 on unbleached bagasse pulp. A recent report by Shah *et al.* (1999) also revealed the

effective release of LDC's and chromophoric material from bagasse pulp, corresponding to brightness increases with a xylanase from an alkalophilic *Bacillus* sp. SAM-3. The influence of xylanase on kraft bagasse pulp was also recently evaluated in CEH bleaching (Jain *et al.*, 2001). The authors reported savings of elemental chlorine up to 18% in conventional CEH bleaching adopted in majority of Indian paper mills, with gain in pulp brightness to a level of 2.5 to 3.5% ISO, and reduction in AOX levels of 20 to 25% in CE stage bleach effluents.

The evaluation of Cartazyme 9704-E (pH 6.5; 3.75 U/g) showed a brightness improvement of 1.0 point to 81.3 % on unwashed bagasse pulp with Cl_2 savings of 25 %, implying that the Cl_2 charges could be reduced further while still maintaining brightness values similar to the control. In comparison, pretreatment of the washed bagasse soda pulp produced Cl_2 saving of 25 % with the same final brightness as the control indicating that further reductions of Cl_2 produced brightness values lower than the control. It therefore appears that the unwashed bagasse soda pulp is more susceptible to xylanase pretreatment than the washed sample.

Pretreatment with Cartazyme 9704 (3.75 U/g) in X-CEH bleaching at reduced hypochlorite charges also displayed a 33.33% reduction in hypochlorite consumption while still maintaining a higher brightness than the control. Even at low enzyme charges the xylanase was able to achieve hypochlorite savings of 16.67 %. Cartazyme 9704-E appears to have great potential in CEH bleaching of bagasse-soda pulp. The enzyme has

shown significant increases in brightness with concomitant reductions of Cl_2 and hypochlorite during CEH bleaching.

Implementation of enzyme technology to the bleach process appears to be the way forward in terms of producing pulps, which are environmentally friendly, and process waters, which can be fully recyclable. It has been reported that reducing chemical consumption during the bleaching process significantly reduce AOX levels by 5 to 20% in bleach plant effluents (Senior and Hamilton, 1992). Many North American and European mills have performed mill trials with xylanases and some have incorporated the enzyme into routine production operations (Wright, 1998). As a part of promoting "cleaner" and environmentally friendly technologies in the pulp and paper industry, biotechnology has been identified as one of the promising and up-coming production paper production options. Enzymatic prebleaching of bagasse soda pulp has been a priority area and the evaluation of various xylanase enzyme preparations available globally as well as that developed indigenously in this study has resulted in immense enthusiasm among leading researchers in the pulp and paper industry. The pulp and paper industry recognizes the importance of biotechnology in the bleach process, and it appears that this technology will play a major role in bleaching processes in the near future.

5.6 CONCLUSIONS

i) The xylanases displayed different degrees of bleachabillity on bagasse soda pulp in X-CEH bleaching.

ii) The unwashed bagasse soda pulp was more susceptible to xylanase bleaching than the washed pulp

iii) In terms of economical efficiency Cartazyme 9704-E proved to be the best xylanase followed by Xylanase P, Ecopulp TX-200C, Pulpzyme HC, *T. lanuginosus* xylanase, and Cartazyme NS-10 in decreasing order of efficiency.

iv) Cartazyme 9704-E displayed the ability to reduce the Cl_2 and hypochlorite consumption by 25 and 33.33%, respectively.

The production of xylanase by SSF and bleaching abilities of these samples are evaluated in chapter six.

CHAPTER SIX: IN SITU SOLID SUBSTRATE FERMENTATION (SSF) AND UTILIZATION OF XYLANASE IN BLEACHING OF BAGASSE SODA PULP

6.1 ABSTRACT

Production of xylanases by nine thermophilic fungi under solid substrate fermentation (SSF) was evaluated. SSF with *Thermomyces langinosus* SSBP was carried out under conditions of varying initial pH and moisture. The optimum pH and moisture content of bagasse pulp for xylanase production by SSF was 7.0 and 83%, respectively. Time course experiments revealed a steady increase in xylanase production until day four, indicating that a four-day incubation period was sufficient for optimal production of xylanases by *T. lanuginosus* SSBP. All the fungal strains investigated produced significantly higher xylanase levels than *T. lanuginosus* SSBP under SSF similar conditions. The strain that appeared to be the most effective was F 980 producing 6037 U xylanase/g bagasse pulp. Evaluation of the bleach enhancing ability of these xylanases indicated strain ATCC 36350 as most efficient, improving the final brightness by 2 points to 84.7 %. *T. lanuginosus* SSBP followed with an improving of 1.9 points over the control at SSF/raw pulp ratio of 1:50. These two organisms produced similar effects on bagasse soda pulp at all SSF/pulp ratios investigated. Strain F 980 was the highest enzyme producer during SSF (6037 U/g), however the bleach-boosting capability was less effective compared to the other strains. Strain ATCC 46882 and ACTT 36350 reduced the Cl_2 consumption by 20 and 30 % at SSF/raw pulp ratios of 1:400 and 1:50, respectively in X-CEH bleaching.

6.2 INTRODUCTION

Hemicellulose is one of the most abundant organic substances, accounting for 5-50% of the dry weight of plant materials (Dekker and Lindner, 1979) and xylan is the most common component of hemicelluloses (Puls and Poutanen, 1989). The endo-β-1,4-xylanases are mainly responsible for the enzymatic hydrolysis of xylan and have attracted considerable research interest because of their potential industrial application (Royer and Nakas, 1989).

Xylanases are currently produced mostly by submerged fermentation (SF), a relatively expensive process even when high producing genetically engineered strains are used (Sheehan and Himmel, 1999). An alternative technology for enzyme production is SSF. SSF is generally defined as the growth of microorganisms on solid materials in the absence or near absence of free water. The substrate, however, must contain enough moisture, which exists in absorbed form within the solid matrix (Pandey, 1992). The history and developments in SSF have been reviewed by several authors (Aidoo *et al.*, 1982; Cannel and Moo-Young, 1980, Stanton, 1978). Selection of a suitable microorganism is one of the most important criteria in SSF. Several groups of microorganisms can grow on solid substrates, and filamentous fungi have the best capability to grow in the absence of free water. However, the vast majority of wild type microorganisms are incapable of producing commercially acceptable yields of the product (Pandey, 1992).

Recent increased awareness of the importance of biological materials as renewable resources has stimulated interest in SSF. SSF holds tremendous potential for the

production of xylanases (Panday *et al.*,1999). The selection of a particular strain, however, remains a tedious task, especially when commercially significant enzyme yields are to be achieved. Pure cultures are generally used in industrial SSF processes to improve the control of the substrate utilization and end-product formation. In nature, SSF is often carried out by mixed cultures in which several microorganisms show development and symbiotic cooperation (Pandey, 1992). Agro-industrial residues are generally considered the best substrates for SSF processes, and enzyme production by SSF is not an exception to that (Panday *et al.*,2000).

The use of abundantly available and cost effective agricultural residues, such as wheat bran, corn cobs, rice, bran, rice husk, and bagasse and other similar substrates, to achieve higher xylanase yields using SSF allows reduction of the overall manufacturing cost of biobleached paper. SSF is attractive because it presents many advantages, especially for fungal cultivations (Weiland, 1988). In SSF, the productivity reactor volume is much higher compared to that of submerged culture (Grajek, 1987). Also, the operation cost is lower because simpler plant, machinery and energy are required (Roche *et al.*, 1994). The disadvantages in large scale process development centre on problems of heat transfer, scale up and process control (Rathbun and Shuler, 1980). Despite these problems, the Japanese researchers have been successful in developing large-scale solid-state fermentations for food and enzyme manufacture (Yamada, 1977).

This study describes the impact of pH and moisture content during SSF on bagasse soda pulp on xylanase production by *T. lanuginosus* SSBP. A comparison of xylanase production levels among 9 thermophilic fungal strains were also evaluated. Samples

of the fermented pulp were used in X-CEH bleaching to examine the bleaching-enhancing abilities of these xylanases.

6.3 MATERIALS AND METHODS

6.3.1 Fungal isolates

The microorganisms under study in SSF on bagasse soda pulp were thermophilic fungi. *Thermomyces lanuginosus* SSBP was provided by Dr S. Singh (ML Sultan Technikon), while the remaining strains were provided by Dr G. Szakacs (Technical University of Budapest, Department of Agricultural Chemical Technology, Budapest, Hungary). The list of the fungal strains investigated is shown in Table 6.1.

Table 6.1. Fungal strains used in the production of xylanase by SSF

Strain	Source of Isolation
T. lanuginosus SSBP	Soil, South Africa
CBS 22463	N/A
ATCC 46882	N/A
ATCC 36350	Bangalore, India
F980	Soil, South Africa
F40	Grape marc compost, Hungary
F31	Soil, Jordan
F80	Soil, Zambia
F 48	Grape marc compost, Hungary

N/A: not available

6.3.2 Growth medium

The growth medium used in SSF for xylanase production consisted of: 5 g/l $NH_4 NO_3$ as nitrogen source, 5 g/l KH_2PO_4, 1 g/l NaCl, 1 g/l $MgSO4.7H_2O$. To this was added 1 ml mineral solution 1 (consisting of 1.6 g/l $MnSO_4$, 3.45 g/l $ZnSO_4$. $7H_2O$ and 2.0 g/l $CoCl_2.6H_2O$) and 1 ml mineral solution 2 (consisting of 5 g/l $FeSO_4.7H_20$). Washed bagasse soda pulp was used as substrate for the SSF process.

6.3.3 Growth conditions

SSF of bagasse pulp with *T. lanuginosus* SSBP was carried out under varying pH and moisture contents. A mass of 5 gram dry weight bagasse soda pulp was used, and fermentations were performed in 500 ml erlenmeyer flasks. The pH varied between 5.0 to 9.0 while the moisture content of the samples was adjusted to 80, 83.3 and 86% by the addition of 25ml, 30ml and 35ml growth medium, respectively. The pH of the samples was adjusted slightly higher than the required pH prior to autoclaving to account for acid production during the autoclaving process. The growth medium was thoroughly mixed with the pulp and autoclaved for 30 min at 120°C. The flasks were inoculated with one-third fungal growth from a petri plate of a five-day old culture. Replicate flasks were incubated at 45°C for a period of 4 days. SSF on bagasse soda pulp with the other thermophilic strains were carried out at pH 7.0, 45°C; and moisture content of 83% for a period of 4 days.

6.3.4 Enzyme extraction

The harvesting of the enzyme was carried out by adding to the fermentation flask a 0.1% solution of Tween 80. A volume of 75 ml Tween 80 was added to the SSF with an initial moisture content of 80 %. Similarly, when SSF was performed at 83 and 86 % moisture contents, 70 and 65 ml Tween 80 was added, respectively. The flasks were left to stand for an hour with occasional stirring. Thereafter the supernatant was separated from the pulp by centrifugation at 10 000 rpm for 10 min. The supernatant was analysed for xylanase activity using the DNS method by Bailey *et al.* (1992) as described in section 3.3.3.

6.3.5 Preparation of SSF samples for biobleaching

The enzymes from replicate flasks after the 4 day incubation period were not harvested. However, the entire contents from the flasks were transferred to foil boats, and left at room temperature for two days to dry. The dried samples were used in the pretreatment of bagasse soda pulp in CEH bleaching. Pretreatment of bagasse soda pulp performed at various SSF/raw pulp ratios and chemical bleaching conditions are described in Table 6.2.

Table 6.2 Conditions for X-CEH bleaching of SSF samples on bagasse soda pulp

Treatment	Charge	Time (min)	Temperature (°C)	pH	Consistency (%)
SSF/ Raw pulp	1:50, 1:100, 1:200, 1:400	180	60	6.0	10
Cl_2 (chlorine)	4.75 %	45	45	ND-	3.2
E (NaOH)	4.5 %	90	70	ND	12
H (NaOCl)	1.5 %	135	70	ND	12

ND: not determined

6.3.6 Brightness of pulp sheets

Brightness was measured as described in section 3.3.8

6.4 RESULTS

6.4.1 Optimization of SSF conditions for the produciton of xylanases by *T. lanuginosus* SSBP

Solid substrate fermentation with *T. lanuginosus* SSBP was carried out under conditions of varying initial pH and moisture content while the temperature remained unchanged at 45°C. The xylanase production under these conditions varied drastically between 400-900 U/g (Table 6.3). The optimum pH and moisture content for xylanase production was 7.0 and 83 %, respectively (Fig 6.1). At acidic pH values, the xylanase production levels were low. However, as the pH was increased towards neutral values (pH 7.0) the production levels increased significantly obtaining a two fold increase as compared to production levels at pH 5.0. Any further increase in the pH beyond pH 7.0 reduced the xylanase production levels appreciably. The moisture content of the fermentation also contributed to the levels of xylanase produced (Table 6.3, Fig. 6.1). It appeared that the moisture content of 83 % was appropriate for the optimal production of xylanases from *T. lanuginosus* SSBP (1060 U/g).

Table 6.3 Effect of pH and moisture content of bagasse soda pulp on xylanase production (U/g) by *T. lanuginosus* SSBP after 4 days at 45°C under SSF conditions

pH	Moisture content (%)		
	80	83	86
5.0	360	440	406
5.4	398	463	480
6.0	495	568	510
6.4	739	744	646
7.0	843	985	845
7.5	801	862	798
8.1	740	720	680
8.4	620	626	600
9.0	515	560	528

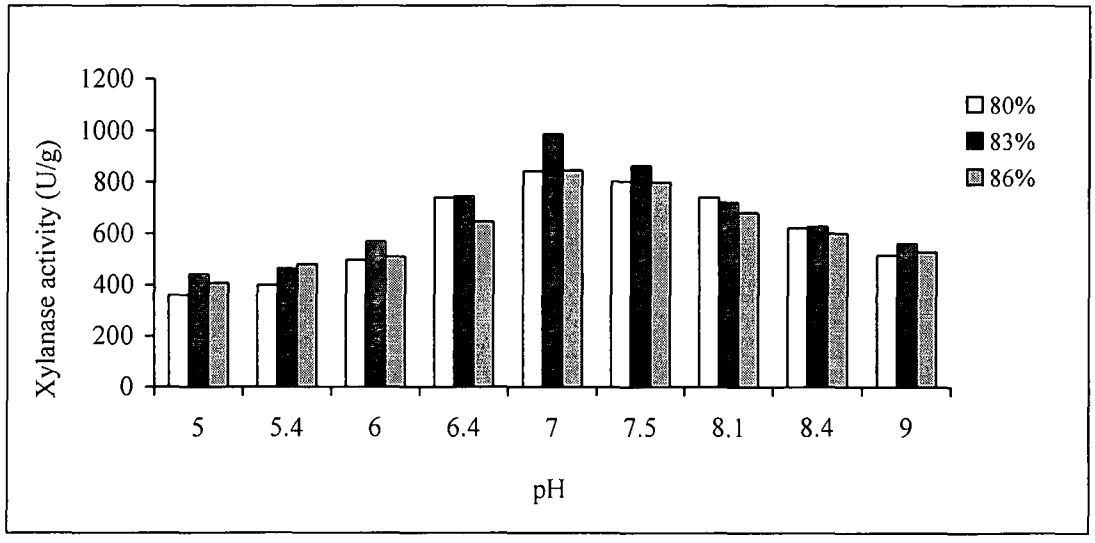


Fig. 6.1 Effect of pH and moisture content of bagasse soda pulp on xylanase production by *T. lanuginosus* SSBP after 4 days at 45°C under SSF conditions

6.4.2 Time course of xylanase production by *T. lanuginosus* SSBP under SSF

The time course experiment revealed a steady increase in xylanase production until day four, thereafter the rate of production remained constant indicating that the four day incubation period was sufficient for optimal production of xylanases by *T. lanuginosus* SSBP (Fig. 6.2). The xylanase concentration on day four was 1060 U/g. It can be concluded that the optimized conditions for xylanase production by *T. lanuginosus* SSBP was pH 7.0, moisture content of 83% and incubation period of four days.

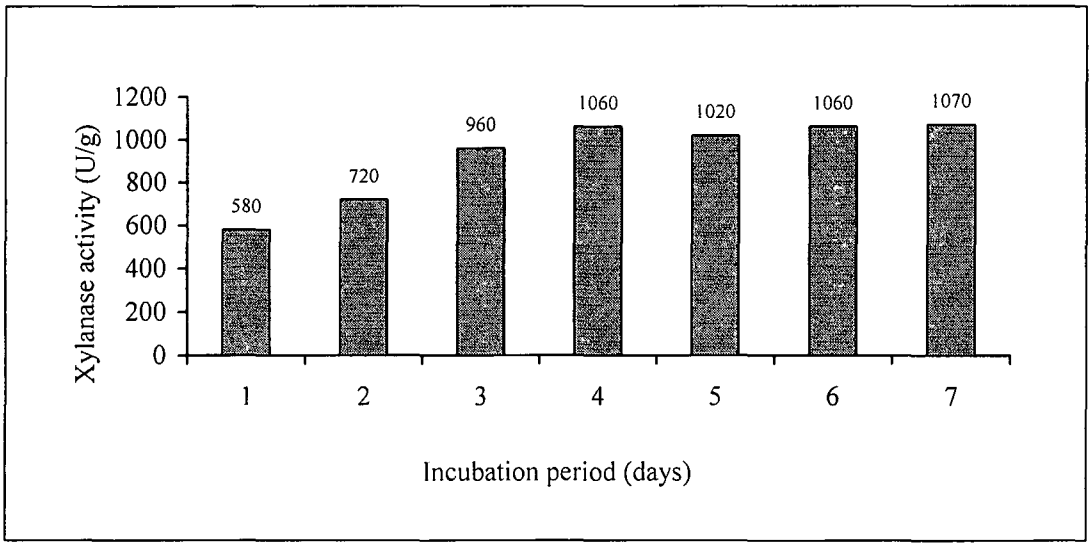


Fig. 6.2 Time course of xylanase production by *T. lanuginosus* SSBP on bagasse soda pulp at 45°C, pH 7.0 and moisture content of 83% under SSF conditions

6.4.3 Production of xylanases by thermophilic fungal strains under SSF

All the fungal strains investigated produced significantly higher xylanase levels than *T. lanuginosus* SSBP under similar SSF conditions. The strain that appeared to be the most effective was F 980 producing 6037 U xylanase/g bagasse pulp (Fig. 6.3). Strain ATCC 46882 also produced significantly high xylanase levels of 5098 U/g. The remaining strains produced xylanase levels in the range of 3000-4000 U/g, with strain F 80 being the least efficient xylanase producer with a concentration of 2958 U/g.

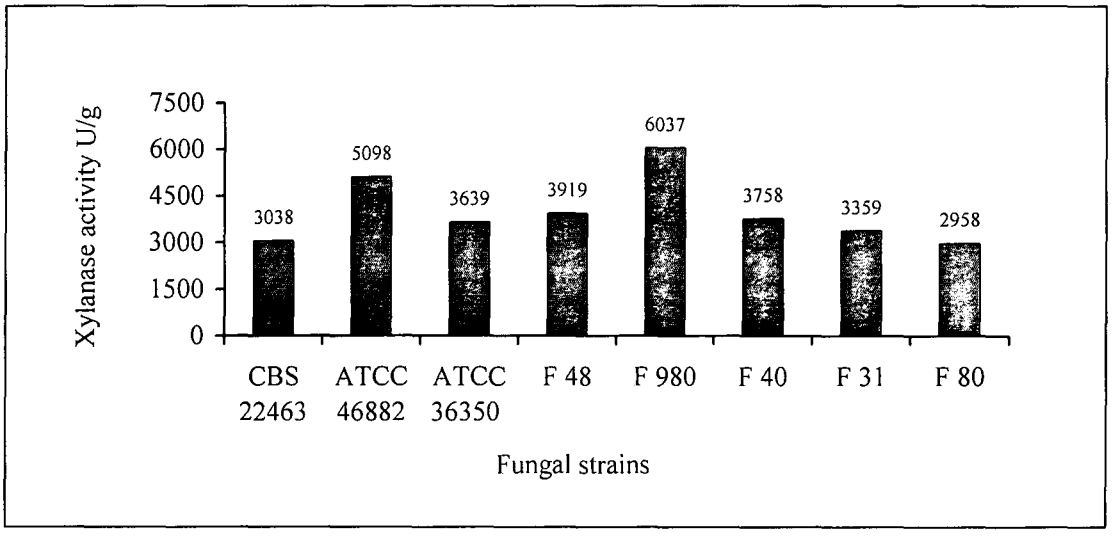


Fig. 6.3 Xylanase production levels by thermophilic fungal strains on bagasse soda pulp at 45°C, pH 7.0 and moisture content of 83% under SSF conditions

6.4.4 Evaluation of SSF xylanases on bagasse pulp in X-CEH bleaching

The SSF pulp samples were bleached in X-CEH bleach sequence at various SSF/raw pulp ratios. The final brightness of the control sample in the CEH bleaching was 82.7 %. Strains ATCC 46882, ATCC 36350 and ATCC 22463 improved brightness by 1.8, 2.0 and 1.8 points to 84.5, 84.7 and 84.5 %, respectively (Table 6.4, 6.5, 6.6, and Fig. 6.4). At higher SSF/raw pulp ratios (1:400), the xylanases still produced higher brightness values than the control, with strain ATCC 46882, ATCC 36350 and ATCC 22463 improving the brightness by 1.2, 1.1 and 0.9 point respectively.

Table 6.4 Influence of SSF xylanase produced by ATCC 46882 on brightness of bagasse pulp bleached in sequence X-CEH

SSF/raw pulp (w/w)	Brightness (%)	Brightness gain over control (points)
Control	82.7	0.0
1:50	84.5	1.8
1:100	84.4	1.7
1:200	84.0	1.3
1:400	83.9	1.2

Table 6.5 Influence of SSF xylanase produced by strain ATCC 36350 on brightness of bagasse pulp bleached in sequence X-CEH

SSF/raw pulp (w/w)	Brightness (%)	Brightness gain over control (points)
Control	82.7	0.0
1:50	84.7	2.0
1:100	84.4	1.7
1:200	84.0	1.3
1:400	83.8	1.1

Table 6.6 Influence of SSF xylanase produced by strain ATCC 22463 on brightness of bagasse pulp bleached in sequence X-CEH

SSF/raw pulp (w/w)	Brightness (%)	Brightness gain over control (points)
Control	82.7	0.0
1:50	84.5	1.8
1:100	84.2	1.5
1:200	83.9	1.2
1:400	83.6	0.9

Strains F 980, F 48 and F 80 improved brightness by 1.6, 1.7 and 1.4 points, respectively at the SSF/raw pulp ratio of 1:50 (Fig. 6.4). The final brightness after X-CEH bleaching was 84.3, 84.4 and 84.1 % with strain F 980, F 48 and F 80, respectively (Table 6.7, 6.8, and 6.9). At a SSF/raw pulp ratio of 1:100 brightness increases of 1.4, 1.6 and 1.3 points were achieved with strain F 980, F 48 and F 80, respectively. Even at ratios of 1:200 brightness increases were 0.9 points and higher with all fungal strains.

Table 6.7 Influence of SSF xylanase produced by strain F 980 on brightness of bagasse pulp bleached in sequence X-CEH

SSF/raw pulp (w/w)	Brightness (%)	Brightness gain over control (points)
Control	82.7	0.0
1:50	84.3	1.6
1:100	84.1	1.4
1:200	83.9	1.2
1:400	83.8	1.1

Table 6.8 Influence of SSF xylanase produced by strain F 48 on brightness of bagasse pulp bleached in sequence X-CEH

SSF/raw pulp (w/w)	Brightness (%)	Brightness gain over control (points)
Control	82.7	0.0
1:50	84.4	1.7
1:100	84.3	1.6
1:200	84.1	1.4
1:400	83.8	1.1

Table 6.9 Influence of SSF xylanase produced by strain F 80 on brightness of bagasse pulp biobleached in sequence X-CEH

SSF/raw pulp (w/w)	Brightness (%)	Brightness gain over control (points)
Control	82.7	0.0
1:50	84.1	1.4
1:100	84.0	1.3
1:200	83.6	0.9
1:400	83.2	0.5

T. lanuginosus SSBP xylanase improved the brightness by 1.8 points to a final brightness of 84.5 % at the SSF/raw pulp ratio of 1:50 (Table 6.12; Fig. 6.5). Strain F 31, at the same ratio improved the brightness by 1.7 points while strain F 40 produced a moderate 1.4 point increase (Fig. 6.5). The superiority of *T. lanuginosus* SSBP xylanase was noted at all SSF/raw pulp ratios investigated. This strain improved the brightness of bagasse soda pulp by 1.5, 1.2 and 1.0 point at ratios of 1:100, 1:200 and 1:400, respectively (Fig. 6.5 to 6.7).

Table 6.10 Influence of SSF xylanase produced by strain F 31 on brightness of bagasse pulp bleached in sequence X-CEH

SSF/raw pulp (w/w)	Brightness (%)	Brightness gain over control (points)
Control	82.7	0.0
1:50	84.4	1.7
1:100	84.1	1.4
1:200	83.8	1.1
1:400	83.6	0.9

Table 6.11 Influence of SSF xylanase produced by strain F 40 on brightness of bagasse pulp bleached in sequence X-CEH

SSF/raw pulp (w/w)	Brightness (%)	Brightness gain over control (points)
Control	82.7	0.0
1:50	84.1	1.4
1:100	83.9	1.2
1:200	83.7	1.0
1:400	83.3	0.6

Table 6.12 Influence of SSF xylanase produced by strain *T. lanuginosus* SSBP on brightness of bagasse pulp bleached in sequence X-CEH

SSF/raw pulp (w/w)	Brightness (%)	Brightness gain over control (points)
Control	82.7	0.0
1:50	84.5	1.8
1:100	84.2	1.5
1:200	83.9	1.2
1:400	83.7	1.0

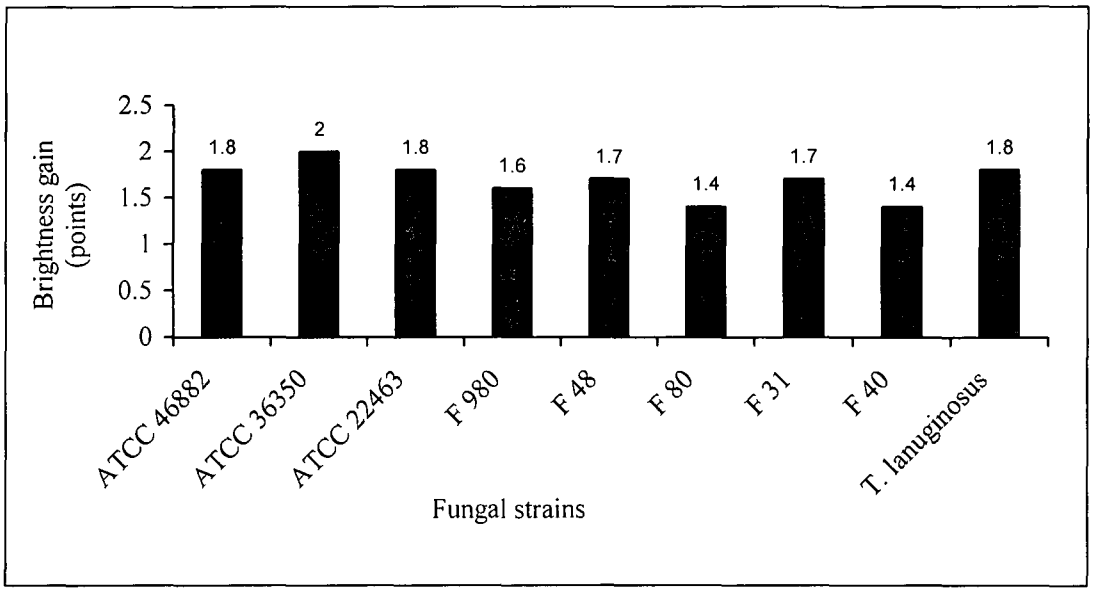


Fig. 6.4 Influence of SSF xylanases produced by thermophilic fungal strains on brightness of bagasse pulp bleached at SSF/raw pulp ratio of 1:50 in sequence X-CEH

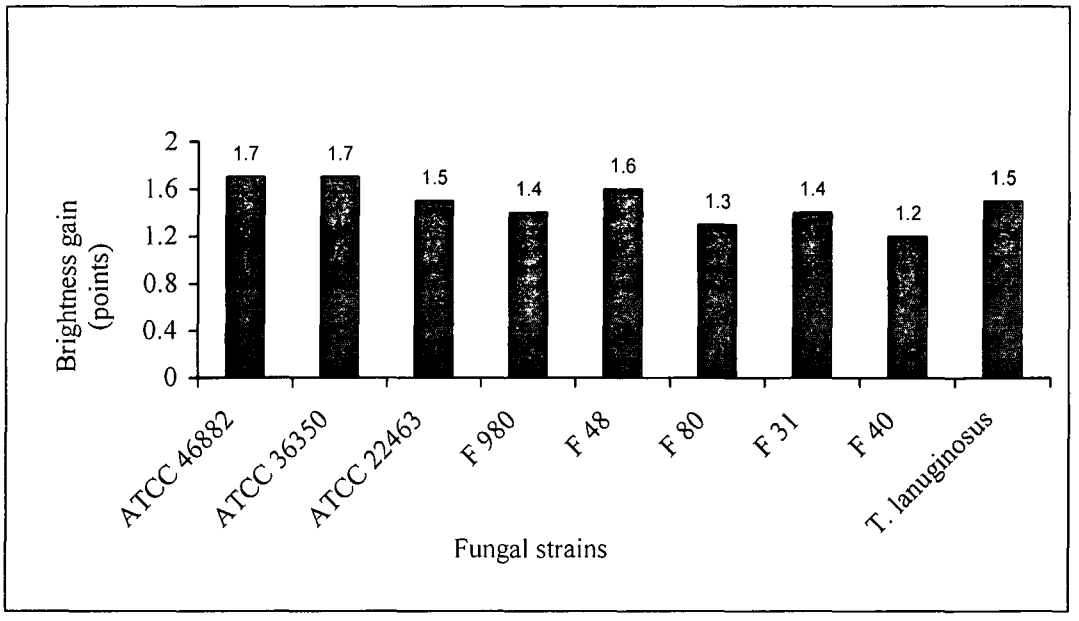


Fig. 6.5 Influence of SSF xylanases produced by thermophilic fungal strains on brightness of bagasse pulp bleached at SSF/raw pulp ratio of 1:100 in sequence X-CEH

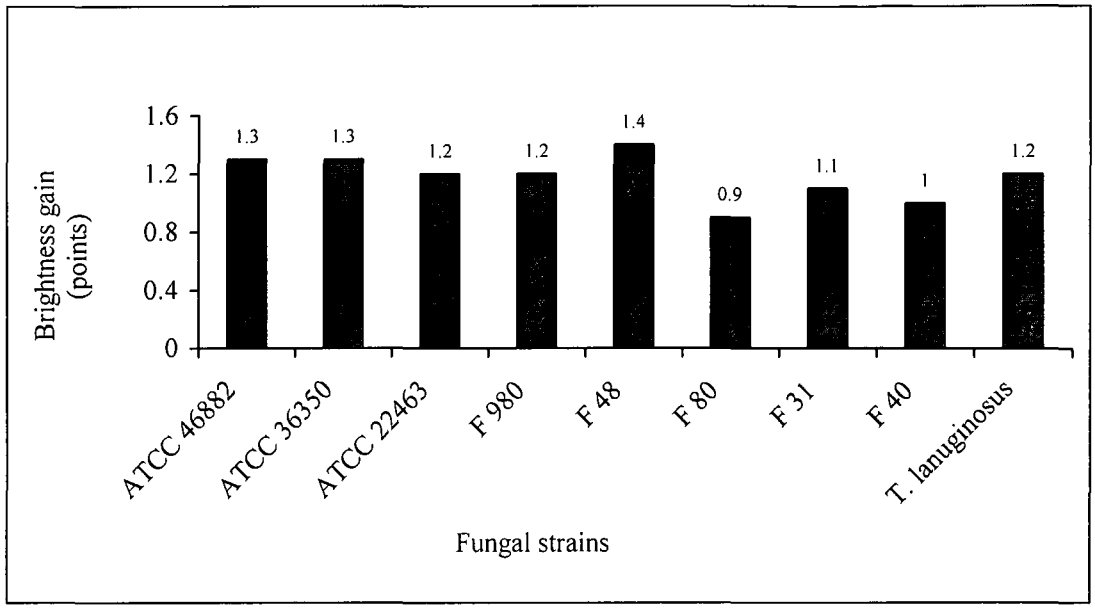


Fig. 6.6 Influence of SSF xylanases produced by thermophilic fungal strains on brightness of bagasse pulp bleached at SSF/raw pulp ratio of 1:200 in sequence X-CEH

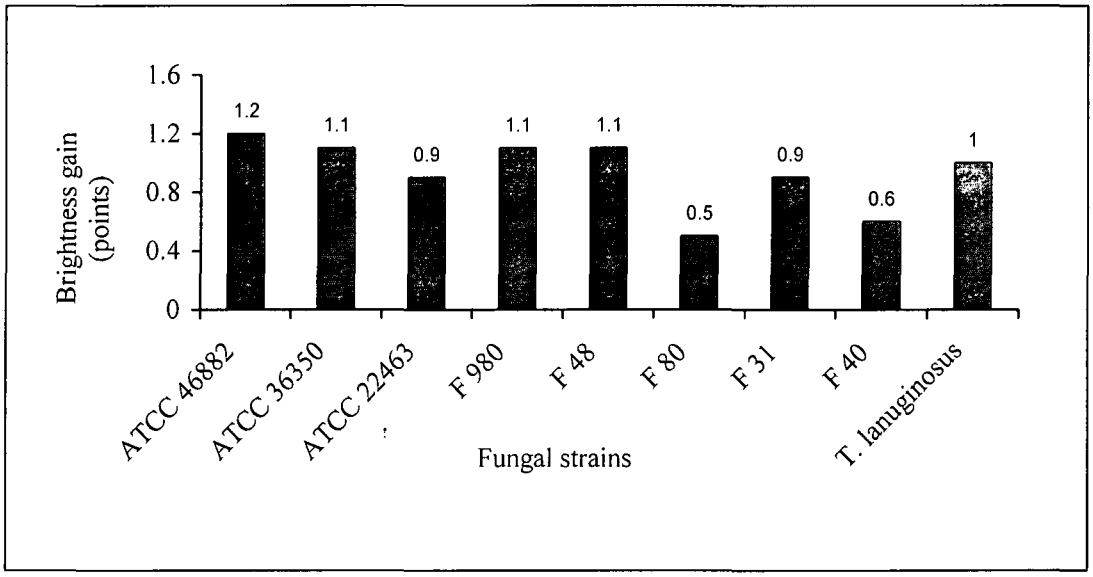


Fig. 6.7 Impact of SSF xylanases produced by thermophilic fungal strains on brightness of bagasse pulp bleached at SSF/Raw pulp ratio of 1:400 in sequence X-CEH

6.4.5 Influence of SSF samples bleached in CEH sequence at reduced Cl_2 charges

Strains ATCC 36350 and ATCC 46882 were evaluated at reduced Cl_2 charges with SSF/raw pulp ratios of 1:50 and 1:400 in CEH bleaching. Both strains were efficient in reducing the Cl_2 consumption by 20% (1:400) maintaining a brightness gain of 0.5 and 0.4 points over the control (Fig. 6.8). Further reductions in Cl_2 produced brightness levels lower than the control reference. However, at the SSF/raw pulp ratio of 1:50, the samples improved the brightness significantly with a concomitant reduction of Cl_2 by 30% (Fig. 6.9).

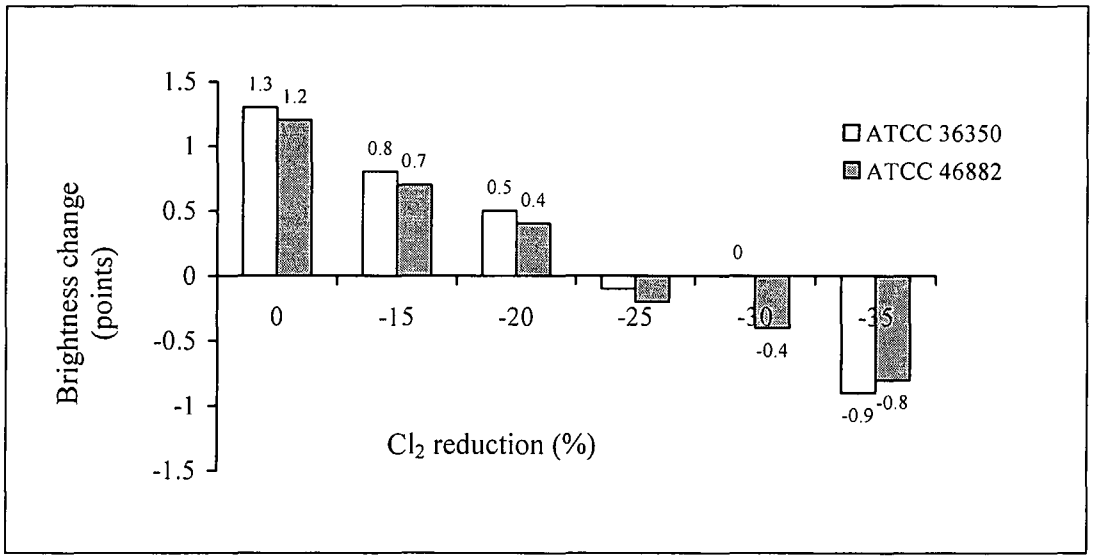


Fig. 6.8 Influence of SSF samples produced by strain ATCC 36350 and ATCC 46882 at SSF/raw pulp ratio of 1:400 on brightness of bagasse pulp bleached in sequence X-CEH at reduced Cl_2 charges

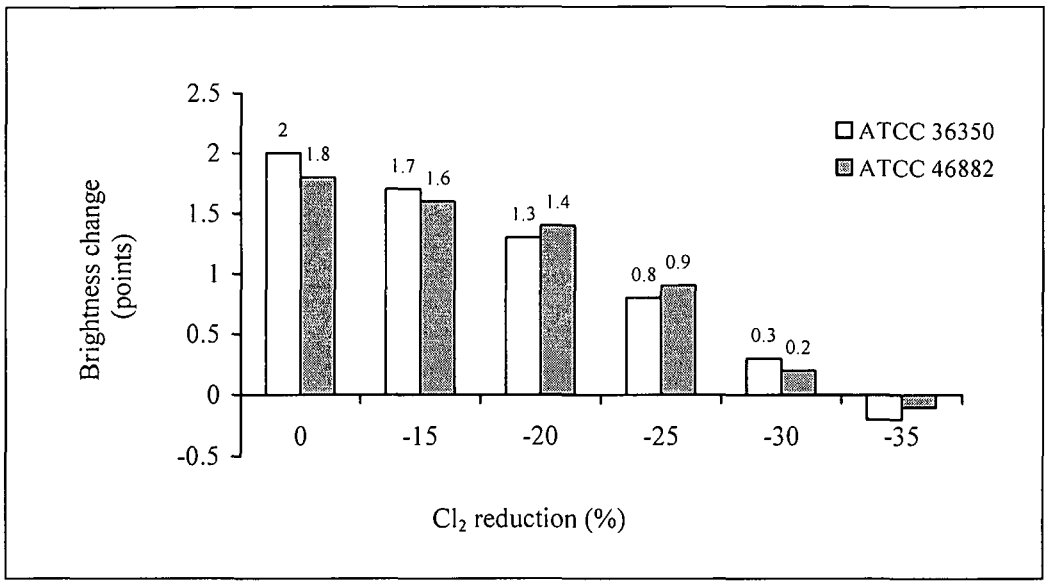


Fig. 6.9 Influence of SSF samples produced by strain ATCC 36350 and 46882 at SSF/raw pulp ratios of 1:50 on brightness of bagasse pulp bleached in sequence X-CEH at reduced Cl_2 charges

6.5 DISCUSSION

With recent advances in SSF technology and the availability of substrate and process selected strains, SSF is becoming a viable alternative for enzyme production. SSF has been used since antiquity, however during the past decade, there has been a significant improvement in the understanding of SSF processes, with the description and modelling of biological and physical phenomena, which led to the economic and practical advantages of SSF being recognized (Durand et al., 1997).

T. lanuginosus strain SSBP produced copious amounts of xylanase under submerged cultivation, however production of xylanases under SSF conditions have not been investigated with this strain. Temperature, moisture, humidity, aeration, and pH are the most important factors, which govern monitoring and controlling of SSF (Pandey, 1992). In this study two parameters, pH variation and moisture content, were evaluated during the fermentation process. This strain produced the highest concentration of xylanases at pH 7.0 and moisture content of 83 % on bagasse soda pulp. At acidic pH values, the xylanase production levels were low, however, at neutral values (pH 7.0) the production levels increased two fold compared to production levels at pH 5.0. The optimum pH for submerged fermentation (SF) with this organism was reported as 6.5 (Singh *et al.*, 2000a), indicating that SSF and SF processes differ with respect to optimal xylanase production levels.

The quantity or properties of the products synthesized by SSF differ from those produced by SF processes. An example of this is a fungal glucosidase produced by SSF exhibited greater degree thermotolerance than the glucosidase produced by SF

(Deschamps and Huet, 1984). Furthermore, some fungi are unable to sporulate viz.,. *Penicillium* sp or produce only low amounts of spores in liquid media viz., *C. minitans* (McQuilken *et al.*, 1997). Therefore spore production by SSF seems the appropriate method in these cases.

The moisture content in SSF is a crucial factor that determines the success of the process. The importance of moisture level in SSF media and its influence on microbial growth and product biosynthesis may be attributed to the impact of moisture on the physical properties of the solid substrate (Raimbault and Alazard, 1980). A higher than optimum moisture level causes decreased porosity, alteration in particle structure, lower oxygen transfer, and enhancement of aerial mycelia (Feniksova, 1960). Likewise, a lower moisture level than optimum leads to reduced solubility of the nutrients of the solid substrate, lower degree of swelling, and a higher water retention (Raimbault and Alazard, 1980).

In SSF using wheat bran and eucaylptus kraft pulp as the primary solid substrates, Streptomyces sp. QG-11-3 (Beg *et al.*, 2000) produced maximum xylanase yield at substrate to moisture ratio of 1:2.5 and 1:3, respectively. However, on increasing or decreasing the moisture level, the xylanase yield marginally decreased. In contrast, a lower solid substrate to moisture level of 1:1 has been reported for maximum xylanase production by *Bacillus* sp. A-009 (Gessesse and Mamo, 1999). An improvement in xylanase production by fungal mixed culture (*Trichoderma reesei* LM-UC4 E 1, *Aspergillus niger* ATCC 10864, and *A. phoenicis* QM 329) using SSF has also been reported (Gutierrez-Correa and Tengerdy, 1998). A higher xylanase yield using SSF

compared with submerged fermentation with wheat straw and sugarcane bagasse has been reported from thermophilic *Melanocarpus albomyces* IIS-68 (Jain, 1995).

Time course experiments indicated a four-day incubation period for optimal production of xylanase by *T. lanuginosus* (1060 U/g). The time taken for maximal production of xylanase varies from 24 h to 30 days depending upon the culture conditions and the organism applied (Ghosh and Nanda, 1993; Rana *et al.*, 1996). *Thermoascus aurantiacus* produced maximum xylanase activity (365.8 U/ml) in liquid medium in 10 days at 45°C on solka floc, while on solid substrate (aspen wood xylan) it required 4 weeks to achieve this level (Yu *et al.*, 1987). However, optimum xylanase production by a thermophilic *Bacillus licheniformis* A99 under SSF required a 72 h incubation period.

The 8 thermophilic strains investigated produced significantly higher xylanase levels than the *T. lanuginosus* strain under similar conditions. The strain that appeared to be the most efficient producer was F 980 (6037 U/g). Reports of other thermophilc fungal production of xylanases include *Humicola lanuginosa*, 2050 U/ml, *Aspergillus fumigatus*, 10 U/ ml, (Kitpreechavanich, 1984), *Sporotrichun thermophile* 15 U/ml (Yoshioka *et al.*, 1981), *Thermoascus aurantiacus* 9.67 U/ml, (Grajek, 1987) and *T. aurantiacus* 6193 U/g (Kalogeris *et al.*, 1998).

The evaluation of SSF samples in bleaching experiments is a relatively new approach to the application of biotechnology in the bleaching process and to our knowledge this is the first report on the application of SSF samples to enhance the bleachability of bagasse soda pulp. *T. lanuginosus* SSF sample displayed an improvement in

brightness of bagasse soda pulp by 1.9 points to a final brightness of 84.52 % at a SSF/raw pulp ratio of 1:50. This organism was effective at all the SSF/raw pulp ratios investigated. Although strain F 980 produced the highest level of xylanase during SSF, xylanase of this strain was not the most efficient brightness enhancer of bagasse soda pulp. This confirms our findings that the level of xylanases produced does not necessarily have a direct correlation with the bleach boosting capabilities of the SSF sample. Strain ATCC 36350 displayed the greatest bleach enhancing abilities, reducing the Cl_2 consumption by 30% with a final brightness higher than the control. This data illustrates the importance of the potential application of SSF samples to the bleaching of bagasse pulp in CEH bleaching, and could pave the way to new and improved bleaching methods.

Bleaching with SSF samples provides an alternative to enzyme-aided bleaching with significant brightening effects on chemical CEH bleaching of bagasse soda pulp. SSF offers distinct advantages over SF including economy of the space needed for fermentation, simplicity of the fermentation media, no requirement for complex machinery, equipment and control systems; greater compactness of the fermentation vessel owing to a lower water volume; greater product yields; reduced energy demand; lesser volume of solvent needed for product recovery; superior yields, absence of foam build up and easier control of contamination due to low moisture level in the system (Arima, 1964; Satyanarayana, 1994; Babu and Satyanarayana, 1995; Narahara *et al.*, 1982; Lonsane *et al.*, 1985).

SSF also has some limitations such as a limited choice of organisms capable of growth at a reduced moisture level, exacting demand for monitoring and control of

parameters such as temperature, pH, humidity, air flow, free oxygen transfer, and removal of CO_2 generated during fermetation, and the need for pretreatment of certain solid substrates (Raimbault and Alazard, 1980; Narahara *et al.*, 1980; Lonsane *et al.*, 1985). SSF production of xylanases is an innovative technology where the fermented substrate is the enzyme source, can be used directly in bleaching processes without a downstream processing. This study has shown the potential of production of xylanases by SSF, and more importantly the efficient bleach enhancing abilities of these SSF samples on bagasse-soda pulp.

6.6 CONCLUSIONS

i) The optimal production of xylanase (1060 U/g) under SSF by *T. lanuginosus* was at pH 7.0, moisture content of 83% after a period of 4 days.

ii) The levels of xylanase produced do not necessarily have a direct correlation with the bleach boosting capabilities of the SSF sample.

iii) Strains ATCC 36350 and ATCC 46882 were efficient in reducing the Cl_2 consumption by 20% and 30% when bleached with SSF/raw pulp ratios of 1:400 and 1:50, respectively.

iv) SSF samples can provide enormous potential application in CEH bleaching of bagasse pulp, improving the final brightness with subsequent reductions in Cl_2 consumption.

The effects of purified xylanases on isolated and fibre-bound hemicellulose of bagasse soda pulp is presented in chapter seven.

CHAPTER SEVEN: EFFECTS OF PURIFIED XYLANASES ON ISOLATED AND FIBRE-BOUND HEMICELLULOSE OF BAGASSE SODA PULP

7.1 ABSTRACT

Three purified xylanases from *Trichoderma viride, Aureobasidium pullulans* and *T. lanuginosus* were evaluated on isolated and fibre-bound hemicellulose of bagasse soda pulp. *T. viride* and *A. pullulans* xylanase displayed pH and temperature optima of 4.5, and 50°C respectively, while *T. lanuginosus* xylanases displayed pH and temperature optima of 6.0 and 65°C, respectively. The xylanases were evaluated individually as well as in combination on extracted hemicelluloses from bagasse pulp, commercial birchwood xylan and untreated bagasse pulp and alkaline-extracted bagasse pulp. There was a gradual increase in the amount of reducing sugars released from the extracted hemicelluloses and commercial birchwood xylan with prolonged incubation period. *T. lanuginosus* xylanase appeared to be the least efficient of the xylanases under study, with reducing sugars at a concentration of 0.19; 0.25; and 0.31 and 0.37 mg/g detected after 1, 2, 3, and 24 h, respectively. Application of *T. viride*, *A. pullulans* and *T. lanuginosus* xylanases in combination produced the highest concentration of reducing sugars in comparison to individual xylanase treatments. Xylanase pretreatment of bagasse pulp at a charge of 5 U/g pulp released greater concentrations of reducing sugars compared to charges of 5 U/g xylan in pulp. At enzymes charges of 5 U/g pulp, reductions of 10 % Cl_2 was attained with *T. lanuginosus* xylanase and 20% with *A. pullulans* and *T. viride* xylanase in X-CEH bleaching. The xylanases when treated in combination produced a

brightness gain of 2.2 points to 87.5 % at full Cl_2 charge and reduced the Cl_2 consumption by 30 % while still maintaining a final brightness 0.9 points higher than the control. The synergistic effect of the xylanases was evident during the biobleaching of bagasse pulp. Xylanase pretreatment of the alkaline-extracted bagasse pulp revealed minimal savings of Cl_2 with a saving of 10 % achieved with *A. pullulans* xylanase. *A. pullulans* xylanase was the most efficient enzyme on xylan substrates as well as on bagasse pulp.

7.2 INTRODUCTION

The composition and structure of xylan are more complicated than that of cellulose and can vary in various wood species. In hardwoods, the major hemicellulose component is the *O*-acetyl-4-*O*-methylglucuronoxylan, whereas in the softwood species the *O*-acetyl-galactoglucomannan is the prominent one (Fengel and Wegener, 1984). The enzymatic degradation of xylan would require the use of endo-xylanases, enzymes, which can randomly split and solubilize xylan polymer. As a result, the penetration of bleaching chemicals into pulp fibres could be improved and therefore lignin removal facilitated.

The mode of action of several endoxylanases has also been studied using both kinetic and end product analysis techniques (Dekker and Richards, 1975; Comtat and Joseleau, 1991). However, few published studies compare the performance of various xylanases on a given substrate. There is also little information on the effect that xylan structure has on the activities of different xylanases. White *et al.* (1998) compared four xylanases in pulp-bleaching applications, and determined that their performances were quite different. It has already been reported that xylanases belong to the glycosyl hydrolase families 10 and 11 (Gilkes *et al.*, 1991). They differ in their efficiency to degrade isolated acetylglucuronoxylans (Biely *et al.*, 1997) or to increase bleachability of kraft pulps (Clarke *et al.*, 1997).

Bagasse pulp contains approximately 30 to 35% hemicelluloses (Blake *et al.* 1971), while hardwood and softwood pulps contain approximately 15 to 30% and 7 to 12%

hemicelluloses, respectively (Beg *et al.*, 2001). Mild treatment of bagasse pulp or hemicelluloses extracted from bagasse pulp with dilute acids produces a mixture of monosaccharides, mainly pentoses, with D-xylose being the main component (Lee and Lin, 1978; Gong *et al.*, 1980). Selective hydrolysis of plant materials is possible because the hemicellulose fraction is hydrolysed preferencially (Lee and Lin, 1978). Hardwood kraft pulp contains more xylan, and smaller xylan macromolecules than softwood kraft pulps (Wong *et al.*, 1996). There is a tendency for hardwood kraft pulp to show higher benefits with regard to chlorine savings during xylanase aided bleaching (Karlsson *et al.*, 1996). The bleach enhancing affects of xylanases on pulp types has been attributed to the hydrolysis of hemicelluloses from pulp. Restrictions in the pentosan removal from pulps have been assigned to retarded accessibility and chemical modification of residual hemicelluose (Paice and Juracek, 1984). The extent of xylan hydrolysis in pulps is dependant on the substrate specificity of the particular enzyme used (Gübitz *et al.*, 1997; Christov *et al.*, 1999) as well as chemical composition and physical state of the xylan substrate (Christov *et al.*, 1997).

It has been demonstrated that xylanases can produce bleach-boosting effect on bagasse soda pulp (Bissoon *et al.*, 2001; Madlala *et al.*, 2000). However, little is known with regards to the correlation between xylan removal and brightness gains from bagasse soda pulp. This study investigates the effects of three purified family 11 xylanases from *Trichoderman viride, Aureobasidium pullulans and Thermomyces lanuginosus* SSBP on extracted and fibre bound xylan from bagasse pulp as well as comercially available birchwood xylan in relation to their biobleaching effects.

7.3 MATERIALS AND METHODS

7.3.1 Purified xylanases

The purified xylanases used in this study are *Trichoderma viride, Aureobasidium pullulans* and *Thermomyces lanuginosus* SSBP, belonging to the Family 11 xylanases.

7.3.2 Xylanase and protein assays

Xylanase was routinely assayed by measuring the RS released from birchwood xylan as described in section 3.3.3. The pH and temperature optima of these xylanases have been determined as shown in Table 7.2. Protein was quantified according to the method of Bradford (1976) using a commercially available assay kit (Bio-Rad, Munich, Germany) calibrated with bovine serum albumin.

7.3.3 Extraction of hemicelluloses from bagasse soda pulp

For chemical removal of hemicelluloses, pulp (100 g) was extracted with 4 000 ml of 20 % degassed KOH under stirring over night (Karlsson *et al.*, 2001). After extraction, the sample was washed with approximately 2 000 ml 20 % alkali and then with plenty of water to neutral pH and dried. The combined alkaline filtrate and washings were then acidified with acetic acid to pH 4.5. Hemicelluoses were precipitated with ethanol (three times the volume of the extract) and the resulting precipitate was then centrifuged off and washed twice with 1 000 ml ethanol. Water was then added and the precipitate was freeze dried.

7.3.4 HPLC analyses of pulp

The monosaccharide content of pulp was determined by high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). Pulp samples were hydrolyzed in two steps with sulphuric acid (Wright and Wallis, 1996), neutralized to pH 7.0 and filtered through a 0.45 μm membrane filter. Monosaccharides were separated on a Dionex Carbopac PA-10 column, preceeded by a Carbopac PA-10 guard column, at a flow rate of 1.0 ml/min and 25°C for 40 min.

7.3.5 Enzymatic treatment of isolated hemicelluloses from bagasse pulp and commercial xylan

Commercial birchwood xylan (Roth, Karlsruhe) and the extracted hemicellulose from bagasse pulp were treated with the three purified xylanases individually and in combination. A 1% substrate solution was used and enzyme charge of 5 U/g xylan applied. The treatment was performed for a period of 24 h, with samples removed and analysed for RS at 0, 1, 2, 3, and 24 h of incubation.

7.3.6 Enzymatic treatment of untreated bagasse soda pulp and alkali-extracted pulp

Duplicate samples of extensively washed pulp (5 g dry weight) of 10% consistency were treated with purified xylanases (5 U/g pulp and 5 U/g xylan in pulp) at the respective optimum conditions. After incubation with enzymes, the samples were heated in boiling water bath for 10 min to denature the enzymes and then filtered through membrane filters (0.45 μm) using small syringes. The filtrates were analysed for reducing sugars.

7.3.7 Reducing sugars

Reducing sugars were determined as described in section 3.3.6

7.3.8 Bleaching conditions

The chemical bleaching conditions on bagasse soda pulp are described in Table 7.1.

Table 7.1 Conditions for CEH bleaching on bagasse soda pulp.

Treatment	Charge (%)	Time (min)	Temperature (°C)	Consistency (%)
C	4.75	45	45	3.2
E	4.5	90	70	12
H	1.5	135	70	12

7.4 RESULTS

7.4.1 Properties of purified xylanases

The xylanases of *T. viride* and *A. pullulans* had a pH and temperature optimum of 4.5, and 50°C respectively, while *T. lanuginosus* xylanases showed maximum activity at pH and temperature of 6.0 and 60°C, respectively (Table 7.2). The enzyme activities varied significantly with *T. lanuginosus* having the lowest activity of 65 U/ml, followed by *A. pullulans* and *T. viride* with activities of 136 and 587 U/ml, respectively. These optimized conditions were implemented during the pretreatment of bagasse soda pulp. No cellulase activity was detected in the enzyme suspensions.

Table 7.2 Characteristics of purified enzymes

Source of xylanase	pH	Temperature (°C)	Activity (nkat/ml)	Activity (U/ml)	Protein (mg/ml)
T. viride	4.5	50	9785	587	0.78
A. pullulans	4.5	50	2245	136	0.62
T. lanuginosus SSBP	6.0	60	1085	65	0.35

7.4.2 Sugar analyses of pulps

Relative sugar composition of untreated bagasse soda pulp and the alkali-extracted pulp was analysed and compared (Table 7.2). The untreated bagasse soda pulp contained 12.5 % xylose of which 4.6 % still remained in the extracted pulp. Mannose and galactose were detected at low quantities in the untreated pulp with no trace amounts of these monosaccharides detected in the extracted pulp. On the other hand arabinose at a concentration of 1.3 % was present in the untreated pulp whereas 0.4 % was detected

after extraction. The glucose concentration in the extracted pulp was higher than in the untreated pulp.

Table 7.3 Relative carbohydrate composition in bagasse pulp

Sample	Glucose (%)	Xylose (%)	Mannose (%)	Galactose (%)	Arabinose (%)
Untreated bagasse pulp	85.5	12.5	0.6	0.1	1.3
Alkaline extracted bagasse pulp	95.0	4.6	0.0	0.0	0.4

The dry weight of the extracted hemicellulose amounted to 25.33 g with 71.98 g of the extracted pulp remaining, indicating a loss of 2.69 g of pulp during the extraction procedure (Table 7.4). The freeze-dried extraction had a moisture content of 10.98 % while the extracted pulp contained 75.05 % moisture. Although a slight loss in yield was detected during the extraction procedure, xylanase treatment was carried out with respect to the percentage xylose present in the extracts and pulp rather than dry weight content of extracted hemicelluloses.

Table 7.4 Mass of extracted pulp and hemicellulose

Sample	Wet weight (g)	Moisture (%)	Dry weight (g)
Extracted hemicellulose	28.45	10.98	25.33
Extracted pulp	288.5	75.05	71.98
Total			97.31

7.4.3 Effect of purified xylanases on RS release from extracted hemicellulose of bagasse pulp and commercial xylan

The three xylanases were evaluated individually and in combination at a charge of 5 U xylanase/g xylan from the extracted bagasse hemicellulose and commercial xylan from birchwood. The release of RS was monitored over a period of 24 h with analysis of samples at 0, 1, 2, 3 and 24 h (Fig. 7.1). There was a gradual increase in the amount of RS released from the extracted hemicellulose with time. *T. lanuginosus* xylanase was the least efficient of the xylanases under study, with RS sugars at a concentration of 0.19; 0.25; and 0.31 and 0.37 mg/g detected after 1, 2, 3, and 24 h, respectively. *A. pullulans* xylanase produced the highest RS concentration in comparison to the individual xylanase applications at all incubation periods. After a period of 3 h the RS released by *A pullulans* and *T. viride* were virtually the same (0.4 mg/g) compared to 25 % reduction detected with *T. lanuginosus* xylanase after the same period. The three enzymes in mixture did produce significantly higher quantities of RS than the individual xylanase treatments. After an incubation period of 3 and 24 h, 0.55 and 0.90 mg/g RS, was detected with the enzyme mixture.

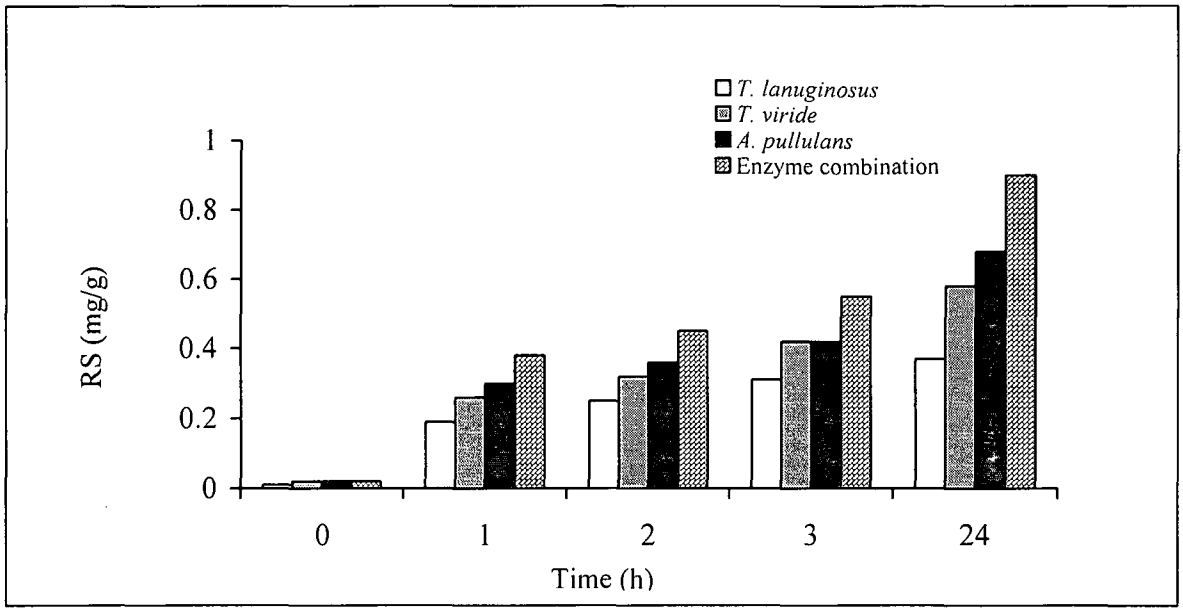


Fig. 7.1 Enzyme-mediated release of RS from extracted hemicellulose of bagasse soda pulp

Xylanase treatment of the commercial birchwood xylan released a higher concentration of RS in comparison to treatment of the extracted hemicelluloses from bagasse pulp. There was a corresponding increase in the release of RS with prolonged incubation. A 25.4 % increase in RS released from birchwood xylan in comparison to the alkaline extracted hemicellulose from bagasse pulp with the application of *A. pullulans* after 24 h was observed. The enzyme combination also produced a high RS concentration during all sampling periods.

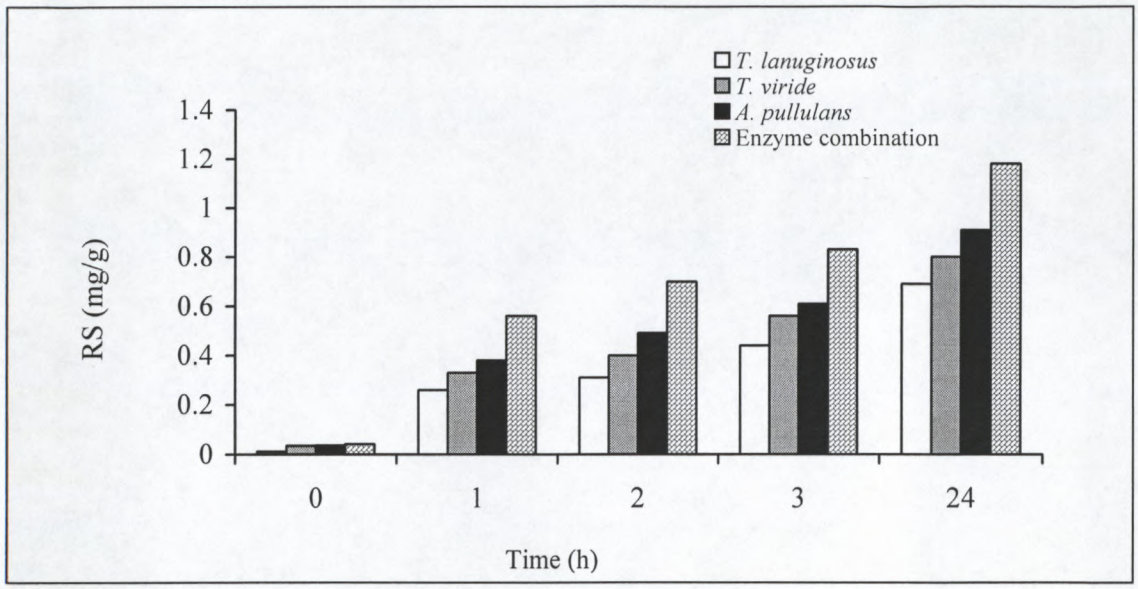


Fig. 7.2 Enzyme-mediated release of RS from commercial birchwood xylan.

7.4.4 Effect of purified xylanases on RS release from alkali-extracted and untreated bagasse pulp

The purified xylanase mediated release of RS from bagasse soda pulp as well as alkali extracted bagasse pulp was monitored after the pretreatment stage. Bagasse soda pulp treated with the individual xylanases produced less RS in comparison to treatment with the combination of the three enzymes (Table 7.5). The RS released at the charge of 5 U/g pulp was greater than enzyme charges of 5 U/g xylan in pulp. This trend was evident with all the xylanases investigated, since a greater mass of pulp was treated to produce a concentration of 5 U/g xylan in pulp. *A. pullulans* xylanase was most effective, followed by *T. viride* and *T. lanuginosus* xylanase in decreasing order of efficiency. The RS released from pulp was lower than that detected on the extracted hemicelluloses and commercial xylan. This could be due to restricted accessibility of the hemicelluloses in the pulp in comparison to the readily available xylan substrate. Treatment of the

extracted pulp did not produce reducing sugars at concentrations of appreciable importance.

Table 7.5 Effect of purified xylanases on RS release from untreated and hemicellulose extracted bagasse pulp

Source of xylanase	RS release (mg/g) from untreated bagasse pulp		RS release from hemicellulose extracted bagasse pulp
	5 U/g pulp	5 U/g xylan in pulp	5 U/g pulp
T. lanuginosus	0.22	0.19	0.05
T. viride	0.27	0.23	0.06
A. pullulans	0.32	0.27	0.06
Enzyme combination	0.35	0.31	0.07

Time 2h, 10 % consistency

7.4.5 Evaluation of purified xylanases on bagasse pulp in X-CEH bleaching

The xylanases were evaluated in X-CEH bleaching at reduced Cl_2 charges to determine the extent of Cl_2 savings brought about by the enzymes. The final brightness achieved with the control samples was 85.3 %. At enzyme charges of 5 U/g pulp, reductions of 10 % Cl_2 was attained with *T. lanuginosus* xylanase and 20% with *A. pullulans* and *T. viride* xylanase (Fig 7.3). Any further reductions in Cl_2 consumption produced brightness levels below the control level. At full Cl_2 charges *A. pullulans* xylanase produced the highest brightness gain of 0.9 points in comparison to 0.5 and 0.3 achieved with *T. viride* and *T. lanuginosus* xylanase, respectively.

Table 7.6 Effect of purified xylanases (5 U/g pulp) on reduction of Cl_2 consumption during X-CEH bleaching of bagasse soda pulp

Cl_2 reduction (%)	Brightness of pulp treated with purified fungal xylanase (%)		
	T. lanuginosus	*T. viride*	*A. pullulans*
0	85.6	85.8	86.2
10	85.5	85.6	86.0
20	85.1	85.4	85.5
30	84.8	85.0	85.1

Control: 85.3%, X charge; 5 U/g pulp; X charge: 5 U/g pulp

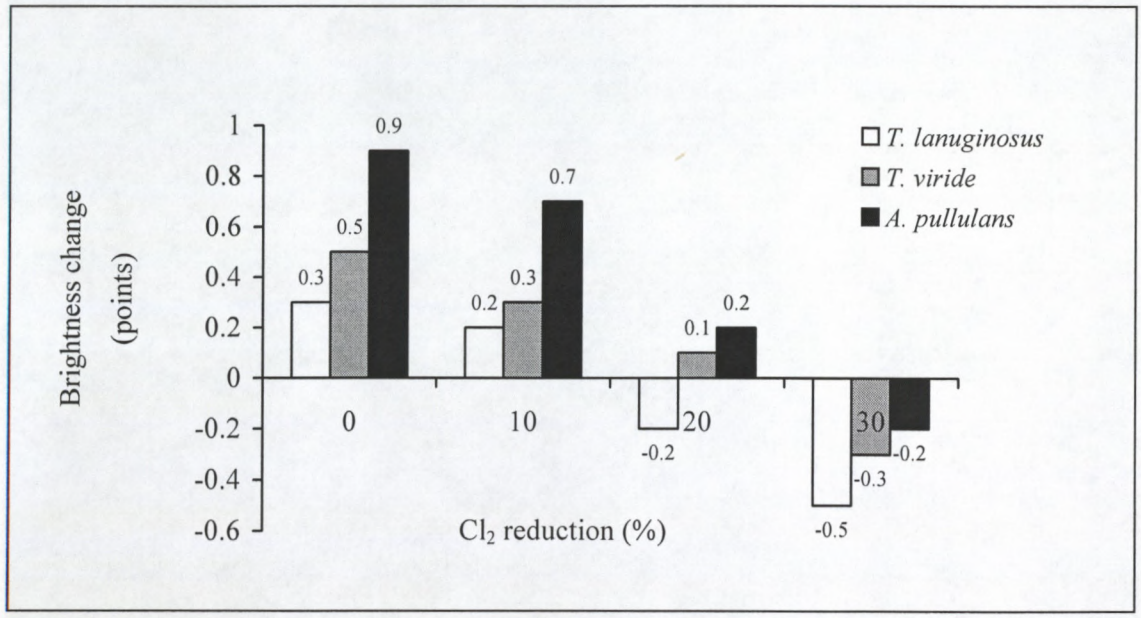


Fig. 7.3 Influence of purified xylanases (5 U/g pulp) on brightness of bagasse soda pulp in sequence X-CEH at reduced Cl_2 charges

The enzymes were not as effective on untreated bagasse pulp at charges of 5 U/g xylan in pulp. Cl_2 savings of 10 % could only be achieved with all three xylanases (Fig. 7.4). The final brightness of the enzyme treated pulp was 85.5, 85.6 and 86.0 % at full Cl_2 charges for *T. lanuginosus, T. viride* and *A. pullulans* xylanases, respectively. (Table 7.7). The brightness gain was 0.2, 0.3, 0.7 with *T. lanuginosus*, *T. viride* and *A. pullulans* xylanases, respectively.

Table 7.7 Effect of purified xylanases (5 U/g xylan in pulp) on reduction of Cl_2 consumption during X-CEH bleaching of bagasse soda pulp

Cl reduction	Brightness of pulp treated with purified fungal xylanase (%)		
(%)	*T. lanuginosus*	*T. viride*	*A. pullulans*
0	85.5	85.6	86.0
10	85.3	85.4	85.7
20	84.8	85.0	85.2
30	84.5	84.7	84.9

Control: 85.3; X charge: 5 U/g xylan in pulp

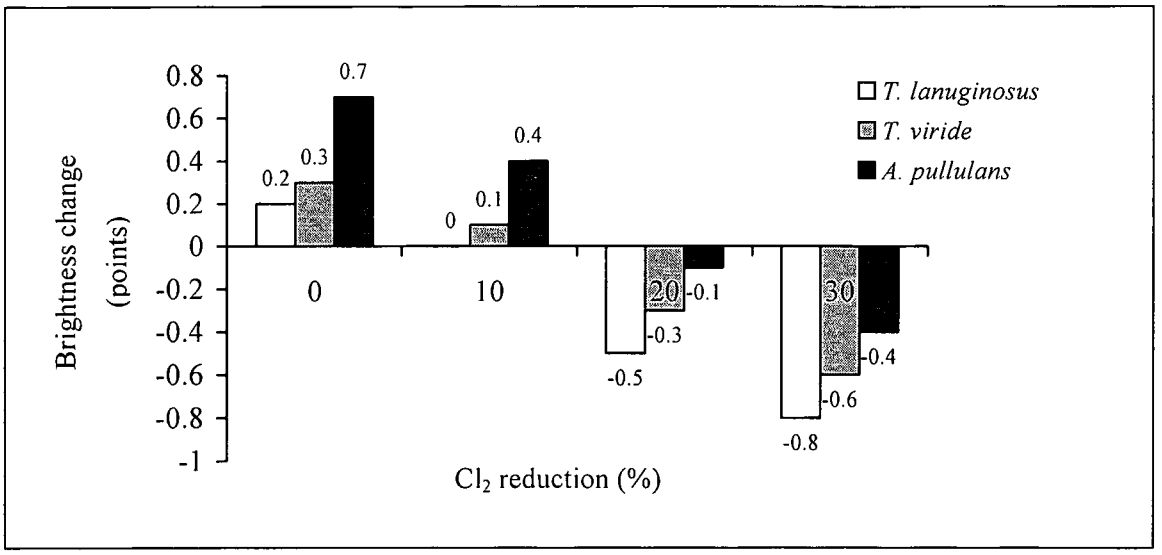


Fig. 7.4 Influence of purified xylanases (5 U/g xylan in pulp) on brightness of bagasse soda pulp in sequence X-CEH at reduced Cl_2 charges

The combined xylanase treatment each at 5 U xylanase/g pulp produced fairly large saving of Cl_2 with concomitant increases in brightness. The enzyme combination produced a brightness gain of 2.2 points to 87. 5 % at full Cl_2 charge. On the other hand the Cl_2 consumption could be reduced by 30 % while still maintaining a final brightness 0.9 points higher than the control (Table 7.8; Fig. 7.5). The enzyme combination displayed a greater efficiency in improving the brightness of bagasse pulp, with subsequent higher reductions of Cl_2 in CEH bleaching.

Table 7.8 Effect of combined treatment with purified xylanases from *T. lanuginosus*, *A.. pullulans* and *T. viride* (5 U/g pulp) on brightness of bagasse soda pulp in X-CEH bleaching

Cl_2 reduction (%)	Brightness (%)
0	87.5
10	87.0
20	86.6
30	86.2

Control: 85.3%; X charge;
5 U each xylanase/g pulp (*T. lanuginosus, A.. pullulans and T. viride*)

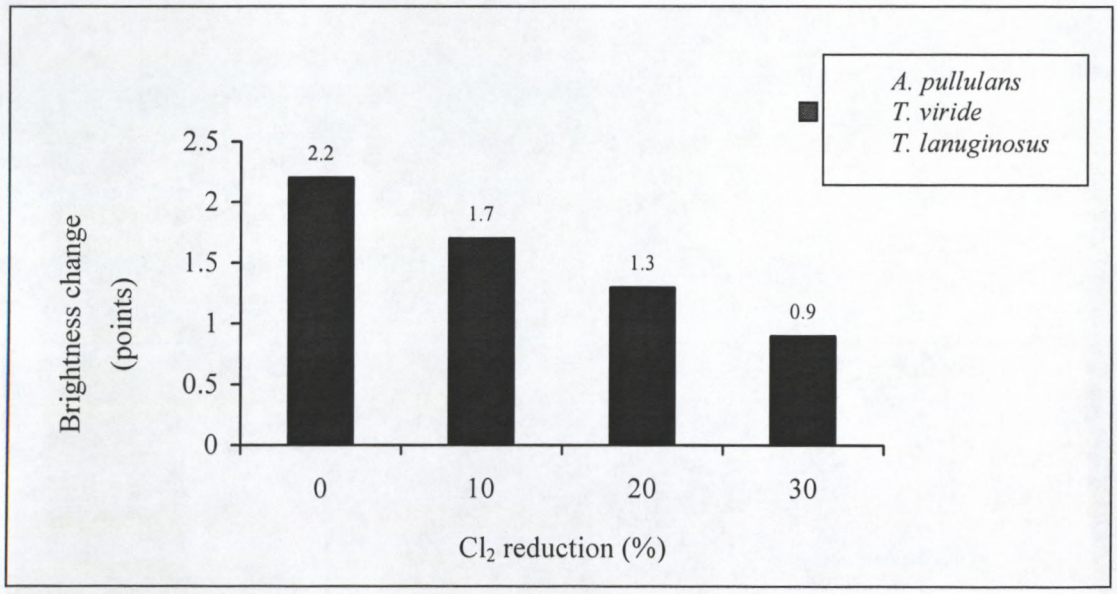


Fig. 7.5 Influence of purified xylanase combination (5 U each xylanase/g pulp) o n brightness of bagasse soda pulp in sequence X-CEH at reduced Cl_2 charges

Evaluation of the bleach enhancing effect of the xylanase combination at a charge of 5 U/g xylan in pulp displayed less bleach boosting capabilities and consequently less Cl_2 savings as compared to the charge of 5 U/g pulp. A 20 % reduction in Cl_2 could be achieved with the enzyme combination (Fig. 7.6). The final brightness of the pulp was 86.2 %, 0.9 brightness points higher than the control at full Cl_2 charge (Table 7.9).

Table 7.9 Effect of combined treatment with purified xylanases from *T. lanuginosus*, *A. pullulans* and *T. viride* (5 U/g xylan in pulp) on brightness of bagasse soda pulp in X-CEH bleaching

Cl_2 reduction (%)	Brightness (%)
0	86.2
10	85.7
20	85.4
30	84.1

Control: 85.3%

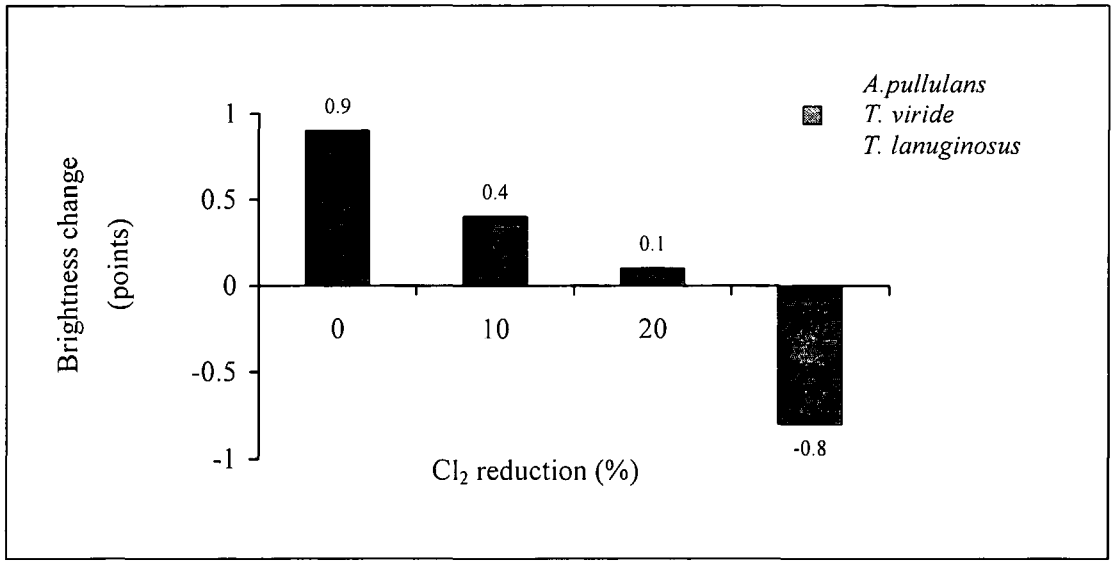


Fig. 7.6 Influence of purified xylanase combination (5 U each xylanase/g xylan in pulp) on brightness of bagasse soda pulp in sequence X-CEH at reduced Cl_2 charges

X-CEH bleaching was also conducted on the alkali-extracted pulp. All the enzymes failed to produce Cl_2 reductions at appreciable levels (Table 7.10). *A. pullulans* xylanase reduced the Cl_2 consumption by 10 % while *T. viride* and *T. lanuginosus* xylanase did not produce Cl_2 savings (Fig. 7.7). The final brightness attained at full Cl_2 charge was 86.2, 86.3, and 86.5 % for *T. lanuginosus*, *T. viride* and *A. pullans* xylanase, respectively.

Table 7.10 Effect of purified xylanases on alkali-extracted bagasse soda pulp in X-CEH bleaching

Cl_2 reduction (%)	Brightness (%)		
	T. lanuginosus	*T. viride*	*A. pullulans*
0	86.2	86.3	86.5
10	86.0	86.1	86.2
20	85.7	85.8	85.9
30	85.4	85.6	85.7

Control: 86.2;
X charge: 5 U/g pulp

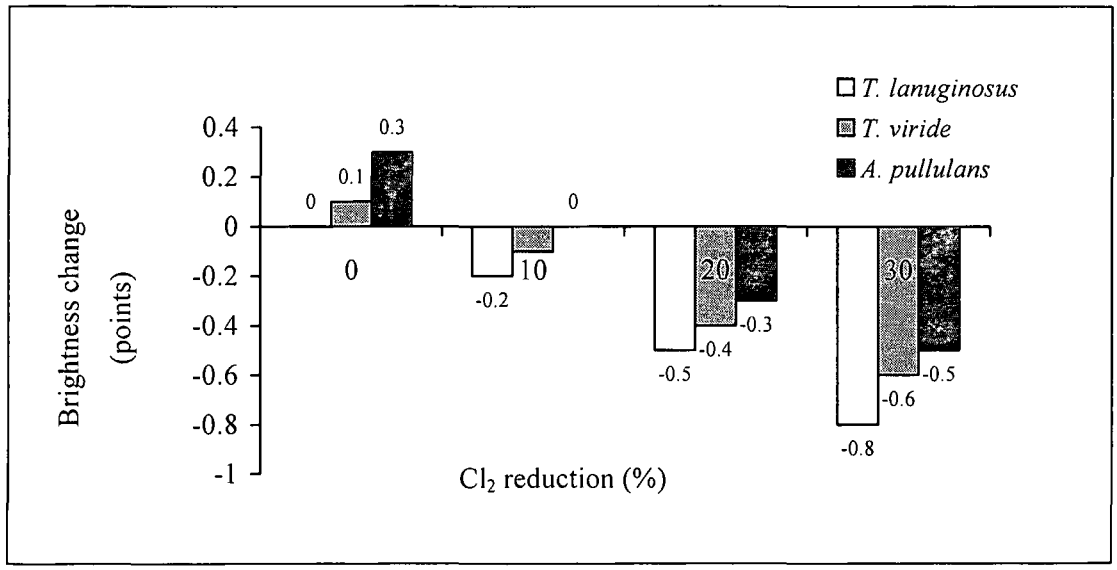


Fig. 7.7 Influence of purified xylanases (5 U/g pulp) on brightness of alkali-extracted bagasse soda pulp in sequence X-CEH at reduced Cl_2 charges

7.5 DISCUSSION

Production of xylanase by many microorganisms is generally associated with the presence of cellulase and vice-versa (Christakopoulos *et al.*, 1996; Sugen and Bhat, 1994; Khandke *et al.*, 1989). However, the availability of cellulase-free xylanase would be of remarkable advantage, especially for bleaching of paper pulp (Viikari *et al.*, 1994). At least two types of xylanolytic enzymes are needed for the hydrolysis of xylan: endo-1.4-β-D-xylanase and β-xylosidase. The purification and biochemistry of xylanases from various fungi has been studied extensively (Lin *et al.*, 1999; Christov *et al.*, 2000; Li *et al.*, 2000), however, research on the hydrolysis mechanisms is complicated by the heterogeneity of the substrate.

The properties of the xylans are determined by the origin of the carbohydrate but also to a great extent by the method of fractionation. Although plant in-situ xylans are insoluble and closely associated with cellulose and lignin, the practical substrates for hydrolysis would in most cases be soluble oligomers (Poutanen *et al.*, 1986). Prior to delignification, extraction of hemicelluloses from plant material with dilute alkali is often incomplete while after delignification the hemicelluloses can be extracted relatively easily by a variety of procedures from the hollocellulose complex.

Grouping of microbial endoxylanases according to physicochemical properties led to the discovery of two endoxylanases, which can be differentiated on the basis of their molecular mass and pI values (Wong *et al.*, 1988). This grouping correlated well with

the classification of endoxylanases into families 10 (formerly family F) and 11 (formerly family G) based on hydrophobic cluster analysis and amino acid sequence homologues (Davies and Henrissat, 1995). Previous studies with endoxylanases of *Streptomyces lividens* (Biely *et al.*, 1993), *Trichoderma reesei* (Biely *et al.*, 1993) and an endoxylanase of *Cryptococcus albidus* (Biely *et al.*, 1997) suggested that high molecular mass/low pI endoxylanases (family 10) exhibited greater catalytic versatility than low molecular mass/high pI endoxylanases (family 11), which hydrolyse the naturally occurring polyaccharides to a greater extent. Also, family 10 xylanases hydrolysed acetyl xylan to a higher degree and released short acetylated oligosaccharides, whereas the degree of hydrolysis of acetyl xylan by family 11 xylanases was rather low, and either acetylated xylobiose (Xyl2) or xylotriose (Xyl3) was hardly found among the products of hydrolysis (Biely *et al.*, 1993, 1997). This study investigated the effect of purified family 11 xylanases on fibres bound and isolated hemicelluloses in relation to their bleach boosting capabilities of bagasse pulp.

Alkaline (KOH) extraction of hemicelluloses from bagasse pulp was not complete since 4.6 % of the 12.5 % xylose present in the untreated pulp remained in the extracted pulp. HPLC analyses indicated that mannose and galactose was successfully removed during the extraction procedure while 30.80% of the arabinose was detected in the extracted pulp. Part of the xylose and arabinose was therefore resistant to the extraction method indicating either inaccessibility of the alkali to the hemicelluloses and/or presence of recalcitrant lignin-carbohydrate complexes.

It is well known that the success of enzyme treatments is dependant upon the particular hydrolysis conditions applied to the substrate. One of the criteria can be used for evaluation of the degree of substrate hydrolysis is the release of RS after treatment with enzymes. RS release from pulp caused by xylanases is indicative of the changes in the pentosan (hemicellulose) content, an important characteristic during the bleaching of pulp types. Evaluation of the purified xylanases for RS release from extracted hemicellulose of bagasse pulp indicated that *A. pullulans* xylanase was most efficient followed by *T. viride* and *T. lanuginosus* xylanase in decreasing order of efficiency. There was a corresponding increase in RS release with prolonged incubation periods. The xylanases when treated in combination, produced the highest levels of reducing sugars detected in the filtrates, indicating effective combined treatment with pronounced influence in comparison to the individual enzyme treatments. This demonstrates the cooperative effects of the xylanases in hydrolyzing the extracted hemicelluloses from bagasse pulp.

Buchert *et al.* (1992) reported a slight synergism between two xylanases from *T. viride* with respect to their action on pine kraft pulp. However, a report by Elegir *et al.* (1995) had revealed greater differences using purified xylanases from *Streptomyces* sp. TUB B-12-2. The enzymes from *T. reesei* were mostly active on acetylglucuronoxylan rather than unsubstituted xylan (Tenkanen *et al.*, 1992). Because xylan in kraft pulp is not acetylated, the difference in their substrate specificity might not have been enough to obtain different effect on pulp bleachability. In contrast, *Streptomyces sp.* TUB B-12-2 xylanases showed greater activity on unacetylated birch xylan than on acetylated xylan; moreover, their action patterns on xylooligosaccharides were completely different from

the activity patterns observed with the *T. reesei* enzyme. These differences in catalytic activities could be responsible for their different capabilities in the biobleaching of kraft pulp and for their apparent synergism (Elegir *et al.*, 1995).

Study of the xylanase action on commercially available birchwood xylan revealed data that contrasted to that of the extracted hemicellulllose from baggase pulp. The commercial xylan was more susceptible to the purified xylanases in terms of RS release compared to the alkali-extracted hemicelluloses from bagasse pulp. This could be due to the nonselective hemicellulose extraction method performed, implying that other extractives and lignins, and the possible presence of residual alkali (KOH) on the extracted hemicellulose, could be a factor that hindered the enzyme hydrolysis efficiency.

The low activity on the extracted hemicelluloses could be due to the low chain length which might be too short to be effectively bound to the active its of the xylanases. This study correlates positively with a report by Bennett *et al.*, (1998) in which the action of endoxylanase from *T. lanuginosus* ACTT 46882 differed on various xylo-oligosaccharides. The report stated that the enzyme had the lowest affinity towards Xyl_3, and Xyl_3 was 250 times less favourable substrate than Xyl_4 and Xyl4 was around 13 times less preferred substrate than Xyl_5. The nature of the fragments liberated from 4-*O*-methylglucuronoxylan, rhodymenan and *O*-acetyl-4-*O*-methylglucuronoxylan indicates that the xylanase of *T. lanuginosus* ATCC 46882 belongs to family 11.

The RS released during xylanase pretreatment of the untreated pulp and the extracted pulp were comparatively lower than that achieved with the extracted hemicelluloses and commercial xylans. This could be attributed to the retarded accessibility of the hemicelluloses in the pulp to xylanase in comparison to the readily available xylan present in the commercial birchwood xylan and extracted hemicelluloses. It has been suggested that the 4-*O*-methylglucuronic acid side chains in xylan cause steric hindrance and prevent binding of the hydrolytic enzymes to the polysaccharide in the immediate vicinity of the branching points (Timell, 1962). It is possible that the absence of glucuronidase in the enzyme preparation is one reason for the low xylose RS released from pulp. Alternatively, the lower hydrolysis potential of the xylanases on hemicellulose in bagasse pulp as compared to the extracted hemicelluloses and commercial xylan could be attributed to the chemical modifications of the hemicelluloses during pulping, resulting in limited recognition of the polysaccharide by the enzyme (Allison *et al.*, 1996).

There was a marked decrease in the concentration of RS with the application of 5 U/g pulp in comparison to 5 U/g xylan in pulp. With the application of 5 U/g xylan in pulp, a greater mass of pulp was utilized to attain the desired concentration. The consequent lower RS concentration appeared to be due to the retarded accessibility of the enzymes in the greater mass of pulp. On the other hand, the combined xylanase pretreatment released RS of higher concentration illustrating the cooperative interaction between the xylanases in hydrolyzing the hemicelluloses in the pulp. Evaluation of the xylanases on the alkali-extracted pulp, produced minimal increases in the reducing sugar release. This

phenomenon could be ascribed to the low xylose concentration of 4.5 % in the extracted pulp.

The bleach boosting capabilities of the purified xylanases indicated that *A. pullulans* xylanase was most effective, with *T. viride* and *T. lanuginosus* xylanase following in decreasing order of bleaching abilities. All three enzymes are from family 11 xylanases indicating a similar pattern of hydrolysis, however, the enzymes did display varying abilities of hydrolysis on isolated hemicelluloses and bagasse pulp. It is therefore proposed that the enzymes could have varying degrees of specificity for the hemicelluloses and *A. pullulans* xylanase could be more specific to the target xylan than the two other xylanases. Besides the specific mode of action of the individual enzymes on the pulp, their molecular mass might also be and important factor, considering the limited accessibility of the hemicellulose in the fibre matrix.

T. viride and *A. pullulans* xylanase reduced the Cl_2 consumption in X-CEH bleaching by 20% at the concentration of 5 U/g pulp, while *T. lanuginosus* produced a 10 % reduction in Cl_2 use. The bleach boosting efficiencies of these individual enzymes on bagasse pulp correlated positively with enzyme treatment on the isolated hemicelluloses and commercial xylan. Treatment of the pulp at a charge of 5 U/g xylan in pulp produced minimal savings of Cl_2 with *T. viride* and *A. pullulans* xylanase and no Cl_2 savings with *T. lanuginosus* xylanase, however, slight increases in brightness in comparison to the control was observed. On the other hand, pretreatment of bagasse pulp with all three xylanases in combination at a charge of 5 U/g pulp produced brightness increases of

significant importance: Cl_2 consumption was reduced by 30 % while still maintaining brightness values 0.3 points higher than the controls. This increase in brightness and reduction in chlorine consumption provides enormous potential for further investigations of xylanase combinations during bleaching. The xylanase combination displayed a synergistic effect in bleaching of bagasse pulp, achieving a greater brightness gain of 2.2 points than the sum of the individual effects of the enzymes, which was 1.8 brightness points. It appears that at appropriate enzyme charges and bleaching conditions the xylanases can produce the desired effects on bagasse pulp.

The enzymes however, were not as effective at charges of 5 U/g xylan in pulp, with savings of 20 % Cl_2 and a final brightness of 85.44 % achieved. This difference in bleaching ability of the xylanases could be ascribed to the inaccessibility of the substrate to the xylanase, resulting in limited hydrolysis of the hemicelluloses and subsequently limited removal of material contributing to the low brightness of unbleached bagasse pulp. Several explanations for the pulp prebleaching effect of hemicellulases have been discussed in the literature during the past few years. Effects of xylanases in prebleaching comprise uncovering lignin from reprecipitated xylan, release of chromophores and increase of the porosity of (Kantelinen *et al.*, 1993; Wong *et al.*, 1996; De Jong *et al.*, 1996).

Assessment of the bleach enhancing effects of purified xylanases on the hemicellulose-extracted bagasse pulp revealed minimal changes in brightness and Cl_2 savings. The initial brightness of the alkali-extracted bagasse pulp was higher than the untreated

bagassse pulp indicating that the removal of hemicelluloses and other extractives contributed to brightness increase. This therefore confirms the assumption that hemicelluloses do contribute to the colour of unbleached bagasse pulp, and there is a correlation between the release of hemicelluloses from bagasse pulp and its bleach boosting capabilities. This is in contrast to the findings (Buchert *et al.*, 1995) that the enzymatic removal of hemicellulose could not improve brightness of softwood sulfite pulps, suggesting that the wood species and pulping method might impact pulp bleachabillity. A report by Clark *et al.*, (1991) also revealed no relation between the extent of hemicellulose removal from radiata pine kraft pulp and improved bleachability. It was also shown that neither differences in pI values nor those in molecular mass of the enzymes could explain their different performances on pulp (Viikari *et al.*, 1993; Elegir *et al.*, 1995). The inefficient bleaching capabilities of the xylanases on the hemicellulose-extracted bagasse pulp can be ascribed to the low concentration of xylose in the pulp, with consequential limited hydrolysis, thereby producing a lower final brightness with minimal savings in Cl_2. This may therefore indicate that the hemicellulose in the untreatred bagasse pulp was a more accessible substrate for xylanases than those of the alkali-extracted bagasse pulp.

Only *A. pullulans* xylanase produced a saving of 10 % Cl_2 on the alkali-extracted bagasse pulp, while *T. lanuginosus* and *T. viride* had no impact in Cl_2 savings. An earlier report showed that hemicellulose removal from dissolving was improved when xylanase was supplemented with other hydrolyzing enzymes such as mannanase and endoglucanase (Gubitz *et al.*, 1997). The endoglucanase treatment probably caused a partial hydrolysis

and loosening of the cellulose structure thereby increasing the hemicellulose accessibility to the synergistic action of xylanase and mannanase on pulp. The extent of xylanase hydrolysis in pulps was also dependant on the substrate specificity of the particular enzyme used (Gubitz *et al.*, 1997) as well as the chemical composition and physical state of the xylan substrate (Christov *et al.*, 1997). The enzyme-mediated removal of xylan was suggested to increase pulp porosity which renders the fibre structure more permeable and enables the bleaching chemicals an easier and smoother penetration and access to lignin (Christov and Prior, 1997). It has also been shown that although dependant on treatment time and xylanase charge, the enzyme hydrolysis on pulp xylan is limited mainly by the partial inaccessibility of the substrate. This could be due to many factors such as enzyme size (Wong *et al.*, 1988) median pore size (Suurnäkki *et al.*, 1997), and accessible surface area of pulp (Stone *et al*, 1969).

The isolated hemicelluloses and commercial birchwood xylan was used as model substrates to find any characteristics of the individual hemicellulases that could be of importance with regard to their effect in bleaching of bagasse soda pulp. *A. pulluans* xylanases was the most efficient enzyme on isolated hemicelluloses from bagasse pulp, commercial birchwood xylan and bagasse pulp. This enzyme produced Cl_2 savings that could not be achieved by *T. lanuginosus* and *T. viride* xylanase alone. Pretreatment with the enzyme combination produced a prominent increase in reducing sugars detected on all the substrates under investigation as well as pronounced bleach boosting capabilities, demonstrating a cohesive mode of action among the enzymes. It is obvious that the overall hydrolysis of heteroxylans of different lignocellulosic origin represent the sum of

the action of several different enzyme activities. In addition to the individual xylanolytic enzymes hydrolyzing the 1,4 glycosidic bonds and acting synergistically to produce xylose from the β-D-xylosidic backbone, enzymes capable of releasing the substituents are also required. The absence of β-xylosidase in these preparations decreased the effectiveness of hydrolyzing the xylans completely. The enzymes that remove the side-groups may also have potential in changing the physico-chemical properties of xylans. It is therefore important to consider the effects of other hemicellulolytic enzymes in synergy with xylanases to investigate the hydrolysis potential on various lignocellulosic substrates. The depolymerization of the hemicelluloses analysed as RS correlated well with the resulting increase in brightness of the pulp. The study demonstrated clearly that the xylanase mediated release of RS as well as compounds contributing to the low brightness of the pulp correlated positively with the bleach enhancing effect with concomitant savings in Cl_2 consumption in CEH bleaching sequence.

7.6 CONCLUSIONS

i) Birchwood xylan was more susceptible to enzymatic hydrolysis than extracted hemicelluloses from bagasse pulp

ii) *A. pullulans* xylanase was the most efficient enzyme when applied to xylan and bagasse pulp, followed by *T. viride* and *T. lanuginosus* xylanase in decreasing order of efficiency.

iii) The xylanases displayed a greater bleach boosting effect on the untreated bagasse pulp than on the extracted pulp.

iv) No synergy among the xylanases was detected during RS release, however synergy was apparent during biobleaching of bagasse pulp.

v) The enzymes in synergy reduced the Cl_2 consumption by 30% demonstrating enormous potential for further investigations of xylanase combinations in bleaching.

The immunolocalization of purified *T. lanuginosus* xylanase on bagasse pulp was evaluated in Chapter eight.

CHAPTER EIGHT: IMMUNOLOCALIZATION OF A PURIFIED *T. lanuginosus* XYLANASE DURING HYDROLYSIS OF BAGASSE PULP.

8.1 ABSTRACT

The extent of diffusion and surface modification of a purified 23.6 kDa xylanase isolated from *Thermomyces lanuginosus* on bagasse pulp was evaluated. Polyclonal anti-xylanase antibodies were raised in two rabbits and in conjunction with immunogold labeling and microscopic studies enzyme diffusion and degradation studies were performed. The purity of the xylanase was confirmed by SDS-PAGE and western blots confirmed the antigen-antibody hybrid on the nitrocellulose membrane. The protein concentration was 0.35 mg/ml with xylanase activity of 65 U/ml. Scanning electron microscopy (SEM) was carried out immediately after labeling with immunogold particles as well as prior to labeling after xylanase treatment. The gold labeling could not be visualized under SEM, however xylanase pretreated bagasse pulp without subsequent labeling revealed distinct differences in the surface architecture of the pulp in comparison to the control. There was a noticeable "tearing-off" effect on the xylanase treated sample in comparison to the smooth compact fibres of the untreated pulp. The degree of abrasion on the fibre surface was not affected by enzyme concentrations, as negligible differences with the application of 5, 50 and 100 U xylanase/gram pulp were evident. Transmission electron microscopy (TEM) revealed xylanase penetration through primary and secondary cell walls into the lumen of the cells. The distribution of the xylanase was homogenous and rapid since after 1 h of enzyme treatment with the pulp, immunolabeling was observed both on the

surface and inside all cell walls. The gold labels were detected prominently on the ends of the fibres possibly due to easy accessibility of the xylans to the enzyme.

8.2 INTRODUCTION

Hydrolysis of woody and non-woody materials have been largely investigated with ligninolytic fungi (Barrasa *et al.*, 1995), but little is known about direct hydrolysis of cell walls by purified enzymes. The role and localization with respect to the fungus during wood degradation are, however, poorly understood, and the few studies performed so far (Garcia *et al.*, 1987; Glen *et al.*, 1983) have not shown conclusively its natural association with lignocellulosic substrates undergoing decay. The degree of fibre porosity (Wong *et al.*, 1988), the related measure of available surface area (Stone *et al.*, 1969) and the medium pore size of fibres (Grethlein, 1985) are key factors influencing the rate and extent of wall polymer breakdown, since they determine the accessibility of a substrate to the enzymes secreted by attacking microorganisms. Spatial orientation of cellulose and xylan in primary and secondary plant cell walls and the covalent linkage of lignin to the noncellulosic polysaccharides are important factors limiting the cell wall hydrolysis of forage (Hatfield, 1990).

Until recently, the in situ localization and demonstration of enzymes involved in plant decay have been carried out by complexing heavy metals with the products produced by enzymatic action. Such reactions can be both unspecific and difficult to interpret (Remond-Zilliox et al., 1997). Recently, more effective and more specific techniques based on the use of antibodies raised against appropriately inoculated antigens (eg., purified enzymes) have been developed. Antibodies have the advantage of having a high specificity for their antigens and can be demonstrated directly or indirectly in situ by

using a variety of immunological detection techniques at both light and electron microscopy levels (Daniel et al., 1989). These techniques can also provide an insight into the problems of enzyme-accessibility in plant cell walls.

Investigations done on the hydrolysis of wheat straw with a purified xylanase obtained from a thermophilic *Bacillus* sp. (Remond-Zilliox. 1996) indicated limited hydrolysis of the straw and the xylanase was absorbed to both xylans and lignins extracted from straw. This indicated that the lignins were competitive components for the adsorption of the xylanase and limited enzymatic diffusion into the substrate could be another factor preventing the enzyme hydrolysis of straw. Bagasse is a non-woody material and a carbohydrate-rich agricultural biproduct that represents a readily available renewable biomass. Studies on the localization of xylanase on pulp are limited and the aim of the present work was to localize purified xylanase during the hydrolysis of bagasse pulp in order to evaluate its degree of diffusion into the fibres as well to investigate xylanase mediated surface changes on bagasse pulp.

8.3 MATERIALS AND METHODS

8.3.1 Preparation of polyclonal serum

The antigen used in the immunization procedure was purified β-xylanase *from T. lanuginosus* SSBP (Lin, 1999). In order to prepare the antigen for inoculation, 1ml of Fruends Incomplete adjuvent (Sigma) was stirred vigorously with a glass rod while adding 1 ml antigen solution dropwise, at a rate of 1 drop per 30 s. The emulsion was ready for inoculation when a drop of the mixture remained as a bead on a distilled water surface. Two rabbits were periodically immunized over a three-month period through intracutaneous injections of the purified xylanase. Serum collected from an initial bleed served as the control. Secondary bleeding was carried out on a weekly basis for three weeks after the final immunization. The serum was collected by centrifugation at 1500 xg for 20 min after incubation at 4°C for 2h. The sera obtained was frozen in 2ml aliquots for use in immunological assays.

8.3.2 Sodium dodecyl sulphite polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the Resolute VMP Chamber in a discontinuous buffer system according to the procedure of Laemmli *et al.* (1970). Proteins samples, together together with a molecualr weight protein marker (Pharmacia LKB, Uppsala, Sweden) were loaded into wells with a Hamilton microlitre syringe. Samples were subjected to electrophoresis at 30 V overnight using 1x electrode buffer (0.25M Tris.HCl, 1.92 M glycine, 1% SDS, pH 8.3). Polypeptides in the gel were simultaneously fixed with methanol/glacial acetic acid and stained with Coomassie Brilliant Blue R250 by

immersing the gel in the staining solution for 4 h with shaking and destaining in a 10 % (v/v) glacial acetic acid/methanol solution and photographed.

8.3.3 Western blotting

Resolved polypeptides from the SDS-polyacrylamide gel were blotted onto a nitrocellulose membrane (Hybond) using a vertical electrophoresis chamber. Electrophoresis was carried out at 0.65 mA for 2h at 4°C for complete fragment transfer. The blot was stained with a working solution of Ponceau S (2 g Ponceau S, 30 g trichloroacetic acid, 30 g sulfosalicyclic acid in 100 ml H2O) in order to ensure successful transfer of protein. Detection was carried out using 100 mg 4-chloro-1-naphthol (Sigma) in 20 ml methanol and 100 ml Tris saline containing 100 µl H_2O_2. The membrane was submerged in the detection reagent and allowed to develop until the desired resolution was obtained. The reaction was terminated by washing in distilled H_2O.

8.3.4 Enzymatic hydrolysis of straw

Hydrolysis of bagasse pulp was performed by purified xylanase (10 U/g) from *Thermomyces lanuginosus* SSBP at 65°C for 3h with occasional kneading. Hydrated samples removed at 0 min, 30 min, and 300 min of hydrolysis were labeled and embedded separately for microscopic studies. Hydrolysed samples were also prepared for viewing under SEM.

8.3.5 Preparation of embedded bagasse pulp

Bagasse pulp after 0 min, 30 min, and 300 min of hydrolysis were fixed for 2h in 4% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at room temperature. Samples were hydrated in an ethanol series and embedded in LR White resin at 50°C overnight. LR White-embedded specimens were cut into ultrathin sections which were collected on nickel grids (Remond-Zilliox *et al.*, 1997).

8.3.6 Immunogold labeling of xylanase

The ultra thin sections on nickel grids as well as xylanase treated pulp were incubated with 0.2 M BSA (bovine serum albumin) for 15 min. They were then incubated for 45 min at room temperature with the primary antibody in a moisture chamber. After thorough washing with PBS the sections were incubated for 30 min with a gold-labeled (10 nm) anti-rabbit immumnoglobulin G conjugate at dilutions of 1:50 and 1:100 in BSA. The samples were then washed thoroughly in PBS, with final washings with water. The sections and directly labeled pulp were viewed under TEM and SEM, respectively. All experiments were accompanied by the following controls: an immunolabeling performed without the primary antibody and an immunolabeling performed in the absence of xylanase hydrolysis in order to check that labeling corresponds only to the *T. lanuginosus* xylanase.

8.3.7 Transmission electron microscopy

After immunolabeling the ultra thin sections were examined under the Phillips CM 120 Biotwin TEM (Holland) at 80 KV.

8.3.8 Scanning electron microscopy

Xylanase pretreated and control handsheet samples were mounted on stubs and sputter-coated with gold palladium (Polloran 5100 sputter coater, Tokyo, Japan) and processed for SEM. Samples were viewed using a Hitachi S-570 scanning electron microscope (Tokyo, Japan) at 10 KV.

8.4 RESULTS

8.4.1 Analyses of purified *T. lanuginosus* xylanase by SDS-PAGE and western Blotting

Protein profiles showed one homogenous band representing the purified xylanase with a molecular weight of approximately 23.6 kDa (Fig. 8.1). The protein concentration was 0.35 mg/ml with a xylanase activity of 65 U/ml.

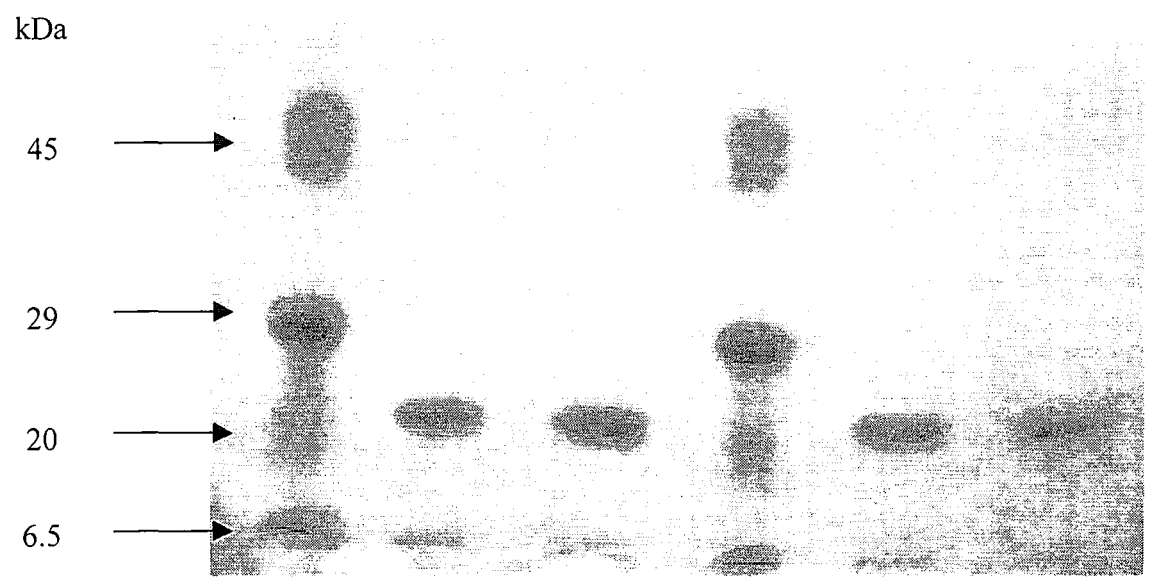


Fig. 8.1 SDS-PAGE of the purified *T. lanu*ginosus xylanase

Detection with chloronaphthol produced an intense blue-black signal at 23.6 kDa (Fig. 8.2) indicating complete transfer of the purified xylanase onto the nitrocellulose membrane. The antibodies were diluted at various ratios to optimize the concentration of the signal produced. This optimized antibody concentration was used during the immunolocalization of xylanase protein on the bagasse pulp. The colour intensity of the blot correlates to the concentration of the antibodies with the darker bands indicating higher antibody concentrations. The optimized antibody concentration used in the immunolocalization was 1:3000

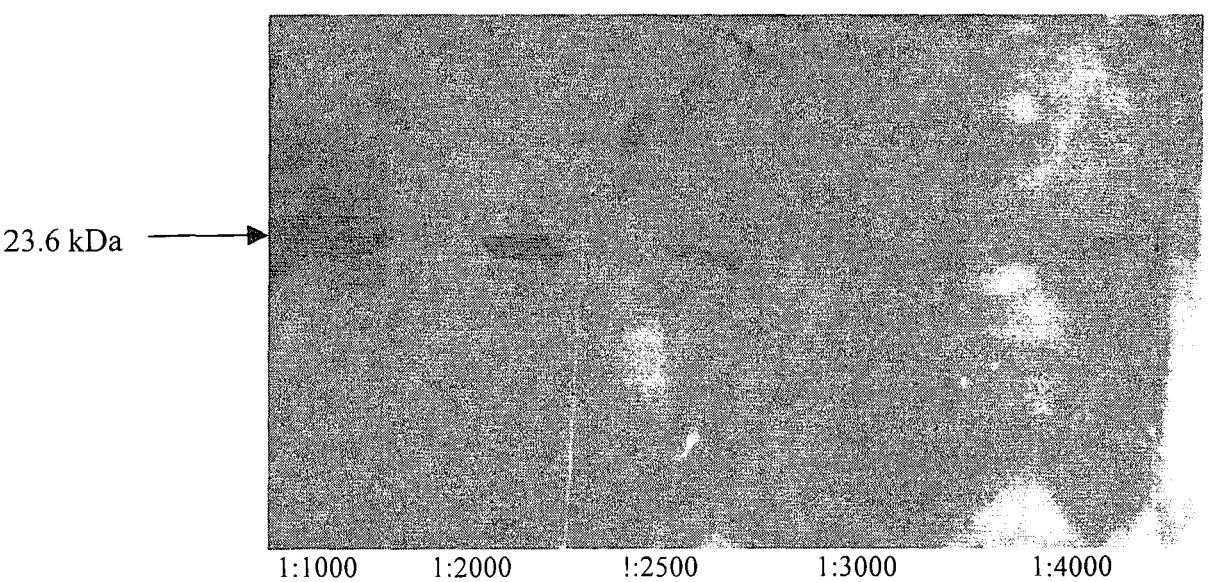


Fig. 8.2 Western blot showing hybridization of the purified xylanase from *T. lanuginosus* and polyclonal antibodies at different dilutions

8.4.2 SEM of xylanase pretreated bagasse soda pulp

The labeled samples had poor resolution and made visualization of the gold labeled xylanase extremely difficult under SEM. It appeared that the gold labels were not large enough to be seen under SEM. However, xylanase treated samples without labeling did show distinct differences in the surface architecture of the pulp in comparison to the control (Fig. 8.3 a and b). There was a noticeable change in the degree of fibrillation of xylanase treated pulp in comparison to the control. The smooth surfaces, intact microfibrils, and compact fibres of the untreated pulp contrast distinctly with the xylanase treated fibres where a "tearing-off" effect was observed.

The degree of abrasion on the fibre surface was not affected by the enzyme concentrations, since no significant differences were evident with the application of 5, 50 and 100 U xylanase/g pulp (data not shown). Nevertheless, xylanase pretreament did produce surface modifications of bagasse pulp without marked fibre disruptions.

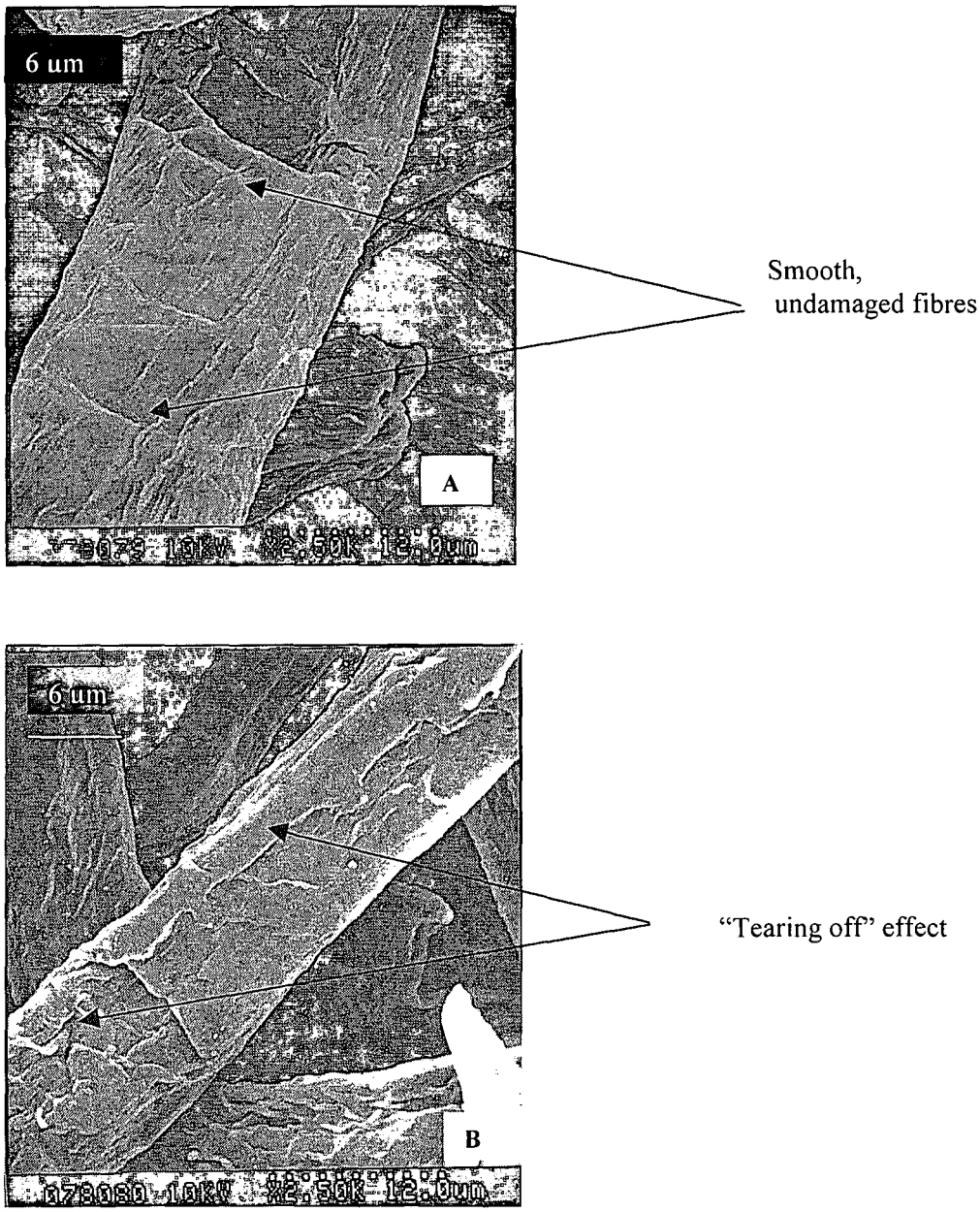


Fig. 8.3 Scanning electron micrographs of bagasse pulp samples showing: (A) Control pulp sample with smooth undamaged fibres, and (B) xylanase treated bagasse pulp (5 U/g pulp)

8.4.3 TEM of immunogold labeled xylanase on bagasse soda pulp

Immunolabeling was observed both on the fibre surface and within the cell walls. Penetration of xylanase was observed through the primary and secondary cell walls into the lumen, which allowed for interaction of the xylanase with the xylans located within the cell walls. (Fig. 8.4 a and b). This indicates that the xylanase having a low molecular weight of (23.6 kDa) was able to penetrate the fibres with relative ease. Localization was also prevalent on the outer surfaces of the fibres shown as dark spots representing the 10 nm gold particles linked to the secondary and primary antibodies. The absence of gold labels in the control sample (no xylanase pretreatment) indicated that nonspecific binding of the gold label during preparation did not occur (Fig. 8.5a).

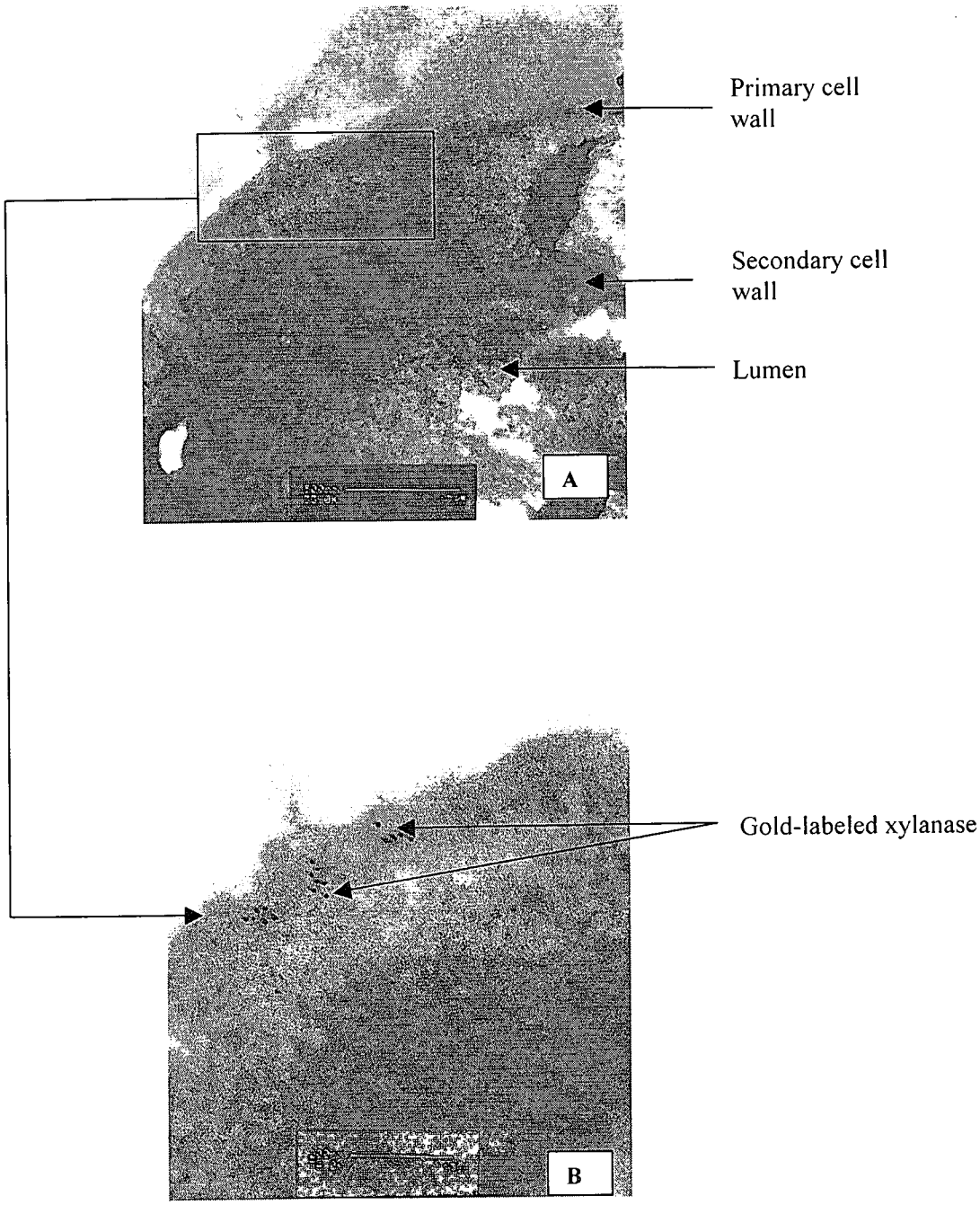


Fig. 8.4 Transmission electron micrograph showing (A) Immunogold localization of *T. lanuginosus* xylanase on bagasse pulp (26k). (B) Magnified section of the primary cell wall with the dark spots indicating the 10 nm particle linked to the xylanase (74k).

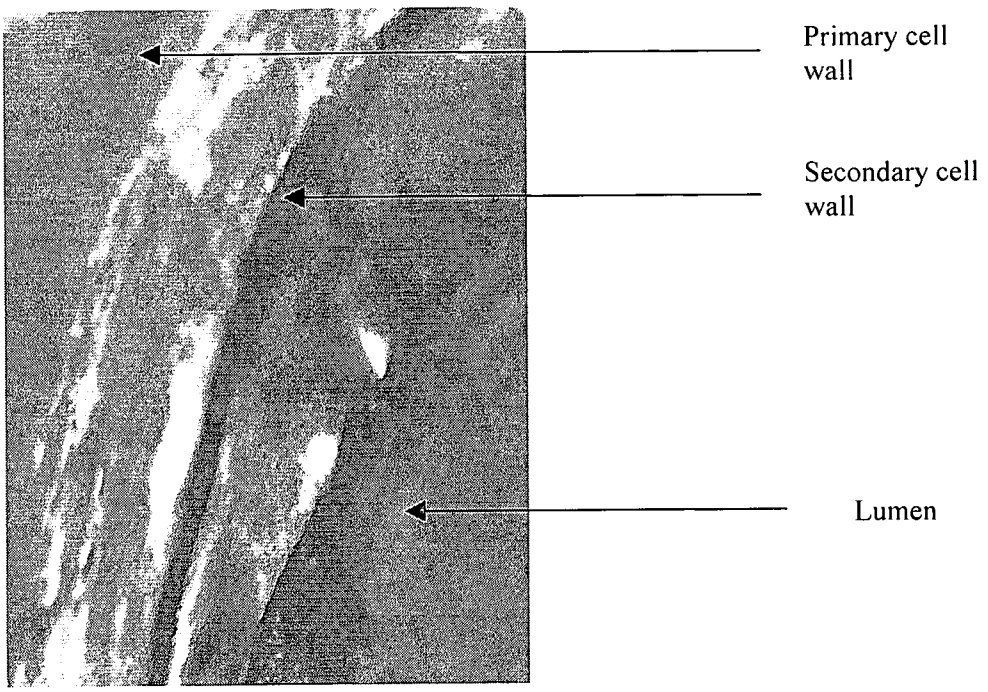


Fig. 8.5 Transmission electron micrograph of untreated bagasse soda pulp showing the absence of gold label within the fibre (26k).

8.5 DISCUSSION

Immunological analyses of the purified xylanase was conducted to investigate the reactivities of the polyclonal antibodies to xylanase from *T. lanuginosus*. Success with the western blotting was confirmed by a positive signal obtained for the purified xylanase implying recognition of the specific antigenic epitopes. Polyclonal antibodies bind to multiple sites on the antigen and therefore have a much higher avidity for the antigen. Together, multiple antibody-antigen reactions provide a multivalent complex that is stable during subsequent analysis. However, polyclonal antibodies produce higher nonspecific backgrounds since multiple interactions lead to the formation of large complexes that are more apt to trap or bind non-specific proteins. Although monoclonal antibodies have the advantage of specificity and may solve some of the problems associated with polyclonal antibodies, their use has several disadvantages, the most important being affinity. Since the antigen is held by only one antibody-antigen interaction, the affinity of the antibody for the antigen is critically important (Hudson and Hay, 1991).

Only a few reports in the literature on SEM of enzyme treated kraft and sulfite pulps are available (Christov *et al.*, 1994; Garg *et al.*, 1998). To the best of our knowledge, this study represents the first report on bagasse pulp. Examination of immunogold-labeled samples under SEM did not provide conclusive results on the location of the xylanases on the fibres. Resolution of the labeled xylanase was poor under SEM, however SEM of xylanase pretreated bagasse pulp prior to labeling did show significant differences in

fibre morphology in comparison to the control. The visual effects of the xylanase on the fibres were not evident at lower magnifications, however changes such as "cracks" and surface fibre "peeling" due to xylanase treatment was evident at higher magnifications. These results correlate with the findings of Pham *et al.* (1995) where surface modifications of kraft pulp after xylanase pretreatment were apparent. The ultrastructure and physico-chemistry of pulp fibres play a major role in determining the intrinsic properties of fibre surfaces, particularly inter-fibre bonding during paper-making (Daniel and Duchesne, 1998). In view of this, a balance has to be achieved in terms of maintaining the strength properties of the paper, by retaining a certain proportion of the hemicelluloses as well producing the desired bleach boosting effects. A report by Garg *et al.* (1998) revealed a marked disruption and separation of kraft pulp fibres with the application of xylanases from *Streptomyces thermoviolaceus.* This suggests that the enzyme not only assisted in surface modification but also penetrated pulp fibres allowing for a much enhanced xylan hydrolysis. The extensive fibre disruption by the *S. thermoviolaceus* xylanase reduced pulp strength properties significantly, due to the large portion of hemicelluloses removed from the pulp In comparison, *T. lanuginosus* xylanase did not produce severe fibre disruptions, however, the fibre surface was visibly affected. These results could provide some indication of xylanase action on bagasse pulp suggesting that the enzyme apparently targeted the xylan located on the outer surfaces of the pulp fibres, thereby increasing fibre pore size, (Kantelinen *et al.*, 1993). It is assumed that the "cracks" and "tearing-off" effect present on the micrographs probably allow for increased diffusion of the residual lignin from the exposed pulp fibres.

SEM studies of xylanase from *Streptomyces* sp. QG-11-3 revealed that enzymatic prebleaching of kraft pulp opened up the pulp structure compared with the smooth surface of the untreated pulp, allowing access of chlorine and other chemicals in the bleaching process (Beg *et al.*, 2000). When this organism was grown on eucalyptus kraft pulp, the mycelia penetrated deep inside the fiber and generated perforations at the of attachment. These observations suggest that the additive effects of xylanase action rendered the pulp fibres more accessible to chemical bleaching agents.

Transmission electron microscopy was the only way to perform an intrawall exploration and provided complementary information on the distribution of the enzyme within the entire thickness of the cell wall. The diffusion of xylanase was rapid, since after 1 hr of enzymatic hydrolysis, immunolabeling was observed both on the surfaces and inside all cell walls. The distribution of xylanases was homogenous, with the label being prominent on the ends of bagasse fibres where the xylans may have been accessible. Studies by Adolph *et al.* (1996) revealed that the localization of xylanase on wheat bran cell walls, subsequently concluded that the enzyme does not penetrate the cell walls. Thus the substrate decomposition occurs mainly on the substrate surface. According to Biely *et al.* (1985), the localization of xylanolytic enzymes in the cell all of microorganisms offers a selection advantage, because of the higher local enzyme concentration. A report by Rémond-Zilliox *et al.*, (1998) showed intense labeling of xylanases on wheat straw stems, with a heterogenous distribution of xylanases on the fragments. Labeling was shown to be more prominent on brittle walls, broken lines, and on the ends of straw fragments possibly due to the presence of large amounts of xylan. In

this study the level of localization of immunolabeling was very similar, irrespective of the incubation period employed, indicating that the adsorption of the xylanase was rapid and that no severe desorption occurred thereafter. The xylanase was localized within the cell walls, signifying that the small size (23.6 kDa) of the protein allowed its easy diffusion into the bagasse fibres. Diffusion is often a limiting factor for hydrolysis in microorganismal attack. Studies on wood degraded with white-rot fungi showed that penetration of lignin peroxidase (42 kDa) in wood cell walls was superficial and limited to decayed regions (Srebotnik *et al.*, 1988). Two hypotheses were proposed: either the lignin peroxidase ws prevented from penetrating wood cell walls because wood pores were too small for enzyme diffusion, or strong enzyme lignin interaction might the pores, although the wood pore size could be slightly greater than the size of the enzyme. Xylanases have been localized on lignocellulosic material with relative ease however, the question of penetrability of ligninase into wood hs not yet been clarified. The fact is that at the present state of preparation methods, only a small amount of extracellular enzyme can be labeled, although ligninase must be present extracellularly, as can easily be proven by an enzyme assay in the culture filtrate (Srebotnik *et al.*, 1988). Studies have shown, that lignin can be oxidized by mediators such as cation radicals, activated oxygen species, Mn (III), suggesting that direct contact between lignin-degrading enzymes and lignin may not be necessary during wood degradation (Haemmerli *et al.*, 1986; Harvey *et al.*, 1986). It is further suggested that lignin degrading enzymes remain associated with the fungal hyphae during decay and are released only in limited amounts (Messner *et al.*, 1987).

Pore sizes in plant cell walls are 3 nm–5 nm (Chesson *et al.*, 1995). Only molecules with effective diameters inferior to those of the pores, equivalent to a globular protein of approximately 19-20 kDa, could freely penetrate and diffuse through the wall. The size of *T. lanuginosus* xylanase being 23.6 kDa, however, allowed for penetration of the cell wall, through the pores, possibly due to initial xylanase activity on the fibre surfaces, resulting in an increase the pore size. A factor that could have hindered the diffusion of xylanases or modify enzyme distribution within the cell wall network is the lignins (Rémond-Zilliox *et al.*, 1998). This study did not investigate the effect of lignins on the diffusibility of xylanases, therefore a future challenge will be to analyse this effect in more detail. Lignins are intimately associated with hemicelluloses, presenting a physical barrier for the enzymatic accessibility of hemicelluloses. One characteristic of forage cell walls is the presence of hydroxycinnamic acids hat bind lignin to xylans via eser and ether bonds (Lam *et al.*, 1990). Chesson (1981) suggested that accessibility of wall polysaccharides to enzymes is dictated by the degree to which they are associated with phenolic polymers. The nature of the complex formed remain unclear, but both a physical incustation, preventing enzyme access to its substrate, and covalent linkages formed between phenolic and carbohydrate residues, inhibiting complete degradation of the polysaccharies, are thought to be important. This study clearly illustrates the degree of surface modification attributed to the xylanases and penetration of these enzymes within the cell wall, hydrolyzing the inner xylan substrates.

8.6 CONCLUSION

i) SEM immediately after xylanase treatment displayed a "peeling" effect on the fibre surface demonstrating fibre modification, which could subsequently promote enhanced chemical bleaching of the pulp.

ii) TEM analysis revealed xylanase penetration through the primary and secondary cell walls into the lumen of the cells, indicating easy penetrability of the enzyme, possibly due to its low molecular weight of 23.6 kDa.

iii) This is a novel study on bagasse pulp representing the importance of immunolocalization of xylanases on the pulp to verify the accessibility of these enzymes to the xylan substrate and subsequently aid in elucidating the mechanism of xylanase-aided bleaching.

CHAPTER NINE: GENERAL DISCUSSION

The pulp and paper industry has gone through major technological changes in the last decades aiming at decreasing the environmental impact associated with their operations. However, these changes have seemingly not been sufficient to attend the strict legislation and to satisfy public opinion. Partial or total mill closure seems to be the current trend in the pulp and paper industry (Ragauskas, 2001)). Interest in using xylanase enzymes for pulp bleaching has increased dramatically in 1999, and in the US and Scandinavian countries, environmental concerns has pressured mills to complete conversion to elemental chlorine free (ECF) bleaching in an economic way. In addition, weak markets for many pulp and paper products have forced mills to lower their bleaching costs (Senior *et al.*, 1999). This has paved the way for the use of enzymes, which can address both needs. Several pulp and paper mills worldwide are evaluating the closed cycle operation, with the goal of developing cost effective technologies, which can satisfy market demands, environmental requirements and public opinion (Tolan, 2001).

South Africa is a valuable international producer of pulp and paper products. The pulp and paper industry in this country is based on the harvesting of managed forests and contributes to at least 3 % of the gross national product. Unfortunately, this industry is also a major polluter. Therefore, the implementation of biotechnology presents an opportunity to minimise the amount of toxic pollutants generated in the manufacture of pulp and paper and improve the existing technology in a cost effective way. (Christov and Prior, 1998) The main cause of concern are the products formed from using these chemicals which include chlorinated organic substances, some of

which are toxic, mutagenic, persistent, and bioaccumulating and cause numerous harmful disturbances in biological systems (Bajpai and Bajpai, 1996).

In Durban (South Africa) where sugar cane is an abundantly grown crop, bagasse is one of the major cheap raw materials available for paper making. Sappi, Stanger utilizes this raw material for the production of fine paper and adopts the CEH bleaching sequence. This produces effluents high in AOX and chloride content, which has only recently become a concern for the pulp and paper industry. Although chlorine is a highly versatile bleaching agent, corrosion of storage tanks and extensive emissions of chlorinated organic compounds in the bleach plant effluent can occur.

Extensive research in biotechnology in the pulp and paper industry has provided a depth of knowledge in the application of enzymes to pulp and papers industries in South Africa. The focus of research in this area as well as developmental work has shifted towards improving the current bleaching process, which includes the reduction of chemical consumption, with consequent reduction of AOX. A project had been established to evaluate and compare the bleach enhancing potential of xylanases from various sources in the existing bleaching process (CEH) as well as other chemical bleaching sequences (ECF and TCF).

***Thermomyces lanuginosus* xylanase**

T. lanuginosus SSBP is a thermophilic hyphomycete, producing a range of thermostable extracellular enzymes, which include β-xylanases, α-galactosidase, α-L-arabinofuranosidase, esterases and β-mannanase (Singh *et al.*, 2000a). In the present study, this organism produced up to 1050 U/ml of xylanase activity in shake-flask

cultures at 50°C after a 7-day incubation period. The organism produced the highest xylanase activity among the *Thermomyces* strains investigated to date (Singh *et al.*, 2000a) and xylanase, which is very active at high alkaline pH values (5.5-9.0) and has an optimum temperature of 70°C (Singh *et al.*, 2000b). These two characteristics make the enzyme extremely stable since xylanases with high thermostability and pH stability are of particular interest for enzymatic hydrolysis of xylan in pulp bleaching.

***T. lanuginosus* xylanase pretreatment of bagasse soda pulp**

This xylanase was optimized with respect to temperature and pH application on bagasse pulp and analyses of the enzyme filtrates indicated noteworthy increases in the reducing sugar, chromophoric material and lignin derived compounds (LDC'S) released in comparison to the control. This was correlated to direct brightening of the pulp as well as reduction in kappa number, displaying enzyme-mediated removal of compounds from the pulp, which contribute to the low brightness of unbleached bagasse pulp. The enzyme therefore possesses important characteristics required in enhancing the bleaching of bagasse pulp as displayed by its effects on the physical properties of the pulp analysed after pretreament. This xylanase was stable on bagasse soda pulp over a wide pH and temperature range with optimum values of 6.0 and 65°C, respectively. Chemical treatment of the enzyme pretreated pulp showed a pronounced bleaching effect with the pulp fibres being more amenable to extraction of residual lignin by NaOH in comparison to the controls. It was also evident that a single bleaching step with H_2O_2, displayed significant brightness gains of approximately 3.8 points to absolute brightness values of 61 %. One of the impediments to elucidating the mechanisms of xylanase aided bleaching is the lack of convenient methods for distinguishing xylan-derived from lignin-derived

chromophores. The challenge provides an excellent opportunity for researchers to reconsider the process determinants, while more thorough analyses are required to determine whether a common target substrate is responsible for the apparently different phenomena that are observed under various process conditions. A major role for xylan-derived chromophores would ensure that xylanase has a unique role in pulp bleaching. Nevertheless, the direct brightening effects of this xylanase has been successfully demonstrated which correlated to the release of coloured material detected in the enzyme filtrate.

The brownstock of bagasse pulp is alkaline (pH 10.5) and it is desired that the enzyme be active at the ambient pH. During the cooking process of the pulp, extensive leaching of alkali is evident even after washing and pH adjustment. In order to achieve a maximum effect, it is essential that the enzyme exert its activity to the interior of the fibres, therefore a broad pH stability of the xylanases especially in the alkaline region is advantageous. A favourable characteristic that *T. lanuginosus* xylanase possesses is that it is stable over a broad pH range between pH 5 and 9 and can be easily introduced into the existing operation. The enzyme also displays thermostability, which is highly attractive characteristic in pulp bleaching. During the initial bleaching sequence of bagasse pulp the temperature of the brown stock is between 60 to 70°C, and *T. lanuginosus* xylanase has an optimum temperature of 65°C, indicating minimal changes to the existing temperature of the brownstock.

Evaluation of xylanases in TCF and ECF bleaching

In view of producing a pulp of high brightness with little or no adverse impact to the environment the evaluation of xylanases in ECF and TCF bleaching processes have

been investigated. The xylanases were compared on equal enzyme charges rather than equal enzyme cost. Xylanase P, when compared to Ecopulp TX-200C, *T. lanuginosus* xylanase and Cartazyme NS-10 at equal enzyme doses as U/g, appeared as the most efficient bleach-enhancing enzyme on bagasse pulp in both ECF (DED) and TCF (OqPaP) bleaching. In DED bleaching (kf of 0.75), a brightness gain of 1.6 points over control could be attained using Xylanase P. Overall, Ecopulp TX-200C *T. lanuginosus* xylanase and Cartazyme NS-10 followed Xylanase P in order of decreasing biobleaching efficiency. This increase in brightness produced by Xylanse P correlated to a reduction of chlorine dioxide by 33% as compared to 20% reduction of the chlorine dioxide consumption achieved with the other three enzyme preparations.

The biobleaching effect induced by xylanase pretreatment depended on both the enzyme and chlorine dioxide charges used and the use of high kappa factors were required for satisfactory bleaching. This could be attributed to the low initial brightness and a relatively low brightness ceiling of bagasse pulp. Implementation of xylanases in ECF bleaching of bagasse pulp has shown remarkable potential for reducing the ClO_2 consumption. On the other hand a higher brightness ceiling achieved after final bleaching will provide a market for paper products that are producing in an environmentally friendly manner.

Mechanical pretreatment of the pulp prior to ECF biobleaching produced a slight increase in final brightness, indicating "activation" of pulp to enzyme and chemical bleaching. It therefore appears that mechanical pretreatment could play an important role in improving the accessibility of the hemicelluloses present in bagasse pulp to

xylanases, however this aspect of the study warrants further investigation. The main criteria which is of great importance with mechanical pretreatment is the energy cost and economical feasibility associated with the entire process. Although brightness gains were observed with the mechanical pretreated pulp, this technology will only be of benefit to the pulp and paper industry if the costs associated with xylanase bleaching and mechanical pretreatment are lower than the current operating bleaching sequences.

Xylanase pretreated bagasse pulp displayed minimal changes to the physical properties in comparison to the controls. Xylanase P and Ecopulp TX-200C increased the tensile strength of the paper with a corresponding increase in the amount of energy required to rupture the test strip. All xylanases however, did decrease the viscosity of the pulps, which could be due to the presence of low levels of cellulase activity. However, analyses of the physical properties of bagasse pulp after full bleaching with xylanase showed negligible changes to the physical properties of the handsheets.

At similar brightness levels, the TCF biobleaching in sequence X-OqPaP was less efficient than the ECF biobleaching in terms of biobleaching effect. The final brightness obtained with this sequence did not reach anticipated levels and further investigations into the chemical charges of the sequence are warranted. However, the xylanases did display bleach-boosting capabilities in TCF bleaching on bagasse soda pulp, with Xylanase P being most efficient. The TCF bleaching sequence under investigation does not provide a suitable alternative to the bleaching process, since brightness levels achieved after final bleaching was lower than anticipated. It appears that chlorine free bleaching agents are not as effective as chlorine based bleaching

agents on bagasse pulp. An alternative is to increase the dose of peracetic acid or hydrogen peroxide in the sequence, however this will not assist with the initial objective, which was to reduce the chemical consumption. Nevertheless, this study indicated that xylanase pretreatment has a great potential in improving the bleachability of bagasse pulp especially in an ECF bleach sequence, and could result in improved bleachability of bagasse pulp. Alternatively, a decrease in chemical consumption was demonstrated, while attaining the target brightness and maintaining the desired physical properties.

Influence of xylanases in CEH bleaching of bagase soda pulp

To our knowledge there are no reports on the evaluation of xylanases on non-woody materials such as bagasse pulp in a CEH bleach sequence and this is the first report thereof. In this study te bleach boosting potential of six xylanases, Xylanase P, Ecopulp TX-200C, Cartazyme NS 10, Cartazyme 9704-E, Pulpzyme HC and *T. lanuginosus* SSBP xylanase on an equal cost basis were investigated on bagasse-soda pulp in CEH bleaching.

Pretreatment of bagasse soda pulp with xylanases revealed that Xylanase P (10 U/g) and Cartazyme 9704-E (5 U/g) were most efficient, reducing the chlorine consumption between 20 to 25 %. The final brightness after CEH bleaching was approximately 76 % and all the xylanases under investigation produced a higher final brightness than the controls with full Cl_2 charges. Ecopulp TX-200C achieved a reduction of 20% Cl_2 while Pulpzyme HC, *T. lanuginosus* SSBP xylanase and Cartazyme NS-10 reduced by Cl_2 charge by 15 %.

Although Xylanase P and Cartazyme 9704-E produced similar Cl_2 saving, more enzyme in terms of U/g of Xylanase P (10 U/g) was applied to achieve similar results as Cartazyme 9704-E (5 U/g). In terms of efficiency from an economical evaluation, Cartazyme 9704-E proves to be the best xylanase.

Evaluation of Cartazyme 9704-E (3.75 U/g) at optimised Cl_2 charges (4.75 %) on unwashed bagasse pulp revealed a brightness improvements 1.0 point to 81.3 % with Cl_2 savings of 25 %, implying that the Cl_2 charges could be reduced further while still maintaining brightness values similar to the control. It was demonstrated that the unwashed bagasse soda pulp was more susceptible to xylanase pretreatment than the washed sample. This phenomenon is difficult to explain, however the use of unwashed pulp could play an important factor in terms of reducing water consumption during washing prior to enzyme treatment. This would be an asset to the pulp and paper industry since excessively large amounts of water are utilized on a daily basis.

Cartazyme 9704 (3.75 U/g) also displayed reduction in hypochlorite consumption by 33.33 % in the XCEH sequence while still maintaining a higher brightness than the control. Even at low enzyme charges the xylanase was able to achieve hypochlorite savings of 16.67 %. Cartazyme 9704-E appears to have great potential in CEH bleaching of bagasse-soda pulp. Implementation of xylanase technology to the bleach process of bagasse-soda pulp is a promising way forward in terms of producing pulps, which are environmentally friendly, and process waters, which could be fully recyclable. Sappi recognizes the importance of biotechnology in the bleach process and the use of xylanases to the current CEH bleaching conducted at the mill will play an important role in bleaching of bagasse pulp in the near future.

Bleaching with SSF xylanase

The bleaching with SSF samples is a relatively new concept in the application of biotechnology in the pulp and paper industry. With recent advances in SSF technology and the availability of substrate and process selected strains, SSF is becoming a viable alternative for enzyme production (Durand *et al.*, 1997). *T. lanuginosus* strain SSBP produced copious amounts of xylanase under submerged cultivation, however production of xylanases under SSF conditions have not been investigated on this strain.

The evaluation of SSF samples in bleaching experiments is a new approach to the application of biotechnology in the bleaching process and this is a novel report on the application of SSF samples to enhance the bleachability of bagasse soda pulp. *T. lanuginosus* SSF samples improved the brightness of bagasse soda pulp by 1.9 points to a final brightness of 84.52 % at a SSF/raw pulp ratio of 1:50. Although strain F 980 produced the highest level of xylanase during SSF, this strain was not the most efficient brightness enhancer of bagasse soda pulp. This confirms our findings that the level of xylanases produced does not necessarily have a direct correlation with the bleach boosting capabilities of the SSF sample. Strain ATCC 36350 displayed the best bleach enhancing abilities reducing the Cl_2 consumption by 30% with a final brightness higher than the control. The data therefore illustrates the important potential application of SSF samples to the bleaching of bagasse pulp in CEH bleaching, and could pave the way to new and improved bleaching methods. Bleaching with SSF samples provide an alternative to direct enzyme-aided bleaching, producing brightness gains of great importance in chemical CEH bleaching of bagasse soda pulp. Although this technology has shown promising potential with huge

success in bleaching on a laboratory scale, the challenge will be to take it a step further, conduct mill trials, and evaluate the economics of this bleaching process.

Bleaching with purified xylanases

This study investigated the effect of purified family 11 xylanases on fibres bound and isolated hemicelluloses in relation to their bleach boosting capabilities of bagasse pulp. Evaluation of the purified xylanases on RS release from extracted hemicellulose of bagasse pulp, and commercial xylan indicated that *A. pullulans* xylanase was most efficient followed by *T. viride* and *T. lanuginosus* xylanase in decreasing order of efficiency. The commercial xylan was more susceptible to the purified xylanases in terms of reducing sugar release than the alkali-extracted hemicelluloses from bagasse pulp. This could be due to the nonselective hemicellulose extraction method carried out, implying that other extractives and lignins that could have been removed hindered the enzyme hydrolysis efficiency.

There was a corresponding increase in reducing sugar release with prolonged incubation periods. The enzymes when treated in combination produced the highest levels of RS detected in the filtrates, indicating effective combined treatment with pronounced influence in comparison to the individual enzyme treatments. However, no synergy was evident in terms of RS release from these substrates.

The low concentration of RS detected in the enzyme treated pulps in relation to the extracted hemicellulose and commercial xylan could be attributed to the inefficient accessibility of the xylanases to the hemicelluloses in the pulp in comparison to the

readily available xylan present in the commercial birchwood xylan and extracted hemicelluloses.

The bleach boosting capabilities of the purified xylanases indicated that *A. pullulans* xylanase was most effective, with *T. viride* and *T. lanuginosus* xylanase following in decreasing order of bleaching abilities. *T. viride* and *A. pullulans* xylanase reduced the Cl_2 consumption in XCEH bleaching by 20% at the concentration of 5 U/g pulp, while *T. lanuginosus* produced a 10 % reduction in Cl_2. The bleach boosting efficiencies of these individual enzymes on bagasse pulp correlated positively with enzyme treatment on the isolated hemicelluloses and commercial xylan.

Treatment of the pulp at a charge 5 U/g xylan in pulp produced minimal savings of Cl_2 with *T. viride* and *A. pullulans* xylanase and no Cl_2 savings with *T. lanuginosus* xylanase. However, pretreatment of bagasse pulp with all three xylanases in combination at a charge of 5 U/g pulp produced brightness increases of significant importance, with reduction in Cl_2 consumption by 30 %. The xylanase combination displayed a synergistic effect in the bleaching experiments, with an increase of 2.2 points in comparison to the sum of the brightness gain achieved with each xylanase amounting to 1.8 points. This phenomenon is of significant interest and the use of xylanase combinations in the bleaching process could play an imperative role in bleaching strategies especially if the individual enzymes target different sites on the xylan chain.

Assessment of the bleach enhancing effects of purified xylanases on the hemicellulose-extracted bagasse pulp revealed minimal changes in brightness and Cl_2

savings. The initial brightness of the hemicellulose extracted bagasse pulp was higher than the untreated bagassse pulp indicating that the hemicelluloses and other extractives removed by the alkali contributed to the low brightness of the pulp. This therefore confirms the assumption that hemicelluloses do contribute to the colour of unbleached bagasse pulp, and there is a correlation between the release of hemicelluloses from bagasse pulp and its bleach boosting capabilities. The inefficient bleaching capabilities of the xylanases on the hemicellulose-extracted bagasse pulp can be ascribed to the low concentration of xylose in the pulp, with consequential limited hydrolysis, thereby producing a lower final brightness with minimal savings in Cl_2. It was also demonstrated that xylan content plays a significant role in xylanase bleaching and the important synergistic effects produced by purified xylanases from *A. pullulans*, *T. viride* and *T. lanuginosus* in CEH bleaching of bagasse pulp.

SEM and TEM studies of xylanase pretreated bagasse pulp

Only a few reports in the literature on SEM of enzyme treated kraft and sulfite pulps are available (Christov *et al*, 1994; Garg *et al.*, 1998). SEM of xylanase pretreated bagasse pulp prior to labeling did show significant differences in fibre morphology in comparison to the control. The visual effects of the xylanase on the fibres were not evident at lower magnifications, however changes such as "cracks" and surface fibre "peeling" due to xylanase treatment was evident at higher magnifications. *T. lanuginosus* xylanase did not produce severe fibre disruptions, however, the fibre surface was visibly affected. These results could provide some indication of xylanase action on bagasse pulp suggesting that the enzyme apparently targeted the xylan located on the outer surfaces of the pulp fibres, thereby increasing fibre pore size, (Kantelinen *et al.*, 1993). It is assumed that the "cracks" and "tearing-off" effect

present on the micrographs probably allowed for increased diffusion of the residual lignin from the exposed pulp fibres, with subsequent bleach enhancing capabilities of the chemicals.

TEM was the only way to perform an intrawall exploration and provided complementary information on the distribution of the enzyme within the entire thickness of the cell wall. The diffusion of xylanase was rapid, since after 1 h of enzymatic hydrolysis, immunolabeling was observed both on the surfaces and inside all cell walls. The distribution xylanases was homogenous, with the label being prominent on the ends of bagasse fibres where the xylans may have been accessible. The level of localization of immunolabeling was very similar, irrespective of the incubation period employed, indicating that the adsorption of the xylanase was rapid and that no severe desorption occurred thereafter. The xylanase was localized within the cell walls, signifying that the small size (23.6 kDa) of the protein allowed its easy diffusion into the bagasse fibres. Diffusion is often a limiting factor for hydrolysis in microorganismal attack. The size of *T. lanuginosus* xylanase being 23.6 kDa, allowed for penetration of the cell wall, through the pores, possibly due to initial xylanase activity on the fibre surfaces, resulting in an increase the pore size. SEM and TEM studies clearly illustrates the degree of surface modification attributed to the xylanases and penetration of these enzymes within the cell wall, hydrolyzing the inner xylans substrates within the bagasse pulp fibres, which could subsequently improved chemical bleaching of the pulp.

An important objective from an industrialists point is keeping the cost of bleaching to a minimum. The economics of enzyme bleaching is comprised of several cost factors

which include enzyme production, maintaining the appropriate temperature, the need for new facilities in the bleaching line and yield loss due to hemicellulose solubilization. The introduction of xylanases does not require substantial capital and excessive changes to the existing bleaching process, and the price of current commercial enzymes does favour its application on a large scale. The target cost of enzyme application has been said to be about 3.5 US $ per tonne in 1995 (Farrell *et al.*, 1996). This value is expected to decrease further as research in this field develops and efficient enzyme production strains are adopted. Usually the price of the enzyme is compared to that of the competing bleach chemicals but factors such as, decreased AOX loading, retention of viscosity and other technical pulp properties, confer additional advantages to xylanases and in such a case it is difficult to specify a price (Buchert *et al.*, 1994). However the environmental implications associated with enzyme applications outweighs the economic gains of bleach chemicals.

This project has clearly demonstrated the importance of xylanase pretreatment on bagasse pulp with remarkably significant advantages when incorporated into chemical bleaching sequences. The enzymes have shown great potential in ECF, TCF and in the existing CEH bleaching sequence. A relatively new approach to bleaching with SSF xylanase has also shown impressive bleach boosting potential with possible implementation in the future. Bagasse pulp appears to be very susceptible to a variety of xylanases, possibly due to the high xylan content in the pulp. In this regard, the implementation of xylanase technology to the bleach process should provide benefits of economic and environmental importance in terms of improving the bleachabilty of bagasse pulp as well as significantly reducing chemical consumption during bleaching. It is obvious that there are numerous possibilities for the application of

xylanase technology in Sappi (Stanger) to the extent that it seems highly likely that this industry will in the near future become dependant on this environmentally benign process.

FUTURE WORK

i) A comparison of the bleach enhancing effect of the purified and crude xylanase from *T. lanuginosus* warrants further study. This investigation will provide important information to the relevance of xylanase purification and its application to pulp.

ii) Mechanical pre-treatment appears to play a positive role in pulp activation, prior to xylanase treatment. Optimisation of mechanical pre-treatment conditions will be performed with emphasis on improving the efficiency of xylanase treatment.

iii) TCF bleaching chemicals and sequences will be evaluated and optimised to produce a final brightness of 80 % on bagasse pulp without affecting the physical properties of the paper.

iv) Cartazyme 9704-E was the most efficient xylanase in X-CEH bleaching of bagasse pulp. This xylanase will be investigated on a larger scale with the intention of introducing the enzyme into a mill trial.

v) The structure of extracted xylan from bagasse pulp will be examined to gain a clearer understanding of the mechanism of xylanase-aided bleaching on this non-woody material.

REFERENCES

Abe, Z. 1987. Prebleaching: Light beatings of pulps before bleaching improves their bleachability. Proceedings of the 4th International Symposium on Wood Pulping Chemistry, April 27-30, Paris, France, Vol. 2, pp. 255.

Aidoo, K. E., R. Hendry, and B. J. B. Wood. 1982. *Advances in Applied Microbiology*, **28**:201-237.

Alam, M., I. Gomes, G. Mohiuddin, and M. Hoq. 1994. Production and characterization of thermostable xylanases by *Thermomyces lanuginosus* and *Thermoascus aurantiacus* grown on lignocelluloses. *Enzyme and Microbial Technology*, **16**:298-302.

Allison, R. W., T. A. Clark, and S. H. Wrathall. 1993a. Pretreatment of radiata pine kraft with a thermophillic enzyme, Part I. Effect on conventional bleaching. *Appita Journal,* **46**:269.

Allison, R. W., T. A. Clark, and S. H. Wrathall. 1993b. Pretreatment of radiata pine kraft with a thermophillic enzyme, Part II. Effect of oxygen, ozone and chlorine dioxide bleaching, *Appita Journal*, **46**:349.

Allison, R. W., and T. A Clark. 1994. Effect of enzyme pretreatment on ozone bleaching. *Tappi Journal*, **77**:127.

Allison, R. W., T. A Clark, and M. J. Ellis. 1995. Process effects on the response of softwood kraft pulp to enzyme assisted bleaching. *Appita Journal*, **48**:201.

Allison, R. W., T. A. Clark, and A. Suurnäkki. 1996. Effect on modified kraft pulping on enzyme assisted bleaching. *Appita Journal*, **49**:18.

Apinis, A. E. 1963. Occurrence of thermophilous microfungi in certain alluvial soils near soils near Nottingham, *Nova Hedgw*, **5**:57-78.

Arima, K. 1964. Microbial enzyme production. **In**: Global impacts of Applied Microbiolgy. M. P. Star, Wiley (eds.). New York, pp. 227-294.

Atchison, J. E. 1993. Bagasse. **In**: Pulp and Paper Manufacture: Secondary fibres and non-wood pulping. F. Hamilton, and B. Leopold (eds.). Joint Textbook Committee of the Paper Industry, pp. 22-70.

Aumont, M., A. Vegega, D. D. Elm, and P. P. Choma. 1996 Low kappa factor bleaching of oxygen delignified hardwood kraft pulp – Xylanase and peroxide applications. *Pulp and Paper Canada*, **97**:72.

Babu, K. R. and T. Satanaryana. 1995. α-Amylase production by thermophilic *Bacillus coagulans* in solid substrate fermentation. *Process Biochemistry*, **30**:305-309.

Bailey, M. J., J. Puls, and K. Poutanen. 1991. Purification and properties of two xylanases from *Aspergillus oryzae*. *Journal of Applied Biotechnology and Applied Biochemistry*, **13**:380-389.

Bajpai, P. and P. K. Bajpai. 1992. Biobleachig of kraft pulp. *Process Biochemistry*, **27**:319-325.

Bailey, M. J., P. Biely, K. Poutanen. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology,* **23**:257–270.

Bajpai P., N. K. Bhardwaj, P. K. Bajpai, and M. B. Jauhari. 1994. The impact of xylanases in bleaching of eucalyptus kraft pulp. *Journal of Biotechnology*, **36**:1.

Bajpai, P. and P. K. Bajpai. 1996. **In**: Advances in Biochemical Engineering/Biotechnology. Scheper, T. (eds.). Springer-Verlag, Berlin, Vol 56, pp.1.

Bajpai, P. 1997. **In**: Advances in Microbiology; Niedleman, S. L., A. L. Laskin (eds.). Academic Press, New York, Vol 43, pp.141.

Bajpai, P. and P. K. Bajpai. 1997. Realities and trends in enzymatic prebleaching of kraft pulp. *Advances in Biochemical Engineering and Biotechnology*, **56**:1-30.

Bajpai, P. 1999. Application of enzymes in the pulp and paper industry. *Biotechnology Progress*, **15**:147-157

Bard, J. W. 1941. Effecting of tannins on alkaline pulping. *Journal of Paper Trends*, **113**:29

Barnoud, F., J. Combat, J. P. Joseleau, F. Mora, and K. Ruel. 1986. Interest in the enzymatic hydrolysis of xylans for modifying the structure of pulp fibres. 3rd International Conference on Biotechnology in the Pulp and Paper Industry, Stolkholm, June 16-19, pp. 70-72.

Beg, Q. K., B. Bhushan, M. Kapoor, and G. S. Hoondal. 2000. Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG-11-3 and its application in biobleaching of eucalyptus kraft pulp. *Enzyme and Microbial Technology*, **27**:459-466.

Beg, Q. K., Kapoor, M. L. Mahajan, and G. S. Hoondal. 2001. Microbial xylanases and their industrial applications. *Applied Microbiology and Biotechnology*, **56**:326-338.

Bennett, N. A., J. Ryan, P. Biely, M. Vrsanska, L. Kremnicky, B. J. Macris, D. Kekos, P. Christakopoulos, P. Katapodis, M. Claeyssens, W. Nerinckx, P. Ntauma, and M. K. Bhatt. 1998. Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of *Thermomyces lanuginosus* ATCC 46882. *Carbohydrate Research,* **306**:445-455.

Bernier, R. L., A. L. Gray, G. P Moser, J. Hamilton, M. Roberge, and D. J. Senior. 1994. Fate of residual xylanase after treatment and bleaching of softwood kraft pulp. *Bioresource Technology*, **50**:79.

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

Biely, P. 1985. Microbial xylanolytic systems. *Trends in Biotechnology*, **3**:286-290.

Biely, P. 1993a. Biochemical aspects of the production of microbial hemicellulases. **In**: Hemicellulose and hemicellulases. Coughlan, M. and G. Hazelwood (eds.). Portland Press, London, pp. 29-50.

Biely, P., M. Vrsanska, L. Kremnicky, M. Tenkanen, K. Poutanen, and M. Hayn. 1993b. Catalytic properties of endo-β-1,4-xylanase of *Trichoderma reesei*. **In**: *Trichoderma reesei* cellulases and other hydrolases. P. Poutanen, and T. Reinikainen (eds.). Fagepaino Oy, Helsinki, pp. 125-135.

Biely, P., D. Kleupfel, R. Morosoli, and F. Shareck. 1993c. *Biochemistry and Biophysics Acta*, **1162**:246-254.

Biely, P., M. Vrsanska, M. Tenkanen, and D. Kleupful. 1997. Endo-β-1,4-xylanase families: Differences in catalytic properties. *Journal of Biotechnology*, **57**:151-166.

Bissoon, S., L. Christov, and S. Singh. 2002. Bleachboosting effects of purified xylanase from *Thermomyces lanuginosus* SSBP on bagasse pulp. *Process Biochemistry*, **37**:567-572.

Blake, J. D., P. T. Murphy, and G. N. Richards. 1971. *Carbohydrate Research*, **16**:49.

Bragger, J. M., R. M. Daniel, T. Coolbear, and H. W. Morgan. 1989. Very stable enzymes from extremely thermophilic archaebacteria and eubacteria. *Applied and Environmental Microbiology* **31**:556-561.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**:248-254.

Brown, J., M. C. Cheek, H. Jameel, and T. W. Joyce. 1994. Medium-consistency ozone bleaching with enzyme pretreatment. *Tappi Journal*, **77**:105.

Buchert, J., M. Ranua, A. Kantelinen, and L. Viikari. 1992a. The role of *Trichoderma reesei* xylanases in the bleaching of pine kraft pulp. *Applied Microbiology and Biotechnology*, **37**:825-829.

Buchert, J., M. Siika-aho, A. Kantelinen, M. Ranua and L. Viikari. 1992b. 6th International Conference on Biotechnology in the Pulp and Paper Industry, Kyoto, Japan, May 27-30.

Buchert, J., M. Tenkanen, and M. Pitkänen, and L. Viikari. 1993. Role of surface charge and swelling on the action of xylanases on birch kraft pulp. *Tappi Journal*, **76**:131.

Buchert, J., M. Tenkanen, A. Kantelinen, and L. Viikari. 1994. Application of xylanases in the pulp and paper Industry. *Bioresource Technology*, **50**:65-72.

Buchert, J., A. Teleman, V. Harjunpaa, M. Tenkanen, L. Viikari, and T. Vuorinen. 1995a. Effect of cooking and bleaching on the structure of xylan in conventional pine kraft pulp. *Tappi Journal*, **78**:125-130.

Buchert, J. and L. Viikari. 1995. Significance of pulp metal profile on enzyme-aided TCF bleaching. *Paperi ja Puu*, **77**:582.

Buchert, J., G. Carlsson, L. Viikari, and G. Ström. 1996. Surface characterization of unbleached kraft pulps by enzymatic peeling and ESCA. *Holzforschung*, **50**:69.

Buchert, J., E. Bergnor, G. Lindblad, L. Viikari, and M. Ek. 1997. Significance of xylan and glucomannan in the brightness reversion of kraft pulps. *Tappi Journal*, **80**:243.

Call, H. P. and I. Mücke. 1994. State of the art of enzyme bleaching and disclosure of a breakthrough process. Proceedings of the International Non-chlorine Bleaching Conference, March 7, Amelia Island, USA.

Cannel, E. and M. Moo-Young. 1980. *Process Biochemistry*, **15**:24-28.

Capps, C. 1995. Enzyme bleaching. *World Paper*, **220**:42.

Chandra, S. K., E. L Hirst, J. K. N. Jones, and E. G. V. Percival. 1950. The constitution of xylan from esparto grass (*stipa tenacissima*). *Journal of Chemical Society*, pp. 1289-1297.

Chang, Y., and H. J Hudson. 1967. Fungi of wheat straw compost I. Ecological studies, *Trans. Br. Mycol. Soc.* **50**:649-666.

Chang, H. and R. L. Farrell. 1996. The effect of xylanase treatment on the bleachability of softwood and hardwood kraft pulps. **In**: Biotechnology in the Pulp and Paper Industry- Recent advances in Applied and Fundamental Research. E. Srebotnik, and K. Messner (eds). Facultas-Universitätsverlag, Wien, pp. 75.

Chauvet, J.-M., J. Comtat, and P. Noe. 1987. Assistance in bleaching of never-dried pulps by the use of xylanases, consequences on pulp properties. Proceedings of the 4th International Symposium on Wood Pulping Chemistry, April 27-30, Paris, France, Vol. 2, pp. 325.

Chen, J., J. Yang., Y. Qu, P. Gao., B. Wang, Q. Zhang, and Q. Yang. 1996. Improvement of wheat straw properties with an alkali-tolerant xylanase from *Pseudomonas* sp. G6-2. ACS Symposium ser. 655:308.

Choi, P. M. K., W. Q. Yean,and D. A. Goring. 1976. Removal of lignin from kraft pulp fibres during washing. *Trans. Techn. Sect. CPPA*, **2**:58

Christov, L. P. and B. A. Prior. 1994. Enzymatic prebleaching of sulfite pulps. *Applied Microbiology and Biotechnology*, **42**:492.

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

Christov, L. P. and B. A. Prior. 1996b. Reduction of active chlorine charges in bleaching of xylanase-treated sulfite pulp. ACS Symposium ser. 655:208.

Christov, L. P. and B. A. Prior. 1997. Bleaching response of sulfite pulps to pretreatment with xylanases. *Biotechnology Progress*, **13**:695-698.

Christov, L. P. and B. A. Prior. 1998. Research in biotechnology for the pulp and paper industry in South Africa. *South African Journal of Science*, **94**:195-200.

Christov, L. P., P. Biely, E. Kaligeris, P. Christakopoulos, B. A. Prior, and M. K. Bhatt. 2000. Effects of purified endo-β-1,4-xylanase of family 10 and 11 and acetyl xylan esterases on eucalypt sulfite dissolving pulp. *Journal of Biotechnology*, **83**:231-244.

Christakopoulos, D. Kekos, B. J. Macris, M. Claeyssens, and M. K. Bhatt. 1996. *Carbohydrate Research*, **289**:91-104.

Clark, T. A., D. Steward, M. E. Bruce, a. G. McDonald, A. P. Singh, and D. J. Senior. 1991. Proceedings of the 45th Appita Annual General Conference, Melbourne, Australia, Vol. 1, pp. 193.

Clark, T. A. and R. W. Allison. 1996. Characterization of process effects in enzyme-assisted bleaching. **In**: Biotechnology in the Pulp and Paper Industry – Recent advances in Applied and Fundamental Research. E. Srebotnik, and K. Messner (eds.). Facultas-Universitätsverlag, Wien, pp. 81.

Clark, J. H., J. E. Rixon, A. Cirula, H. J. Gilbert, and G. P. Gilbert. 1997. Family 10 and Family 11 xylanases differ in their capacity to enhance the bleachability of hardwood and softwood paper pulps. *Applied and Microbiology and Biotechnology*, **48**:177-183.

Colodette, J.L., U. P. Singh, A. K. Ghosh, B. Dhasmana, and R. P. Singh. 1994. Development of a new bleaching system based on the use of oxygen derived chemicals. Tappi Proceedings International Environmental Conference, April 17-20, Portland, USA, Vol. 2. pp. 513.

Comtat, J. and J. P. Joseleau. 1981. Mode of action of a xylanase and its significance for the structural investigation of the branched L-arabino-D-glucurono-D-xylan from redwood (*Sequoia sempervirens*). *Carbohydrate Research*, **95**:101-112.

Cooney, D. C., and R. Emerson. 1964. Thermophilic fungi, An account of their biology, activities and classification, Publ. Freeman and Co. San Francisco, pp. 180.

Cooney O. G. and R. Emerson. 1968. Thermophillic fungi: An account of their biology, activities and classification. pp. 80-88. W.H. Freeman and Company.

Coughlan, M. P. and G. P. Hazlewood. 1993. β-1.4-D-Xylan-degrading enzyme systems: Biochemistry, molecular biology, and applications. *Biotechnology and Applied Biochemistry*, **17**:259-289.

Craveri, R., P. L. Manachni, and A. Craveri. 1964. Eumiceti presenti nel suolo. *Annali di microbiologia ed enximologia*, 13-26.

Crisan, E. V. 1959. The isolation and identification of thermophilic fungi. MSc thesis, Perdue University.

Crisan, E. V. 1969. Isolation and culture of thermophilic fungi. Thompson Institute for Plant Research, **22**:291-301.

Crisan, E. V. 1973. Current concepts of thermophilism and thermophilic fungi. *Mycologia*, **63**:1171-1198.

Cuevas, W. A., A. Kantelinen, P. Turner, B. Bodie, and S. Leskinen. 1996. Purification and characterization of novel mannanases used in pulp bleaching. **In**: Biotechnology in the pulp and Paper Industry – Recent advances in Applied and Fundamenatal research. E. Srebotnik, and K. Messner (eds.). Facultas-Universitätsverlag, Wien, pp. 123.

Daneault, C., C. Leduc, and J. L. Valade. 1994. The use of xylanases in kraft pulp bleaching. *Tappi Journal*, **27**:125.

da Silva, R., D. K. Yim, and Y. K. Park. 1994. Purification and characterization of thermostable xylanases from thermophilic *Humicola* sp. and their application in pulp improvement. *Microbiological Reviews*, **25**:112.

Davis, S., B. Gysin, J. Casimir, W. Zimmerman. 1992. **In**: Xylans and Xylanases. Visser, J., G. Beldman, M. A. Kuster-van Someren, and A. G. J. Voragen (eds.). Elsevier, Amsterdam, pp. 551.

de Jong, E., K. K. Y. Wong, L. A. Martin, S. D Mansfield, F. M. Gama, and J, Saddler. 1996. The molecular mass distribution of the materials solubilized by xylanase treatment of Douglas-fir kraft pulp. ACS Symposium Ser. 655:44.

de Jong, E., K. K. Y. Wong, and J. N Saddler. 1997. The mechanism of xylanase prebleaching of kraft pulp: An examination using model pulps prepared by depositing lignin and xylan on cellullose fibres. *Holzforschung*, **51**:19.

de la Rosa., F. T. Vidal, A. L. Torres, and J. F. Colom. 2001a. The impact of xylanases in bleaching of Kenaf AQ pulp. Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry. June 4-8, Finlandia Hall, Helsinki, Finland, pp. 207-208.

de la Rosa., F. T. Vidal, A. L. Torres, C. Barba, D. Montané, X. Farriol, and J. F. Colom. 2001b. The use of xylanases on bleaching of *Miscanthus sinensis*. Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry. June 4-8, Finlandia Hall, Helsinki, Finland, pp. 209-210

Du Manoir, J. R., J. Hamiltom, D. J. Senior, R. L. Bernier, J. E. Grant, L. E. Moser, and P. Dubelsten. 1991. Biobleaching of kraft pulps with cellulase-free xylanase. Proceedings of International Pulp Bleaching Conference, June 11-14, Stockholm, Sweden, Vol. 2, pp. 123.

Dekker, R. F. H. and W. A. Lindner. 1979. Bioconversion of hemicellulose: Aspects of hemicellulase production by *Trichoderma reesei* QM9414 and enzyme saccharification of hemicellulose. *South African Journal of Science*, **75**:65-71.

Dekker, R. F. H. and G. N Richards. 1975. Purification, properties and mode of action of hemicellulase I produced by *Ceratocystis paradoxa*. *Carbohydrate Research*, **39**:97-114.

Deschamps, F. and M. C. Huet. 1984. β-Glucosidase production in agitated solid fermentation, study of its properties. *Biotechnology Letters*, **6**:451-456.

Dubey, A. K. and B. N. Johri. 1987. Xylanolytic activity of thermophilic *Sporotrichum* sp. and *Myceliophthora thermophilum*. Proceedings of the Indian Academy of Science, (Plant Science), **97**:247-255.

du Manoir, J. R., J. Hamilton, D. J. Senior, R. L. Bernier, J. E. Grant, L. E. Moser, and P. Dubelsten. 1991. Biobleaching of kraft pulps with cellulase-free xylanase. Proceedings of the International Pulp Bleaching Conference, June 11-14, Stockholm, Sweden, Vol. 2, pp. 123.

Dunlop-Jones, N., and V. Gronberg. 1995. Recent developments in the application of xylanase enzymes in elemental chlorine-free (ECF) and total chlorine-free (TCF) bleaching. *Pulp and Paper Canada*, **96**:20

Dunlop, R. W., B. Wang, B. Ball, P. Kumar, A. B. Roullo, C. J. Falk, and K. W. Norman. 1996. A method for selecting xylanases useful for bleach boosting Eucalyptus kraft pulp. **In**: Biotechnology in the pulp and Paper Industry – Recent Advances in Applied and Fundamental Research, E. Srebotnik, and K. Messner (eds.). Facultas-Universitätsverlag, Wien, pp. 111.

Durand, A., S. Almanza, R. Renaud, J. Maratray. 1997. *Agro-Food-Ind. Hi-Tech.*, **8**:39-42.

Eda, S., A. Ohnishi, K. Kato. 1976. Xylan isolated from the stalk of *Nicotiana tabacum*. *Agric. Biol. Chem.*, **40**:359-364.

Eligir, G., M. Sykes, and T. W. Jeffries. 1995. Different and synergistic action of *Streptomyces* endoxylanases in prebleaching of kraft pulps. *Enzyme and Microbial Technology*, **17**:954.

Eligir, G., M. Sykes, and T. W. Jeffries. 1996. Mechanism of Streptomyces xylanases in the prebleaching of kraft pulp. **In**: Biotechnology in the pulp and Paper Industry – Recent advances in Applied and Fundamental Research, (eds.) E. Srebotnik and K. Messner, Facultas-Universitätsverlag, Wien, pp. 87

Elisashvili, V. I. 1993. Biosynthesis and properties of cellulases and xylanases of higher Basidiomycetes. *Prekladnaya Biokhimya Microbiologiya*, **29**:340-352.

Emerson, R. 1968. Thermophilic fungi. **In**: The Fungi. G. C Ainsworth and A. S Sussman (eds). Vol. 3, Acad. Press, New York, pp. 105-128.

Eremeeva, T., T. Bykova, and A. Treimanis. 1995. The accessibility and the properties of wood and pulp residual hemicelluloses and lignin in different fibre wall layers. Proceedings of the 8th International Symposium on Wood Pulping Chemistry, June 6-9, Helsinki, Finland, Vol. III, pp. 225.

Eriksson, K-E. L., R. A. Blanchette, and P. Ander. 1990. Microbial and enzymatic degradation of wood and wood components. *Springer-Verlag, Berlin. ISBN*, **3**:540-516.

Eriksson, K-E. L. 1991. Three approaches to reduce the environmental impact of the pulp and paper industry. *Science Progess. Oxford*, **75**:175-189.

Eriksson, K-E. L, and J. L. Yang. 1993. Proceedings of the Tappi Environmental Conference, March 28-31, Boston, MA, Tappi Press: Atlanta, GA, Book 2, pp. 627-632.

Eriksson, K-E. L. 1998. Past success and future possibilities for biotechnology in the pulp and paper industry. 7th International Conference on Biotechnolgy in the Pulp and Paper Industry, Vancouver, BC Canada, June 16-19.

Farrell, R. L., L. Viikari, and D. Senior. 1996a. Enzyme treatments of pulp. **In**: Pulp Bleaching-Principles and Practice. C. W. Dence, and D. W. Reeve (eds.). Tappi Press, Atlanta, USA, pp. 145.

Farrell, R. L., P. Biely, and D. L. McKay. 1996b. Production of hemicellulase by the fungus Penicillium kloeckeri. **In**: Biotechnology in the pulp and Paper Industry – Recent advances in Applied and Fundamenatal research. E. Srebotnik, and K. Messner (eds.), Facultas-Universitätsverlag, Wien, pp. 69.

Fastén, H. 1993. TCF pulp at ASPA mill – History and Future. Proceedings of Non-chlorine Bleaching Conference, Merch 14-18, Hilton Head, USA, Paper No. 18.

Favis, B. D., J. M. Willis, and D. A. I. Goring. 1983. The effect of electrolyes on the leaching of lignin from unbleached kraft pulp fibres. *Journal of Wood Chemistry and Technology*, **3**:9.

Fengel, D. and G. Wegener. 1984. Wood:Chemistry, Ultrastructure, Reactions. Walter de Gruyer, Berlin.

Feniksova, R. V., A. S. Tikhomrova, and B. E. Rakhleeva. 1960. Conditions for forming amylase and pectinase in surface culture of *Bacillus subtilis*. *Mikrobiologica*, **29**:745-748.

Fergus, C. L. 1964. Thermophilic and thermotolerant moulds in mushroom composting during peak heating. *Mycologia*, **56**:267-284.

Folke, .J. 1995. International NCB Conference. Amelia Island, March 5-8.

Forber, C. L. 1992a. Twenty-first century bleaching will be a challenging lottery. *Pulp and Paper*, **66**:90.

Forber, C. L. 1992b. Chlorine and chlorine dioxide replacements in kraft pulp bleaching: Emerging technologies or laboratory curiosities? Tappi Poceedings of Pulping Conference, Nov 1-5, Boston, USA, Vol 3, pp. 1209.

Freiermuth, B., A. Seisto, and A. Gaertner. 1994. A quick test for xylanase enzyme activiity using Cibacron Red dyed pulp. Proceedings of the International Pulp Bleaching Conference, June 13-16, Vancouver, Canada, pp. 109.

Frühauf, S., G. Gamerith, and G. Ditzelmüller. 1996. Treatment of kraft and sulfite pulps with hemicellulases: A comparison of hydrolytic and bleaching properties. **In**: Biotechnology in the pulp and Paper Industry – Recent advances in Applied and Fundamental Research. E. Srebotnik and K. Messner (eds.). Facultas-Universitätsverlag, Wien, pp. 115.

Furman, G. S., and W. F. W. Lonsky. 1988. Charge-transfer complexes in kraft lignin. Part 2: Contribution to colour. *Journal of Wood and Chemical Technology*, **8**:191

Garg A. P., J. C. Roberts, and A. J. McCarthy. 1996. Biobleaching effect of *Streptomyces thermoviolaceus* xylanase preparations on birchwood kraft pulp. *Enzyme and Microbial Technology*, **18**:261-267.

Garg A. P., J. C. Roberts, and A. J. McCarthy. 1998. Bleach boosting effect of cellulase-free xylanase of *Streptomycetes thermoviolaceus* and its comparison with two commercial enzyme preparations on birchwood kraft pulp. *Enzyme and Microbial Technology*, **22**:594-598.

Gebler, J., N. R., Gilkes, M. Claeyssens, D. B. Wilson, P. Beguin, W. W. Wakarchuk, D. G. Kilburn, R. C. Miller, R. A. Warren, S. G. Withers. 1992. Stereoselective hydrolysis catalysed by related β-1,4-glucanases and β-1,4-xylanases. *Journal of Biological Chemistry*, **276**:12559-12561

Gellerstedt, G. and J. Li. 1995. Xylan degradation products from birch kraft pulp. Proceedings of the 8th International Symposium of Wood Pulping Chemistry, June 6-9, Helsinki, Finland, Vol 1, pp 533.

Gellerstedt, G. 1996. An HPLC method for the quantitative determination of hexeneuronic acid groups in chemical pulps. *Carbohydrate Research*, **294**:41-51.

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

Gessesse, A, and G. Mamo. 1998. Purification and characterization of an alkaline xylanase from alkaliphilic *Micrococcus* sp. AR-135. *Journal of Indian Industrial Microbiology and Biotechnology*, **20**:210-214.

Gessesse, A, and G. Mamo. 1999. High level xylanase production by an alkalophilic *Bacillus* sp. by using solid state fermentation. *Enzyme and Microbial Technology*, **25**:68-72.

Ghosh, M. and G. Nanda. 1993. Thermostability of β-xylosidase from *Aspergillus sydowii* M. G-49. *FEMS Microbiology Letters*, **117**:151-156.

Gibson, K. 1992. Enzymatic bleach boosting. Proceedings of the 25th Pulp and Paper Annual Meeting, ABTCP, Nov 23-27, Sao Paulo, Brazil, pp. 47.

Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. Miller Jr, and R. A. J. Warren. 1991. Domains in microbial β-1,4-glycanases: sequence conservation, function and enzyme families. *Microbiology Reviews*, **55**:303-315.

Gliese, T., S. Kleemann, and K. Fischer. 1996. Enzymatic bleaching of organosolv-pulp. **In**: Biotechnology in the Pulp and Paper Industry – Recent Advances in applied and Fundamental Research. E. Srebotnik, and K. Messner (eds.). Facultas-Universitätsverlag, Wien, pp. 63.

Godfrey, T. 1983. The application of enzymes in industry. **In**: Industrial Enzymology. Godfrey, T. and J. Reichelt (eds.). Macmillan Publ. Ltd., Basingstoke, pp. 340-351.

Gomes, J., I. Gomes, W. Kreiner, H. Esterbauer, M. Sinner, and W. Steiner. 1993a. Production of high level of cellulase-free and thermostable xylanase by a wild strain of *Thermomyces lanuginosus* using birchwood xylan. *Journal of Biotechnology*, **30**:283-297.

Gomes, J., H. Purkarthofer, M. Hoyn, M. Kapplmuller, and W. Steiner. 1993b. Production of a high level of cellulase-free xylanase by the thermophilic fungus *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. *Applied Microbiology and Biotechnology*, **39**:700-707.

Gomes, D. J., I. Gomes, and W. Steiner. 1994. Factors influencing the induction of endoxylanase by *Thermoascus aurantiacus* and partial characterization of the enzyme. *Journal of Biotechnology*, **33**:87-94.

Goncalves, A. R., D. S. Ruzene, C. M. Andrade, M. C. Damaso, and N. Pereira Jr. 2001. Bleaching of ethanol/water-bagasse pulps by using xylanase from *Thermomyces lanuginosus*. Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry. June 4-8, Finlandia Hall, Helsinki, Finland, pp. 205-206

Gong, C. S., L. F. Chen, M. C. Flickinger, L. C. Chiang, and G. T. Tsao. 1980. *Applied and Environmental Microbiology*, **41**:430.

Grajek, W. 1987a. Comparative studies on the production of cellulases by thermophilic fungi in submerged and solid state fermentation. *Applied and Microbial Biotechnology*, **26**:126-129.

Grajek, W. 1987b. Production of D-xylanases by thermophilic fungi using different methods of culture. *Biotechnology Letters*, **9**:353-356.

Grant, R. 1996. Paper improves by taking more proteins. *Pulp and Paper International*, **38**:38.

Gregoy, P. H., M. E. Lucey, G. N Festenstein, and F. A. Skinner. 1963. Microbiological and biochemical changes during the moulding of hay. *Journal of General Microbiology*, **33**:147-174.

Gronberg, V., S. Rose., D. Moody, and N. Dunlop-Jones. 1995. Biobleaching at high temperature. Proceedings of the 8th International Symposium on Wood Pulping Chemistry, June 6-9, Helsinki, Finland, Vol. II, pp. 385.

Gübitz, G. M., W. Schnitzhofer, H. Balakrishnan, and W. Steiner. 1996. Two mannanases from *Sclerotium rolfsii* in total chlorine-free bleaching of softwood kraft pulp. *Journal of Biotechnology*, **50**:181.

Gübitz, G. M., T. Lischnig, D. Stebbing, J. N. Saddler. 1997. Enzymatic removal hemicellulose from dissolving pulps. *Biotechnology Letters*, **19**:491-495.

Gutierrez-Correa, M, and R. P. Tengerdy. 1998. Xylanase production by fungal mixed culture solid substrate fermentation on sugarcane bagasse. *Biotechnology Letters*, **20**:45-47.

Haarhoff, J., C. J. Moes, C. Cerff, W. J. van Wyk, G. Gerischer, and B. J. H. Janse. 1998. Characterization and biobleaching effect of hemicelulases produced by thermophilic fungi. 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, BC, Canada, June 16-19, **C:**35-39.

Hagström-Näsi, C., R. Järvinen, J. Sundquist, and K. –M. Moilanen. 1987. Determination and characterization of the leachable lignin from unbleached kraft pulps. Proceedings of the 4th International Symposium on Wood Pulping Chemistry, April 27-30, Paris, France, Vol. 2, pp. 131.

Haltrich, D., B. Nidetzky, K. D. Kulbe, W. Steiner, and S. Zupancic. 1996. *Bioresource Technology*, **58**:137-161.

Hamilton, J., D. J. Senior, A. Rodriguez, D Santiago, J. Szwec, and A. j. Raugauskas. 1996. Improvements in ECF bleaching: Use of activated oxygen species and xylanases. *Tappi Journal*, **79**:231.

Harazona, K., R. Kondo, K. Sakai, and K. Tsuchikawa. 1996. Application of ligninolytic enzymes to bleaching of kraft pulp (III) – Bleaching of kraft pulp with manganese peroxidase combined with lignin peroxidase or xylanase. *Japanese Tappi Journal*, **50**:58.

Hartler, N. and H. Norrstrom. 1969. Light absorbing properties of pulp and pulp components. Kraft pulp. *Tappi*, **52**:1712.

Henrissat, B. and A. Bairoch. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Journal of Biochemistry*, **293**:781-788.

Hernadi, A., I. Lele, P. Zavodszky, A Nemeth and S. Kamondi. 2001. Application of xylanases from *Thermotoga maritima* in the biobleaching of straw pulp. Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry. June 4-8, Finlandia Hall, Helsinki, Finland, pp. 211-212

Hoffman, G. C. 1991. Enzymatic pulp bleaching technology. *Paper Age*, **106**:26.

Hortling, B., M. Korhonen, J. Buchert, J. Sundquist and L. Viikari. 1994. Leachability of lignin from kraft pulp after xylanase treatment. *Holzforschung*, **56**:245-249

Hoq, M. M., C. Hempel, and W. Deckwer. 1994. Cellulase-free xylanase by *Thermomyces lanuginosus* RT9: Effect of agitation, aeration, and medium components on production. *Journal of Biotechnology*, **37**:49-58.

Ito, K., H. Ogasawara, T. Sugimoto, and T. Ishikara. 1992. Purification and properties of acid stable xylanases from *Aspergillus kawachii*. *Journal of Bioscience, Biotechnology and Biochemistry*, **56**:1338-1340.

Jager, A., M. Sinner, H. Purkarthofer, H. Esterbauer, and G. Ditzelmuller. 1992. **In:** Biotechnology in the Pulp and Paper Industry. Kuwahara, M., and M. Shimada (eds.). UNI Publishers Co, Tokyo, pp. 112.

Jahan, M. S., G. Mohiuddin, S. H. Talukder, and M. Rashid. 2001. Xylanase bleaching of Non-wood Pulps. Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry. June 4-8, Finlandia Hall, Helsinki, Finland, pp. 214.

Jain, A. 1995. Production of xylanase by thermophilic fungus *Melanocarpus albomyces* IS-68. *Process Biochemistry*, **30**:705-710.

Jain, K. K., R. M. Mathur, and A. G Kulkarni. 2001. Enzymatic prebleaching of pulps:Challenges and opportunities in Indian Pulp and Paper Industry. Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry. June 4-8, Finlandia Hall, Helsinki, Finland, pp. 201-202.

Jean, P., J. Hamilton, and D. j. Senior. 1994. Mill trial experiments with xylanase: AOX and chemical reductions. *Pulp and Paper Canada*, **95**:126.

Jeffries, T. W. and M. Davis. 1998. Mechanisms for kappa reduction and colour removal. 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, BC Canada June 16-19, **C**:41-43.

Jiménez, L., E. Navarro, J. L. Ferrer, F. Lopez and J. Ariza. 1999. Biobleaching of cellulose pulp from wheat straw with enzymes and hydrogen peroxide. *Process Biochemistry*, **35**:149-157.

Johnson, A. P. 1994. Fitting together the ECF-TCF jigsaw. *Appita Journal*, **47**:243.

Johri, B. N, and T. Satyanarayana. 1986. Thermophilic moulds: Perspective in basic and applied research. *Indian Review of Life Science,* 6;75-100.

Joseleau, J. P., J. Comtat, and K. Ruel. 1992. Chemical structure of xylans and their interaction in plant cell walls. **In:** Xylans and Xylanases. J. Visser, G. Beldman, M. A. Kuster-van Someren, and A. G. J. Voragen (eds.). Elsevier, Amsterdam.

Juracek, L. and M. Paice. 1992. Saving bleaching chemicals and minimizing pollution with xylanase. Proceedings of the International Symposium of Pollution Prevention in the Manufacture of Pulp and Paper - Opportunities and Barriers, Washington D.C.

Kalogeris, E., P. Christakopoulos, D. Kekos, and B. J. Macris. 1998. Studies on the solid state production of thermostable endoxylanases from *Thermoascus aurantiacus*: Characterization of two isoenzymes. *Journal of Biotechnology*, **60**:155-163.

Kantelinen, A., M. Ratto, J. Sundquist, M. Ranua, L. Viikari, and M. Linko. 1988. Hemicellulases and their potential in bleaching. Proceedings of the International Pulp Bleaching Conference, Orlando, FL, Tappi, pp. 1-5.

Kantelinen, A., J. Sundquist, M. Linko, and L. Viikari. 1992. The proposed mechanism of enzymatic bleaching of kraft pulps with xylanases. *Holzforschung*, **47**:318-324.

Kantelinen, A., B. Hortling, J. Sundquist, M. Linko and L. Viikari. 1993. Proposed mechanism of enzyme aided bleaching of kraft pulp with xylanases, *Holzforchung,* **47**:318-324.

Karlsson, O., and U. Westermark. 1996. Evidence for chemical bonds between lignin and cellulose in kraft pulps. *Journal of Pulp and Paper Science*, **22**:397.

Karlsson, O., B. Pettersson, and U. Westermark. 2001. The use of cellulases and hemicellulases to study lignin-celluloses as well as lignin-hemicellulose bonds in kraft pulps. *Journal of Pulp and Paper Science*, **27**:196-201.

Kerr, A. J. and D. A. I. Goring. 1975. The role of hemicellulose in the delignification of wood. *Canadian Journal of Chemistry*, **53**:952.

Khandke, K. M., P. J. Vithayathil, and S. K. Murthy. 1989. *Achives in Biochemistry and Biophysics*, **274**:491-500.

Kibblewhite, R. P. and T. A. Clark. 1996. Enzymatic modification of radiata pine kraft fibre and handsheet properties. *Appita Journal*, **49**:390.

Kitpreechavanich, V., M. Hayashi, and S Nagai. 1984. Purification and properties of endo-1,4-β-xylanase from *Humicola lanuginosa*. *Journal of Fermentation Technology*, **62**:415-420.

Kleupfel, D., N. Daigneault, R. Morosoli, and F. Shareck. 1992. Purification and characterization of a new xylanase (xylanase C) produced by *Streptomyces lividens* 66. *Applied Microbiology and Biotechnology*, **36**:626-631.

Kondo, R., K. Harazono, and K. Sakai. 1994. Bleaching of hardwood kraft pulp with manganese peroxidase secreted from *Phanerochaete sordida* YK-624. *Applied and Environmental Microbiology*, **60**:4359.

Koponen, R. 1991. Enzyme systems prove their potential. *Pulp and Paper International*, **33**: 20-25.

Kormelink, F. J. M., M. J. F. Searle-Van Leewen, T. M. Wood and A. G. J. Voragen. 1993. Purification and characterization of three endo-1,4-β-xylanases and one β-xylosidase from *Aspergillus awamori*. *Journal of Biotechnology*, **27**:249-265.

Kovasin, K. K. and P.O. Tikka. 1992. Superbatch cooking results in superlow kappa numbers. Paper presented at the *4th International Conference of new Available techniques and Current trends*, Bologna, Italy, 19-22 May.

Kringstad, K. P. and K. Lindstrom. 1984. Spent liquors from pulp bleaching. *Environmental Science Technology*, **18**:236.

Kukkonen, K. and I. Reilama. 1993. Experiences and conclusions with TCF bleaching at Metsä-Botnia. Proceedings of Non-chlorine Bleaching Conference, March 14-18, Hilton Head, USA.

Kulkarni. N, and M. Rao. 1996. Application of xylanase from alkaliphilic *Bacillus* sp. NCIM 59 in biobleaching of bagasse pulp. *Journal of Biotechnology*, **51**:167-173.

Lahtinen, T., P. Ojapalo, A. Laukkanen, and D. Senior. 1993. Enzymes in RCF bleaching – Use of xylanase at temperatures suitable for 100% ClO_2 bleaching sequence. Preprints Spring Conference CPPA, Pacific Coast Western Branches, May 26-30, Whistler, Canada, pp. 2-3.

Laine, J., P. Stenius, G. Carlsson, and G Ström. 1994. Surface characterization of unbleached kraft pulps by means of ESCA. *Cellulose*, **1**:145.

Laine, J., J. Buchert, L. Viikari, and P. Stenius. 1996. Characterization of unbleached kraft pulps by enzymatic treatment, potentiometric titration and polyelectrolyte adsorption. *Holzforschung*, 50:208.

Lavielle, P., M. Koljonen, P. Piiroinen, R. Koponen, D. Reid, and R. Fredriksson. 1992. Three large scale uses of xylanases in kraft pulp bleaching. Proceedings of the 4th International Conference of New Available Techniques and Current trends, pp. 203-15

Lavielle, P. 1993. Xylanase prebleaching technology – an innovative answer to chlorine-less and chlorine-free bleaching of kraft pulps. Proceedings of EUCEPA International Environmental Symposium, April 27-28, Paris, France, Vol. 1, pp. 151

Ledoux, P., R. Detroz, E. deBuyl, J. Shetty, N. Troughton, and J. R. Presley. 1993. Use of bacterial xylanase in chlorine-free bleaching sequences. Tappi Proceedings Pulping Conference, Nov 1-3, Atlanta USA, Vol. 3, pp. 1057.

Leduc, C., C. Daneault, P. Delaunois, C. Jaspers,and M. Pennickx. 1995. Enzyme pretreatment of kraft pulp to reduce consumption of bleach chemicals. *Appita Journal*, **48**:435.

Lee. Y. Y., C. M. Lin, T. Johnson, and R. P Chambers. 1978. *Biotechnology and Bioengineering Symposium*, 8:75.

Leite, M., T. Eremeeva, A. Treimanis, V. Egle, T. Bykova, L. Purina, and U. Viesturs. 1995. The effects of xylanase type enzymes on the different layers on birch wood organosolv-pulp fibre walls. *Acta Biotechnology*, **15:**199.

Li, J., M. G. Paice, J. M. McLeod, and L. Jurasek. 1996. Bleachability improvements of kraft pulp by alkaline leaching and xylanase treatment. *Journal of Pulp and Paper Science*, **22**:207.

Li, K., P. Azadi, R. Collins, J. Tolan, J. S. Kim, and K- E. L. Eriksson. 2000. Relationship between activities of xylanases and xylan structures. *Enzyme and Microbial Technology*, **27**:89-94.

Lin, J., L. M. Ndlovu, S. Singh, and B. Pillay. 1999. Purification and biochemical characteristics of β-D-xylanase from thermophilic fungus, *Thermomyces lanuginosus* SSBP. *Journal of Biotechnology and Applied Biochemistry*, **30**:71-79.

Lindberg, B., K. –G. Rosell, and S. Svensson. 1973. Position of the O-acetylgroups in birch xylan. *Svensk. Papperstid*, **76**:30-32.

Loginova, L. G., and A. E. Kosmachev, R. S. Golovacheva, and I. M. Seregina. 1962. Issledovanie termofil'nol microflory gory yangan, Tan ha Yuzhnom Urale, *Mikrobiologiya*, **31**:1080-1086.

Lonsane, B. K., N. P. Ghildyal, S. Budaitman, and S. V. Ramakrishna. 1985. Engineering aspects of solid substrate fermentation. *Enzyme and Microbial Technology,* **7**:258-265.

Lundgren, K. R., L. Bergkvist, S. Högman, H. Jöves, G. Eriksson, T. Bartfai, J. van der Laan, E. rosenberg, and Y. Shoman. 1994. TCF mill trial on softwood pulp with Korsnäs thermostable and alkaline stable xylanase T6. *FEMS Microbiology Reviews*, **13**:365.

Luonteri, E., M. Tenkanen, M. Siika-aho, J. Buchert and L. Viikari. 1996. α-arabinosidases of Aspergillus terreus and their potential in pulp and paper applications. **In**: Biotechnology in the pulp and Paper Industry – Recent advances in Applied and Fundamenatal research. E. Srebotnik, and K. Messner (eds.). Facultas-Universitätsverlag, Wien, pp. 119.

Luthi, E., N. B. Jasmat, and P. L. Bergquist. 1990. Xylanase from the extremely thermophilic bacterium *Caldocellum saccharolyticym*: Overexpression of the gene in *E. coli* and characterization of the gene product. *Applied and Environmental Microbiology*, **56**:2677-2683.

Maat, J., M. Roza, J. Verbakel, H. Stam, M. J. Santos da Silva, M. Bosse, M. R. Egmond, M. L. D. Hagemans, R. F. M. Gorcom, J. G. M. Hessing, C. A. M. J. J. Hondel, and C. Rotterdam. 1992. **In**: Xylans and Xylanases. Visser, J., G. Beldman, M. A. Kusters-van Someren, and A. G. J. Voragen (eds.). Elsevier, Amsterdam, p. 349-360..

Madlala, A. M., S. Bissoon, S. Singh and L. Christov. 2001. Xylanase-induced reduction of chlorine dioxide consumption during elemental chlorine-free bleaching of different pulp types. *Biotechnology Letters*, **23**:345-351.

Maheshwari, R., P. T. Kamalam. 1985. Isolation and culture of thermophilic fungi, *Melanocarpus albomyces* and factors influencing the production and activity of xylanase. *Journal of General Microbiology*, **131**:3017-3027.

Maheshwari, R., P. T. Kamalam, and P. V. Balasubrahmanyam. 1987. The biogeography of thermophilic fungi. *Current Science*, **56**:151-155.

Maheshwari, R. 1997. **In**: The ecology of thermophilic fungi. K. K. Janardhanan, C. Rajendran, K. Natarajan and D. L. Hawksworth (eds.). *Tropical mycology,* Oxford and IBH publishers, Dehli, pp. 277-289.

Maheshwari, R., G. Bharadwaj, and M. K. Bhat. 2000. Thermophilic Fungi: Their Physiology and Enzymes. *Microbiology and Molecular Biology Reviews*, **64**:461-488.

Margartitis, A., and R. Merchant. 1983. Biotechnology and Bioengineering Symposium, **13**:299-314.

Matsuo, M., T. Yashui, and T. Kobayashi. 1975. Production and saccharification action for xylan of xylanase from *Malbranchea pulchella* var. *sulfurea* no 48. *Nippon Nagaikagaikagaku Kaishi*, **49**:263-270.

McCleary, B. V. 1986. Application of endolytic hydrolases in the hydrolysis of xylan. *International Journal of the Biology of Macromolcules*, **8**:349-354.

McDonough, T. J. 1995. Recent advances in bleached chemical pulp manufacturing technology. Part 1: Extended delignification, oxygen delignification, enzyme applications, and ECF and TCF bleaching. *Tappi Journal*, **78**:55.

McQuiken, M. P., S. P. Budge, and J. M. Whipps. 1997. Production, survival and evaluation of liquid culture produced inocula of *Coniothyrium minitans* against *Sclerotina sclerotiorum*. *Biocontrol Science Technology*, **7**:23-36.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry,* **31**:238-244.

Merchant, R., F. Merchant, and A. Margaritis. 1988. Production of xylanase by the thermophilic fungus, *Thielavia terrestris*. *Biotechnology letters*, **10**:513-516.

Montgomery, R., F. Smith, and H. C. Srivastava. 1956. Structure of corn hull hemicellulose I. Partial hydrolysis and identification of 2-*O*-(α-D-glucopyranosyluronic acid)-D-xylopyranose. *Journal of the American Chemical society*, **78**:2837-2839.

Mora, F., J. Comtat, F. Barnoud, F. Pla, and P. Noe. 1986. Action of xylanases on chemical pulp fibres. Part I: Investigations on cell wall modifications. *Journal of Wood and Chemistry Technology*, **6**:147.

Munk, N., A. M. Nissen, T. Vollmond, and H. Lund. 1993. Bleach boosting with xylanases: Recent research results. Proceedings of the 47th Appita Annual General Conference, April 19-23, Rotorua, New Zealand, Vol 2, pp 257.

Narahara, H., Y. Koyama, T. Yoshida, S. Pichangkura, R. Ueda, and H. Taguchi. 1982. Growth and enzyme production in a solid state culture of *Aspergillus oryzae*. *Journal of Fermentation Technology*, **60**:311-319.

Nelson, S. L., K. K. Y. Wong, J. N. Saddler and R. P Beatson. 1995. The use of xylanase for peroxide bleaching of kraft pulps derived from different softwood species. *Pulp and Paper Canada*, **96**:42-45

Niku-Paavola, M.-L., M. Ranua, A. Suurnäkki, and A. Kantelinen. 1994. Effects of lignin modifying enzymes on pine kraft pulp. *Bioresource Technology*, **50**:73.

Orgill, B., and W. Pichler. 1992. Recent developments in non-wood fibre pulping and bleaching. Proceedings of the 2nd international Nonwood Fibre Pulping and Paper making Conference, April 6-9, Shanghai, China, Vol. 1, pp. 277.

Paice, M. G. and L. Juracek. 1984. Removing hemicellulose from pulps by specific enzymatic hydrolysis. *Journal of Wood Chemistry and Technology*, **4**:187-198.
Paice, M., M. Berier, and L. Juracek. 1988. Viscosity enhancing bleaching of hardwood kraft pulp with xylanase from a cloned gene. *Biotechnology and Bioengineering*, **32**:235-239.

Paice, M. G., R. Bourbonnais, and I. D. Reid. 1995. Bleaching of kraft pulps with oxidative enzymes and alkaline hydrogen peroxide. *Tappi Journal*, **78**:161.

Pandey, A. 1992. Recent process developments in solid-state fermentation. *Process Biochemistry*, **27**:109-117.

Panday, A., P. Selvakumar, C. R. Saccol, and P. Nigam. 1999. Solid state fermentation for the production of industrial enzymes. *Current Science*, **77**:149-162.

Panday, A., C.R. Soccol, and D Mitchell. 2000. New developments in solid state fermentation: I-bioprocesses and products. *Process Biochemistry*, **35**:1153-1169.

Patel, R. N., A. C. Grabski, and T. W. Jeffries. 1993. Chromophore release from kraft pulp by purified *Streptomyces roseiscleroticus* xylanases. *Applied Microbiology and Biotechnology,* **39**:405-412.

Pederson, L. S., P. Kihlgren, A. M. Nissen, N. Munk, J. C. Holm, and P. P. Choma. 1992. Proceedings of the Tappi Pulping Conference, Boston, M. A, Nov 1-5, Tappi Press: Atlanta, GA, Book 1, pp. 31.

Pham, P. L., Y. Alric, and M. Delmas. 1995. Incorporation of xylanase in total chlorine-free bleach sequences using ozone and hydrogen peroxide. *Appita Journal*, **48**:213-217

Pigman, W. W., and W. R. Csellak. 1948. A study of the origin of kraft colour. *Technical Association of Paper*, **31**:393.

Poutanen, K., J. Puls, and M. Linko. 1986. The hydrolysis of steamed birchwood hemicellulose by enzymes produced by *Trichoderma reesei* and *Aspergillus awamori*. *Applied Microbiology and Biotechnology*, **23**:487-490.

Prasad, D. Y., N. R. M. Rao, K. S. Rajesh, T. T. Praburaj, and T. W. Joyce. 1996. Enzymes improve the bleachability of bagasse mechanical pulp. *Tappi Journal*, **79**:133-138.

Puls, J. and K. Poutanen. 1989. Mechanisms of enzyme hydrolysis of hemicelluloses (xylans) and procedures for determination of the enzyme activities involved. **In**: Enzyme

systems for lignocellulose degradation. Coughlan, M. P (ed.). Elsevier Applied Science, London, pp151-165.

Puls, J. and J. Schuseil. 1993. Chemistry of hemicelluloses: relationship between hemicellulose structure and enzyme required for hydrolysis. **In**: Hemicelloses and Hemicellulases. Coughlan, M. P., and G. P. Jazlewood, (eds.). Portland Press, London, pp. 1-27.

Purkarthofer, H., M. Sinner, and W. Steiner. 1993. Cellulase-free xylanase from *Thermomyces lanuginosus*: Optimization of production in submerged and solid-state culture. *Enzyme and Microbial Technology* **15:**677-682.

Pyke, D. G., and D. J. McKenzie. 1996. Substitution of chlorine for chlorine dioxide in canadian bleached chemical pulp mills. *Pulp and Paper Canada*, **97**:27.

Ragauskas, A. J., K. M. Pou, and A. K. Cestnino. 1994. Effect of xylanase pretreatment procedures on non-chlorine bleaching. *Enzyme and Microbial Technology*, **16**:492.

Ragauskas, A. J. 2001. Biotechnology in the Pulp and Paper Industry: A Challenge for Change. Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry, June 4-8, Finlandia Hall, Helsinki, Finland, pp.15-16.

Raimbault, M. and D. Alazard. 1980. Culture method to study fungal growth in solid fermentation. *European Journal of Applied Microbiology and Biotechnology*, **9**:199-209.

Ramesh, M. V. and B. K. Lonsane. 1989. Solid state fermentation for production of higher titres of thermostable α-amylase with two peaks for pH optima by *Bacillus licheniformis* M27. *Biotechnololgy Letters*, **11**:49-52.

Rana, B. K., B. N. Johri, and I. S. Thakur. 1996. Formation and activities of xylan-hydrolysing enzymes of *Humicola griesea* var. *thermoidea*. *World Journal of Microbiology and Biotechnology*, **17**:114-118.

Rathburn, B.and M. L. Shuler. 1980. *Biotechnology and Bioengineering*, **22**:2219.

Reeve, D. W. 1989. Bleaching Technology. *Pulp and Paper Manufacture*, **5**:391.

Reichelt, Jr. 1983. The application of enzymes in industry. **In**: Industrial Microbiology. Godfrey, T. and J. Reichelt (eds.). Macmillan Publishers Ltd, Basingstoke, pp. 210-220.

Roberts, J. C., A. J. McCarthy, N. Flynn, and P. Botha. 1990. Modification of paper properties by the pretreatment of pulp with *Saccharomonospora viridis* xylanase. *Enzyme and Microbial Technology*, **12**:210-213.

Robinson, J. V. 1975. A summary of reflectance equations for application of the Kubelka-Munk theory to optical properties of paper. *Tappi Journal*, **58:**152.

Roche, N., C. Desgragnes, and A. Durand. 1994. Study on the solid state production of a thermostable α-L-arabinofuranosidase of *Thermoascus aurantiacus* on sugar beet pulp. *Journal of Biotechnology*, **38**:43-50.

Roncero., M. B, T. Vidal, A. L. Torres and F. J Colom. 1996. Use of xylanases in the totally chlorine free bleaching of Eucalyptus kraft pulp. **In**: Enzymes for Pulp and Paper Processing. Jeffries T. W, L. Viikari (eds.). ASC Symposium series 655, American Chemical Society, Washington, pp 219-227.

Roncero, M. B., A. L. Torres, J. F. Colom, T. Vidal. 2000. Effects of xylanase treatment on fibre morphology in totally chlorine free bleaching (TCF) of Eucalyptus pulp. *Process Biochemistry*, **36**:45-50.

Royer, J. C., and J. P. Nakas. 1989. Xylanase production by *Trichoderma longibrachiatum*. *Enzyme and MIcrobial Technology*, **11**:405-411.

Rydholm S. A. 1965. *Pulping processes.* Wiley-Interscience, New York.

Saake, B., T. Clark, and J. Puls. 1995. Investigations on the reaction mechanism of xylanases and mannanses on sprucewood chemical pulps. *Holzforschung*, **49**:60.

Sabharwal, H. S. and R. A. Young. 1996. International agro-fibre research initiative. *Tappi Journal*, **79**:66-67.

Satyanarayana, T. 1994. Production of bacterial extracellular enzymes by solid state fermentation. **In**: Solid State Fermentation. A. Pandey (ed.). Wiley Eastern Ltd, New Delhi, India, pp. 122-129.

Schwartz, H., J. L. McCarthy, and H. Hibbert. 1940. The colouring matter of kraft and its influence in bleaching. *Paper Trends Journal*, **111**:30.

Scott, G. M., M. Akhtar, M. J. Lentz, and R. E. Swaney. 1998. Engineering aspects of fungal pretreatment for wood chips. **In**: Environmental Friendly Pulping and Bleaching Methods, New York.

Senior, D. J. and J. Hamilton. 1992. Bleaching with xylanases brings biotechnology in reality. *Pulp and Paper*, **66**:111.

Senior, D. J., J. Hamilton, and R. L. Bernier Jr. 1992. Use of *Streptomyces lividens* for biobleaching of kraft pulps. *Progress in Biotechnology*, **7**:555-557.

Senior, D. J. and J. Hamilton. 1993. Xylanase treatment of the bleaching of softwood kraft pulps: the effect of chlorine dioxide consumption. *Tappi Journal*, **76**:200.

Senior, D. J., J. Hamilton, T. Pasi and T. Jaana. 1999. Enzyme use can lower bleaching costs, aid ECF conversions. *Pulp and Paper,* **73**:59.

Shah, A. K., S. S. Sidid, A. Ahmad, and M. V. Rele. 1999. Treatment of bagasse pulp with cellulase-free xylanases from an alkalophilic *Bacillus* sp. SAM-3. *Bioresource Technology*, **68:**133-140.

Shah, A. K., D. Cooper, R. Adolphson and K.- E. L. Eriksson. 2000. Xylanase treatment of oxygen-bleached hardwood kraft pulp at high temperature and alkaline pH levels gives substantial savings in bleaching chemicals. *Journal of Pulp and Paper Science*, **26**:8-11.

Sheehan, J., and M. E. Himmel. 1999. *Progress Biotechnology*, **15**:817-827.

Shoham, T., Z. Zosim., and E. Rosenberg. 1993. Partial decolourization of kraft pulp at high temperature and at high pH values with an extracellular xylanase from *Bacillus stearothermophilus*. *Journal of Biotechnology*, **30**:123.

Silva, M. R. and J. L. Colodette. Impact of the bleaching process on kraft pulp mill closure. 1997. Proceedings of the Fifth Brazilian Symposium on the Chemistry of Lignins and other Wood Components. 31 August to 5 December, Bourbon Hotel, Curitiba. P R, Brazil, pp 193-203.

Simpson, H. D., U. R. Haufler, and R. M. Daniel. 1991. *Journal of Biochemistry*, **277**:413.

Singh R. P. 1993. Bleaching. **In**: Pulp and Paper Manufacture: Secondary fibres and non-wood pulping. F. Hamilton, and B. Leopold (eds.). Joint Textbook Committee of the Paper Industry, pp. 234-237.

Singh, S., B. Pillay, and B. A. Prior. 1995. Characterization of high activity xylanase from a thermophilic *Thermomyces lanuginosus* SSBP. 6th International Conference on Biotechnology in the Pulp and Paper Industry, Vienna, June 11-15, pp. 238.

Singh, S., B. Pillay, V. Dilsook, and B. Prior. 2000a Production and properties of hemicellulases by a *Thermomyces lanuginosus* strain. *Journal of Applied Microbiology*, **88**:975-982.

Singh, S., B. Pillay, and B. A. Prior. 2000b. Thermostability of β-xylanases produced by different *Thermomyces lanuginosus* strains. *Enzyme and Microbial Technology*, **26**:502-508.

Smith, A. J., R. R. Gustafson, and W. T. McKean. 1993. Enhanced lignin removal in brownstock washing. *Tappi Journal*, **76**:81.

Smook, G. A. 1992. Handbook for Pulp and Paper Technologist, 2nd Edition, Anges Wilde Press, Vancouver, Canada.

Stanton, W. R. 1978. **In**: Global impacts of applied microbiology. W. R. Stanton (ed.), Kualalumpur, Malaysia, pp. 180-189.

Sterker, P. S., R. L. Farrell, and H.-m. Chang. 1991. Chlorine-free bleaching with Cartazyme^{TM} HS treatment. Proceedings of the International Pulp Bleaching Conference, June 11-14, Stockholm, Sweden. Vol. 2. pp. 93.

Stone, J. E. and A. M. Scallan. 1968. The effect of component removal upon the porous structure of the cell wall of wood. Part III. A comparison between the sulfite and kraft processes. *Pulp and Paper Magazine Canada*, **69**:288.

Stone, J. E., A. M. Scallan, E. Donefer, E. Ahlgren. 1969. Digestibility as a simple function of a molecule of similar size to a cellulase enzyme. **In**: Cellulases and their Applications. Hajny, G. J. and R. T. Reese (eds.). American Chemical Society, Washington, DC, pp. 219-24.

Subrahmanyam, A. 1999. *Thermophilic moulds in Biotechnology*. **In**: Ecology and Distribution. B. N Johri, T. Satyanarayana, and J. Olsen (eds.). 13-42.

Sugden, C. and M. K. Bhatt. 1994. *World Journal of Microbiology and Biotechnology*, **10**:444-451.

Sunna, A. and G. Antranikian. 1997. Xylanolytic Enzymes from Fungi and Bacteria. *Critical Reviews in Biotechnology*, **17**:39-67.

Suurnäkki, A., A. Kantelinen, J. Buchert, L. Viikari. 1994. Enzyme-aided bleaching of industrial softwood kraft pulps. *Tappi Journal*, **77**:111.

Suurnäkki, A., T. A. Clark, R. W Allison, L. Viikari, and J. Buchert. 1996. Xylanase and mannanase-aided ECF and TCF Bleaching. *Tappi Journal*, **79**:111.

Tan, L. U. L, P. Mayers, and J. N. Saddler. 1987. Purification and characteristics of a thermostable xylanase from a thermophilic fungus, *Thermoascus aurantiacus*. *Canadian Journal of Microbiology*, **33**:689-692.

Teleman, A., V. Harpunpä, M. Tenkanen, J. buchert, T. Hausalo, T. Dragenberg, and T. Vuorinen. 1996. Characterization of 4-deoxy-β-L-*threo*-hex-4-enopyranosyluronic acid attached to xylan in pine kraft pulp and pulping liquor by ^{1}H and ^{13}C NMR spectroscopy. *Carbohydrate Research*, **272**:55.

Tenkanen, M., J. Puls, and K. Poutanen. 1992. Two major xylanases of *Trichoderma reesei*. *Enzyme and Microbial Technology*, **14**:566-574.

Timell, T. E. 1964. Wood hemicelluloses I. *Advances in Carbohydrate Chemistry*, **19**:247-302.

Tolan, J. S. 1992. The use of enzymes to enhance pulp bleaching. Tappi Proceedings of Pulping Conference, Nov 1-5, Boston, USA, Vol. 1. pp. 13.

Tolan, J. S. and R. V. Canovas. 1992. The use of enzymes to decrease the Cl_2 requirements in pulp bleaching. *Pulp and Paper Canada*, **93**(5):39.

Tolan, J. S. 1995. Pulp quality impact of contaminating cellulase in commercial xylanase preparations. *Journal of Pulp and Paper Science*, **21**:132.

Tolan, J. S., D. Olson, and R. E Dines. 1995. Survey of xylanase enzyme usage in bleaching in Canada. *Pulp and Paper Canada*, **96**: 107-110.

Tolan, J. S., D, Olson, and R. E. Dines. 1996. Survey of mill usage of xylanase. ASC Symposium, serial 665:24-35

Tolan, J. S. 2001. How a mill can ge more benefit out of its xylanase treatment. Proceedings of the 8th International conference on Biotechnology in the Pulp and Paper Industry. June 4-8, Finlandia Hall, Helsinki, Finland, pp.81.

Tuohy, M. G., C. D. Laffey, and M. P. Coughlan. 1994. Characterization of the individual components of the xylanolytic enzyme system of *Talaromyces emersonii. Bioresource Technology*, **50**:37-42.

Turner, J. C., P. S. Sterker, B. J. Burns, H. C. Howard, M, A, Alonso, and J. L. Andres. 1992. Bleaching with enzymes instead of chlorine-mill trials. *Tappi Journal*, **75**:83.

Uffen, R. L. 1997. Xylan degradation : A glimpse at microbial diversity. *Journal of Industrial Microbiology and Biotechnology*, **19**:1-6.

Vicuña, R., M. C. Yeber, and M. Osses. 1995. Bleaching of radiata pine kraft pulp with the Enzone process. *Journal of Biotechnology*, **42**:69.

Vidal, T., A. L. Tores, J. F. Colom, and J. Siles. 1997. Xylanase bleaching of eucalyptus kraft pulps – an economical ECF process. *Appita Journal*, **50:**144.

Viikari, L., M. Ranua, A. Kantelinen, M. Linko, and J. Sundquist. 1986. Bleaching with enzymes. Protocol of the 3rd International Conference on Biotechnology in the Pulp and Paper Industry, Stockholm, pp. 67-69.

Viikari, L., M. Ranua, A. Kantelinen, M. Linko. and J. Sundquist. 1987. Application of enzymes in bleaching. Proceedings of the 4th International Symposium on *Wood and Pulping Chemistry*, Vol. 1, pp. 151-154.

Viikari, L., M. Tenkanen, J. Buchert, M. Ratto, M. Biely, M. Siika-aho, and M. Linko. 1993. Hemicellulases for Industrial Applications. **In**: Bioconversion of Forest and Agricultural Plant Residues. pp. 131-182. Saddler, J. N. (ed.). CAB International, Oxford.

Viikari, L., A. Kantelinen, J. Sundquist, and M. Linko. 1994. Xylanases in bleaching: From an idea to the industry. *FEMS Microbiological Reviews*, **13**:335-350

Viikari, L., A. Suurnaki, and J. Buchert. 1996. Enzyme aided bleaching of kraft pulps: Fundamental mechanisms and prcatical applications. **In**: Jeffries, T. W, L. Viikari (eds). Enzymes for Pulp and Paper Processing. ACS Symposium series 655, Washington, pp. 15-24.

Weiland, P. 1988. Principles of solid state fermentation. **In**: Treatment of hemicellulosics with white-rot fungi (Eds.) Zadrazil, F., Reiniger, P. Elsevier, London, pp 64-76.

Whistler, R. L. and E. L. Richards. 1970. Hemicelluloses. **In**: The Carbohydratres. Pigman, W. and D. Horton (eds.). Vol. 2a, Academic Press, New York, pp. 447-469.

Wilcox, P. R. and D. A. I. Goring. 1990. The effect of pH on the leaching of lignin on kraft and chemimechanical pulps. *Cellul. Chem. Technol.*, **24**:735.

Wong, K. K. Y., L. U. L. Tan, and J. N. Saddler. 1988. Multiplicity of ß-1,4-xylanase in microorganisms: function and applications. *Microbiological Reviews*, **52**:305-317.

Wong, K. K. Y. and J. N. Saddler. 1992. *Trichoderma* xylanases, their properties and application. *Critical Reviews in Biotechnology*, **12**:413.

Wong, K. K. Y., S. L. Nelson and J. N. Saddler. 1996. Xylanase treatment for the peroxide bleaching of oxygen delignified kraft pulps derived from three softwood species. *Journal of Biotechnology*, **48**:137

Wong, K. K. Y., E. de Jong, J. N Saddler, and R. W. Allison. 1997a. Mechanisms of xylanase-aided bleaching of kraft pulp. Part 1. *Appita*, **50**:415-422

Wong, K. K. Y., E. de Jong, J. N Saddler, and R. W. Allison. 1997b. Mechanisms of xylanase-aided bleaching of kraft pulp. Part 2 Target substrates. *Appita Journal*, **50**:509-518.

Wong, K. K. Y., L. A. Martin, F. M. Gama, J. N. Saddler, and E. de Jong. 1997c. Bleach boosting and direct brightening by multiple xylanase treatments during peroxide bleaching of kraft pulps. *Biotechnology and Bioengineering*, **54**(4):312-318.

Wong. K. K. Y., R. W. Allison, and S. Spehr. 2001. Effects of alkali and oxygen extractions of kraft pulp on xylanase-aided bleaching. *Journal of Pulp and Paper Science*, **27**(7): 229-234.

Woodward, J. 1984. Xylanases: functions, properties and applications. *Top. Enzyme Ferment. Biotechnol.* **8**:9-30.

Wright, J. D. 1998. Future directions and research needs of the pulp and paper industry. 7th International Conference on Biotechnology in the Pulp and Paper Industry, Vancouver, BC Canada, June 16-19, **A**:3-6.

Wright, P. J. and A. F. A Wallis. 1996. Rapid determination of carbohydrates in hardwoods by high performance anion exchange chromatography. *Holzforschung*, **50**:518-524.

Yamada., K. 1977. Japan's most advanced industrial fermentation technology and industry. International Technical Information Institute, Tokyo.

Yamasaki, T., S. Hosoya., C.-L. Chen, J. S. Gratzl, and H.-m. Chang. 1981. Characterization of residual lignin in kraft pulp. Proceedings of the International Symposium od Wood Pulping Chemistry, June 9-12, Stockholm, Sweden, Vol II, pp. 34.

Yang, J. L. and K. E.-L. Eriksson. 1992. Use of hemicellulolytic enzymes as one stage in bleaching of kraft pulps. *Holzforschung*, **46**:481.

Yang, J. L., G. Lu, and K. E.-L. Eriksson. 1992. The impact of xylanases on bleaching of kraft pulps. *Tappi Journal*, **75**:95-101.

Yang, J. L., D. H. Cates, S. E. Law, and K.- E. L. Eriksson. 1994. Bleaching of softwood kraft pulps with the Enzone process. *Tappi Journal*, **77**(3):243.

Yoshioka, H., N. Nagato., S. Chavanich, N. Nilubol, and S. Hayashida. 1981. Purification and properties of thermostable xylanase from *Talaromyces byssochlamydoides* YH-50. *Agric. Biol. Chem*, **45**:2425-2432.

Yu. E. K. C., L. U. L. Tan, M. K. H. Chan, L. Deschatelets, and J. N. Saddler. 1987. Production of thermostable xylanase by a thermophilic fungus *Thermoascus aurantiacus*. *Enzyme and Microbial Technology*, **9**:16-24.

Yu, X., J. L. Minor, R. H. Atalla, M. Labbauf, and R. L. Farrell. 1998. Effect of hemicellulases on unbleached softwood kraft pulp. Proceedings of the International Pulp Bleaching Conference, June 13-16, Vancouver, Canada, pp. 63.

Zamost. B. L., H. K. Nielsen, and R. L Starnes. 1991. Thermostable enzymes for industrial applications. *Journal of Industrial Microbiology*, **8:**71-82.

Zheng, L., Y. Du. J. Zhang. 2000. Biobleaching effect of xylanase preparation from an alkalophilic *Bacillus* sp. on ramie fiber. *Biotechnology Letters*, **22**:1363-1367.

Zeikus, J. G., C. Lee, and B. C. Saha. 1991. Applications of hemicellulases in the food, feed, and pulp and paper industries. ACS Symposium, pp.36-51.

Zimbo, M., and T. E. Timell. 1967. Studies on a native xylan from Norway spruce (*Picea abies*) I. Isolation and constitution. *Svensk Papperstid*, **70**:695-701.

Zimmermann, W. 1992. Bacterial degradation of hemicelluloses. *Metal Ions Biol. Syst*, **28**:357-398.

Ziobro, G. C. 1990. Origin and nature of kraft colour: 2. The role of bleaching in the formation of the extraction stage effluent colour. *Journal of Wood and Chemical Technology*, **10**:151.