FUNGAL UTILISATION OF PULP MILL WASTE WATER FOR XYLANASE PRODUCTION

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M.Sc. (UFS)

Submitted in fulfilment of the requirements for the degree

PHILOSOPHIAE DOCTOR

In the Faculty of Natural and Agricultural Sciences, Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State, Bloemfontein, South Africa

May 2005

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“Every great advance in science has issued from a new audacity of imagination.....”

John Dewey (1859-1952)

I dedicate this thesis to my parents.

Their patience and unwavering support has been my pillar of strength.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to:

Prof. J.C. du Preez for his never ending support, invaluable guidance and constructive criticism during the course of the study. I would really like to thank him for not giving up on me, even in the darkest of times.

Prof. L. Christopher for his insightful contribution, especially in matters pertaining to pulp and paper.

Prof. S.G. Killian for the suggestions offered during our group discussions.

Prof. G. Szakacs for assistance in the initial screening of fungal strains

Mr. P.J. Botes for his technical assistance with the chromatographic analyses

Ms. Y. Makaum for handling the oddest of requests and being patient with me in the lab and Ms. L. Steyn for her invaluable assistance with my bioreactor work.

Dr. A. Hugo for his assistance in the statistical analyses

Fellow students and staff in the Fermentation and Sappi Biotechnology Groups.

The staff and students of the Extreme Biochemistry Group for their invaluable contribution with the SDS page and zymogram analyses.

My family and friends for being there for me throughout the study and for their much needed words of encouragement and support.

Ms. L. Myburg and Ms. W. Ras for always having a quick solution to my financial dilemmas.

The National Research Foundation, Water Research Commision, Sappi Saiccor as well as the department of Microbial, Biochemical and Food Biotechnology, for financial assistance.

The Lord God Almighty without whom this work would have not come to pass.

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CHAPTER 1

INTRODUCTION

1. Introduction

The pulp and paper industry is a major industrial sector utilising huge amounts of lignocellulosic material and water. This industry is also a major polluter, generating vast amounts of effluents containing chlorinated compounds. These waste waters cause slime growth, scum formation, colour problems as well as impact negatively on the aesthetic beauty of the environment (Pokhrel and Viraraghavan, 2004). As a result of growing public awareness of these pollutants, pulp and paper industries have been forced to find alternative, more environmentally friendly methods for producing their products, one such alternative being the application of enzymes in the pulp and paper manufacturing process (Viikari *et al*., 1986, 1987; Christov and Prior, 1997).

Wood is the major raw material used in the pulp and paper industry. The wood is broken down to separate the cellulose and non-cellulose fractions by chemically dissolving the raw material so as to form a pulp slurry which is subsequently dried on a paper machine to produce a paper sheet. To produce the required quality of paper or paper-associated products, the addition of dyes, coating materials or preservatives may occur during the paper making process (Thompson *et al*., 2001).

More than half of the world's pulp is produced in North America, where the pulp and paper industry ranks as the fifth largest in the U.S. economy. In Western Europe, pulp production amounts to about 20 % of the world's total supply, with the Nordic countries contributing more than 64 % of this. The third most important producer of paper is reported to be Japan, contributing more than 12 % of the world's total production (Thompson *et al*., 2001). In Africa, South Africa is the largest manufacturer of paper and the biggest paper supplier and in 1992 was ranked as the tenth largest producer of pulp and 22nd biggest supplier of paper and board in the world (Bethlehem, 1995; Christov and Prior, 1998).

Xylan, the most abundant hemicellulose, is a major structural polysaccharide in plant cells (Prade, 1995). Due to its structural heterogeneity, the enzymatic degradation of xylan to its monomer, xylose, involves a battery of cooperatively acting enzymes (Biely, 1985; Puls *et al*., 1987; Poutanen *et al*., 1991; Subramaniyan and Prema, 2002). Among these hydrolytic enzymes, endo-1,4-ß-D-xylanases and ß-D-xylosidases have the most important activities. Xylanases are produced by a plethora of organisms (Dekker and Richards, 1976) and microbial xylanases are the preferred catalysts for xylan hydrolysis due to their diverse

characteristics. Filamentous fungi are particularly interesting producers of xylanases since they secrete the enzyme and their enzyme levels are much higher than those of yeasts and bacteria (Haltrich *et al*., 1996; Kulkarni *et al*., 1999).

The production of xylanases can be induced by xylan hydrolysis products such as xylose, xylobiose and xylotriose (Hrmová *et* al., 1986; Wang *et al*., 1992; Piñaga *et* al., 1994; Zhao *et al*., 1997). These products, being low-molecular weight compounds, can easily enter the cell and induce xylanase production. Even though xylose is a potent inducer of xylanase production (Gosh and Nanda, 1994; Purkarthofer and Steiner, 1995), there are, however, cases where the presence of xylose in the cultivation medium results in catabolic repression of xylanase synthesis (Hoq *et al*., 1994; Kadowaki *et al*., 1997). Similarly, the presence of glucose in the culture medium can also lead to repression of xylanase synthesis (Chandra and Chandra, 1995; Kadowaki *et al*., 1997).

Basic factors to be considered for the efficient production of xylanolytic enzymes include the choice of an appropriate inducing substrate, an optimum medium composition as well as optimum culture conditions. Small-scale laboratory experiments frequently use low-molecular weight compounds directly derived from xylan and purified xylans for the evaluation of xylanase production. However, for large-scale production processes, these substrates are unsuitable due to their cost, leading to increased enzyme production costs. One alternative would be to use inexpensive lignocellulosic substrates such as corn cobs, wheat bran or straw, rice or barley husks, hay or wood hydrolysates (Bailey and Poutanen, 1989; Kadowaki *et al*., 1997; Nascimento *et al*., 2002; Xiong *et al*., 2004). The use of these substrates, however, often requires pretreatment (Pham *et al*., 1998; Shah and Madamwar, 2005). In the case of wood hydrolysates, pretreatment would serve to remove potential inhibitory compounds (Olsson *et al*., 1995; Olsson and Hahn-Hägerdal, 1996; Jönsson *et al*., 1998; Larsson *et al*., 1999; Miyafuji *et al*., 2003). Environmental factors such as culture pH, temperature, aeration rate and agitation intensity play a significant role in the production of xylanases (Smith and Wood, 1991; Bailey and Viikari, 1993; Gomes *et al*., 1994; Hoq *et al*., 1994; Singh *et al*., 2000; Anthony *et al*., 2003). The nitrogen source as well as its concentration in the culture medium has also been reported to have a significant effect on xylanase production (Smith and Wood, 1991; Haapala *et al*., 1994; Pham *et al*., 1998).

Current data on the physico-chemical properties of xylanases mostly stem from studies conducted on bacterial and fungal enzymes (Sunna and Antranikian, 1997). Endoxylanases

from bacterial and fungal sources exhibit varying optimum temperatures, between 40 and 60 °C and are usually stable over a wide pH range of pH 3 to 10, with a pH optimum in the range of 4 to 7 (Kulkarni *et al*., 1999). The production of multiple forms of xylanases by bacteria and fungi is well documented (Biely *et al*., 1985; Elegir *et al*., 1994; Dobozi *et al*., 1992; Tsujibo *et al*., 1997; Kormelink *et al*., 1993; Wong *et al*., 1988) and this phenomenon has been attributed to the hydrolysis of the complex structure of heteroxylans which would require the action of multiple xylanases with overlapping but different specificities (Wong *et al*., 1988).

Cellulase-free xylanases have a wide range of potential biotechnological applications. On an industrial scale, they are produced for use as bleaching agents in the pulp and paper industry (Vicuna *et al*., 1995; Kulkarni and Rao, 1996), as food and beverage additives (Hang and Woodmans, 1997) and are also used in the poultry industry (Zhang *et al*., 2000). In addition, they are used in the bioconversion of lignocelluloses to sugar or ethanol (Sun *et al*., 2002). One of the stages of pulp bleaching involves the use of chlorine or chlorine dioxide, thereby resulting in the formation of chlorinated organic compounds. The resultant effluents are highly coloured and can cause serious environmental problems, as some of these compounds are toxic and mutagenic (Easton *et al*., 1997; Brusick, 1987; Ali and Sreekrishnan, 2001). The application of cellulase-free xylanases to the pulp bleaching process can significantly reduce the usage of bleach chemicals and at the same time enhance the pulp brightness, thereby reducing the amount of chlorinated compounds in the bleach effluents (Viikari *et al*., 1986; Christov *et al*., 1999, 2000; Dhillon *et al*., 2000).

1.1. Objectives of the study

The main production cost of many industrial enzymes is attributed to the growth substrate, which accounts for approximately 30 to 40 % of the process costs (Hinnman, 1994). There are numerous reports on xylanase production using various lignocellulosic materials, but none with spent sulphite liquor (SSL), a waste water of the pulp and paper industry, as carbon substrate. SSL is readily and abundantly available from a South African based pulp and paper company, which is one of the worlds largest producers of dissolving pulp, producing up to 600 000 tons of pulp per annum. The overall objective of this study was, therefore, to evaluate SSL as an alternative, inexpensive and abundant carbon substrate for xylanase production. The produced xylanases would be evaluated for their biobleaching efficacy and the reduction in bleaching chemicals, if any, determined. The envisioned

ultimate long-term goal of this study is that the application of xylanases produced from SSL in biobleaching would, hopefully, lead to the production of environmentally acceptable waste waters containing considerably lower amounts of chlorinated toxic compounds compared to the conventional bleaching processes.

With the overall objective in mind, the first task was to determine the chemical properties of the SSL, followed by the evaluation of several filamentous fungi (previously evaluated using bleach plant effluent (Christov *et al*., 1999)) for xylanase production, using this waste water as carbon substrate. Crude enzyme preparations from the best xylanase-producing strains would then be evaluated in biobleaching studies to determine the savings in bleaching chemicals, namely chlorine dioxide (Chapter 3). The second task was to evaluate the effect of environmental factors on xylanase production. Thus, the effects of culture pH and agitation rates on xylanase production in batch culture with SSL as carbon substrate were evaluated (Chapter 4). The effect of easily metabolisable sugars is well documented and since SSL is known to have a high biological oxygen demand, which is associated with the sugar content of the waste water, the third task was to evaluate repression of xylanase production using fed-batch cultures with SSL as carbon substrate (Chapter 5). Also, SSL being a wood hydrolysate, it may contain inhibitory compounds that adversely affect its utilisation as a carbon substrate. The removal of potential inhibitors in wood hydrolysates has been shown to be necessary and this may be achieved by different pretreatment procedures. The fourth task, therefore, was to evaluate the effect of two SSL pretreatments, namely ultrafiltration and overliming, on xylanase production (Chapter 6).

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CHAPTER 2

LITERATURE SURVEY

Contents

2. Literature survey

2.1. Manufacture of pulp and paper

2.1.1. Process description

The process of papermaking involves five basic steps, which include debarking, pulping, bleaching, washing and finally paper and paper products. Debarking involves the removal of bark from the woody material and conversion of the raw materials into smaller pieces called chips (Ali and Sreekrishnan, 2001). The next step is the pulping stage, which involves the removal of most of the lignin and hemicellulose from the wood chips, resulting in a cellulose-rich fibrous mat. Pulping processes are generally classified as mechanical, chemical, chemo-mechanical or thermo-mechanical (Pokhrel and Viraraghavan, 2004). In mechanical pulping, the wood is processed in a grinder, which physically separates the fibres. However, the quality of the pulp is of low grade, since it is highly coloured and contains short fibres. Chemical pulping, as the name suggests, involves the use of chemicals to break the wood chips into a fibrous mass. The wood chips are cooked with appropriate chemicals in an aqueous solution at elevated temperature and pressure. Chemical pulping can be carried out in either alkaline or acidic media. In the kraft pulping process, the wood chips are cooked in a solution of Na(OH) and NaS_2 and this is currently the most widely used process. In the sulphite pulping process, the wood chips are cooked in a mixture of sulphurous acid and bisulphide ions (HSO_3^-) (Pokhrel and Viraraghavan, 2004). In chemo-mechanical pulping (CMP), the wood chips are firstly treated with chemicals prior to mechanical treatment, whereas thermo-mechanical pulping (TMP) involves steaming the chips prior to mechanical pulping. In some cases, including a chemical treatment step during the steaming stage modifies TMP and this is referred to as chemi-thermomechanical pulping (CTMP) (Pokhrel and Viraraghavan, 2004). After pulping, the resultant pulp is washed by passing the pulp through a series of washers and screens at high temperatures so as to remove the dissolved lignin and chemicals in the pulp.

Unbleached kraft pulps have a low brightness as a result of the brown coloured lignin. Thus, to obtain a certain degree of whiteness of the pulp, a bleaching step is employed to remove the colour associated with the remaining residual lignin. This involves applying successive bleaching stages and the most reactive bleaching chemicals include elemental chlorine (C), ozone (Z), peroxy acids, chlorine dioxide (D) and oxygen (O), sodium hypochlorite (H) and hydrogen peroxide (P) (Buchert *et al*., 1994). The various types of pulp bleaching include elemental chlorine bleaching which uses chlorine and hypochlorite to brighten the pulp, elemental chlorine free bleaching (ECF) where the chlorine is replaced

with chlorine dioxide and hypochlorite is omitted, totally chlorine free bleaching (TCF) where the use of chlorinated bleaching agents are replaced with bleaching agents such as oxygen and peroxide (EPA fact sheet, 1997). Bleaching agents and colour are removed from the bleached pulp in the washing stage and generally involves the use of an alkali and hence this process is also referred to as the alkali extraction stage (Ali and Sreekrishnan, 2001).

After the alkali extraction stage, the pulp is diluted with water and mixed with appropriate fillers such as china clay, titanium dioxide or calcium carbonate, water soluble optical brighteners and sizing agents such as rosin and starch. Water is subsequently drained from this mixture by vacuum drying, pressing and finally passing the paper sheet through a series of steam-heated cylinders, resulting in the final product, namely paper and paper products (Ali and Sreekrishnan, 2001; Thompson *et al*., 2001).

2.1.2. Xylanases in the pulp and paper industry

Although microorganisms have long been employed in waste treatment, it has only recently been discovered that microbial enzymes can be useful in the processing of pulp and paper. The application of enzymes in this industry has, however, progressed slowly due to the difficulty of degrading wood and pulp (Kirk and Jeffries, 1996). In the last two decades the number of possible applications of enzymes in pulp and paper manufacture has grown steadily with several becoming or approaching commercial use. These include enzymatic bleaching with xylanases, pitch removal using lipases and freeness enhancement using cellulases and hemicellulases (Kirk and Jeffries, 1996).

The application of xylanolytic enzymes in the pulp and paper industry has been considered as one of the most important new biotechnological applications (Viikari *et al*., 1994). In the last few years, public awareness has increased concerning the environmental impact of wastewaters arising from the pulp and paper industry, especially with the formation of toxic organic chlorines as a result of chlorine usage in the bleaching of pulp (Viikari *et al*., 1994; Coughlan and Hazlewood, 1993). Xylanases have been shown to play an important role in debarking, deinking of recycled fibres and also in the purification of cellulose for the preparation of dissolving pulp (Jager *et al*., 1992). Figure 2.1 shows a schematic illustration of how a biobleaching process could be incorporated in the conventional process.

2.1.2.1. Enzyme-aided bleaching

The removal of the residual lignin to produce bright or completely white finished pulp requires bleaching of the pulp and this involves the use of large amounts of chlorine-based chemicals. To minimise the use of chlorine-based chemicals, other options may be employed and these include oxygen delignification, prolonged cooking times as well as the substitution of chlorine dioxide for chlorine, hydrogen peroxide and ozone (Beg *et al*., 2001). Most of these methods are, however, cost intensive thus the use of enzymes has provided a simple and economic alternative to reduce the use of chlorine and other bleaching chemicals. The main enzyme required in the delignification of kraft pulp has been shown to be xylanase (Kantelinen *et al*., 1988; Paice *et al*., 1988; Viikari *et al*., 1994). However, it has also been shown that xylanases in conjunction with other enzymes such as mannanases, lipases and α-galactosidases improve the effect of the enzymatic treatment of kraft pulp (Wong and Saddler, 1992; Gubitz *et al*., 1997; Clarke *et al*., 2000). Bleaching of pulp with xylanases is reported to be more effective than with lignin-degrading enzymes and this is simply because the lignin is cross-linked mostly to the hemicellulose, and the hemicellulose is more readily depolymerised than lignin (Subramaniyan and Prema, 2002).

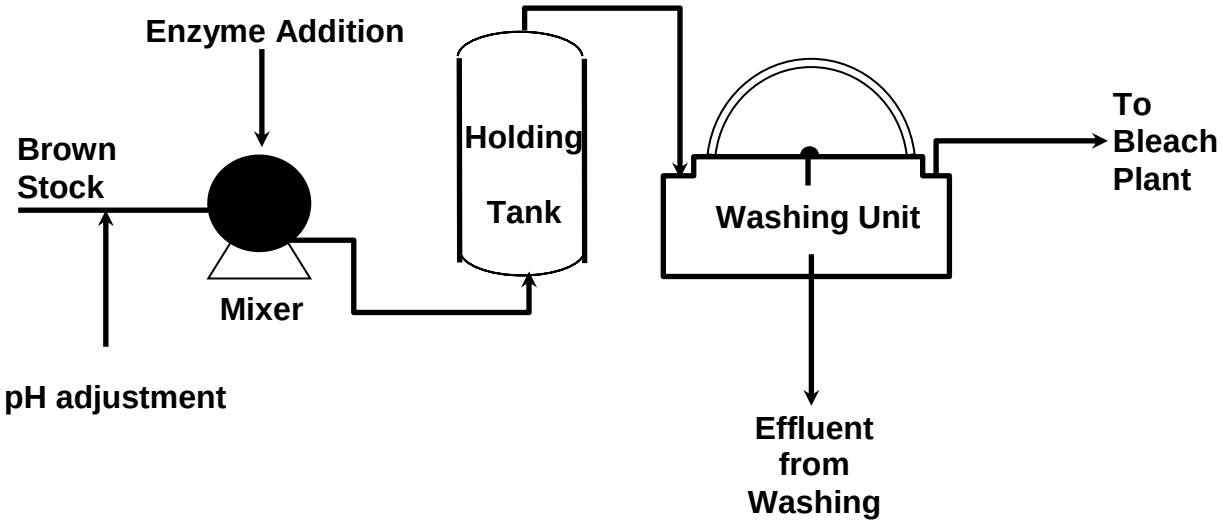

Figure 2.1. Schematic illustration of an enzymatic bleach boosting process (Nissen *et al*., 1992).

2.1.2.2. Mechanism of enzyme-aided bleaching

The exact mechanism by which xylanases facilitate the bleaching of pulps is not fully understood. There are, however, two major hypotheses that could explain this phenomenon. One of the hypotheses suggests that xylanases change the structure of the pulp by increasing the pulp porosity, thereby rendering the pulp more permeable to the extraction of lignin. Thus, xylanases act mainly on the relocated and reprecipitated xylan on the surface of the kraft pulp fibres and reprecipitation occurs when nearly all of the xylan side groups have been cleaved off, resulting in a very resistant chemical structure (Kantelinen *et al*., 1991; Kantelinen *et al*., 1993). Further support for this finding was provided when it was shown that accessory enzymes splitting off the xylan side groups only had a limited effect in hydrolysis and bleaching tests (Kantelinen *et al*., 1988; Kantelinen *et al*., 1993).

The other hypothesis that has been suggested to explain the mechanism of enzyme-aided bleaching is the possible release of chromophores associated with carbohydrates. Thus, the extractability of residual lignin is enhanced by the cleavage of the carbohydrate portion of the lignin-carbohydrate complex, resulting in smaller residual lignin molecules which are easier to remove (Viikari *et al*., 1986; Wong and Saddler, 1992). The proposed mechanism of enzyme-aided bleaching is schematically shown in Figure 2.2. It can thus be said that both types of phenomena are involved in the enzymatic pretreatment of kraft pulp, thereby resulting in improved delignification.

2.1.2.3. Effect of enzyme-aided bleaching

The advantages of using enzymes are dependent on the chemical bleaching sequence employed as well as the residual lignin content of the pulp. Some bleaching sequences incorporating an enzyme stage as well as primary objectives for employing a particular bleaching sequence have been summarised by Viikari *et al*. (1994). Pulp pretreatment with xylanases has been reported to result in either a higher final brightness, a lower kappa number or a lower bleaching chemical consumption (Viikari *et* al., 1986, 1987; Vicuna *et al*., 1997; Bim and Franco, 2000; Dhillon *et al*., 2000; Christov and Prior, 1996, 1997; Christov *et al*., 1999, 2000). Pretreatment with xylanase can reduce the requirement for oxidising chemicals by up to 20 to 40 % (Viikari *et al*., 1986, 1987; Vicuna *et al*., 1997).

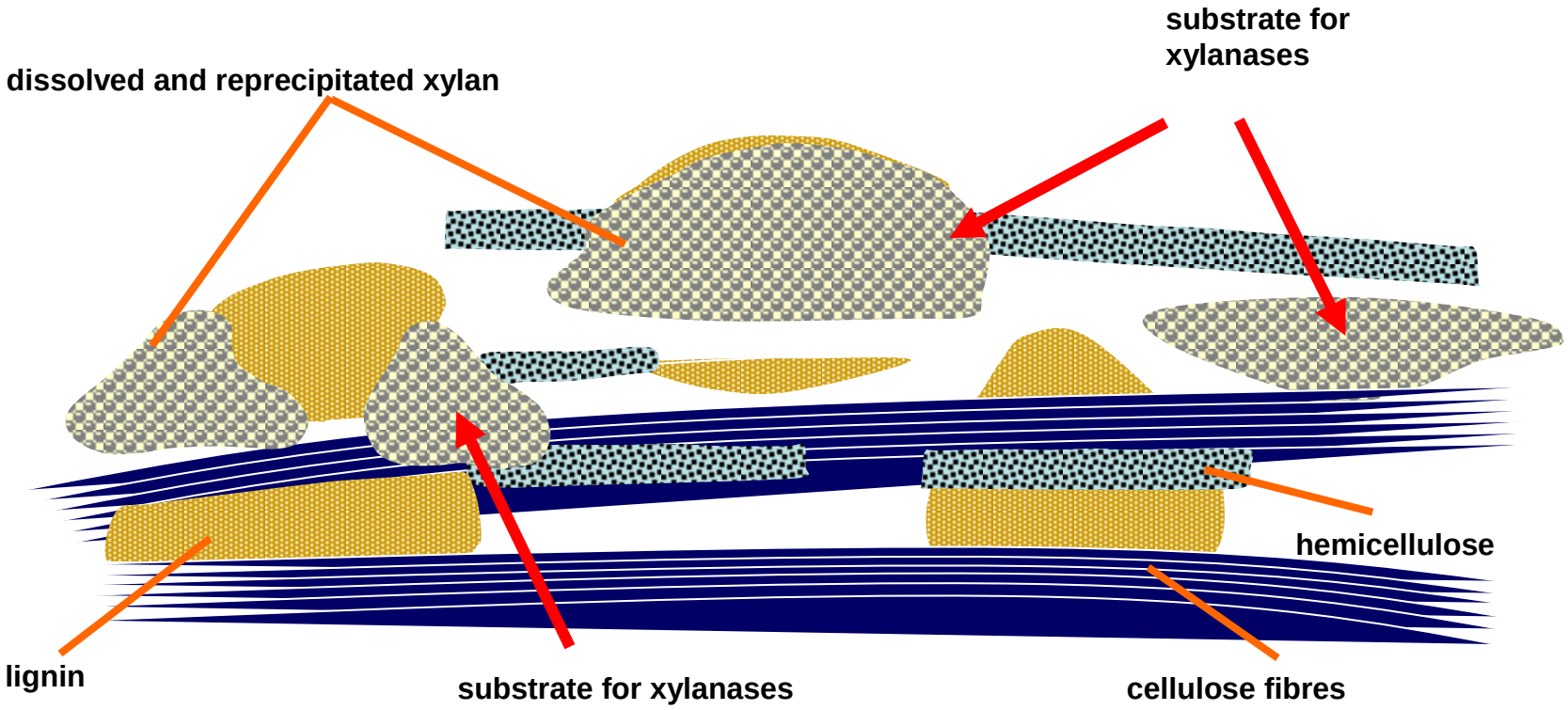

Figure 2.2. Schematic representation of a proposed mechanism of enzyme-aided bleaching (Redrawn from Viikari *et al*., 1994).

The bleachability of Eucalyptus kraft pulp was evaluated by Vicuna *et al*. (1997) using commercial xylanases, namely Ecopulp, Cartazyme NS-10 and Pulpzyme HC. Xylanase treatment of *E. globulus* and *E. nitens* pulps obtained by conventional cooking resulted in a saving of up to 12 and 14.7 % of ClO_2, respectively. However, xylanase treatment of *E. globulus* pulps that had been oxygen-delignified resulted in a greater decrease in ClO_2 consumption of up to 40 %. The enhanced performance of the xylanases with the kraft-oxygen pulps was attributed to a higher accessibility of the xylans in these pulps. Similarly, Dhillon *et al*. (2000) reported a 20 % reduction in chlorine consumption when a xylanase produced by *Bacillus circulans* was used in the pretreatment of Eucalyptus kraft pulp. A higher viscosity of the kraft pulp was also obtained and this was indicative of the hydrolysis of low DP xylans in the pulp (Dhillon *et al*., 2000). The pretreament of kraft pulp from a xylanase from *Bacillus pumilus* also resulted in a significant reduction in the kappa number with no effect on the viscosity of the pulp, implying that the xylanase did not affect the pulp fibres (Bim and Franco, 2000). In general, results obtained from laboratory studies and mill trials using kraft pulp have shown that about a 20 to 25 % reduction in total chlorine for hardwoods and 10 to 15 % for softwoods can be achieved with the application of xylanases (Viikari *et al*., 1987; Buchert *et al*., 1992).

Only a few reports on enzyme-aided bleaching of sulphite pulps have been published, however (Christov and Prior, 1996, 1997; Christov *et al*., 1999). This could be attributed to the fact that sulphite pulps do not contain reprecipitated xylan due to the harsh cooking conditions (Viikari *et al*., 1994). Also, the presence of acid resistant residual acetyl and 4-*O*-methylglucuronic acid groups act as barriers against the adsorption and intercrystalisation of xylan onto the cellulose micromolecules, thereby making the residual xylan in sulphite pulps less accessible since it is localised mainly in the secondary cell walls (Christov *et al*., 2000). It has, however, been shown that xylanase treatment of sulphite pulps can also result in increased bleachability of the pulps (Christov and Prior, 1996, 1997; Christov *et al*., 1999, 2000).

To obtain the desirable bleaching effect, one of the major requirements is that the xylanase preparation should be completely free of cellulase activity (Subramaniyan and Prema, 2000; Techapun *et al*., 2003). The presence of cellulase activity in the xylanase preparations may lead to serious economic implications such as loss of cellulose, diminished pulp quality and increased effluent treatment costs. Other factors that should be taken into consideration include pH and temperature optima, and stability of the enzyme. Xylanase pretreatment in

mills takes place in storage tanks where the pulp is at high temperatures of over 60 °C and at alkaline pH values of between 8 to 10 (Beg *et al*., 2001; Techapun *et al*., 2003). Thus, enzymes that are active at high temperatures and alkaline pH values have great potential as they can be introduced at different stages of the bleaching process without requiring adjustments to pH or temperature.

2.1.3. Other xylanase applications

Other potential applications for xylanases are summarised in Table 2.1. Xylanases have also been shown to play a major role in the conversion of the xylan present in wastes from agricultural and food industries into xylose, clarification of juices, and wine, extraction of coffee, plant oils and starch (Biely, 1985; Wong and Saddler, 1993), improvement in the efficiency of agricultural silage production (Wong and Saddler, 1992) and production of fuel and chemical feedstocks (Linko *et al*., 1989).

2.2. Pulp and paper mill waste waters

The pulp and paper industry is a highly water intensive industry and is ranked third in terms of freshwater withdrawal in the world, after the primary metals and chemical industries (Kallas and Munter, 1994). The consumption, however, varies with the type of paper being produced (Thompson *et al*., 2001) and this water eventually reappears in the form of effluent. Owing to the various combinations of technologies available that may be employed in the manufacture of pulp and paper, it is highly improbable that two paper mills will discharge identical effluents. Unfortunately, wastewaters emanating from this industry are highly polluted and are characterised by a high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), suspended solids, toxicity and colour, when untreated or poorly treated effluents are discharged into receiving waters (Ali and Sreekrishna, 2001; Pokhrel and Viraraghavan, 2004). In the USA, the pulp and paper industry is considered as the third largest polluter and in Canada it has been estimated that this industry is responsible for approximately 50 % of all wastes dumped into its waters (Sinclair, 1990). Although some pollutants in these effluents are naturally occurring wood extractives such as tannins, resin acids and lignin, compounds such as chlorinated lignins are xenobiotic, having been formed during the pulping and paper making process. Aptly put, pulp and paper mill effluents end up turning into a Pandora’s box of waste chemicals (Peck and Daley, 1994).

Table 2.1. Other potential industrial applications for xylanases (Adapted from Collins *et al*., 2005)

Market	Industry	Application	Function	*Reference*
Food	Fruit and vegetable processing, brewing, wine production	Fruit and vegetable juices, nectars, purees, oils (e.g., olive oil, corn oil) and wines	Improves maceration and juice clarification, reduces viscosity. Improves extraction yield and filtration, process performance and product quality.	Wong and Saddler, 1993; Bhat, 2000
	Baking	Dough and bakery products	Improves elasticity and strength of the dough, thereby allowing easier handling larger loaf volumes and improved bread texture.	Maat *et al*., 1992
Feed	Animal feeds	Monogastric (swine and poultry) and ruminant feeds	Decreases the content of non-starch polysaccharides, thereby reducing the intestinal viscosity and improving the utilisation of proteins and starch. Improves animal performance, increases digestability and nutritive value of poorly degradable feeds, e.g., barley and wheat.	Bhat, 2000; Mathlouthi *et al*., 2002
Technical	Starch	Starch-gluten separation	Reduces batter viscosity, improves gluten agglomeration and process efficiency.	Bhat, 2000
	Textiles	Retting of flax, jute, ramie, hemp, etc.	Enzymatic retting, reduces/replaces chemical retting methods.	Sharma, 1987; Beg *et al*., 2001
	Bioremediation/Bioconversion	Treatment of agricultural, municipal and food industry wastes	Treatment/recycling of wastes. Production of fermentable products, renewable fuel (bioethanol) and fine chemicals	Prade, 1995; Mielenz, 2001; Saha, 2003

2.2.1. Spent sulphite liquor

2.2.1.1. Origin

One of the waste products of the wood pulping industry is spent sulphite liquor (SSL), also referred to as sulphite waste liquor (SWL). The SSL originates from the sulphite pulping process involving the separation of the cellulosic constituents of wood from the non cellulosic fraction. Treatment conditions involve the use of high temperature and pressure in an aqueous sulphurous acid solution of either calcium of magnesium bisulphite (Holderby and Moggio, 1960). Due to the acidic conditions in this type of pulping process, the less resistant hemicellulose component of the wood chips is also hydrolysed. Short chains are solubilised to monosaccharides in the cooking liquor and the hydrolysis of acetyl groups on the hemicellulose fraction results in the formation of acetic acid. Furfural may also be formed from xylose degradation, whereas levulinic and formic acids may result from glucose degradation via hydroxymethyl furfural (Hinck *et al*., 1985).

2.2.1.2. Composition

The general composition of SSL includes lignosulphonates, acetic acid as well as carbohydrate hydrolysis products. SSL from different mills varies considerably and this is due to the type of wood used and degree of cooking required to produce the desired pulp quality with hardwood liquors having a higher proportion of sugars (up to 30 %) than softwood liquors (15 to 22 %). Similarly, the acetic acid content of SSL may also be higher in hardwood liquors. The types of sugars found in the SSL are also a function of the type of wood used, with softwood yielding up to 75 % hexoses, whereas hardwoods would yield as much as 70 % pentoses (Holderby and Moggio, 1960).

2.2.1.3. Utilisation of SSL

SSL is used in a number of different applications such as in concrete technology (Georgescu *et al*., 1997), the recovery for fuel value through evaporation and burning at the mill, preparation of lignosulphonate compounds and derivatives for various industrial applications (Holderby and Moggio, 1960) and in microbial fermentation for the production of different metabolites including ethanol (Kosaric *et al*., 1981; Rousseau *et al*., 1992; Taherzadeh *et al*., 2003) and single cell protein (Mckee and Quicke, 1977; Chaudry *et al*., 1977; Streit *et al*., 1987).

2.3. Xylan: occurrence and structure

The major wood plant wall constituents comprise cellulose, hemicellulose and lignin and constitute 97 to 99 % of the total dry mass of woods, of which 65 to 75 % are polysaccharides. Other polymeric compounds found in smaller quantities include starch, pectin, ash and extractives (Fengel and Wegener, 1989). The relative amounts and chemical composition can, however, differ in different plant species. Table 2.2 summarises the general compositions of softwood and hardwood. Lignin is an aromatic polymer synthesised from phenylpropanoid precursors and can be divided into guaiacyl lignins and guaiacylsyringyl lignins, and can be differentiated by the substituents on the phenylpropanoid skeleton (Adler, 1977). Softwoods generally contain more lignin than hardwoods (Table 1). Cellulose is a high molecular weight linear monopolysaccharide of ß-1,4-linked D-glucose units (Fan *et al*., 1982). Cellulose molecules can sustain linearity not only in solid state but also in solutions and this is made possible by the linear cellulose chains easily forming intracellular hydrogen bonds between the proton of the C3-OH and the C5 oxygen atom of the neighbouring glucose ring.

Table 2.2. Average composition (%) of softwoods and hardwoods (Saka, 1991).

	Cellulose	Hemicellulose	Lignin
Softwood	38-52	16-27	26-36
Hardwood	37-57	20-37	17-30

In lignocellulosic biomass, hemicellulose; a complex of heteropolymeric carbohydrates including xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galagtose, D-glucose and D-mannose) and arabinogalactan (heteropolymer of D-galactose and D-arabinose) is the linking material between cellulose and lignin (Shallom and Shoham, 2003). The structure and composition of hemicelluloses differ in softwoods and hardwoods. Softwood hemicelluloses are mannan rich polymers and contain small amounts of xylan. By contrast, hardwood hemicelluloses are xylan rich polymers containing insignificant amounts of mannan. Also, hemicelluloses of hardwoods are more acetylated than those of softwoods (Fengel and Wegener, 1989; Saka, 1991; Sakakibara, 1991).

Xylan is the second most abundant polysaccharide in nature (Biely, 1985; Prade, 1995) and is typically located in the secondary cell wall of plants and can also be found in the primary cell wall in monocots (Wong *et al*., 1988). Xylans mostly occur as heteropolysaccharides and the backbone, comprising of 1,4-linked ß-D-xylopyranosyl residues, can be substituted to varying degrees with glucuronopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or *p*-coumaroyl side chain groups (Whistler and Richards, 1970; Kulkarni *et al*., 1999; Li *et al*., 2000).

2.3.1. Hardwood xylan

Hardwoods from angiosperms contain large quantities of xylan, varying from between 15 to 30 % of the cell wall content (Puls, 1997). The xylan in hardwoods exists as *O*-acetyl-4-*O*-methylglucuronoxylan (Figure 2.3) and exhibits an average degree of polymerisation (DP) of between 150 and 200 (Collins *et al*., 2005). Every tenth xylose unit carries a 4-*O*-methylglucuronic acid residue, which is α-(1-2)-linked to the xylan backbone. Hardwood xylans are highly acetylated and the acetylation may occur at either the C-3 or the C-2 position (Woodward, 1984; Eriksson *et al*., 1990). The partial solubility of xylan in water is as a result of the presence of these acetyl groups that are, however, readily removed when the xylan is subjected to alkali extraction (Dekker, 1989).

2.3.2. Softwood xylan

The xylan content in softwoods is lower than that in hardwoods, varying from between 7 to 10 % of the cell wall content (Puls, 1997) and exists as arabino-4-*O*-methylglucuronoxylan (Figure 2.4). Softwood xylans are shorter than hardwood xylans with an average DP of between 70 and 130 and are also less branched. The 4-*O*-methylglucuronic acid content is higher that that of hardwoods and these residues are attached to the C-2 position. Softwood xylans are rarely acetylated and instead of acetyl groups, α-L-arabinofuranose units are linked by α-1,3-glycosidic bonds to the C-3 position of the xylose residue.

Figure 2.3. Schematic representation of the composition of hardwood xylan (*O*-acetyl-4-*O*-methylglucuronoxylan). Numbers on the figure indicate carbon atoms at which substitutions take place. Ac: Acetyl group; α-4-*O*-Me-GlcA: α-4-*O*-Methylglucuronic acid (From Sunna and Antranikian, 1997).

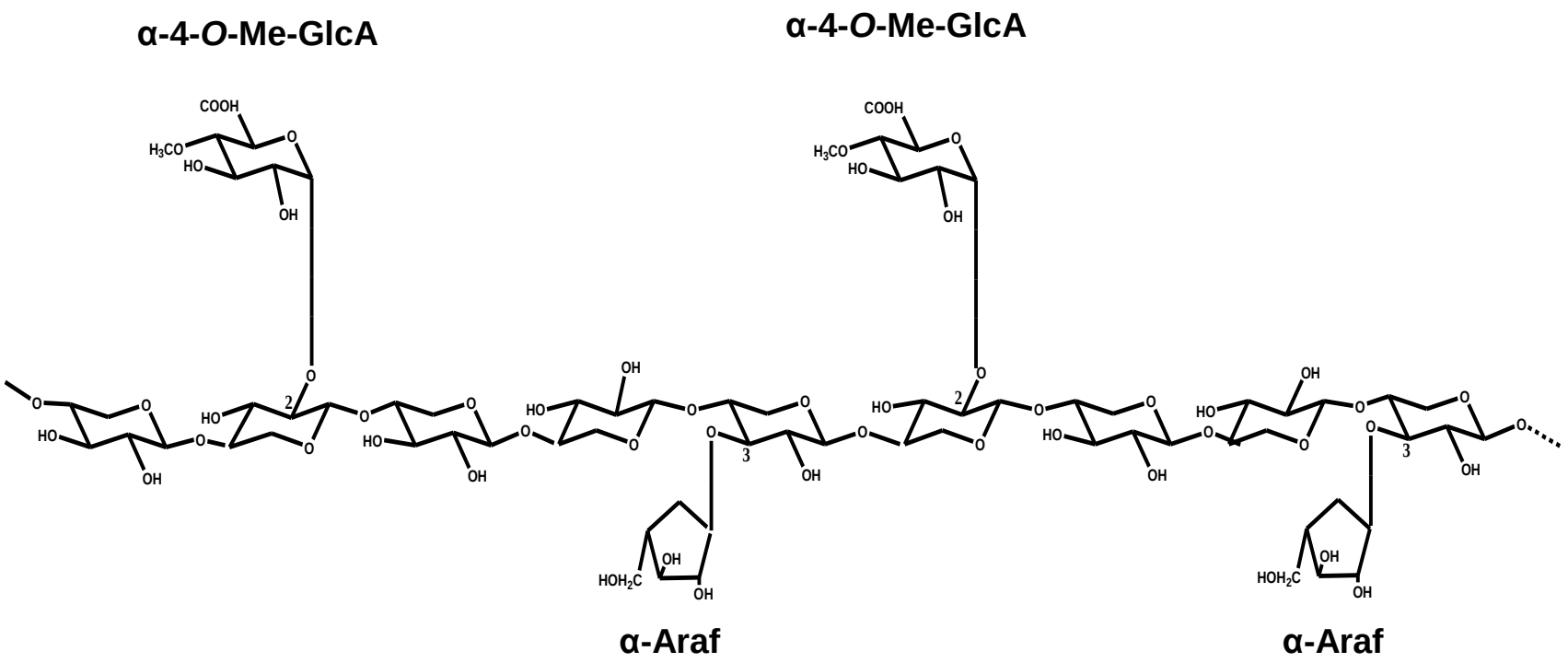

Figure 2.4. Schematic representation of the composition of softwood xylan (arabino-4-*O*-methylglucuronoxylan). Numbers on the figure indicate carbon atoms at which substitutions take place. α-Araf: α-Arabinofuranose; α-4-O-Me-GlcA: α-4-*O*-Methylglucuronic acid (From Sunna and Antranikian, 1997).

2.4. Xylanolytic enzymes

It is clear, from the previous sections, that xylan is a heterogeneous as well as complex polysaccharide. As a result, the complete enzymatic hydrolysis of xylan requires a variety of cooperatively acting enzymes (Biely, 1985; Puls *et al*., 1987; Erikson *et al*., 1990; Sunna and Antranikian, 1997; Subramaniyan and Prema, 2002; Collins *et al*., 2005). This complex enzyme system includes endoxylanases, ß-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, acetylxylan esterases, ferulic acid esterases and *p*-coumaric acid esterases. Figure 2.5 depicts a hypothetical fragment of plant heteroxylans comprising all the major structural features of the polysaccharide and the points of action of the various xylanolytic enzymes. The cooperative action of all these enzymes results in the conversion of xylan to its constituent sugars. Also, due to the presence of different substituent groups in the backbone and side chain of heteroxylans, complete degradation of such a complex polysaccharide may involve synergistic action between the different components of the xylanolytic enzyme system. The enzymatic hydrolysis of xylan as well as the xylanolytic enzyme systems of a variety of microorganisms has been extensively reviewed by Biely (1985), Dekker (1985), Wong *et al*. (1988), Eriksson *et al*. (1990), Sunna and Antranikian (1997), Subramaniyan and Prema (2002), to name but a few.

2.4.1. Enzymatic hydrolysis of xylan

2.4.1.1. ß-1,4-Endoxylanase

The crucial enzyme for xylan depolymerisation is ß-1,4-endoxylanase (1,4-ß-D-xylan xylohydrolase, EC 3.2.1.8) which cleaves the internal glycosidic linkages of the heteroxylan backbone, eventually resulting in the formation of xylotriose, xylobiose and xylose (Dekker and Richards, 1976). The end products resulting from xylan hydrolysis have been used to differentiate endoxylanases and have, therefore, been classified as either debranching or nondebranching, depending on whether or not they liberate free arabinose in addition to cleaving the main chain linkages (Dekker and Richards, 1976; Reilly, 1981). Many organisms are able to produce both debranching and nondebranching endoxylanases and examples of arabinose-cleaving endoxylanases include those from *Neurospora crassa* (Mishra *et al*., 1984), *Aspergillus niger* (Takenishi and Tsujisaka, 1975) and *Trichoderma koningii* (Wood and McCrae, 1986). The underlying physiological significance for the production of both debranching and nondebranching endoxylanases by various microorganisms is not clear but it could, however, be postulated that possession of both

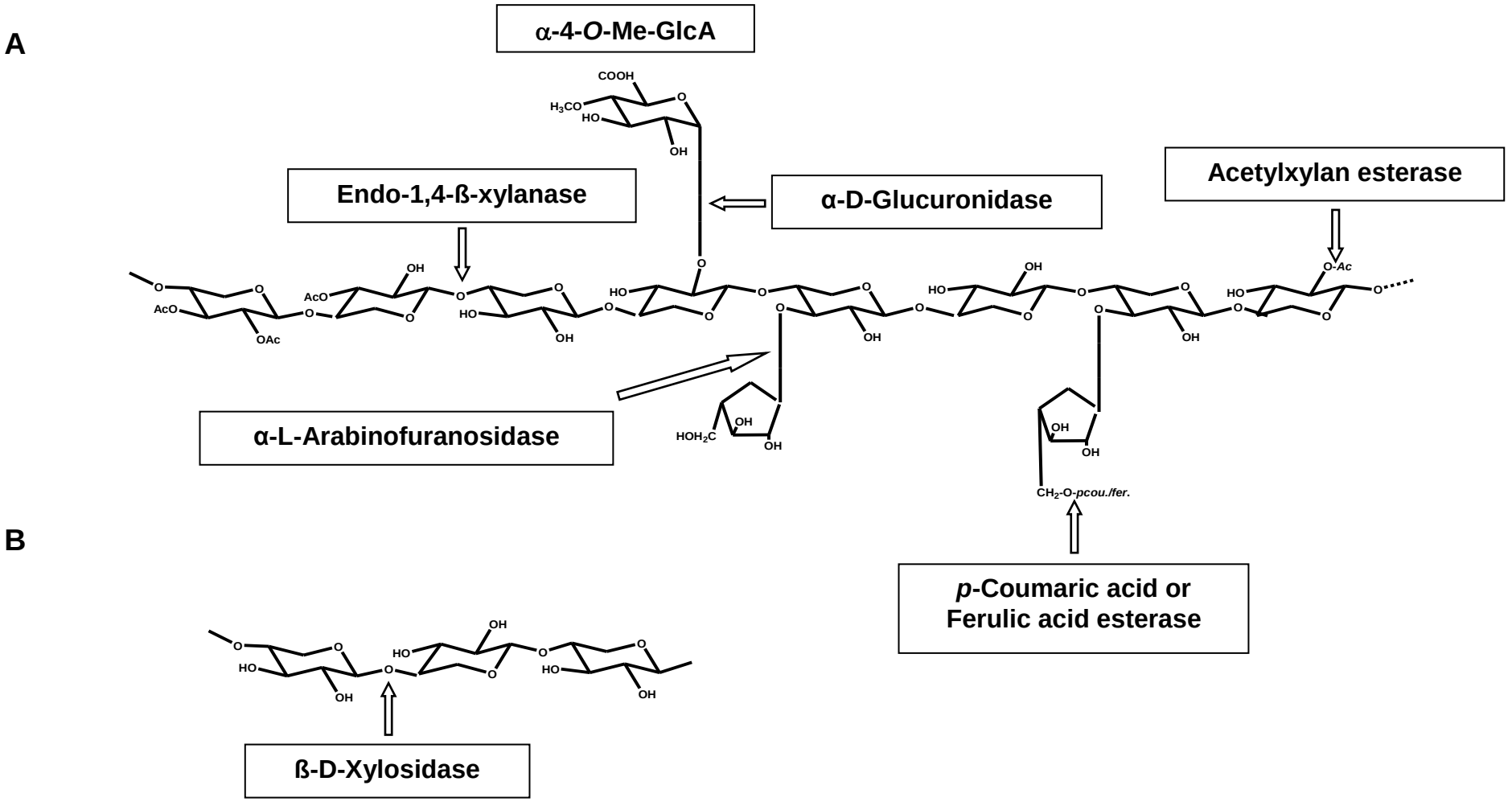

Figure 2.5. A schematic representation of xylanolytic enzymes involved in xylan degradation (A), and the hydrolysis of xylooligosaccharide by ß-xylosidase (B). Ac: Acetyl group; α-4-*O*-Me-GlcA: α-4-*O*-Methylglucuronic acid; α-Araf: α-Arabinofuranose; *p-cou*.: *p*-Coumaric acid; *fer*.: Ferulic acid (Modified from Sunna and Antranikian, 1997).

types of enzymes is required for maximal efficiency in the hydrolysis of xylan (Takenishi and Tsujisaka, 1975; Wong *et al*., 1988).

2.4.1.2. Xylanase multiplicity

Many microorganisms have been shown to produce multiple xylanases (Biely *et al*., 1985; Bailey *et al*., 1991; Ito *et al*., 1992) which may have diverse physicochemical properties, structures, specific activities, yields as well as overlapping but dissimilar specificities. The production of multiple xylanases may lead to an increased efficiency and extent of xylan hydrolysis, since not all xylosidic linkages in the substrate are equally accessible to xylan degrading enzymes due to the complex structure of xylans (Wong *et al*., 1988). Typical examples of microorganisms producing multiple xylanases include *A. niger*, reported to produce 15 xylanases (Biely *et al*., 1985), *T. viride*, reported to secrete 13 (Biely *et al*., 1985), *A. oryzae*, from which two (Bailey *et al*., 1991) and *A. awamori*, from which three xylanases have been purified (Kormelink *et al*., 1993). The multiplicity of xylanases could be attributed to a number of factors which include differential mRNA processing, postsecretional modifcation as a result of proteolytic digestion, posttranslational modification such as glycosylation and autoaggregation (Biely *et al*., 1985; Coughlan *et al*., 1993). Multiple xylanases may also be the product resulting from different alleles of the same gene or the product of independent genes (Wong *et al*., 1988; Hazlewood and Gilbert, 1993).

2.4.1.3. Biochemical characteristics of xylanases

Wong *et al*., (1988) proposed two groups for the classification of xylanases, based on their physicochemical characteristics. These groups included xylanases with a low-molecular weight (≤30 kDa) and basic pI and those with a high-molecular weight (>30 kDa) and acidic pI. It should, however, be noted that several exceptions to this general pattern have been reported (Filho *et al*., 1993; Matte and Forsberg, 1992) and up to about 30 % of the presently identified xylanases cannot be classified by this system (Sunna and Antranikian, 1997). Xylanases have basically been classified into two families. The high-molecular weight xylanases (> 30 kDa) with low pI values have been classified as glycanase family 10, formerly family ‘F’, whereas the low-molecular weight xylanases (≤ 30 kDa) with high pI values (8 to 9.5) belong to the glycanase family 11, formerly family ‘G’ (Henrissat, 1991; Henrissat and Bairoch, 1993). Biely *et al*. (1997) conducted an extensive study on the differences in catalytic properties of the xylanase families and concluded that in contrast to family 11 xylanases, family 10 xylanases are capable of attacking the glycosidic linkages next to the branch points and toward the non-reducing end. Family 10 xylanases require two

unsubstituted xylopyranosyl residues, whereas the xylanases of family 11 require three unsubstituted consecutive xylopyranosyl residues.

Most fungal xylanases exhibit an optimum pH for xylan hydrolysis of around pH 5 and are normally stable between pH values of 2 and 9 (Buchert *et al*., 1994). Bacterial xylanases, however, exhibit generally slightly higher pH optima than fungal xylanases (Buchert *et al*., 1994; Subramaniyan and Prema, 2002). Although fungi have generally been reported to produce xylanases at an initial cultivation pH lower than 7, there are some exceptions. An alkali-tolerant xylanase from *A. fischeri* (Chandra and Chandra, 1995) was reported to exhibit remarkable pH stability at pH 9. Similarly, the xylanase from *Cephalosporium* was reported as exhibiting activity over a broad pH range of between 6.5 to 9.0 (Bansod *et al*., 1993).

The optimum temperature varies from between 40 and 60 °C for xylanases from bacterial and fungal sources. Fungal xylanases are, however, reported to be less thermostable than bacterial xylanases (Kulkarni *et al*., 1999). One of the most thermostable xylanases, shown to have a half life of 20 min at 105 °C, is from a thermophilic *Thermotoga* species (Bragger *et al*., 1989). Among the fungi, xylanase from *T. aurantiacus* has been reported to be stable at 70 °C for 24 h with a half-life of 54 min at 80 °C. Other thermostable xylanases include those from *Paecilomyces variota* (Krishnamurthi and Vithayathil, 1989) and from *Talaromyces byssochlamydoides* (Yoshika *et al*., 1981).

2.4.1.4. Other xylanolytic enzymes

ß-D-Xylosidases (ß-D-xyloside xylohydrolase, EC 3.2.1.37) hydrolyse short xylooligosaccharides and xylobiose from the nonreducing end to liberate xylose and may be mono-, di- or tetrameric proteins. ß-Xylosidases are produced by a variety of microorganisms and are relatively large enzymes, ranging from 60 to 360 kDa, some of which have been purified from bacteria, yeast and filamentous fungi and characterised (Sunna and Antranikian, 1997). ß-Xylosidases, contrary to xylanases which are secreted by cells into the surrounding medium, may be extracellular or cell bound depending on the organism and cultivation conditions. In almost all bacteria and yeasts this enzyme is cell associated, whereas in filamentous fungi these enzymes remain associated with the mycelium during the early stages of growth and are later secreted into the medium in the later incubation cycle; this occurs either by secretion or as a result of cell lysis (Kumar and Ramon, 1996; Lenartovicz *et al*., 2002; Subramaniyan and Prema, 2002).

Reese *et al*. (1973) evaluated a wide variety of genera including bacteria, phycomycetes, ascomycetes, basidiomycetes and filamentous fungi and reported that ß-xylosidase is an inducible enzyme. Andrade *et al*. (2004) evaluated ß-xylosidase production by *A. versicolor* using different carbon substrates and reported that the presence of xylan or xylose in the culture medium resulted in induction of the enzyme. Glucose, however, was reported to repress ß-xylosidase production. Kumar and Ramon (1996) also reported similar results on ß-xylosidase induction by using *A. nidulans*.

Most ß-xylosidases show a high activity toward xylobiose and xylotriose and generally no activity toward xylan with the activity toward xylooligosaccharides often decreasing rapidly with increasing chain length (Matsuo and Yasui, 1984; van Doorslaer *et al*., 1985). However, there are some reports of ß-xylosidases that are able to slowly hydrolyse xylan to produce xylose (Dekker and Richards, 1976). Many ß-xylosidases have transferase activity in addition to direct hydrolase action and this normally results in products of higher molecular weight than that of the substrate. Transfer reaction may also result in the formation of both ß-1,3- and ß-1,4- bonds.

α-L-Arabinofuranosidases (AFs) are defined as enzymes that hydrolyse the terminal nonreducing residues from arabinose-containing polysaccharides. L-Arabinofuranosyl residues are widely distributed in heteropolysaccharides such as α-L-arabinofuranosides, arabinans, arabinoxylans and arabinogalactans (Eriksson *et al*., 1990). Arabinases can either be of the exo-type (AFs, EC 3.2.1.55), which hydrolyse terminal nonreducing residues and can hydrolyse 1,3- and 1,5-α-arabinosyl linkages of arabinan or of the endo-type (endo-1,5-α-L-arabinases, EC 3.2.1.99), which degrade arabinan in an endo-fashion and are active only toward linear arabinans (Saha, 2000). A variety of microorganisms including fungi, actinomycetes and bacteria have been reported to produce these enzymes (Dekker and Richards, 1976) and although AFs are part of microbial xylanolytic systems necessary for complete breakdown of arabinoxylans and could represent potential rate limiting enzymes in xylan degradation especially with substrates form agricultural residues, only a few of these enzymes have been isolated and characterised (Saha, 2000; Chacon-Martinez *et al*., 2004; Matsumura *et al*., 2004). The microbial production of AFs seems to be induced, among others, by arabinan, arabinose, arabitol and wheat bran. Induction of AFs with an *A. niger* strain occurred with arabinose and arabitol but not with xylose or xylitol (van der Veen *et al*., 1992, 1993). Arabitol, in particular, was reported to play an important role in the induction of AFs in *A. nidulans* (van der Veen and Visser, 1993). Also, several rumen bacterial isolates

investigated by Williams and Withers (1982) yielded high AFs activities on arabinose or arabinose-containing polysaccharides but AF production was repressed in the presence of glucose. Most arabinosidases exist as monomers, but dimeric, tetrameric and octameric forms have also been found with the molecular weights of reported enzymes varying significantly, ranging from 53 kDa to 495 kDa (Eriksson *et al*., 1990). Multiple forms of AFs have been detected in the culture broths of *streptomyces* (Zimmermann, 1989), *A. nidulans* (Ramon *et al*., 1993), *A. niger* (Rombouts *et al*., 1988) and *A. terreus* (Luonteri *et al*., 1995). Microbial AFs exhibit a broad range of pH and temperature dependence, with pI values ranging from 3.6 to 9.3, optimum pH and temperature values from 2.5 to 6.9 and 40 to 75 °C (Coughlan *et al*., 1993; Kaji, 1984; Lee and Forsberg, 1987).

The role of α-glucuronidases (EC 3.2.1.139) in the hydrolysis of xylan is to release 4-*O*-methyl-D-glucuronic acid (MeGlcA) residues linked α-1,2-glycosidically to the main chain of the polysaccharide (Eriksson *et al*., 1990). α-Glucuronidase activity has been detected in the culture filtrates of both fungi and bacteria (Dekker, 1985; Puls *et al*., 1987; Khandke *et al*., 1989; Johnson *et al*., 1989), although most organisms secrete only low levels of this enzyme. The first α-glucuronidase was purified from *Thermoascus aurantiacus* (Khandke *et al*., 1989) and this enzyme was able to liberate MeGlcA from both polymeric xylan and xylooligosaccharides at similar rates. The substrate specificities of α-glucuronidases has been reported to differ depending on the enzyme source. The majority of described α-glucuronidases do not have the ability to liberate MeGlcA directly from the xylan polysaccharide but can release acid residues that are linked to a single D-xylopyranosyl residue or to the nonreducing terminal xylopyranosyl residues of xylooligosaccharides (Fontana *et al*., 1988; Siika-aho *et al*., 1994; de Vries *et al*., 1998; MacKenzie *et al*., 1987). Thus, for the liberation of substantial amounts of MeGlcA from xylans, α-glucuronidases need to act synergistically with backbone-hydrolysing xylanase. α-Glucuronidases of *Penicillium chrysosporium* (Castanares *et al*., 1995) and *A. tubigensis* (de Vries *et al*., 1998) have also been reported to exhibit low activity towards polymeric xylan. The various α-glucuronidases described thus far differ in cellular localisation. In some species the enzyme has been reported to be confined to the cells and is said to be localised internally. Examples include enzymes of *Clostridium stercorarium* (Bronnenmeier *et al*., 1995) and *A. niger* (Uchida *et al*., 1992). Microorganisms in which the enzyme is localised extracellularly include *Agaricus bisporus* (Puls *et al*., 1987), *A. tubigensis* (de Vries *et al*., 1998), *T. reesei* (Siika-aho *et al*., 1994) and *T. viride* (Dekker, 1983).

Microbial acetylxylan esterases (EC 3.1.2.72, AcXEs) are involved in the deacetylation of partially acetylated 4-*O*-methyl-D-glucuronoxylan or its fragments thereof, generated by the action of xylanases (Matsihubova and Biely, 2004). Recent studies on the mode of action of AcXEs from *S. commune* (Biely *et al*., 1986) and *T. reesei* (Biely *et al*., 1997) on fully or partially acetylated methyl ß-D-xylopyranoside revealed that the enzymes, in general, deacetylated positions 2 and 3 and these positions were compatible with their function in the biodegradation of hemicellulose. Deacetylation, therefore, makes the xylopyranosyl residues of the main xylan chain more accessible to degradation by xylanases (Biely *et al*., 1986). Confirmation of the existence of esterases was demonstrated by Frohwein *et al*. (1963) where these enzymes were shown to release acetic acid from the deacetylation of acetyl-mannose, acetyl-glucose, acetyl-maltose and acetyl-cellobiose. However, Biely *et al*. (1985, 1986) was the first to report on the existence of AcXEs in fungal cultures where the acetyl esterases of *T. reesei*, *A. niger*, *S. commune* and *Aureobasidium pullulans* were found to have a high capacity to deacetylate steamed xylan. Biely *et al*. (1988) also evaluated the production of AcXEs by *T. reesei* and *S. commune* on different carbon substrates and reported low enzyme activities on low-molecular weight substrates such as glucose, xylose and cellobiose. On polymeric substrates such as xylan, cellulose and their mixture, however, highest activities of AcXEs were obtained. Other substrates shown to induce significant levels of AcXE production include wheat bran (Johnson *et al*., 1988) and solka floc (Poutanen *et al*., 1987).

Ferulic and *p*-coumaric acids are linked to xylan by ester bonds and are involved in the crosslinking of cell wall polysaccharides and lignin (Faulds *et al*., 1997; Garcia *et al*., 1998). The cleavage of these bonds and the subsequent removal of feruloyl and *p*-coumaroyl groups from the xylan are helpful in the removal of lignin (Subramaniyan and Prema, 2002). Ferulic acid esterases cleave the ester linkages between arabinose side chains and ferulic acids in xylan, whereas *p*-coumaric acid esterase cleaves the ester linkage between arabinose and *p*-coumaric acid (Sunna and Antranikian, 1997). MacKenzie *et al*. (1987) first described the presence of these phenolic esterases and suggested that these enzymes perform a similar function to alkali in the deesterification of plant tissues. Ferulic acid esterase production by *Streptomyces olivochromogenes* was reported to be highest with oat spelts xylan as carbon substrate (Mackenzie *et al*., 1987) and only low constitutive levels of this enzyme were produced in the absence of lignocellulosic substrate. Johnson *et al*. (1988) partially purified three feruloyl esterases from this organism. *S. commune* was

reported to produce high levels of ferulic acid esterase with Avicel cellulose, higher than with oat spelts or wheat bran as carbon substrates (Mackenzie and Bilous, 1988).

2.5. Production of microbial xylanases

2.5.1. Microbial xylanases

There are numerous reports on microbial xylanases that date as far back as 1960. These reports, however, placed prime importance on plant pathology-related studies (Subramaniyan and Prema, 2002). It was only during the 1980s that the impact of xylanases in the area of biobleaching began receiving attention (Viikari *et al*., 1986). Microbial xylanases are quite widespread among bacteria, yeasts as well as filamentous fungi (Haltrich *et al*., 1996; Sunna and Antranikian, 1997; Kulkarni *et al*., 1999; Subramaniyan and Prema, 2002) and these xylanase-producing microorganisms have been found to inhabit a diverse and wide ecological niche, typically including environments where plant materials accumulate and deteriorate, and can also be found in the rumen of ruminants (Prade, 1995; Subramaniyan and Prema, 2002).

The two main methods that can be used to produce xylanases include solid-state cultivation systems and submerged liquid cultivation systems. Most research has been conducted using submerged cultures as this type of cultivation allows for better control over parameters such as the degree of aeration, culture pH and temperature of the medium, in addition to control over environmental factors required for the optimum growth of microorganisms. The solid-state cultivation system, however, has received renewed interest in recent years and has often been employed in the production of xylanases. Renewed interest is as a result of a number of economic and engineering advantages, such as the nature of substrate, lower aeration pressures as well as the absence of vigorous agitation. A major problem with this type of culture system is the evolution of heat, especially in a scaled-up process. Thus, for xylanase production, most research has used submerged culture systems in which it is easier to control the environmental parameters. This literature review focuses on xylanase production in submerged culture, as this was the method used to investigate xylanase production in this study.

Fungal xylanases from the genera *Aspergillus* and *Trichoderma* are amongst the most studied and best characterised xylanases. Production and characterisation of xylanases

from several *Aspergillus* strains include those of *A. niger* (Frederick *et al*., 1981; 1985; Fournier *et al*., 1985), *A. terreus* (Hrmová *et al*., 1989; 1991), *A. tubigensis* (De Graaff *et al*., 1992), *A. nidulans* (Piñaga *et al*., 1994; Taneja *et al*., 2002), *A. sydowii* (Gosh and Nanda, 1994), *A. fumigatus* (Anthony *et al*., 2003) and *A. versicolor* (Carmona *et al*., 2005). *Trichoderma* strains reported to produce xylanases include *T. viride* (Hashimoto *et al*., 1971; Labavitch and Greve, 1983; Biely *et* al., 1985; Gibson *et al*., 1987), *T. harzianum* (Tan *et al*., 1985a; 1985b; Wong *et al*., 1986a,1986b; Seyis and Aksoz, 2005), *T. longibrachiatum* (Royer and Nakas, 1989), *T. koningii* (Wood and McCrae, 1986), *T. reesei* (Kolarova and Farkas, 1983; Tenkanen *et al*., 1992; Xiong *et al*., 2005). In addition to studies conducted on xylanases produced by *Aspergillus* and *Trichoderma*, studies have also been conducted with many other fungi such as *Thielaviopsis basicola* (Gosh and Deb, 1988), *Penicilluim canescens* (Gaspar *et al*., 1997), *Talaromyces emersonii* (Tuohy and Coughlan, 1992), *T. laniginosus* (Gomes *et al*., 1993; Purkathofer and Steiner, 1994; Xiong *et al*., 2004; Li *et al*., 2005), to name but a few.

Bacterial xylanases have also been produced, isolated and characterised, with the most often studied bacterial xylanases being those originating from the genera *Bacillus* (Esteban *et al*., 1982; 1983; Bocchini *et al*., 2002; Poon *et al*., 2003; Chang *et al*., 2004; Lama *et al*., 2004; Sa-Pereira *et al*., 2004; Heck *et al*., 2005) and *Streptomyces* (Marui *et al*., 1985; Nakajima *et al*., 1984; Nascimento *et al*., 2002; Techapun *et al*., 2002, 2003; Wang *et al*., 2003; Ito *et al*., 2004).

Different genera of yeast have also been reported to produce extracellular xylanases, such as *Cryptococcus albidus* (Notario *et al*., 1979; Biely *et al*., 1980), *Aureobasidium pullulans* (Leathers *et al*., 1986; Leathers, 1986, 1989) and *Pichia stipitis* (Lee *et al*., 1987; Gorgens *et al*., 2005).

2.5.2. Xylanase biosynthesis

The microbial production of xylanases has been shown to be inducible (Hrmová *et al*., 1986; Piñaga *et al*., 1994; Zhao *et al*., 1997). In the regulation of xylanase synthesis, it is the low-molecular mass fragments resulting from xylan hydrolysis that play a key role since xylan, being a high molecular mass polymer, cannot penetrate the cell wall. These xylan hydrolysis products include xylose, xylobiose, xylotriose and other oligosaccharides (Wang *et al*., 1992; Zhao *et al*., 1997). There are a number of reviews describing the generally accepted mechanism by which xylanase regulation is believed to occur (Haltrich *et al*., 1996; Kulkarni

et al., 1999). Apparently, low levels of the hydrolysing enzymes are constitutively formed and secreted, thereby partially hydrolysing the substrate and liberating small amounts of the hydrolysis products. The uptake of these compounds by the organism will signal the presence of the polymeric substrate, thus triggering the synthesis of larger quantities of the enzyme required for the complete breakdown of the substrate. Of the xylan hydrolysis products, xylose is the smallest molecule and can easily enter the microbial cells and induce xylanase synthesis. The other products are, however, larger and their transportation into the cell may be problematic.

Subramaniyan and Prema (2002) offered two plausible explanations for the direct induction by these larger molecules based on reports by Wang *et al*. (1992) and Gomes *et al*. (1994). One explanation was that xylo-oligomers formed due to xylan hydrolysis are directly transported into the cell matrix where they are further degraded by intracellular ß-xylosidases that release xylose residues in an exo-fashion. The second explanation offered was that the xylo-oligomers are hydrolysed by hydrolytic transporters possessing exo-ß-1,4-bond cleaving proteins, such as the ß-xylosidases, into monomers as they are transported through the cell membrane into the cell matrix. Either way, the end product xylose leads to enhanced xylanase production levels. A diagrammatical representation, redrawn from Kulkarni *et al*. (1999), showing the possible factors affecting xylanase induction, is shown in Figure 2.6. In the hypothetical model, constitutive xylanases degrade xylan to xylooligosaccharides and xylobiose. These compounds are then taken up into the cell and, in turn, induce other xylanase genes. Inducible xylanases further degrade xylan to xylooligosaccharides and xylobiose. The ß-xylosidases, produced either consitutively or by induction, convert xylobiose to xylose and may also transglycosylate it to XylB1-2Xyl and GlcB1-2Xyl. These compounds are subsequently taken up by the cell and act as additional inducers of genes encoding for xylanolytic enzymes.

Xylobiose, together with other xylooligosaccharides, has been reported to be a potent inducer in a range of microorganisms (Yasui *et al*., 1984; Royer and Nakas, 1989; Gosh and Nanda, 1994; Piñaga *et al*., 1994). Likewise, xylose can also be an effective inducer of xylanase synthesis (Leathers *et al*., 1986; Lopez *et al*., 1998; Liu *et* al., 1998; Gosh and Nanda, 1994), although in some cases xylose in the culture medium may result in catabolite repression of xylanase synthesis (Haltrich and Steiner, 1994; Ishihara *et al*., 1997; Sachslehner *et al*., 1998; Liu *et al*., 1999). Similarly, the presence of glucose in the culture

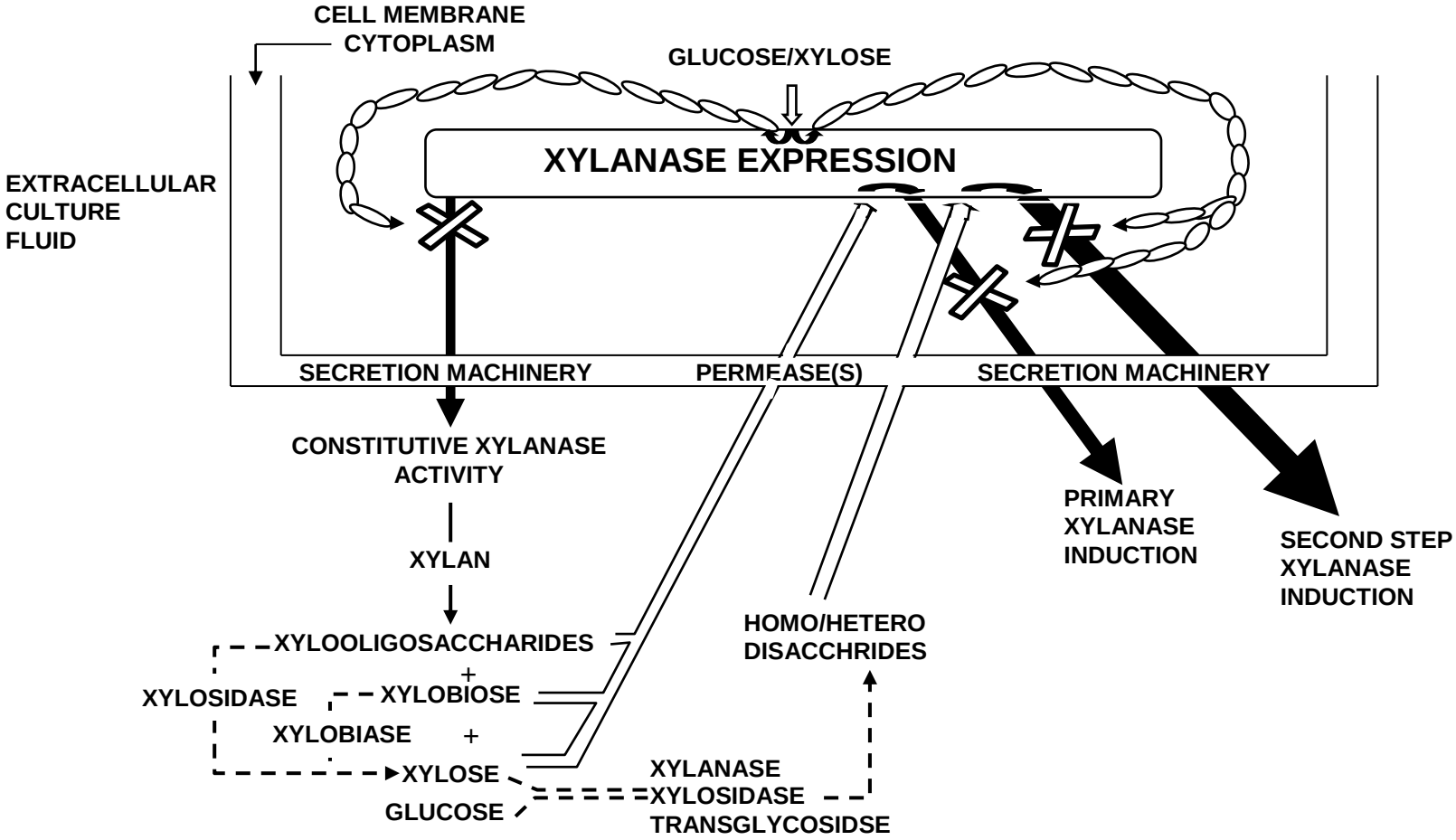

Figure 2.6. A hypothetical model showing the regulation of xylanase biosynthesis (Redrawn from Kulkarni *et al*., 1999)

medium has been reported to repress xylanase synthesis (Leathers *et al*., 1986; Hoq *et al*., 1994; Piñaga *et al*., 1994; de Souza *et al*., 2001; Prathumpai *et al*., 2004). The synthesis of xylanase has also been shown to be inducible by ß-methyl-D-xylopyranoside, a non-metabolisable, structural analogue of xylobiose. This substrate was shown to effectively induce xylanase synthesis in *Aspergillus sydowii* (Gosh and Nanda, 1994), *Thermoascus aurantiacus* (Gomes *et al*., 1994), *Aureobasidium pullulans* (Leathers *et al*., 1986) and in *Cryptococcus flavus* (Yasui *et al*., 1984).

Practically all xylanase producing microorganisms also produce cellulases and vice versa. Reports in the literature as to whether or not cellulolytic and xylanolytic enzyme systems are under separate or common regulatory control varies. Eriksson and Goodell (1974) proposed that in *Polyporus adustus*, a white rot fungus, cellulases, mannases and xylanases are under a single common regulatory gene. However, Hrmová *et al*. (1986) suggested that in *T. reesei*, the synthesis of cellulases and xylanases are under the control of separate regulatory genes. The formation of xylanase was selectively induced by xylan and xylobiose, whereas sophorose induced the production of both cellulase and xylanase. Similarly, in *A. terreus*, xylan degrading enzymes could be selectively induced by using either xylan, xylobiose or xylose. In the presence of cellulose or cellobiose, both cellulase and xylanase enzymes were produced (Hrmová *et al*., 1989). The induction of xylanase in the presence of cellulosic substrates may be attributed to contaminating xylan, which is present in commercially available cellulose (Hrmová *et al*., 1986).

Xylanase repression with glucose as carbon substrate in *A. nidulans* was investigated by Piñaga *et al*. (1994) and it was found that xylanase production only began after the glucose concentration in the medium decreased to ca. 0.1 % (w/v) from an initial concentration of 1 % (w/v). This result was confirmed by evaluating the production of xylanase using a medium containing 1 % oat spelts xylan and 0.1 % (w/v) glucose and under these conditions no carbon catabolite repression was detected (Piñaga *et al*., 1994). Liu *et al*. (1999) also reported similar results, where xylanase production by *Trichosporon cutaneum* was repressed when the glucose concentration was above 0.1 % (w/v) in the culture medium.

2.6. Factors affecting the production of microbial xylanases

In addition to the carbon substrate and culture medium composition, a wide range of environmental parameters determine the production of microbial xylanases. Some of these parameters that will be discussed further in this section include the culture pH, temperature, aeration rate as well as agitation intensity.

2.6.1. Selection of an inducing substrate

One of the basic factors affecting the production of xylanolytic enzymes is the choice of an appropriate inducing substrate which, besides providing the necessary inducing compounds for xylanase production, also serves as carbon and energy source. Purified xylans and low-molecular weight compounds derived thereof have been reported to be excellent substrates for xylanase production and significantly high xylanase activities with xylan as substrate have been reported (Bailey and Viikari, 1993; Fernández-Espinar *et al*., 1992; Costa-Ferreira *et al*., 1994; Kadowaki *et al*., 1997; Bakir *et al*., 2001; Nascimento *et al*., 2002; Anthony *et al*., 2003; Lama *et al*., 2004; Xiong *et al*., 2004; Seyis and Aksoz, 2005). Similarly, xylose has also been reported to be a potent inducer of xylanase production (Purkarthofer and Steiner, 1995; Zhao *et al*., 1997), although in some cases it can act as repressor in the production of xylanase (Hoq *et al*., 1994; Kadowaki *et al*., 1997). Other substrates reported to induce xylanase production also include cellulose (Panda, 1989; Sadana *et al*., 1980) and lactose (Haltrich *et al*., 1995; Haapala *et al*., 1994), amongst others. ß-Methyl-D-xyloside (ßMX), a synthetic analogue of xylobiose, has been used as alternative inducer for the production of xylanase. Bailey and Poutanen (1989) showed that the addition of ßMX to the culture medium resulted in significantly higher xylanase activities and a linear relationship between xylanase activity and ßMX concentration in the medium, from 0 to 5 g l^{-1} with *A. oryzae* was shown. Simao *et al*. (1997) also showed that ßMX significantly enhanced xylanase production in *A. tamarii* and that this organism was able to produce biomass with ßMX as the sole carbon substrate. This was, however, contrary to results reported by Gosh and Nanda (1994) and Biely *et al*. (1980) where ßMX alone was not able to support growth but could induce xylanase production in the respective cultures, thereby functioning as a non-metabolisable inducer.

Due to the high cost of commercially available purified xylans, alternative substrates for xylanase production have been investigated. Agricultural by-products containing cellulose, hemicellulose and lignin could serve as inexpensive sources for xylanase production and since hemicellulose constitutes the second most abundant polymer in nature, utilisation of

agricultural by-products and other natural compounds by xylanolytic microorganisms may provide an alternative towards lower production costs. Several inexpensive hemicellulosic substrates such as wheat bran, corn cob, sugar cane bagasse, barley husk, hay , rice straw, have been evaluated for the production of xylanases by various microorganisms, including yeasts, bacteria and filamentous fungi (Haltich *et al*., 1996; Kadowaki *et al*., 1997; Nascimento *et al*., 2002; Xiong *et al*., 2004).

Nascimento *et al*. (2002) evaluated xylanase production by a *Streptomyces* strain using different lignocellulosic substrates such as wheat bran, wheat germ, brewer's spent grain, corn cobs and recycling paper mill sludge. High xylanase activities were obtained with wheat bran, wheat germ and brewer's spent grain as carbon substrates, although higher activities were obtained with larchwood xylan. Xylanase production, however, was repressed by glucose and xylose. *A. tamarii* (Kadowaki *et al*., 1997) was shown to produce high xylanase activities with corn cob powder and wheat bran as carbon substrate, although activities were lower than with oat spelts xylan. However, *T. lanuginosus* was shown to produce significantly higher xylanase activities with corn cobs than with xylan as carbon substrate (Xiong *et* al., 2004). Similarly, Chandra and Chandra (1995) reported higher xylanase activities with *A. fischeri* when using a lignocellulosic substrate, namely wheat bran, than with xylan as carbon substrate. Wheat bran was also found to induce appreciable levels of xylanase activity in several *Aspergillus* strains evaluated by Bailey and Poutanen (1989). Addition of deacetylated xylan to the wheat bran-containing medium slightly enhanced xylanase production by *A. oryzae*, whereas the addition of ßMX to the culture medium increased xylanase production significantly (Bailey and Poutanen, 1989).

2.6.1.1. Pretreatment of lignocellulosic substrates

The utilisation of lignocellulosic substrates for xylanase production may, in some cases, require pretreatment with the aim of making the substrate more available to the microorganisms by increasing accessibility of the cellulosic or hemicellulosic substrate to enzymatic attack. One of the widely accepted methods for solubilising lignin from the lignocellulosic substrates involves treatment with mild alkali with 1 M NaOH (Shah and Madamwar, 2005). Pham *et al*. (1998) reported higher xylanase activities with alkali treated lignocellulosic substrates than with birchwood xylan as carbon substrate. These substrates included alkali treated corn cobs, which yielded the highest xylanase activities, as well as treated wheat straw and corn leaf. These findings suggested that for the strain used by Pham *et al*. (1998), pretreatment of corn cobs, wheat straw and corn leaf was a prerequisite

for enhanced xylanase production. Contrary to the findings of Pham *et al*. (1998), Shah and Madamwar (2005) observed a slight negative effect of alkali treatment of wheat straw and corn cobs on the production of xylanase by *A. phoenicis*. Similarly, xylanase production by *A. ochraceus* was also observed to be affected negatively by alkali treatment of the substrate (Biswas *et al*., 1988).

Steam-hydrolysis has also been used in the pretreatment of lignocellulosic substrates. Bakir *et al*. (2001) found that xylanase production by *Rhizopus oryzae* was three to seven times higher with agricultural by-products that had been steam-hydrolysed than with untreated substrates. The particle size of certain lignocellulosic substrates has also been shown to have a significant impact on the production of xylanases by some microbial strains. It was reported that in the production of xylanase by *T. lanuginosus* with corn cobs as substrate, a particle size of 2 to 7 mm was optimal, whereas if the same substrate was used as a fine powder, a significant decrease in xylanase activity of more than three-fold occurred. Also, a reduction in the particle size of wheat or barley straw from between 2 and 3 to 0.20 and 0.25 mm resulted in a decrease in enzyme yields by *Sporotrichum thermophile* (Sugden and Bhat, 1994). To explain this phenomenon, Biswas *et al*. (1988) suggested that pretreatment increased the available surface area, thereby enhancing the consumption of substrate and concurrent release of monomeric sugars leading to the repression of xylanase synthesis.

2.6.1.2. Effect of nitrogen

Mechanisms governing the formation of extracellular proteins are influenced by the availability of precursors for protein synthesis (Kulkarni *et al*., 1999). Nitrogen sources used in the xylanase production medium have been shown to have a dramatic influence on enzyme production. Also, the nitrogen source can significantly affect the pH of the culture medium during the course of cultivation (Haapala *et al*., 1994). For a strain of *Bacillus polymyxa*, it was shown that organic nitrogen generally induced greater xylanase production than inorganic nitrogen, with almost no xylanase activity detected when the strain was grown in media containing inorganic nitrogen sources (Pham *et al*., 1998). Similarly, for several other fungi, reports show that the addition of a complex nitrogen supplement resulted in higher xylanase production than when inorganic nitrogen sources were used (Smith and Wood, 1991; Fernández-Espinar *et al*., 1992; Gaspar *et al*., 1997). Good xylanase activities have generally been obtained when peptones or yeast extract has been used in the production medium. Yeast extract was shown to yield the best results for the production of xylanase activities by *B. polymyxa* (Pham *et al*., 1998). This was in agreement with results

reported by Piñaga *et al*. (1993) where yeast extract was found to be the best nitrogen source for this strain. For a strain of *A. foetidus*, proteose peptone was shown to be the best source of nitrogen for xylanase production (Shah and Madamwar, 2005). Other alternative nitrogen sources that have been shown to successfully enhance xylanase production include cotton seed flour, corn steep liquor as well as soybean meal (Haltrich and Steiner, 1994).

2.6.1.3. Other medium components

In addition to the carbon and nitrogen sources, a typical medium for xylanase production will also contain several mineral salts and some metallic ions. Mineral salts would generally comprise KH_2PO_4, K_2HPO_4, $MgSO_4$, $CaCl_2$, NH^{4+} or NO_3^-salts, whereas metallic ions would comprise components of a trace element solution, which would include Fe^{2+}, CO^{2+} and Zn^{2+} (Gomes *et al*., 1993). However, there are no reports in the literature regarding the effects of nutrients, other than the carbon and nitrogen sources, on xylanase production.

2.6.2. Culture conditions

2.6.2.1. Effect of culture pH

The pH value of a cultivation medium has been shown to be an important parameter which significantly affects the production of xylanases. Purkarthofer *et al*. (1993) reported that a cultivation pH of 7.5 resulted in increased xylanase production by *T. lanuginosus*. It was also noted that at this pH xylanase production began earlier and the enzyme production phase lasted longer. As the optimum pH of xylanase activity of *T. lanuginosus* is 6.5, it was suggested that at cultivation pH 7.5, there was a slower release of soluble hydrolysis products from the substrate (corn cob xylan). Conducting hydrolysis experiments at pH 6.5 and 7.5 and investigating the concentration of reducing sugars verified this hypothesis. The authors found that the concentration of xylose after 6 h of hydrolysis at pH 7.5 was 85 % of that released at pH 6.5. Similarly, Royer and Nakas (1989) found that the optimum pH for xylanase and cellulase production by *T. longibrachiatum* of between 6.5 and 7.7 was higher than that for optimum xylanase activity and also suggested that the high xylanase production pH may result in a relatively slow release and utilisation of soluble end-products of cellulose and xylan hydrolysis.

Bailey and Viikari (1993) found that it was essential to decrease the pH of the cultivation medium to below 3.5 for efficient xylanase production by *A. fumigatus*. A cultivation in which the pH was maintained between 2.7 and 3.0 yielded higher xylanase activities than the cultivation in which the pH was not controlled, resulting in an increase in culture pH to 6.5.

However, Anthony *et al*. (2003), working with an alkali-tolerant *A. fumigatus* strain, reported results contrary to those of Bailey and Viikari (1993), finding maximal xylanase production at a cultivation pH of 5.0. This alkali-tolerant strain was found to produce xylanase in a wide pH range, from pH 4 to 10.

2.6.2.2. Effect of cultivation temperature

A significant part of the cost of optimal enzyme production is associated with maintaining a constant fermentation temperature. The effect of temperature on microbial xylanase production is highly variable and not only affects the growth rates but may also affect the levels of xylanase produced. Smith and Wood (1991) showed that for *A. awamori*, xylanase production was similar in the range of 25 to 35 °C with the optimal xylanase productivity occurring at 35 °C. However, at temperatures above 35 and below 25 °C, a marked decrease in xylanase production was observed. At 40 and 20 °C, there was a 50 % and 33 % decrease in xylanase production, respectively. Castro *et al*. (1997) also reported that cultivation temperature had a significant effect on the growth and xylanase production of a newly isolated *Aspergillus* strain. This *Aspergillus* strain showed better growth at 37 than at 45 °C, but a higher xylanase activity was obtained at 45 than at 37 °C with the specific activity being five-fold higher at the higher temperature. However, the xylanases produced at 37 °C exhibited a higher temperature optimum of 80 °C compared to 50 °C for the xylanases produced at 45 °C. This result was surprising, since it was expected that the enzymes produced at the higher temperature would have a higher temperature optimum. On the other hand, Kvesitadze *et al*. (1994) reported that xylanases produced by *Allescheria terrestris* at a cultivation temperature 48 °C exhibited a higher thermostability than those produced at 40 °C. The reason for the difference in thermostability is not clear but it was suggested that the high thermostability exhibited by the xylanases produced at 48 °C may in some way be related to the structural characteristics of the enzyme or it could be that different enzymes with differing properties are produced at the different cultivation temperatures.

2.6.2.3. Effect of aeration

Amongst the many environmental factors affecting xylanase production, the aeration rate has been shown to have a significant effect on xylanase production (Hoq *et al*., 1994; Palma *et al*., 1996; Singh *et al*., 2000; Reddy *et al*., 2002). Varying the aeration rates from 0.5 to 1.5 vvm in a 15 l stirred tank bioreactor during cultivation of *T. lanuginosus* cultivations, xylanase production was found to be highest at an aeration rate of 1 vvm. Slightly lower

xylanase activities were obtained at 0.5 vvm and even lower at 1.5 vvm (Hoq *et al*., 1994). Also, at all the agitation rates evaluated, aeration at 1 vvm resulted in a higher total dry mass, increased specific growth rates as well as reduced cultivation times required for the maximum xylanase concentration to be reached. The higher final biomass concentrations obtained with 1 vvm were attributed to the increase in the mass transfer rate of oxygen (Hoq *et al*., 1994). Similarly, Reddy *et al*. (2002) reported that the optimum aeration rate using a 30 l stirred tank bioreactor for xylanase production by *T. lanuginosus* SSBP was 1 vvm and above this rate the xylanase activity declined sharply. Xylanase production by *Penicillium janthenellium* using a 4 l bioreactor was also reported to be adversely affected by increasing the aeration rate, with optimum xylanase production occurring at 0.2 vvm (Palma *et al*., 1996).

2.6.2.4. Effect of agitation intensity

As a result of the high apparent viscosities and non-Newtonian behavior of broths in many fungal fermantations, the use of high agitation speeds to provide adequate mixing and oxygen transfer is often necessary. However, due to mycelial damage at high stirrer speeds, the acceptable range of speeds is limited, thereby resulting in oxygen transfer limitations as well as volumetric productivity limitations (Amanullah *et al*., 1999). A screening of process variables by Milagres and Lacis (1991) in a *Penicillium janthinellum* cultivation indicated that agitation intensity was an important variable. Similarly, there are a number of reports indicating that agitation plays an important role in xylanase production by filamentous fungi (Palma *et al*., 1996; Lejeune and Baron, 1995; Purkarthofer *et al*., 1993; Singh *et al*., 2000; Gomes *et al*., 1994; Hoq *et al*., 1994). The impeller tip velocity often represents the shearing force of a stirred tank fermentor; however, almost all studies use the agitation rate as the sole measure of shear stress, thereby making it difficult to draw any meaningful comparisons between studies, especially when different vessel sizes were employed in different studies. Therefore, the use of impeller tip speed as representative of the shear stress is preferable for comparative purposes (Mukataka *et al*., 1988; Gibbs *et al*., 2000).

Hoq *et al*. (1994) evaluated the effect of varying the agitation intensity in a 15 l bioreactor from 100 to 300 rpm on xylanase production by *T. lanuginosus* RT9 and reported that the highest xylanase activity was obtained with the agitation speed of 200 rpm. Higher or lower agitation speeds resulted in a decrease in xylanase production by the strain. It was suggested that at the low agitation rate (100 rpm) there was a mass transfer limitation that resulted in low xylanase activities, whereas at the higher agitation rate (300 rpm), the shear

force effect may have caused the low xylanase activities. At this high agitation rate, the authors observed that the fungal hyphae were highly branched and suggested that this could have been the cause for a decrease in the production of xylanase. Purkathofer *et al*. (1993) reported similar effects of agitation intensity on xylanase production by *T. lanuginosus*. Palma *et al*. (1996) also reported a decrease in xylanase production by *P. janthinellum* with increasing agitation intensity using a 4 l Microferm bioreactor. It was reported that intensive agitation altered the morphology of the organism and this could have led to the reduction in xylanase production. Singh *et al*. (2000) evaluated the effect of agitation rate and dissolved oxygen tension on the production of hemicellulases by *T. lanuginosus* SSBP using a 15 l bioreactor and the results indicated that agitation intensity affected xylanase production with maximal xylanase production occurring at a low stirrer speed of 400 rpm. High stirrer speeds of up to 1 400 rpm were found to curtail xylanase production and this was attributed to the effect of hydrodynamic stress.

2.7. Concluding remarks

The pulp and paper industry is one of the major environmental polluters and utilises huge amounts of water. However, due to public pressure and legisaltion, this industry is forced into finding alternative methods for pulp and paper manufacture to yield more environmentally friendly waste waters; hence the application of xylanases in bleaching, which is considered as one of the most important and new biotechnological applications. This literature survey showed that xylanase application in this industry leads to improved bleachability resulting in an increased final brightness, a decrease in chemical consumption and a reduction in the environmental loading of both kraft and sulphite pulps, although most research as well as the large scale industrial applications have been conducted using kraft pulp.

The cost of xylanase production for large scale application is one of the limiting factors. The costs of the carbon substrate and/or inducing substrate, as well as the additional medium components largely determine the economics of xylanase production. Therefore, to minimise production costs, a relatively cheap, abundant and inexpensive substrate would be appropriate. However, care should be taken in the selection of an inducing substrate so that it does not result in the repression of xylanase production, as has been shown to occur with some carbon substrates such as glucose and, in some cases, xylose. Although xylose is

suitable for microbial growth, it may result in the repression of xylanase production. The utilisation of lignocellulosic substrates may be one such alternative for reducing xylanase production costs. Also, the utilisation of pulp mill waste waters such as spent sulphite liquor, which is abundantly available and exhibits a high BOD, may be the key in bringing down xylanase production costs. It was with this in mind that the current study of evaluating spent sulphite liquor as carbon substrate for xylanase production was initiated.

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CHAPTER 3

XYLANASE PRODUCTION BY FUNGAL STRAINS ON SPENT SULPHITE LIQUOR

An abridged version of this chapter has been accepted for publication:

Zawadi A. Chipeta, James C. du Preez, George Szakacs and Lew Christopher (2005). Xylanase production by fungal strains on spent sulphite liquor. *Appl Microbiol Biotechnol* **(in press).**

Contents

3.1. Abstract

Xylanase production by seven fungal strains was investigated using concentrated spent sulphite liquor (SSLc), xylan and D-xylose as respective carbon substrates. An SSLc-based medium induced xylanase production at varying levels in all of these strains, with *Aspergillus oryzae* NRRL 3485 and *Aspergillus phoenicis* ATCC 13157 yielding activities of 164 and 146 U ml^{-1}, respectively; these values were higher than those obtained on xylan or D-xylose with the same fungal strains. Whereas xylan induced high activities in some strains, up to 322 U ml^{-1} xylanase activity with *Aspergillus foetidus* ATCC 14916, xylose induced little or no xylanase activity in the fungal strains evaluated. *A. oryzae* and *A. phoenicis* yielded high biomass concentrations with SSLc as carbon substrate and the high concentrations were attributed to the utilisation of additional non-sugar carbon present in the SSLc, as was indicated by the total organic carbon results. Electrophoretic and zymogram analysis indicated three xylanases from *A. oryzae* with molecular weights of approximately 32, 22 and 19 kDa, whereas *A. phoenicis* produced two xylanases with molecular weights of about 25 and 21 kDa. Crude xylanase preparations from these *A. oryzae* and *A. phoenicis* strains exhibited optimal activities at pH 6.5 and 5.0 and at 65 and 55 °C, respectively. The *A. oryzae* xylanolytic activity was stable at 50 ºC in a pH range of 4.5 to 10. The crude xylanase preparations from these *A. oryzae* and *A. phoenicis* strains had negligible cellulase activity and their application in the biobleaching of hardwood pulp reduced chlorine dioxide consumption by 20 to 30 % without sacrificing brightness.

3.2. Introduction

Fungal endo-β-xylanases are finding use as a pre-bleaching aid to chemical pulp bleaching (Viikari *et al*., 1994; Onysko, 1993). It has been shown that application of these enzymes effected significant increases in the brightness of kraft pulps or reduced the usage of active chlorine (Paice *et al*., 1989; Bailey and Viikari, 1993). Recently, it was shown that these enzymes were also beneficial in the biobleaching of sulphite pulps (Christov and Prior, 1996; 1997; Christov *et al*., 1999). Xylanase preparations used in pulp bleaching have to be free of cellulolytic enzymes to avoid deterioration of strength properties and to minimize yield loss (Bailey and Viikari, 1993; Viikari *et al*., 1994).

Spent sulphite liquor (SSL) results from the delignification of wood chips in an aqueous solution of acid bisulphites with an excess of SO_2, resulting in the solubilisation of lignin and leaving the wood cellulose largely undegraded (Mueller and Walden, 1970). This effluent may contain 11 to 14 % solids, depending on the type of wood and the cooking process used (Mueller and Walden, 1970; McKee and Quicke, 1977). The solids fraction comprises mainly 65 to 70 % lignosulphonates, 20 to 30 % pentose and hexose sugars and 6 to 10 % polysaccharides, galacturonic and acetic acid, resins, unconsumed bisulphite and ash (Mueller and Walden, 1970; McKee and Quicke, 1977).

Whereas SSL has been used as carbon feedstock for the production of yeast (Mueller and Walden, 1970; Chaudry *et al*., 1977; McKee and Quicke, 1977) and filamentous fungal biomass (Pretorius and Lempert, 1993a,b,c) as well as ethanol (Kosaric *et al*., 1981; Taherzadeh *et al*., 2003), the use of SSL for enzyme production, particularly xylanases, has not previously been investigated. However, a recent study on xylanase production using bleach plant effluent as carbon source, reported activities of less than 6 U ml^{-1} (Christov *et al*., 1999).

This study evaluated the production of xylanases by seven strains of the genera *Aspergillus* and *Gliocladium* using SSL that had been concentrated at a pulp mill as carbon feedstock. The properties of the crude xylanase preparations as well as their efficacy in the pre-bleaching of hardwood cellulose pulps were also determined.

3.3. Materials and methods

3.3.1. Fungal strains

The filamentous fungi used in this study included six *Aspergillus* strains (*A. oryzae* NRRL 1808 (ATCC 12892) and NRRL 3485 (ATCC 46244), *A. niger* ATCC 10864, *A. foetidus* ATCC 14916, *A. phoenicis* ATCC 15555 and ATCC 13157) and *Gliocladium viride* CBS 658.70. The same strains, excluding *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157, had previously been investigated using bleach plant effluent as carbon substrate (Christov *et al*., 1999). The cultures were maintained on Sabouraud-dextrose agar slants (Biolab Diagnostics, Midrand, South Africa) and stored at 4 °C with subculturing at 12-week intervals.

3.3.2. Media and cultivation conditions

The SSL was supplied in a concentrated form (designated SSLc) from an acid sulphite pulp mill in South Africa, where it had been concentrated approximately five-fold by a three-step evaporation process. The respective carbon sources used included the above SSLc diluted ten-fold with distilled water to give 11 to 13 g total sugars l^{-1} (depending on the SSLc batch), oat spelts xylan (10 g l^{-1}; Sigma Chemical Co., St. Louis, MO, USA) and D-xylose (10 g l^{-1}; Sigma). The other medium constituents were (g l^{-1}): citric acid, 0.25; $(NH_4)_2SO_4$, 5; K_2HPO_4, 5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.02 and yeast extract, 10 as well as 1 ml of a trace element solution (du Preez and van der Walt, 1983). These media were adjusted to pH 6.0 prior to autoclaving. The inoculum was prepared by inoculating each of 500 ml Erlenmeyer flasks containing 100 ml of the appropriate medium with 5 ml of a fungal spore suspension of ca. 10^7 spores ml^{-1}, washed from a 5-day old Sabouraud dextrose agar plate culture with 10 ml of a 0.05 M KH_2PO_4 solution containing 0.1 % Tween 80. These flasks were incubated at 210 rpm on a rotary shaker at 30 °C for 48 h and 5 ml were subsequently transferred to each of a second group of similar sterile shake flasks. The xylanase activity and biomass concentration of these cultures were determined over a 5-day incubation period.

3.3.3. Analytical methods

3.3.3.1. Sugar composition

The sugar composition of the SSL, SSLc and culture supernatants was determined using a Dionex 4000i HPLC (Dionex Corp., Sunnyvale, CA, USA) equipped with a pulse amperometric detector and gold electrode, using a 250 x 4 mm (I.D.) PA1 Carbopac column (Dionex Corp.) with inlet and oven temperatures of 30 °C and 1.8 mM NaOH as eluent at a flow rate of 1 ml min^{-1} with D-xylose, D-galactose (both Sigma), D-glucose (Saarchem, Wadeville, South Africa) and L-arabinose (Merck, Darmstadt, Germany) as standards.

3.3.3.2. Acetic acid determination

Samples were acidified with formic acid (7.5 % v/v) prior to injecting into a Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Atlanta, GA, USA) equipped with a flame ionisation detector and a 150 x 2 mm (I.D.) glass column packed with 80-100 mesh Porapak Q (Waters Associates, Milford, MA, USA), using inlet and oven temperatures of 180 °C and a nitrogen carrier gas flow rate of 3 ml min^{-1}.

3.3.3.3. Other analyses

The chemical oxygen demand was determined by a standard spectrophotometric method (Anon., 1997). Total polyphenols in the effluent and in the culture media were determined by the prussian blue assay method (Graham, 1992) and the total organic carbon was determined at the Institute for Ground Water Studies, University of the Free State, Bloemfontein. Total and suspended solids were determined by drying aliquots of the effluent and its sediment after centrifugation at 13 800 x g for 10 min, respectively, to constant mass at 105 ºC.

3.3.3.4. Enzyme assays

Xylanase activity was determined using the dinitrosalicylic acid (DNS) assay method with birchwood xylan (Sigma) as substrate (Bailey *et al*., 1992). The crude enzyme solution was diluted with a 0.05 M, pH 6.0 citrate buffer, incubated with the substrate at 50 ºC for 5 min and the reaction subsequently stopped by the addition of DNS followed by boiling for 5 min and cooling in ice water prior to reading the absorbance at 540 nm using a DU 7500 spectrophotometer (Beckman Instruments, Fullerton, CA, USA).

Cellulase activity was determined using the filter paper assay (FPU) method with Whatman No. 1 filter paper (Whatman International Ltd, Maidstone, UK) as substrate (Ghose, 1987). The culture supernatant (0.5 ml) was incubated with substrate in a 0.05 M Na-acetate buffer (pH 4.8) for 1 h at 50 ºC and the liberated sugars assayed using the DNS assay. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar (xylose or glucose equivalents) per minute of reaction time.

3.3.3.5. Biomass determination

The fungal biomass concentration was gravimetrically determined after centrifugation of triplicate 5-ml aliquots of the respective cultures, washing twice with equal volumes of distilled water and drying at 105 ºC. In the presence of particulate xylan in the xylan-based medium, the fungal biomass was estimated from a standard curve of biomass versus protein concentration, using the biuret protein assay (Herbert *et al*., 1971).

3.3.4. Effect of pH and temperature on activity of xylanases

The effect of pH on xylanase activity was determined by using different reaction buffers (0.05 M citrate for pH 4.0, 5.0, 5.5, 6.0; 0.05 M phosphate for pH 6.5, 7.0, 8.0; and 0.1 M carbonate-for pH 9.0, and 10.0) at 50 ºC. The temperature optima of the crude xylanase

preparations were established by assaying at reaction temperatures of 40 to 80 ºC in a 0.05 M citrate buffer at pH 6.0. The pH stability of the xylanases was assessed by diluting the crude enzyme preparation in appropriate buffers at different pH values (as above) and incubating at 50 ºC for 30 and 90 min prior to determining the residual activity at pH 6.0 and 50 ºC. The thermal stability of the xylanases was similarly determined following incubation at temperatures ranging from 40 to 70 ºC in 0.05 M citrate buffer at pH 6.0. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar (xylose equivalents) per minute.

3.3.5. SDS-PAGE and zymogram analysis

The supernatants of centrifuged *A. oryzae* and *A. phoenicis* cultures with xylan and SSLc as carbon substrates were lyophilised and a 2 to 5 mg aliquot used for separation of the extracellular proteins by SDS-PAGE using a 12.5 % polyacrylamide gel. Prior to loading, the protein samples were incubated at 37 ºC (to avoid complete denaturation) for 1 h. After electrophoresis, the protein bands were stained with 0.2 % Coomassie Brilliant Blue R250 (MERCK, Merck KGaA, Darmstadt, Germany) to allow visualisation. SDS-PAGE broad range molecular weight standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as markers.

Zymogram samples were subjected to electrophoresis in a 12.5 % SDS-PAGE gel containing 0.1 % Remazol Brilliant Blue R-D-xylan (RBB xylan, Sigma) as soluble substrate for the xylanases. After electrophoresis the gel was washed twice for 1 h each in 0.02 M Tris-HCl buffer at pH 7.5 containing 0.7 % (w/v) Triton X-100 followed by a 20 min washing in the same buffer without Triton X-100. After washing, the gel was incubated at 50 ºC in 0.05 M citrate buffer at pH 6.0 until clearing zones were observed, then soaked in a 0.1 % Congo red (Saarchem, Merck Laboratory Supplies (Pty) Ltd, Gauteng, South Africa) solution for 15 min followed by destaining with 1 M NaCl. To clearly visualise the xylanase active bands, the gel was soaked in 5 % acetic acid which contrasted the active bands against a dark blue background.

3.3.6. Evaluation of xylanases in biobleaching

Oxygen-delignified soda-aq hardwood (Eucalyptus) pulp, prepared by cooking the wood chips in a solution of 20 % NaOH and 0.1 % anthraquinone, was obtained from a South African paper mill. The shake flask supernatants of *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157, grown on xylan and SSLc as respective carbon substrates, served as crude

xylanase preparations that were applied to the pulp. The *A. phoenicis* xylanase was applied at an enzyme charge of 10 U g^{-1} of dry pulp equivalent at pH 6.0 and 50 °C, whereas pH 7.0 and 70 °C were used in the case of the *A. oryzae* xylanase. A pulp consistency of 10 % was maintained and the aliquots were incubated for 2 h. Pulp samples were chemically bleached after enzyme treatment using a D_1ED_2 bleaching sequence (D_1 and D_2, chlorine dioxide at 2.63 and 1.315 % (w/w) active chlorine, respectively; E, alkali extraction with 0.7 % (w/w) NaOH). Subsequently, handsheets were prepared and brightness measured in triplicate using a brightness meter (Technidyne Colour Touch 2 model ISO, Technidyne Corporation, New Albany, Indiana, USA).

3.4. Results

3.4.1. Chemical composition of the spent sulphite liquor

The SSLc effluent had a high total sugar concentration of 145.2 g l^{-1} with a D-xylose content of more than 80 % of the total sugars, whereas arabinose, galactose, and glucose constituted the remainder of the total sugars (Table 3.1). Concentrating the SSL resulted in only a slight increase in the acetic acid content, indicating that most of the initial acetic acid content was lost through evaporation. The COD value was very high, indicative of the presence of a considerable amount of non-sugar organic material. The SSLc had a high viscosity (not determined) due to the high total solids content of which 2.4 % were suspended solids (Table 3.1).

3.4.2. Fungal growth and xylanase production

The SSLc induced xylanase production in all of these fungal strains, with major strain-specific differences evident (Table 3.2). Only strains *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 exhibited significantly higher xylanase activities on SSLc than on xylan (Table 3.2), a known inducer of xylanase activity (Bahkali, 1996; Siedenburg *et al*., 1998). On xylan, the highest xylanase activity of 322 U ml^{-1} was obtained with *Aspergillus foetidus* ATCC 14916. D-xylose effected no or little induction of xylanase activity. All of the strains exhibited negligible cellulase activity with xylan or SSLc as carbon feedstock. Further work focused on the two strains that exhibited the highest xylanase activities on SSLc, namely *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157.

Table 3.1. Composition and chemical properties (Mean values of duplicate determinations) of spent sulphite liquor

Property	SSL	SSLc
Xylose (g l^{-1})	23.6	119.0
Glucose (g l^{-1})	2.2	10.9
Arabinose (g l^{-1})	0.9	4.2
Galactose (g l^{-1})	2.4	11.1
Total sugars (g l^{-1})	29.1	145.2
Acetic acid (g l^{-1})	10.3	12.6
COD (mg l^{-1})	2.0 x 10^5	1.1 x 10^6
Suspended solids (g l^{-1})	10	18
Total solids (g l^{-1})	145	753
Total polyphenols (g l^{-1})	14	139
pH	2.7	3.3

SSL Spent sulphite liquor
SSLc Spent sulphite liquor concentrate
COD Chemical oxygen demand

Figures 3.1 and 3.2 depict typical cultivation profiles of these two strains grown on xylan and SSLc as respective carbon sources. Both xylose and glucose were readily utilised in the SSLc-based medium and after 24 h of cultivation the concentration of these sugars had decreased to below 0.2 g l^{-1}. The acetic acid was depleted after 72 and 48 h of cultivation of *A. oryzae* and *A. phoenicis*, respectively. With *A. oryzae*, the pH of the media with xylan and SSLc as carbon substrates increased to final values of pH 8.3 and 7.8, respectively (Figure 3.1). Growth of *A. phoenicis* resulted in the pH of the xylan-based medium decreasing to pH 3.8, whereas in the SSLc-based medium it increased to pH 6.2 (Figure 3.2).

Table 3.2. Xylanase activities (U ml^{-1})[a] produced by selected fungi on different carbon sources

Fungus	Carbon substrate		
	SSLc	Xylan	D-Xylose
Aspergillus oryzae NRRL 3485	172 (± 27.6)	110 (± 21.6)	27 ± (3.4)
Aspergillus oryzae NRRL 1808	50 (± 10.2)	130 (± 19.0)	4 (± 1.2)
Aspergillus phoenicis ATCC 13157	173 (± 25.6)	80 (± 16.2)	21 (± 1.6)
Aspergillus phoenicis ATCC 15555	11 (± 1.2)	42 (± 6.3)	0
Aspergillus foetidus ATCC 14916	32 (± 10.6)	322 (± 33.1)	9 (± 2.0)
Aspergillus niger ATCC 10864	7 (± 1.6)	31 (± 12.6)	3 (± 0.9)
Gliocladium viride CBS 658.70	10 (± 3.0)	23 (± 5.2)	0

[a] Mean values and standard deviations (in parentheses) of four experiments

SSLc Spent sulphite liquor concentrate, diluted ten-fold

3.4.3. Growth parameters for A. oryzae *and* A. phoenicis

The growth parameters of the above two strains are shown in Table 3.3. *Aspergillus oryzae* grew markedly faster than *A. phoenicis* in both culture media, with a significantly higher maximum specific growth rate on xylan than in the SSLc-based medium. The maximum volumetric rates of xylanase production by both strains were, however, higher with SSLc than with xylan as carbon substrate. The xylanase yield per g biomass, however, indicated that xylan was the stronger inducer of xylanase production (Table 3.3).

The higher maximum volumetric rates of xylanase production obtained with SSLc as carbon substrate could be as a result of the higher biomass concentrations reached in the SSLc-based medium. The total organic carbon (TOC) analyses showed that about 28 and 29 % of the TOC was utilised by *A. oryzae* and *A. phoenicis*, respectively, during growth in the SSLc-based media (Table 3.4). However, the total sugars utilised by *A. oryzae* and *A. phoenicis* accounted for only 57 and 58 %, respectively, of the TOC utilised, suggesting that non-sugar carbon in the SSLc accounted for 43 to 42 % of the TOC utilised (Table 3.4).

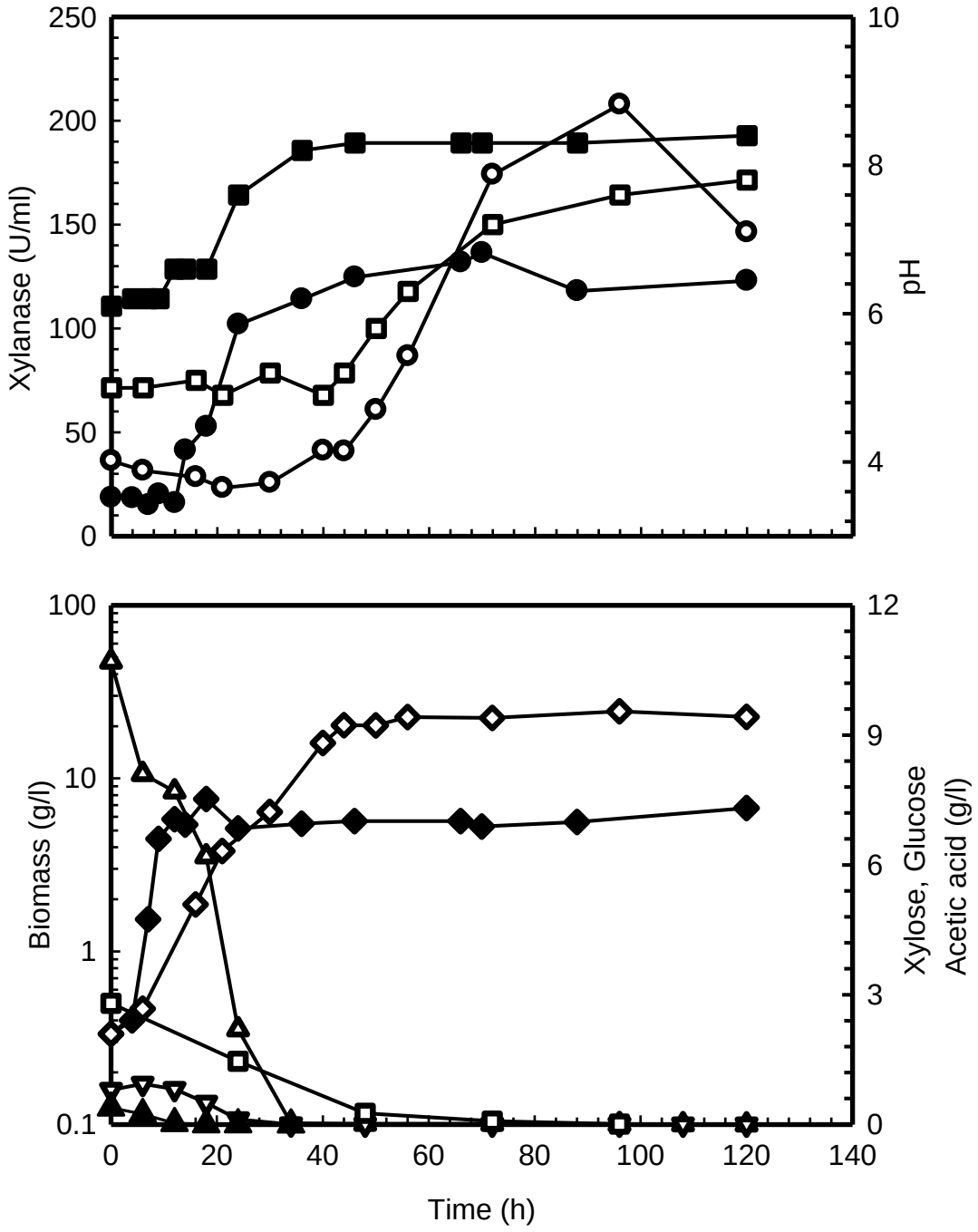

Figure 3.1. Typical cultivation profiles of *A. oryzae* NRRL 3485 with xylan (solid symbols) and SSLc (open symbols) as the respective carbon sources in shake flask cultures at 30 °C. Symbols: ●, ○ xylanase activity; ◆, ◊ biomass concentration; ■, □ pH; ▲, △ xylose; ▽ glucose; □ acetic acid.

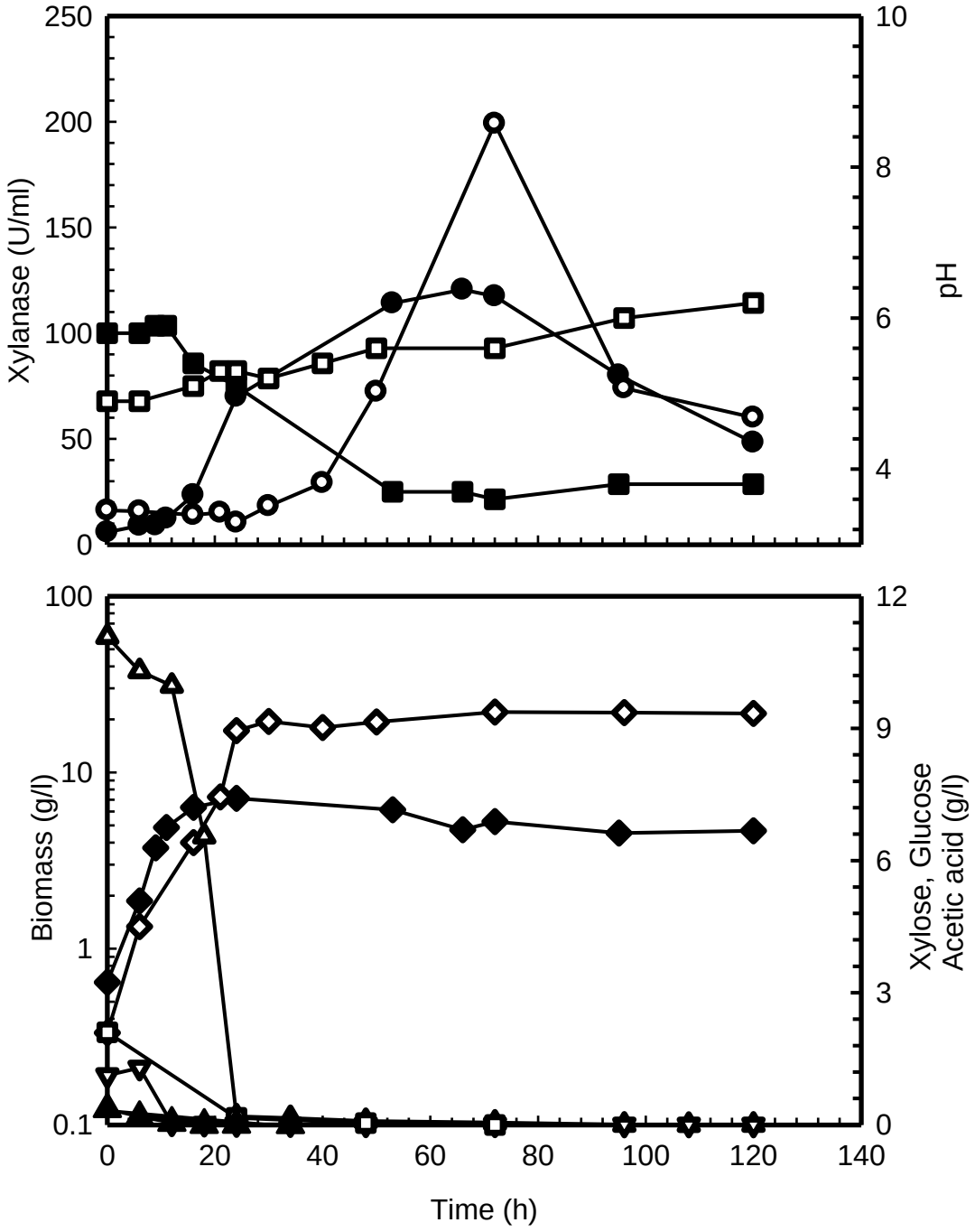

Figure 3.2. Typical cultivation profiles of *A. phoenicis* ATCC 13157 with xylan (solid symbols) and SSLc (open symbols) as the respective carbon sources in shake flask cultures at 30 °C. Symbols: ●, ○ xylanase activity; ◆, ◊ biomass concentration; ■, □ pH; ▲, △ xylose; ▽ glucose; □ acetic acid.

Table 3.3. Growth parameters of *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 in shake flask cultures with xylan and SSLc as respective carbon substrates

Growth Parameter[a]	***A. oryzae***		***A. phoenicis***	
	Xylan	SSLc	Xylan	SSLc
Xylanase (U ml^{-1})	110 (± 21.6)	172 (± 27.6)	80 (± 16.2)	173 (± 25.6)
Cellulase (FPUs)	0.1 (± 0.001)	0.4 (± 0.001)	0.2 (± 0.001)	0.2 (± 0.002)
Biomass (g l^{-1})	6.9 (± 0.61)	21.17 (± 2.93)	6.8 (± 0.70)	19.47 (± 2.19)
μ_{max} (h^{-1})	0.326 (± 0.02)	0.198 (± 0.08)	0.178 (± 0.02)	0.137 (± 0.01)
Q_p^{max} (U ml^{-1} h^{-1})	3.51 (± 0.14)	4.18 (± 0.21)	2.79 (± 0.26)	4.04 (± 0.33)
$Y_{p/x}$ (U g^{-1})	15 940 (± 2130)	8 120 (± 827)	11 760 (± 1228)	8 880 (± 2147)

[a] Mean values and standard deviations (in parentheses) of three experiments

FPUs Filter paper units

SSLc Spent sulphite liquor concentrate, diluted ten-fold

Q_p^{max} Maximum volumetric rate of xylanase production, calculated from the maximum slope of the enzyme activity versus time curve

$Y_{p/x}$ Xylanase yield coefficient, U g^{-1} biomass

μ_{max} Maximum specific growth rate

Table 3.4. Total organic carbon (g l^{-1}) [a] of culture supernatants of *Aspergillus oryzae* and *Aspergillus phoenicis* grown in SSLc-based medium

TOC_i	TOC_r	TOC_u	Sugar carbon utilised[b]	Non-sugar organic compounds utilised
Aspergillus oryzae NRRL 3485				
33.8	24.5	9.3	5.3	4.0
Aspergillus phoenicis ATCC 13157				
33.5	23.9	9.6	5.6	4.0

[a] Mean values of duplicate determinations

[b] Organic carbon utilised based on the total sugar content of the medium obtained by HPLC analysis

TOC_i Initial total organic carbon in culture supernatant

TOC_r Residual total organic carbon in culture supernatant at end of cultivation

TOC_u Total organic carbon utilised during growth

3.4.4. Physico-chemical properties of the xylanases

The optimal temperatures of the crude enzyme preparations of *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 under the standard assay conditions were 65 and 50 ºC and the optimum pH values were 6.5 and 5.0, respectively (Figures 3.3A and B). The xylanase activity of *A. oryzae* was stable between pH 4.5 and 10, whereas that of *A. phoenicis* was stable between pH 4.0 and 8.5, losing up to 25 % of its activity at pH 9.0 after 90 min of incubation (Figure 3.4). Xylanases from both strains were not stable above 40 ºC (Figures 3.5A and B) with the *A. oryzae* xylanase losing almost 50 % of its activity at 60 ºC after 5 min of incubation, whereas the *A. phoenicis* xylanase lost up to 70 % of its activity under similar conditions.

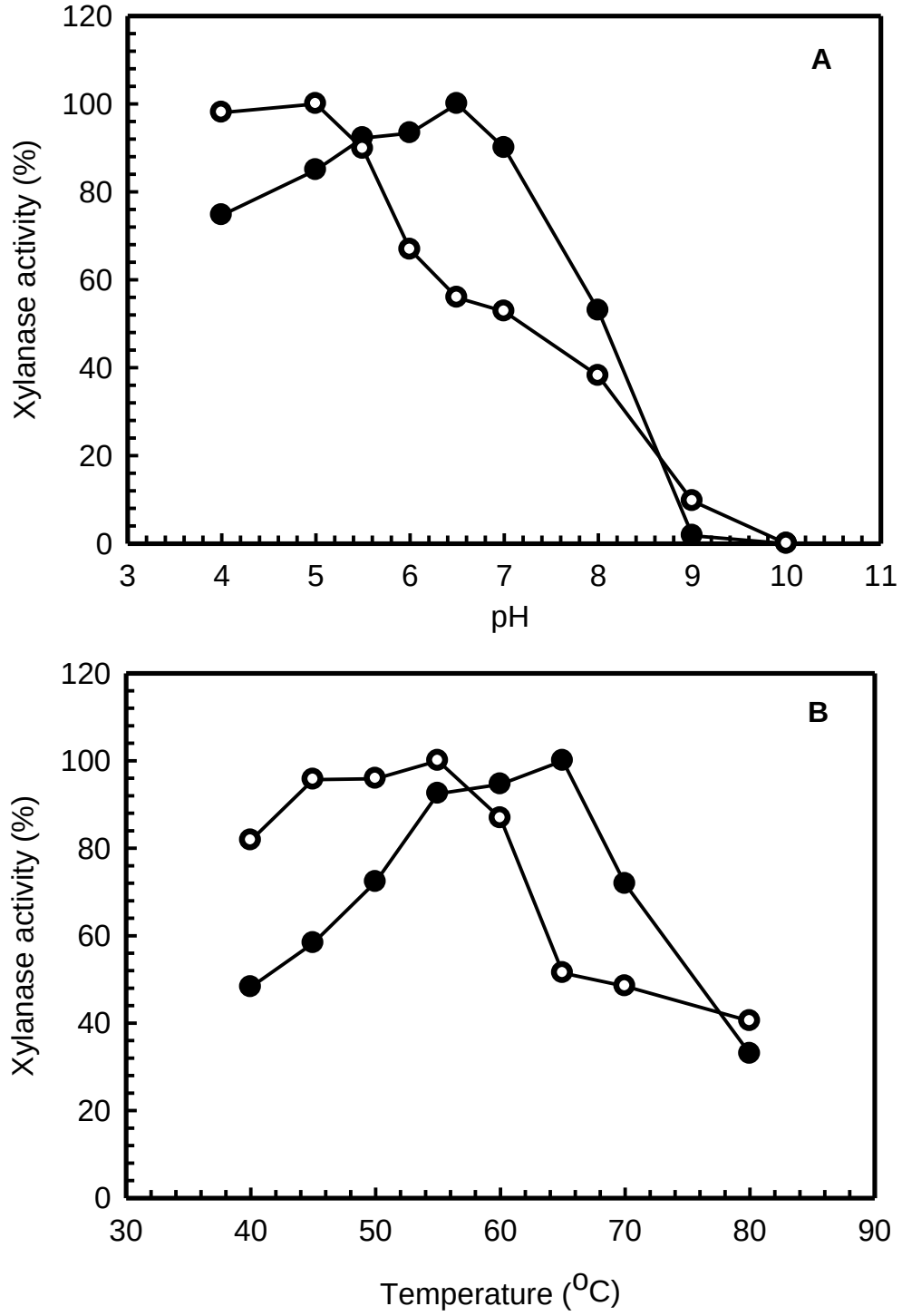

Figure 3.3. Xylanase activity of *A. oryzae* NRRL 3485 (●) and *A. phoenicis* ATCC 13157 (○) grown on SSLc as carbon source as a function of the assay pH (A) and assay temperature (B). Mean values of triplicate determinations are shown.

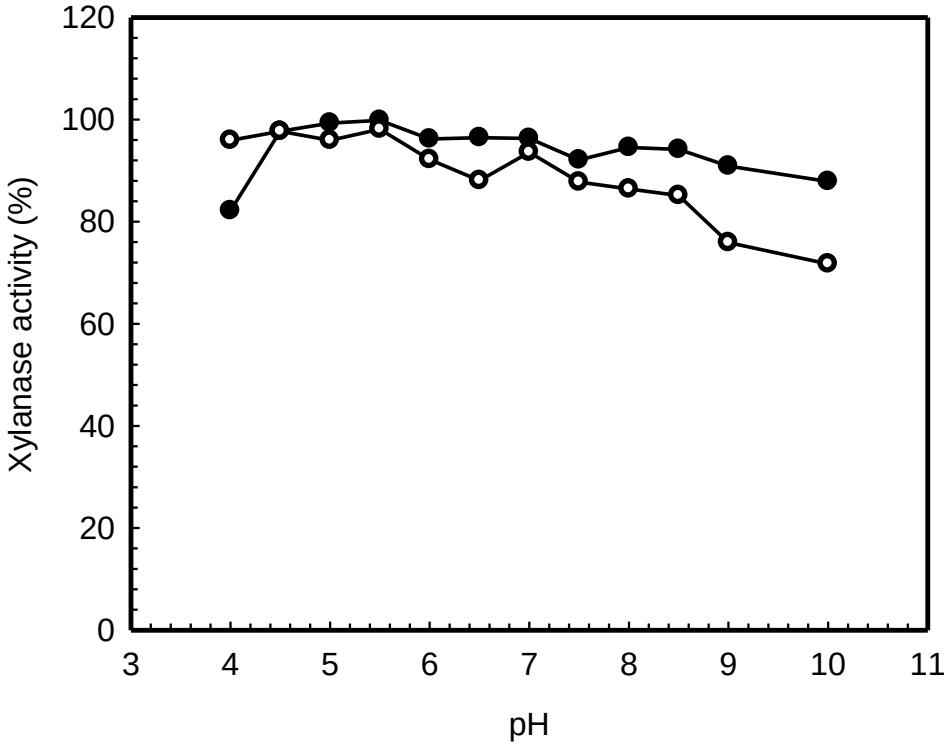

Figure 3.4. Effect of pH on xylanase activity of *A. oryzae* NRRL 3485 (●) and *A. phoenicis* (○) grown on SSLc as carbon source. The residual activity was determined after incubation for 30 min at different pH values. Mean values of triplicate determinations are shown.

The zymograms of the *A. oryzae* and *A. phoenicis* crude enzyme preparations revealed three and two xylanase bands, respectively (Figure 3.6). By comparing the zymogram activity bands to the SDS-PAGE stained bands and marker proteins, the molecular weights of the *A. oryzae* xylanases were 32, 22 and 19 kDa, whereas the *A. phoenicis* xylanases were 25 and 21 kDa, respectively (Figure 3.6).

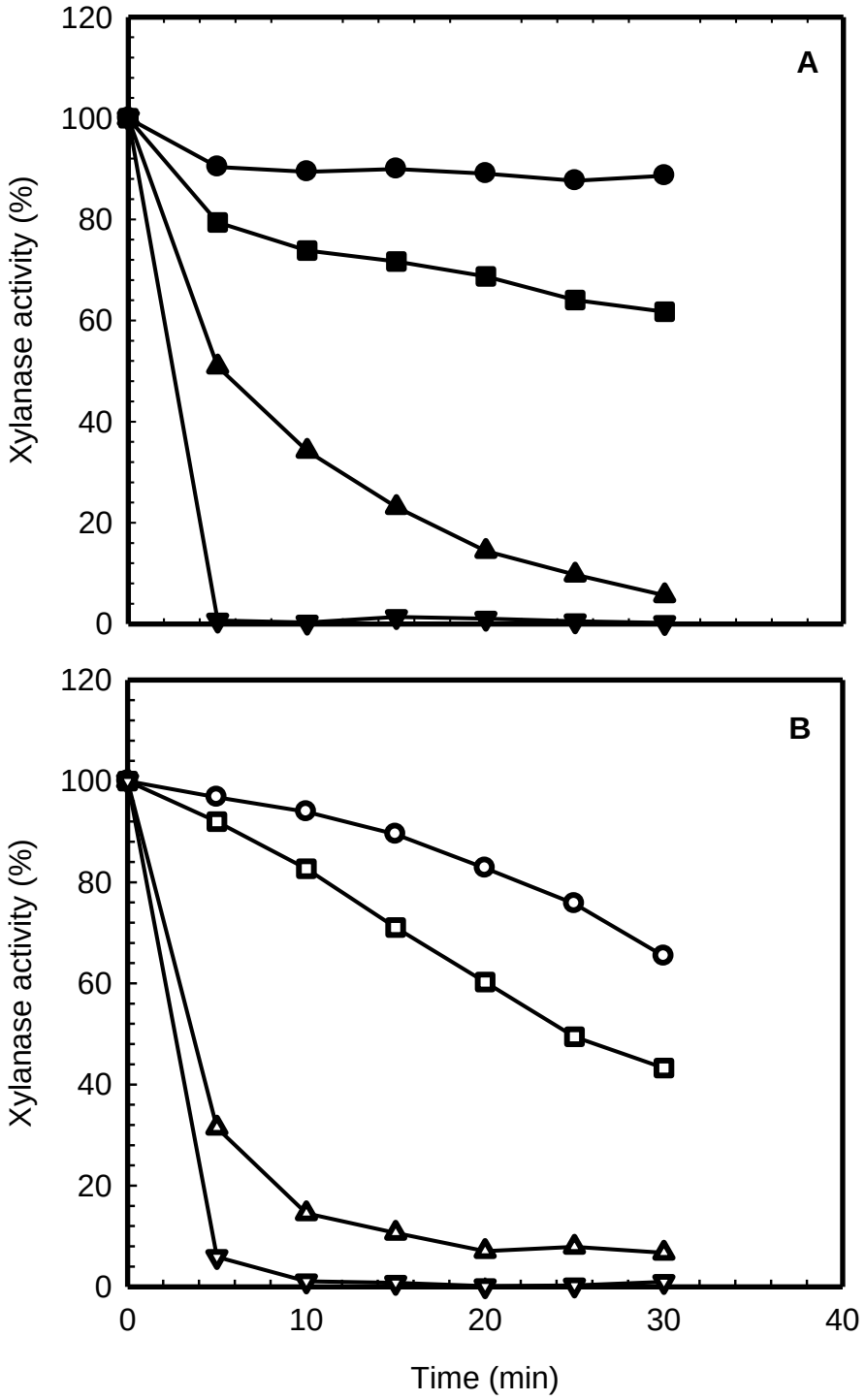

Figure 3.5. Effect of temperature on xylanase activity of *A. oryzae* NRRL 3485 (A) and *A. phoenicis* ATCC 13157 (B) grown on SSLc as carbon source. The residual activity was determined after incubation at different temperatures. Symbols: ●, ○ 40 ºC; ■, □ 50 ºC; △, ▲ 60 ºC; ▽, ▼ 70 ºC. Mean values of triplicate determinations are shown.

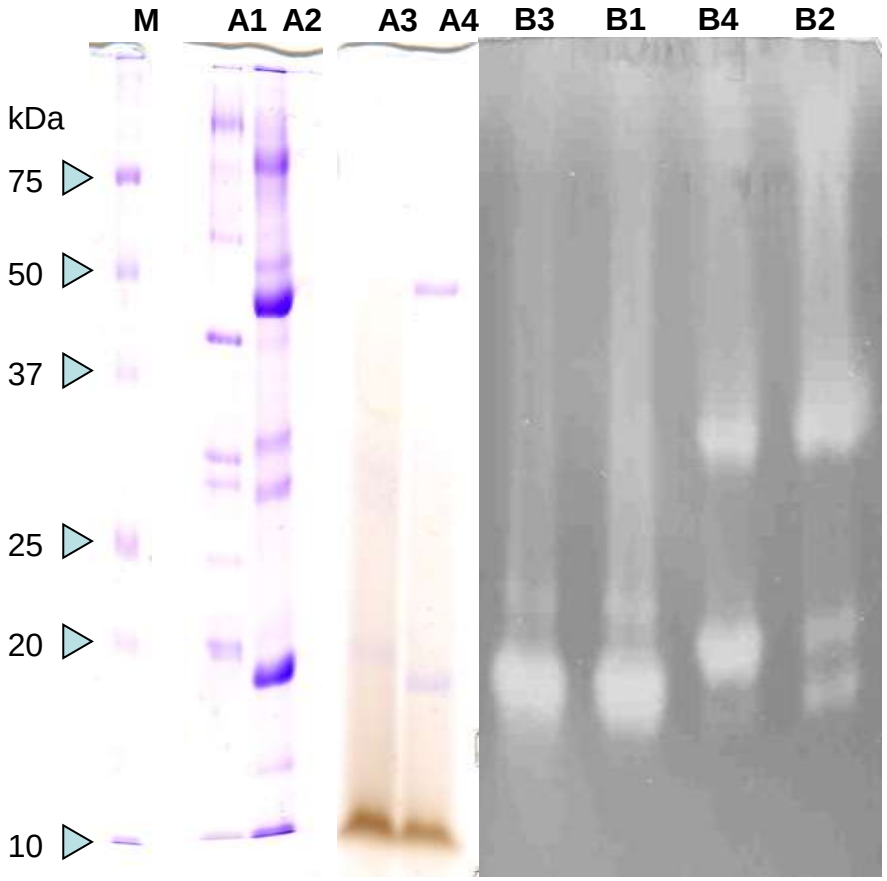

Figure 3.6. SDS-PAGE (lanes A) and zymogram (lanes B) analysis of xylanase preparations from *A. oryzae* NRRL 3485 (lanes A2, A4, B2, B4) and *A. phoenicis* ATCC 13157 (lanes A1, A3, B1, B3) with xylan (lanes A1, A2, B1, B2) and SSLc (lanes A3, A4, B3, B4) as carbon substrates.

3.4.5. Biobleaching of cellulose pulp

Oxygen-delignified soda-aq pulp was pretreated with crude xylanases from *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 grown on xylan and SSLc, respectively. Using the full chlorine dioxide charge, a brightness gain was achieved with both xylanases (Figure 3.7). However, the crude xylanase of *A. phoenicis* grown in the SSLc-based medium produced

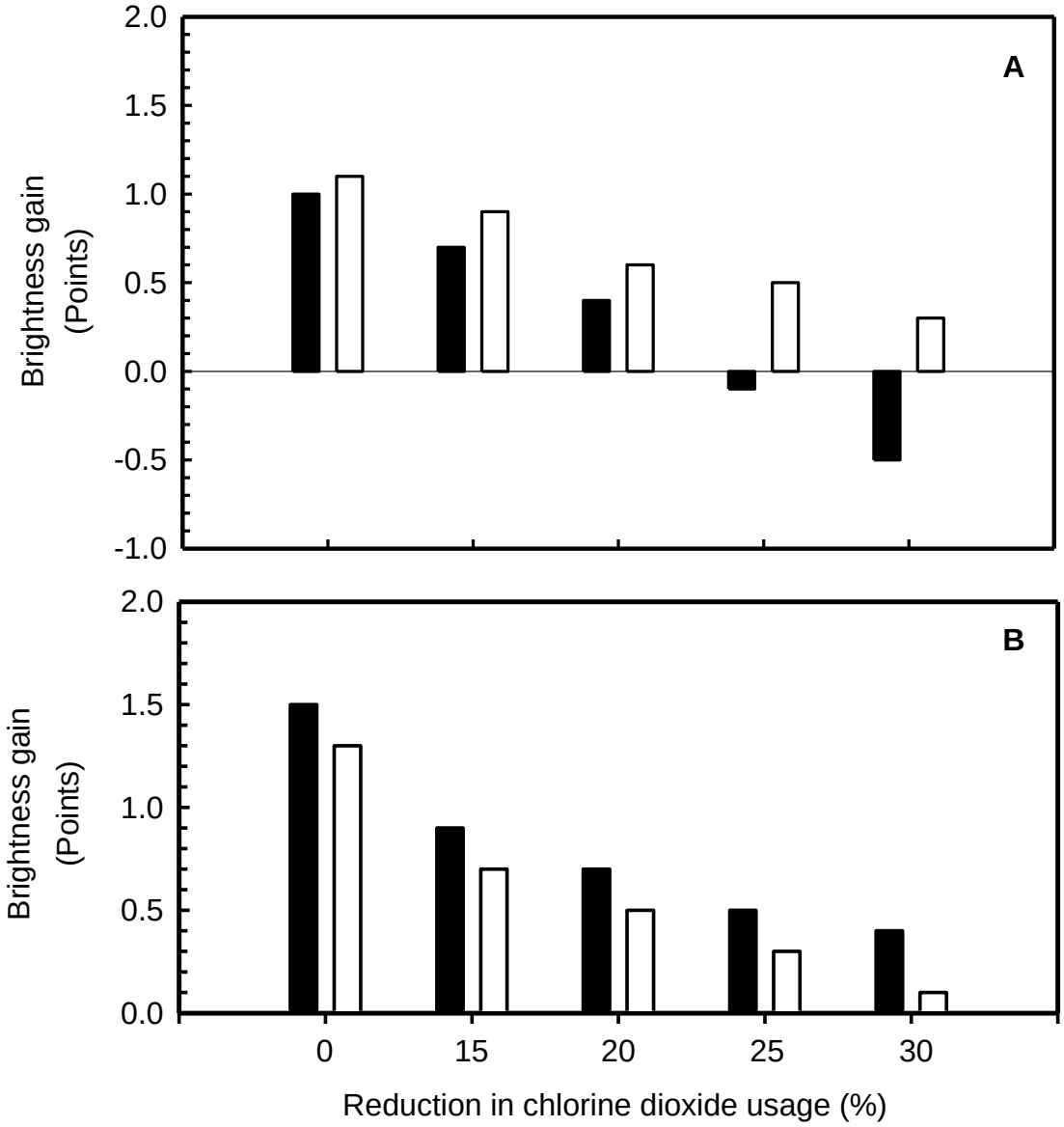

Figure 3.7. Impact of biobleaching on oxygen-delignified soda aq pulp using xylanases of *A. oryzae* NRRL 3485 (A) and *A. phoenicis* ATCC 13157 (B) grown with the SSL concentrate (solid bars) and xylan (open bars) as respective carbon substrates on chlorine dioxide consumption. The enzyme charge was 10 U per g dry pulp.

the highest gain of 1.5 brightness points over the control. Alternatively, a 30 % reduction in the chlorine dioxide charge could be attained without sacrificing brightness. The application of the xylanases of *A. oryzae* and *A. phoenicis* grown on xylan resulted in gains of 1.1 and 1.3 brightness points, respectively, over the control (Figure 3.7). Overall, the *A. phoenicis* xylanase was more efficient in pulp bleaching than the *A. oryzae* xylanase. Furthermore, the

xylanase of *A. phoenicis* grown in the SSLc-based medium had a greater bleaching potential than the xylanase obtained with xylan as carbon substrate (Figure 3.7).

3.5. Discussion

Xylan is known to be a good inducer of xylanase production (Leathers *et al*., 1986; Bahkali, 1996; Siedenburg *et al*., 1998); accordingly, high xylanase activities were obtained on xylan as carbon substrate (Table 3.2). Furthermore, relatively high xylanase activities were produced by certain strains of *Aspergillus* using a cheap raw material, namely spent sulphite liquor concentrate, as carbon feedstock without the addition of an expensive inducer such as xylan. The xylanase activities reported in this chapter with *A. oryzae* and *A. phoenicis* using an SSLc-based medium were higher than those reported elsewhere for *Aspergillus* species grown on other agricultural raw materials such as sugarcane bagasse, wheat bran, wheat straw or rice straw (Gawande and Kamat, 1999; Biswas *et al*., 1988; Gosh *et al*., 1993).

Although easily metabolisable sugars such as xylose and glucose are known to repress xylanase production (Bahkali, 1996; Siedenburg *et al*., 1998; De Souza *et al*., 2001; Ponce-Noyola and de la Torre, 2001), there are some reports of high xylanase activities using xylose as substrate (Leathers *et al*., 1986; Chandra and Chandra, 1995). Xylanase production by *A. tamari* was repressed by the addition of xylose or glucose to the culture medium and enzyme production continued only after the depletion of these sugars (Gawande and Kamat, 1999). Similar findings were reported for *A. awamori* (Siedenberg *et al*., 1998). Figures 3.1 and 3.2 showed that rapid xylanase production coincided with a decrease in the total sugar concentration of the medium to low levels, suggesting derepression of xylanase production at these low sugar concentrations. By contrast, on xylan the xylanase activity started to increase much earlier in the cultivation. Seeing that all of the seven fungal strains investigated here produced no or very low xylanase activities on D-xylose as sole carbon substrate and in all cases higher xylanase activities with the SSLc as carbon feedstock than on xylose (Table 3.2), it is unlikely that the xylose component of the SSLc could have been the main inducing compound, suggesting that an unknown compound in the SSLc acted as inducer.

Although the SSLc had a high total sugar content, it also contained acetic acid which is toxic to microbial cells. The effect of weak acids such as acetic acid on microbial growth is well

documented. It is the undissociated acid that is toxic due to the fact that it freely diffuses across the plasma membrane; therefore, the inhibitory effect of acetic acid is pH-dependent (Brown and Booth, 1991; Axe and Bailey, 1995; Nigam, 2001). Comparison of the maximum specific growth rates on xylan and the SSLc-based medium (Table 3.3) suggested that the acetic acid present in the SSLc may have exerted some inhibitory effect, despite its relatively low concentration in the medium and the use of a culture pH close to neutral. It is also possible that other compounds in the SSLc may also have contributed to the inhibition of the growth rate.

The high biomass concentrations obtained with *A. oryzae* and *A. phoenicis* in the SSLc-based medium were unexpected (Table 3.3). Being a wood hydrolysate, SSL contains wood minerals, carbohydrate decomposition products (acetic acid, hydroxymethyl furfural and furfural) and lignin degradation products that comprise primarily monomeric and polymeric phenolic compounds (Frazer and McCaskey, 1989). Thus, some of these organic compounds might serve as additional carbon substrate for growth. Although it is known that aspergilli can degrade the phenolic as well as polysaccharide components present in a mixture of soluble lignocarbohydrate components, some controversy exists regarding the degradation of lignin (Iyayi and Dart, 1982; Milstein *et al*., 1983). It has, for instance, been shown that *A. niger* possessed extracellular enzymes that hydrolysed phenolic compounds and this species has been used for the removal of phenolic compounds and the colour reduction of olive mill waste waters (Duarte and Costa-Ferreira, 1994). Although the difference in the total polyphenol content of the SSLc-based medium before (17.95 ± 0.21 g l^{-1}) and after (16.85 ± 0.07 g l^{-1}) cultivation was small, it suggested some degradation of polyphenolic compounds. The TOC analyses (Table 3.4) provided evidence of the utilisation of non-sugar organic compounds present in the SSLc-based medium, where the non-sugar organic compounds utilised by *A. oryzae* and *A. phoenicis* amounted to 43 and 42 %, respectively, of the TOC analysis values. Thus, it seems that the utilisation of unidentified non-sugar organic compounds in the SSLc accounted for the high fungal biomass concentrations found.

The use of high temperatures during the pulping and bleaching processes dictates the use of xylanases active at a relatively high pH and temperature (Viikari *et al*., 1994). Xylanases from members of the genus *Aspergillus* typically display a pH optimum of 4.0 to 6.0 and a temperature optimum of about 50 °C (Duarte and Costa-Ferreira, 1994). Two xylanases from *A. oryzae* VTT-D-85248 that were purified and characterised also exhibited

characteristics typical of the genus *Aspergillus*, with both xylanases exhibiting a pH optimum of 5.0 and one of the xylanases being almost fully stable at 50 ºC, whereas the other was completely inactivated under similar conditions (Bailey *et al*., 1991). While the pH and temperature optima of the *A. phoenicis* ATCC 13157 crude xylanase preparation was typical of fungal xylanases, the *A. oryzae* NRRL 3485 xylanase exhibited unusually (for *Aspergillus*) high pH and temperature optima of pH 6.5 and 65 °C and showed a better pH stability than the *A. phoenicis* xylanases, thereby rendering this enzyme of potential industrial importance.

Zymogram analysis confirmed that multiple xylanases were present in all crude enzyme preparations, with *A. oryzae* producing three and *A. phoenicis* two low molecular weight xylanases on both xylan and SSLc carbon substrates. Most xylanolytic fungi produce at least two xylanases (Wong *et al*., 1988). This has also been reported for some *Aspergillus* species (Kormelink *et al*., 1993; Anthony *et al*., 2003), including *A. oryzae* VTT-D-85248, which produced two xylanases with respective molecular weights of 28 and 26 kDa that were purified and characterised by Bailey *et al*., (1991). It has been suggested that the production of multiple xylanases may reflect the need to produce xylanases with different specificities which would be able to act on either highly substituted, unsubstituted and/or differentially soluble substrates (Ryan *et al*., 2003). These differential specificities were clearly demonstrated by Bailey *et al*., (1991) with the two above xylanases from *A. oryzae* VTT-D-85248, designated xylanase I and xylanase II. Both these xylanases yielded similar hydrolysis products with partially soluble beech 4-*O*-methylglucuronoxylan (DP ca. 200), but xylanase II hydrolysed insoluble beech xylan (DP 30 to 40) more efficiently than xylanase I, whereas only xylanase I was able to hydrolyse insoluble, unsubstituted long-chain birch xylan (DP ca. 200).

The results demonstrated that the SSL concentrate had potential as carbon feedstock for the production of fungal xylanases suitable for application in pulp biobleaching. The application of the crude xylanase preparations obtained from two *Aspergillus* strains, at a charge of 10 U per g pulp, resulted in a reduction in the usage of chlorine dioxide of up to 30 % without compromising pulp brightness. The use of this inexpensive and abundant waste liquor as carbon substrate and inducer of xylanase activity could reduce the costs of xylanase production and enhance the economic viability of biobleaching technology. Furthermore, the utilisation of industrial waste waters such as SSL to produce xylanases for on-site use at the paper mills would contribute to the development of effluent-free technology and impact positively on the environment.

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CHAPTER 4

EFFECT OF CULTIVATION PH AND AGITATION RATE ON GROWTH AND XYLANASE PRODUCTION BY *ASPERGILLUS ORYZAE*

Contents

4.1. Abstract

The effect cultivation parameters such as pH and agitation rate on xylanase production by *Aspergillus oryzae* in bioreactor cultures were evaluated using spent sulphite liquor (SSLc) as carbon substrate. Oat spelts xylan was used as control carbon substrate in the evaluation of the effect of cultivation pH on xylanase production. In an SSLc-based medium, the xylanase activities increased with increasing culture pH, reaching 199 U ml^{-1} at pH 7.5 but decreased to 160 U ml^{-1} at pH 8.0. The biomass concentration, however, decreased with increasing culture pH from 10.1 g l^{-1} at pH 4.0 to 4.9 g l^{-1} at pH 8.0. With xylan as carbon source, the xylanase activity profile displayed a similar trend, although at the higher pH values considerably higher xylanase activities of up to 289 U ml^{-1} were reached without a drastic decrease in the biomass concentration. The maximum specific growth rate with SSLc as carbon substrate increased with increasing cultivation pH, reaching 0.391 h^{-1} at pH 7.5, which was higher than with xylan as carbon substrate. Similarly, the maximum volumetric rate of xylanase production and the xylanase yield coefficient also increased with increasing cultivation pH with both SSLc and xylan as carbon substrates. These parameters, however, were higher with xylan than with SSLc as carbon source. As with the xylanase activities, the β-xylosidase activities were higher with xylan than with SSLc. Cellulase activities in all the culture supernatants were, however, below 0.07 U ml^{-1}. SDS-PAGE and zymogram analysis of the crude extract of the culture supernatant revealed three xylanase bands of 32, 22 and 19 kDa. All three xylanases were produced across the culture pH range evaluated, as was shown by the zymograms of the crude xylanase preparations. Stirrer speeds within the range of 400 to 800 rpm, corresponding to an impeller tip velocity of 1.56 to 3.12 m s^{-1}, had no significant effect on the xylanase activity nor on the maximum volumetric rate of xylanase production. The highest biomass concentration of 3.5 g l^{-1} was reached at a stirrer speed of 400 rpm, whereas the highest maximum specific growth rate of 0.265 h^{-1} was obtained at 800 rpm. Similarly, the highest xylanase yields were obtained at the high agitation rates. These results indicated that the concentrated spent sulphite liquor constituted a suitable carbon feedstock for xylanase production by the *Aspergillus* strain in submerged cultivation. It was also clearly shown that the shear stress imposed on the organism by the range of stirrer speeds used here did not have a significant effect on xylanase production.

4.2. Introduction

The efficient production of xylanolytic enzymes is determined by a wide range of cultivation parameters that include the inducing substrate, medium composition, temperature, pH, dissolved oxygen tension, agitation as well as the inoculum. It is, therefore, necessary to investigate the optimal conditions of cultivation providing the highest rate of product formation when considering the development of scale-up strategies of a bioprocess. The cost of the substrate is one of the limiting factors in the economics of an enzyme production process. Several inexpensive lignocellulosic substrates such as corn cobs, wheat straw or bran, hay and rice straw (Gomes *et al.*, 1993; Haltrich *et al.*, 1993; Purkarthofer *et al.*, 1993), compared to pure substrates such as xylan, have been evaluated for xylanase production. Some of these substrates gave significantly better results than the pure substrates and could, therefore, be realistic alternatives to the expensive carbon sources.

For various xylanase-producing microorganisms, the pH value during cultivation has been shown to be of tremendous importance for enzyme production (Dubeau *et al.*, 1987; Royer and Nakas, 1989; Purkarthofer *et al.*, 1993; Bailey *et al.*, 1993). The cultivation pH influenced xylanase production in various *Aspergillus* strains. *A. fischeri* fxn1 was shown to grow well over a wide pH range (pH 5.0 to 10.0) with maximal xylanase activities between pH 6.0 to 10.0 (Chandra and Chandra, 1995). Similarly, *A. fumigatus* AR1 produced xylanase over a wide cultivation pH range from pH 4.0 to 10.0 with maximal xylanase activities obtained at pH 5.0 (Anthony *et al.,* 2003). Contrary to these findings, Bailey and Viikari (1993) found that a decrease in the pH of the cultivation to below 3.5 was essential for the efficient production of xylanase by a strain of *A. fumigatus* VTT-D-82195. Fungal xylanases are generally associated with cellulases (Steiner *et al.*, 1987). However, the selective production of xylanase is possible and was demonstrated in the case of *Trichoderma,* where a low cultivation pH of 4.0 favoured cellulase production, whereas a cultivation pH of between 6.0 and 7.0 favoured xylanase production (Bailey *et al.,* 1993).

One other cultivation parameter implicated in influencing xylanase production in submerged culture is the agitation rate. For many fungal cultivations, high viscosities and non-Newtonian behaviour of culture broths necessitate the use of high agitation rates to provide adequate mixing and oxygen transfer. Mycelial damage at high agitation rates, however, can limit the acceptable range of speeds and consequently the volumetric productivity of the culture. Several papers report on the effect of agitation in combination with the aeration rate

and dissolved oxygen tension (Hoq *et al*., 1994; Palma *et al*., 1996; Singh *et al*., 2000; Techapun *et al*., 2003). It has been shown that xylanase production by a number of fungal strains was influenced by the agitation rate (Palma *et al*., 1996; Singh *et al*., 2000; Techapun *et al*., 2003). On the other hand, Amanullah *et al.* (1999) found that the agitation speed had no significant effect on the production of protein by a recombinant strain of *Aspergillus oryzae*. The above findings, therefore, seem to suggest that optimal agitation conditions are unique for each microbial strain and process.

In this study, the production of xylanase by *Aspergillus oryzae* at different culture pH values as well as the influence of agitation intensity in batch culture was evaluated. Results on xylanase production with concentrated spent sulphite liquor as an inexpensive carbon substrate, as well as xylan, are presented. The effect of cultivation pH on xylanase multiplicity in the above fungal strain was also determined.

4.3. Materials and methods

4.3.1. Fungal strain

The fungal strain used in this study was *Aspergillus oryzae* NRRL 3485 and was maintained on Sabouraud-dextrose agar (Biolab Diagnostics Ltd., Midrand, South Africa) slants and stored at 4 ºC with subculturing at 12-week intervals.

4.3.2. Effect of cultivation pH

4.3.2.1. Culture conditions

Batch cultivations were carried out at 30 °C for 60 or 120 h, using a 15 l Biostat C bioreactor (B. Braun Biotech International, Melsungen, Germany) with a working volume of 8.5 l. A 500 ml mycelial inoculum was added after the *in situ* sterilisation of 8.0 l medium. The dissolved oxygen tension (DOT) in the culture was monitored with a polarographic pO_2 electrode (Mettler Toledo, Halstead, UK) and automatically controlled at $\geq$ 30 % of saturation by increasing the air flow rate between 0.03 to 8.0 std l min^{-1} and adjustment of the stirrer speed between 400 to 800 rpm. The pH was controlled at pH 4.0, 5.0, 6.0, 7.0, 7.5 and 8.0 by automatic titration with 5 N KOH or 5 N H_2SO_4. Cultivations where the pH was not controlled during growth of the fungal strain were also included in the evaluation.

4.3.2.2. Inoculum and culture medium

The inoculum was prepared by inoculating each of five 500 ml Erlenmeyer flasks containing 100 ml of a xylan-based medium with 5 ml of a fungal spore suspension (ca. 10^7 spores ml^{-1}) washed from a 5-day old Sabouraud dextrose agar (Biolab Diagnostics Ltd., Midrand, South Africa) plate culture that had been incubated at 30 ºC with 10 ml of a 0.05 M KH_2PO_4 solution containing 0.1 % Tween 80. These flasks were incubated at 210 rpm on a rotary shaker at 30 ºC for 48 h.

Spent sulphite liquor from an acid sulphite pulp mill in South Africa, was supplied in a concentrated form (designated SSLc) and was used diluted twenty-fold with distilled water as the main carbon source. Oats spelts xylan (10 g l^{-1}; Sigma Chemical Co., St. Louis, MO, USA) was used as control carbon substrate for xylanase production. The cultivation medium comprised (l^{-1}): citric acid, 0.25 g; $(NH_4)_2SO_4$, 5 g; K_2HPO_4, 5 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.02 g and yeast extract (Biolab), 10 g. Also added was 1 ml of a trace element solution (du Preez and van der Walt, 1983) as well as 1 ml Dow Corning 1520 Silicone antifoam (Dow Corning product, Dow Corning Corporation, USA, repackaged by BDH Laboratory Supplies, Poole, England).

4.3.2.3. Enzyme assays

Xylanase and cellulase activities were determined as described in Chapter 3 using the dinitrosalicylic acid (DNS) method (Bailey *et al.*, 1992) and filter paper assay (FPU) method (Ghose, 1987), respectively.

The β-xylosidase activity was assayed as described by Lenartovicz *et al.*, (2002) where 1 ml of *o*-nitrophenyl-β-D-xylopyranoside (0.1 % in 0.05 M citrate buffer, pH 5.4) was incubated with 0.1 ml of the culture filtrate at 50 ºC for 10 min and the released *o*-nitrophenol subsequently determined at 410 nm. A unit of enzyme activity was defined as the amount of enzyme producing 1 mol of *o*-nitrophenol per min under the above conditions.

Protease activity was determined using a modified method of Sarath *et al.* (1994), using 2 % azocasein (Sigma) as substrate. A 0.15 ml enzyme solution was incubated with 0.25 ml substrate for 30 min at 30 ºC, after which 1.2 ml of a 10 % TCA solution was added to stop the reaction. Samples were centrifuged at 15 000 rpm for 5 min and 1.2 ml subsequently transferred into tubes containing 1.4 ml 1 M NaOH and the absorbance of the solution determined at 440 nm. A unit of protease activity was defined as the amount of enzyme

required to produce an absorbance change of 1.0 in a 1-cm cuvette. The absorbancies of all the enzyme samples were spectrophotometrically determined using a DU 7500 spectrophotometer (Beckman Instruments, Fullerton, CA. USA).

4.3.2.4. Other analyses

The sugar composition as well as acetic acid concentration of the culture supernatants were determined as described in Chapter 3.

The fungal biomass concentration was determined gravimetrically after filtration using GF 50 glass fibre filter papers (Schleicher and Schuell GmbH, Dassel, Germany) of duplicate aliquots of the respective cultures, washing twice with equal volumes of distilled water and drying at 105 ºC to constant weight.

4.3.2.5. SDS-PAGE and zymogram analysis

Extracellular proteins in the culture supernatants of *A. oryzae* with SSLc as carbon substrate using different cultivation pH values were separated by SDS-PAGE using a 12.5 % polyacrylamide gel as described in Chapter 3.

Zymogram samples were subjected to electrophoresis and the identification of xylanase active bands identified following the procedure outlined in Chapter 3.

4.3.3. Effect of agitation rate

4.3.3.1. Culture conditions

The effect of agitation rate on xylanase production was investigated in batch culture using the bioreactor described in section 4.3.2.1. The vessel had four baffles with one propeller impeller and three Rushton disc turbine impellers, each measuring 74.4 mm in diameter, fitted equidistantly on the stirrer shaft with the propeller at the top. The dissolved oxygen tension (DOT) was maintained at or above 25 % of saturation by automatic control of the aeration rate in the range of 0.05 to 8.0 std l min^{-1} while maintaining a constant agitation speed. Different stirrer speeds of 400, 500, 600 and 800 rpm were evaluated separately in batch cultures. These stirrer speeds corresponded to impeller tip velocities of 1.56, 1.95, 2.34, and 3.12 m s^{-1}, calculated as Πnd_i, where n is the stirrer speed and d_i is the impeller diameter (Mukataka *et al*., 1988). All cultivations were carried out at 30 °C with the culture pH controlled at pH 7.5 by automatic titration with 5 N KOH and 5 N H_2SO_4. Samples were harvested at various time intervals over a 72 h cultivation period for determination of the

xylanase activity and biomass concentration as described in Sections 4.3.2.3 and 4.3.2.4, respectively.

4.3.3.2. Inoculum and culture medium

The fungal inoculum was prepared as described in Section 4.3.2.2, where each of five 500 ml Erlenmeyer flasks containing 100 ml of a xylan-based medium was inoculated with 5 ml of a fungal spore suspension (ca. 10^7 spores ml^{-1}). Spent sulphite liquor concentrate (SSLc) was used as carbon substrate and was diluted 40-fold with distilled water. The medium composition was also similar to that described in Section 4.3.2.2.

4.4. Results

To determine the optimal cultivation pH for xylanase production by *A. oryzae*, several bioreactor cultivations at different cultivation pH values were performed with SSLc as the carbon substrate. Oat spelts xylan (Sigma) was used as control substrate since it is a known inducer of xylanases (Siedenburg *et al.*, 1998; Bahkali, 1996).

4.4.1. Growth parameters for A. oryzae *at different cultivation pH values*

The growth parameters for the *A. oryzae* strain with SSLc as carbon substrate at the different cultivation pH values are summarised in Figure 4.1. The maximum volumetric rate of xylanase production, xylanase activity, xylanase yield as well as the maximum specific growth rate increased with an increase in cultivation pH. Above pH 7.5 these parameters decreased significantly. The maximum specific growth rate increased from 0.19 h^{-1} at pH 4.0 to 0.39 h^{-1} at pH 7.5, dropping to 0.12 h^{-1} at pH 8.0. Similarly, the maximum volumetric rate of xylanase production increased from 2.24 U ml^{-1} h^{-1} at pH 4.0 to 13.1 U ml^{-1} h^{-1} at pH 7.5. The biomass concentration, however, was highest at pH 4.0 but decreased as the culture pH was increased. As a result of the decrease in biomass concentration and increase in xylanase activity at the higher cultivation pH values, there was a significant increase in the xylanase yield per gram biomass, from 7 000 U g $biomass^{-1}$ at pH 4.0 to 35 000 U g $biomass^{-1}$ at pH 7.5. The cellulase activity at all the cultivation pH values was below 0.07 U ml^{-1} with the protease activity reaching a high of 0.85 U ml^{-1} at pH 6.0. The extracellular β-xylosidase activity was highest at cultivation pH 4.0, reaching 0.36 U ml^{-1} and was found to decreased with increasing culture pH, reaching activities of only 0.18 U ml^{-1} at pH 7.5.

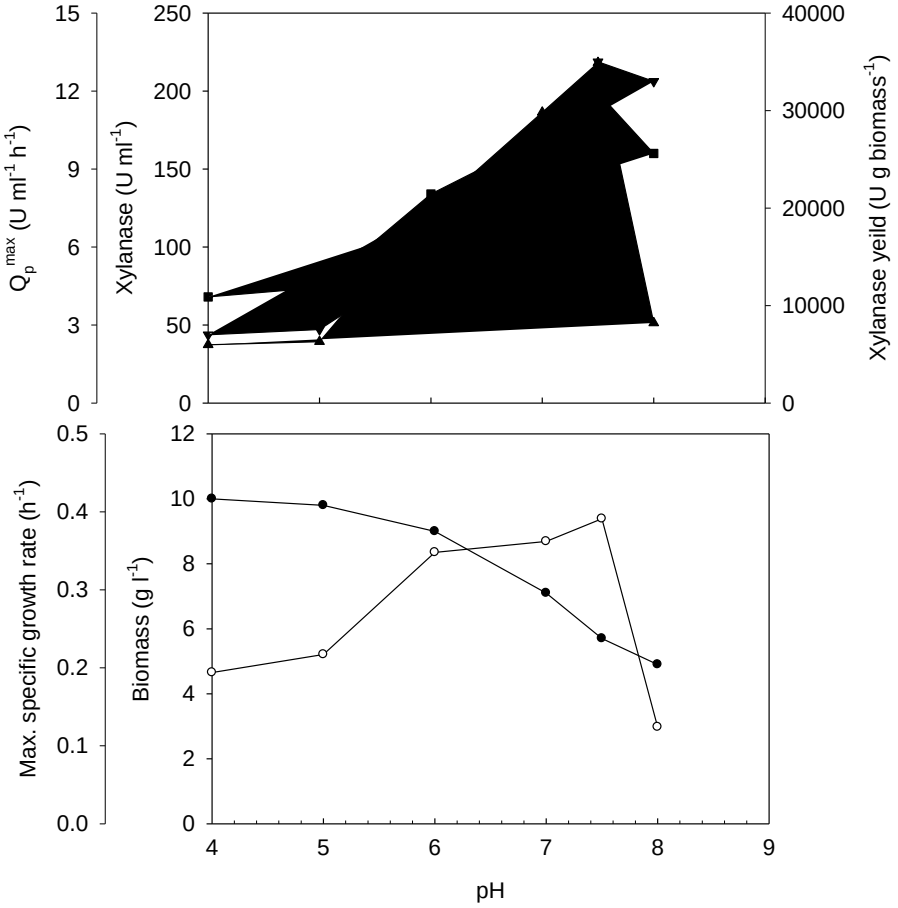

Figure 4.1. Growth parameters of *A. oryzae* NRRL 3485 with SSLc as carbon substrate in bioreactor cultures at 30 ºC as a function of the cultivation pH. Symbols: ■ xylanase activity; ▲ maximum volumetric rate of xylanase production (Q_p^{max}); ▼ xylanase yield; ● biomass; ○ maximum specific growth rate.

Without cultivation pH control, the maximum specific growth rate in the SSLc-based medium was 0.356 h^{-1}, which was similar to the growth rate at pH 7.0. However, the maximum volumetric rate of xylanase production as well as xylanase yield were similar to the values obtained at pH 6.0, namely 5.79 U ml^{-1} h^{-1} and 11 800 U g $biomass^{-1}$, respectively. The final biomass concentration and xylanase activity in the culture without pH control were 6.0 g l^{-1} and 153 U ml^{-1}, respectively. These values were lower than those obtained at a controlled pH of 7.5. The culture pH increased from an initial cultivation pH of 5.7 to a final pH of 8.4 at the end of the cultivation.

Table 4.1 shows the growth parameters of *A oryzae* with xylan as carbon substrate at different cultivation pH values. As with the SSLc as carbon substrate, there was a significant increase in xylanase activity, volumetric rate of xylanase production and xylanase yield from pH 4.0 to 7.5. However, these parameters were higher than with SSLc as carbon feedstock (Figure 4.1). There was, however, no significant change in the maximum specific growth rate, which remained fairly constant, unlike with the SSLc where it increased significantly from pH 4.0 to 7.5. Cultivation on xylan without pH control yielded a slightly higher maximum specific growth rate of 0.187 h^{-1} than cultures with pH control. The maximum volumetric rate of xylanase production and xylanase yield were, however, similar to those at pH 4.0. As with SSLc as carbon source, low ß-xylosidase, cellulase as well as protease activities were obtained with the xylan substrate.

Table 4.1. Growth parameters (mean values of two experiments) of *A. oryzae* NRRL 3485 with xylan as carbon substrate in bioreactor cultures at 30 ºC and different cultivation pH values.

Parameter	**Cultivation pH**			
	4.0	6.0	7.5	No pH control
Xylanase (U ml^{-1})	187	286	289	153
Biomass (g l^{-1})	6.2	6.7	6.1	6.0
Cellulase (FPUs)	0.06	0.04	0.07	0.06
β-Xylosidase (U ml^{-1})	0.48	0.07	0.24	0.14
Protease (U ml^{-1})	0.93	0.73	1.24	0.83
μ_{max} (h^{-1})	0.13	0.14	0.14	0.187
Q_p^{max} (U ml^{-1} h^{-1})	8.3	8.4	18.3	8.17
$Y_{p/x}$ (U g $biomass^{-1}$)	30 000	43 000	47 000	25 000

Q_p^{max} Maximum volumetric rate of xylanase production, calculated from the maximum slope of the enzyme activity versus time curve

$Y_{p/x}$ Xylanase yield coefficient

μ_{max} Maximum specific growth rate

4.4.2. Cultivation profiles

4.4.2.1. SSLc as carbon substrate

Figure 4.2 shows the cultivation profiles of *A. oryzae* in the SSLc-based medium at pH 4.0 and 7.5 for comparison. At pH 4.0, growth and enzyme production were considerably slower than at pH 7.5 (note that the time scales in Figure 4.2 differ). The highest xylanase activity of 199 U ml^{-1} was obtained at pH 7.5, whereas cultivation at pH 4.0 yielded the lowest activity of 69 U ml^{-1} (Figure 4.2). At both pH values, rapid xylanase production commenced only after the total sugar concentration in the culture had decreased to low levels. A prolonged growth lag phase was observed at a cultivation pH of 4.0, lasting up to 51 h, at which time the acetic acid concentration in the culture had decreased to a minimal level, with

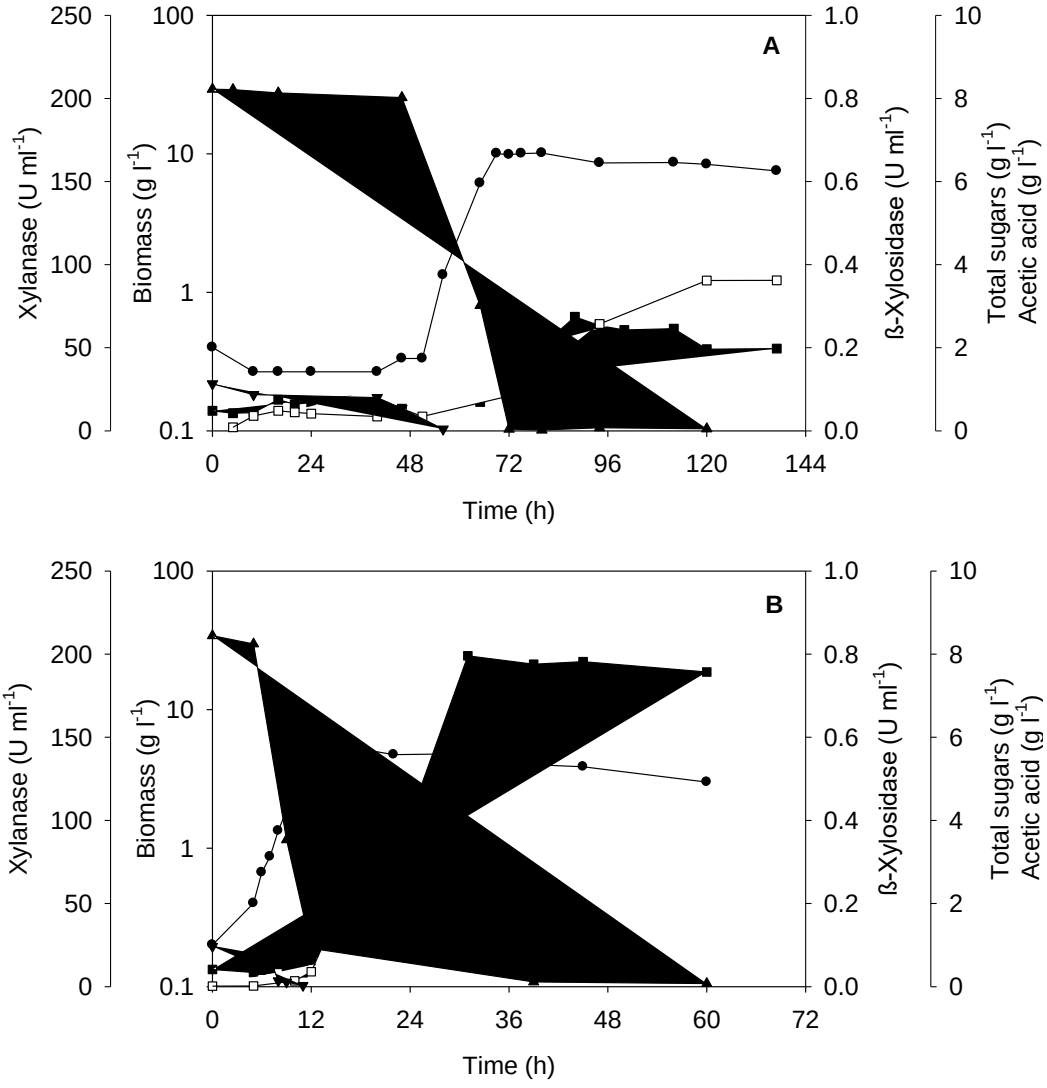

Figure 4.2. Typical cultivation profiles of *A. oryzae* NRRL 3485 with SSLc as carbon substrate in bioreactor cultures at 30 ºC and pH 4.0 (A) and pH 7.5 (B). Symbols: ● biomass; ■ xylanase; □ β-xylosidase; ▲ total sugars; ▼ acetic acid.

extracellular xylanase activity detected only after 65 h of cultivation. Cultivation at pH 4.0 yielded β-xylosidase activities of up to 0.36 U ml^{-1}, whereas only 0.18 U ml^{-1} was obtained at pH 7.5 (Figure 4.2). Cellulase production at all the cultivation pH values was negligible, namely below 0.07 U ml^{-1}.

4.4.2.2. Xylan as carbon substrate

Cultivation profiles of *A. oryzae* with xylan as carbon substrate at pH 4.0 and 7.5 are shown in Figure 4.3 for comparison. Xylanase activities at these two pH values followed a trend similar to those obtained with SSLc as carbon substrate, with the highest xylanase activity of 289 U ml^{-1} obtained at pH 7.5, whereas 187 U ml^{-1} were obtained at pH 4.0 (Table 4.1). The cultivation profile at pH 4.0 with xylan as carbon substrate did not exhibit any prolonged

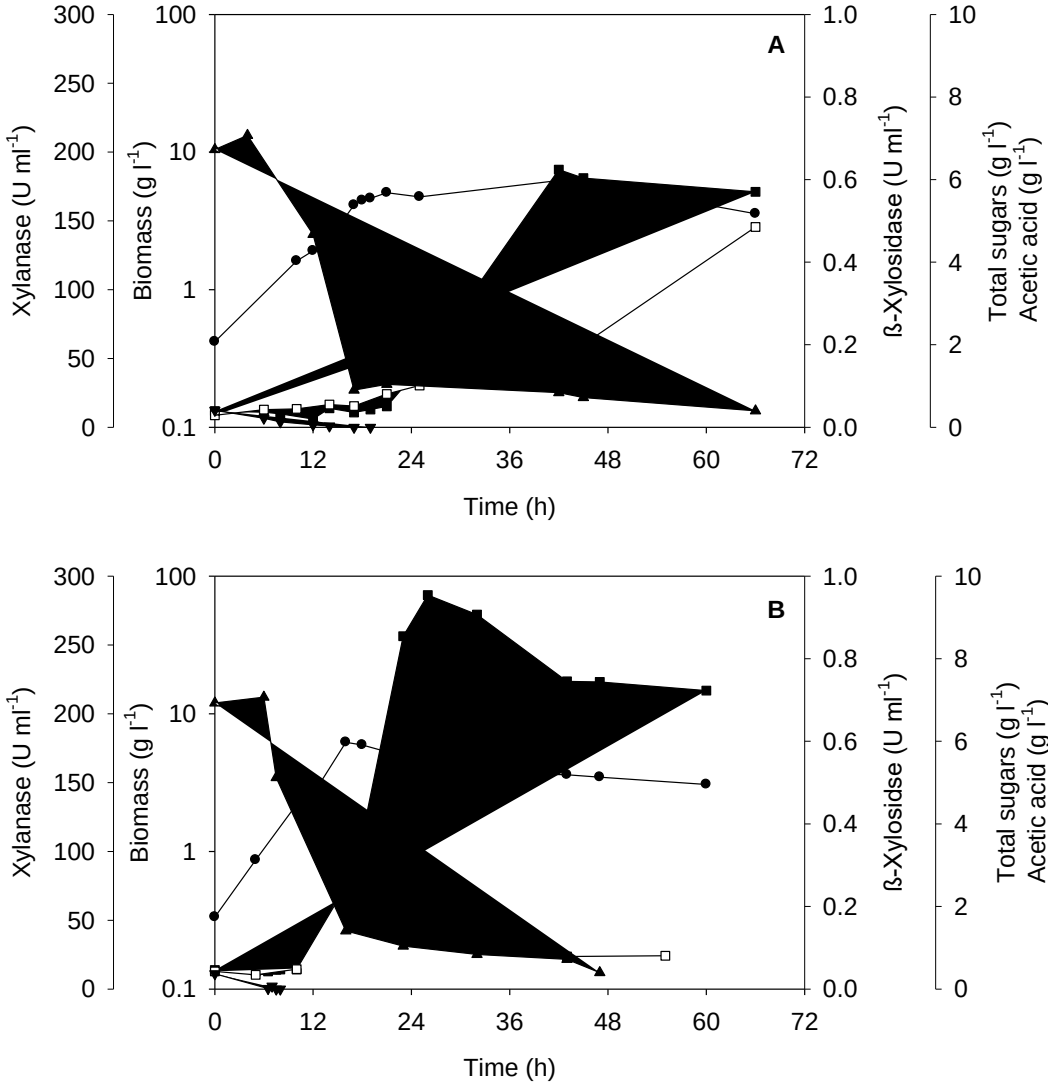

Figure 4.3. Typical cultivation profiles of *A. oryzae* NRRL 3485 with xylan as carbon substrate in bioreactor cultures at 30 ºC and pH 4.0 (A) and pH 7.5 (B). Symbols: ● biomass; ■ xylanase; □ β-xylosidase; ▲ total sugars; ▼ acetic acid.

growth lag phase and xylanase production began much earlier (Figure 4.3A) than was the case with SSLc as carbon substrate (Figure 4.2A). β-Xylosidase activities were significantly greater at pH 4.0 (0.49 U ml^{-1}) than at pH 7.5 (0.25 U ml^{-1}), and were also greater than the

values recorded in the SSLc-based medium. Cellulase activities at these two cultivation pH values were negligible (Table 4.1), as was also found with the SSLc-based medium. The protease activity was highest at cultivation pH 7.5 reaching 1.24 U ml^{-1}.

4.4.3. SDS-PAGE and zymograms

The zymogram for *A. oryzae* showed that all three xylanase isozymes were produced at the cultivation pH values of 4.0, 6.0, 7.5 and 8.0 (Figure 4.4). The molecular weights of these xylanase isozymes were 32, 22 and 19 kDa, respectively. A sample obtained from cultivation without pH control also showed the presence of the three xylanase bands.

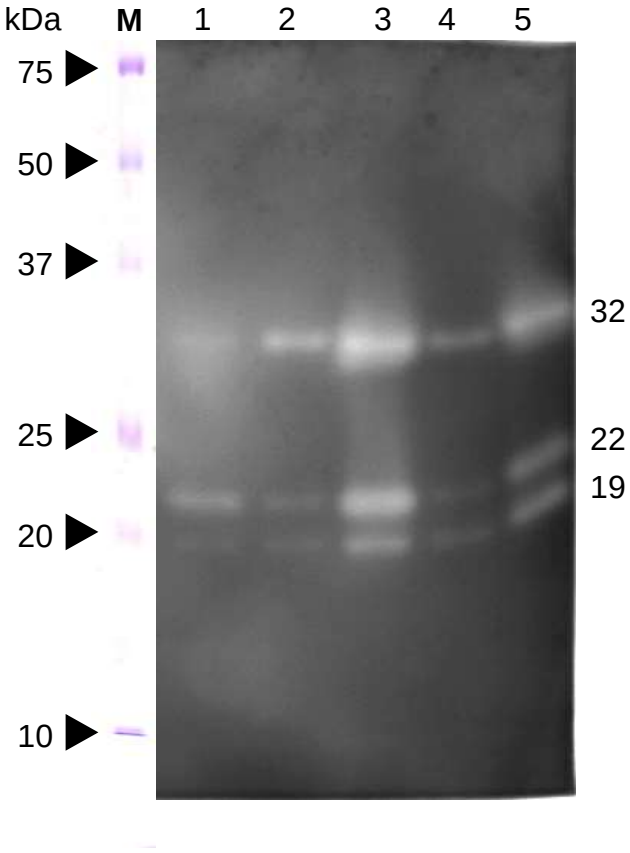

Figure. 4.5. Zymogram analysis of crude xylanase preparations from *A. oryzae* NRRL 3485 with SSLc as carbon source in bioreactor cultures at 30 ºC and cultivation pH values of 4.0 (lane 1), 6.0 (lane 2), 7.5 (lane 3), 8.0 (lane 4) and without pH control (lane 5).

4.4.4. *Effect of agitation rate*

Growth parameters of *A. oryzae* under various stirrer speeds in batch culture are shown in Table 4.2. Stirrer speeds between the range of 400 to 800 rpm were selected because, in controlling the DOT at or above 25 % during batch cultivation, the stirrer speed operated within this range. It was, therefore, deemed necessary to determine the effect of agitation rate within this range on xylanase production. Increasing the stirrer speed from 400 to 800 rpm did not have any apparent effect on the xylanase activity nor on the maximum volumetric rate of xylanase production, which remained relatively constant at the different

Table 4.2. Growth parameters (mean values of duplicate experiments) of *A. oryzae* NRRL 3485 in batch culture at different stirrer speeds with SSLc as carbon substrate.

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

Q_P^{max} Maximum volumetric rate of xylanase production, calculated from the maximum slope of the enzyme activity versus time curve

$Y_{p/x}$ Xylanase yield coefficient, U g biomass^{-1}

μ_{max} Maximum specific growth rate

agitation rates. The final biomass concentration was, however, slightly higher at 400 and 500 rpm than at 600 and 800 rpm, with up to 3.5 g biomass l^{-1} at 400 rpm. Despite the slightly higher biomass concentrations at these low agitation rates, the maximum specific growth rates were the greatest at stirrer speeds of 600 and 800 rpm, reaching a μ_{max} value of 0.265 h^{-1} at 800 rpm. Similarly, the xylanase yield was greater at the higher agitation rates.

4.5. Discussion

This study demonstrated conclusively that the concentrated spent sulphite liquor could serve as carbon substrate as well as inducer for the production of essentially cellulase-free xylanases by *A. oryzae*. Xylan, which is a known powerful inducer of xylanase (Siedenburg *et al.*, 1998; Bahkali, 1996), yielded higher volumetric xylanase activities as well as xylanase yields (U g biomass^{-1}) than SSLc as carbon substrate, however.

Xylanase production by *A. oryzae* increased with increasing culture pH to reach a maximum activity at pH 7.5 with both xylan and SSLc as respective carbon substrates. The rate of xylanase production was also much higher at this pH value, which decreased considerably at pH 8.0. Purkarthofer *et al.* (1993) also showed that xylanase production by a *T. lanuginosus* strain was favoured by a high cultivation pH of up to pH 7.5. Xylanase production by other fungal strains such as *Thermomyces lanuginosus* (Purkarthofer *et al.*, 1993), *Trichoderma reesei* (Bailey *et al.*, 1993), *T. reesei* RUT C-30 (Xiong *et al.*, 2004) and *Aspergillus fumigatus* (Bailey and Viikari, 1993) was also shown to be greatly affected by the cultivation pH. A rather high pH of up to pH 7.0 was essential for the production of a high xylanase activity by *T. reesei* (Bailey *et al.*, 1993). At this high pH there was a slower release of soluble hydrolysis products from the corn cob xylan, which may have led to increased xylanase levels. It has been shown for other fungi that the optimal culture pH for xylanase production is generally higher than the optimal pH value for xylan hydrolysis (Royer and Nakas, 1989). The above findings are in contrast to those of Bailey and Viikari (1993), who reported that a cultivation pH of below 3.0 was essential for the enhancement of xylanase production by *A. fumigatus*. It is, therefore, obvious that optimal enzyme production parameters for one fungal strain do not necessarily apply to others.

The effect of xylose on xylanase production is well documented, with some reports showing that xylose was a good inducer (Leathers *et al.*, 1986; Chandra and Chandra, 1995), whereas others showed that xylose repressed xylanase production (Siedenburg *et al.*, 1998; De Souza *et al.*, 2001), suggesting that repression of xylanase production by xylose is strain specific, probably also dependent on the xylose concentration, and cannot be generalised. The cultivation profiles shown in Figures 4.2 and 4.3 show that rapid xylanase production commenced only after the total sugar concentration in the culture medium had decreased to low levels, suggesting derepression of xylanase synthesis at these low sugar concentrations. D-xylose constituted over 80 % of the total sugars in the SSLc; therefore, xylanase

repression could be attributed to the D-xylose present in the SSLc-based medium (Chapter 3).

The inhibitory effect of acetic acid on microbial growth is well documented and is pH-dependent (Axe and Bailey, 1995; Nigam, 2001). At pH 4.0 with SSLc as carbon substrate, a prolonged lag phase lasting up to 51 h was observed, which ended as the acetic acid concentration in the medium decreased to minimal levels. The cultivation profile at pH 4.0 (Figure 4.2A) suggested that the acetic acid present in the SSLc exerted a major negative effect on initial growth, despite its relatively low concentration of ca. 1.13 g l^{-1} in the culture medium. However, once the acetic acid in the medium had been assimilated, fungal growth commenced and a high biomass concentration of up to 10.1 g l^{-1} was obtained, which was higher than at pH 7.5 where up to 5.7 g l^{-1} was obtained.

β-Xylosidase activities produced by the *A. oryzae* strain used in this study were comparable to the activities produced by other *Aspergillus* strains such as *A. fumigatus*, *A. terreus* and *A. tamarii* (Bailey and Poutanen, 1989; Kadowaki *et al.*, 1997). β-Xylosidases may be extracellular or cell bound and in almost all bacteria and yeasts these enzymes are cell associated (Biely, 1985). The β-xylosidases of filamentous fungi are also associated with the mycelium during the early phases of growth and can be released later into the medium, either by secretion or as a result of cell lysis, hence the recovery of β-xylosidases in exracellular fractions (Wong and Saddler, 1992; Rizzatti *et al.*, 2001; Lenartovicz *et al.*, 2002). Bailey and Poutanen (1989) evaluated β-xylosidase production by a number of *Aspergillus* strains. Whereas *A. oryzae* VTT-D-85248 and *A. foetidus* VTT-D-71002 produced significant activities of up to 70 and 110 nkat ml^{-1} (4.2 and 6.6 U ml^{-1}), respectively, *A. fumigatus* VTT-D-82195 and *A. terreus* VTT-D-82209 produced very low levels of β-xylosidase of not more than 10 nkat ml^{-1} ($\leq$ 0.6 U ml^{-1}). These low enzyme levels were attributed to the enzyme probably being intracellular or cell bound in these two strains.

Owing to the complex structure of heteroxylans, the xylosidic linkages in the substrate are not equally accessible to xylan degrading enzymes. Xylan hydrolysis, therefore, requires the action of multiple xylanases with overlapping but different specificities (Wong *et al.*, 1988). The culture filtrate of *A. niger* was found to comprise 15 xylanases, *T. viride* 13 and *Talaromyces emersonii* between 11 and 13 xylanases (Biely *et al.,* 1985; Coughlan *et al.*, 1993). Multiple xylanases have been purified from culture filtrates of *A. niger* (Wong *et al.*, 1988), *A. kawachii* (Ito *et al.*, 1992), *A. awamori* (Kormelink *et al.*, 1993) and *A. oryzae*

(Bailey *et al.*, 1991). Two low molecular weight xylanases (28 and 26 kDa) were purified from an *A. oryzae* VTT-D-85248 strain by Bailey *et al.* (1991). It was also determined that the xylanase and cellulase activities of this strain were associated with different proteins. It would therefore be possible, when producing xylanases for treatment of cellulose pulps, to minimise cellulase production by using a suitable substrate and/or cultivation conditions. The zymogram prepared from crude xylanase preparations of the *A. oryzae* NRRL 3485 strain used in this study revealed three low molecular weight xylanases that were produced across all the cultivation pH values (Figure 4.4). This is, however, contrary to the findings of Anthony *et al.* (2003) and Xiong *et al.* (2004), who found that *A. fumigatus* AR1 and *T. reesei* RUT C-30, respectively, responded to the cultivation pH by modifying the enzyme production patterns, with *A. fumigatus* AR1 producing multiple xylanases at pH 9.0, whereas a single xylanase was produced at pH 5.0 (Anthony *et al.*, 2003). Similarly, Xiong *et al.*, (2004) found that *T. reesei* RUT C-30 produced xylanase I and II at pH 4.0, whereas cultivation at pH 6.0 resulted in the production of xylanase II and III. These findings clearly demonstrated that some fungi modified their enzyme production patterns according to the culture pH, but this trait also appeared to be strain specific.

The effect of agitation intensity on xylanase production has been investigated and in a number of studies this was conducted in combination with the aeration rate (Techapun *et al*., 2003), dissolved oxygen tension (Singh *et al*., 2000) and cultivation pH (Purkarthofer *et al*., 1993). Almost all studies use agitation rates as the sole measure of shear stress and ignore the effects of the impeller dimensions. This renders meaningful comparisons between studies difficult, especially when they have been conducted at different vessel scales. The use of the impeller tip velocity of the impeller would be more suited to represent the shearing strength (Gibbs *et al*., 2000).

Results presented in the current study clearly show that the stirrer speed within the range of 400 to 800 rpm, corresponding to impeller tip velocities of 1.56 to 3.12 m s^{-1}, did not have any apparent effect on the xylanase activity of the *A. oryzae* strain. These results correlate well with those of Amanullah *et al.* (1999) who reported that α-amylase and amyloglucosidase (AMG) production using a recombinant strain of *A. oryzae* were not affected by increasing the agitation rate and that the expression of homologous and heterologous proteins remained independent of stirrer speed in the range of 550 to 1000 rpm. There are, however, numerous reports to the contrary. Singh *et al.* (2000) showed that at a low stirrer speed of 400 rpm with xylose as carbon substrate, *Thermomyces lanuginosus*

SSBP produced the highest xylanase activity and that higher agitation rates had a negative effect on xylanase production. The negative effect of a high agitation rate on xylanase production was also confirmed by Purkarthofer *et al.* (1993) and Hoq *et al.* (1994) who conducted studies with *T. lanuginosus* DSM 5826 and *T. lanuginosus* RT9, respectively. Similarly, Mukataka *et al*. (1988) evaluated the effect of impeller tip velocity on cellulase activity and found that the tip velocities affected the activities differently. Filter paper cellulase activity reached a maximum at 1.0 m s^{-1}, carboxymethyl cellulase activity at 0.7 m s^{-1}, whereas β-glucosidase reached a maximum at 1.4 m s^{-1}.

The decrease in xylanase production at high stirrer speeds has been attributed to the effects of hydrodynamic stress which may cause hyphal disruption and leakage of intracellular compounds (Purkarthofer *et al.*, 1993; Singh *et al.*, 2000). Shear stress as a result of agitation intensity has also been reported to cause morphological as well as physiological changes in some filamentous fungi, resulting in decreased xylanase production (Palma *et al.*, 1996; Techapun *et al.*, 2003).

Although the xylanase activity was not affected by agitation intensity in the current study, it was found that at the higher stirrer speeds of 600 and 800 rpm lower biomass concentrations but slightly higher maximum specific growth rates were obtained than at 400 and 500 rpm. These results are consistent with observations made by Hoq *et al.* (1994) for *T. lanuginosus* RT9. Hoq *et al.* (1994) suggested that at low stirrer speeds, oxygen limitation may have slowed down the growth rate but, on the other hand, the low shear stress may have aided a higher final biomass concentration, whereas at higher stirrer speeds the sufficient conditions facilitated a higher growth rate but the increased shear stress might result in lower biomass concentrations. The high xylanase yields per g biomass at the high stirrer speeds of 600 and 800 rpm was mainly due to the decreased biomass concentrations, as the volumetric xylanase activities remained fairly constant over the range of stirrer speeds evaluated.

The results presented here clearly demonstrated that xylanase production by *Aspergillus oryzae* NRRL 3485 was markedly affected by the culture pH. A high pH of 7.5 favoured xylanase production, whereas cultivation at pH 4.0 had an adverse effect on xylanase production. This trend was observed with both carbon substrates used, namely SSLc and xylan. The cellulase activities, however, were detected in negligible amounts through out all the culture pH ranges on both carbon substrates. It was also shown that xylanase activities were independent of agitation rates in the range of 400 to 800 rpm. These results indicated

that the concentrated spent sulphite liquor was a convenient substrate for the production of essentially cellulase-free xylanases by *A. oryzae*, being a cheap carbon feedstock as well as serving as an inducer for xylanase synthesis and that cultivation of *A. oryzae* at the agitation rates between the ranges evaluated would not have any detrimental effect on xylanase.

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CHAPTER 5

EFFECT OF XYLOSE-LIMITED FED-BATCH CULTIVATIONS ON XYLANASE PRODUCTION BY *ASPERGILLUS ORYZAE*

Contents

5.1. Abstract

The use of fed-batch cultures to minimise the effect of xylose repression of xylanase production by *Aspergillus oryzae* was investigated. Xylose repression of xylanase production was minimised in fed-batch culture operating at a constant feed rate of 130 g medium h^{-1} (corresponding to 2.38 g xylose h^{-1}), producing a xylanase activity of up to 210 U ml^{-1} compared to 90 U ml^{-1} in batch culture. Xylanase production was further enhanced to 260 U ml^{-1} by increasing the $(NH_4)_2SO_4$ concentration in the medium. A concomitant increase in biomass as well as protease activity of up to 26 g l^{-1} and 2.39 U ml^{-1}, respectively, was also observed. At this feed rate the residual xylose concentration in the culture remained below 0.13 g l^{-1}. The biomass increased linearly, indicating that growth was limited by the feed rate. The current study clearly showed that the shear stress imposed on the organism by the range of stirrer speeds used here, did not have a significant effect on xylanase production. Fed-batch cultures demonstrated that a growth rate-limiting xylose concentration enhanced xylanase production. The nitrogen concentration was also shown to play a significant role in the production of xylanase by the *A. oryzae* strain.

5.2. Introduction

Different microbial systems are currently under investigation for the production of cellulase-free xylanases using strategies based on either controlling the cultivation conditions, genetic manipulation or the development of downstream processing techniques for the mixed broth containing xylanase and cellulase (Panda, 1989; Bailey and Viikari, 1993; Amanullah *et al.*, 1999; Levin and Forchiassin, 1998). The role of xylose as inducer for various microorganisms is not consistent, however. In some cases it can act as inducer (Hrmová *et al*., 1989; de Graaff *et al*., 1992; Purkarthofer *et al*., 1993) and in other cases as repressor (Hrmová *et al*., 1986; Piñaga *et al*., 1994; Kadowaki *et al*., 1997). Xylanases are inducible by certain low-molecular weight substances structurally related to the natural substrate. Xylan, xylobiose, xylose, arabinose and synthetic xylosides are common specific inducers (Purkarthofer and Steiner, 1995).

The synthesis of many enzymes is subject to catabolite repression, i.e. their synthesis is prevented by the presence of a rapidly utilised carbon source (Aunstrup *et al*., 1979). Easily metabolisable carbohydrates repress the synthesis of enzymes related to catabolism of

alternative carbon substrates, thereby ensuring the utilisation of the most favoured carbon substrate present (Ruijter and Visser, 1997). The expression of xylanases in fungi is also subject to regulation by catabolite repression and the accumulation of reducing sugars has been reported to have a negative effect on xylanase production (Gosh and Nanda, 1994; Leathers *et al*., 1986; de Souza *et al*., 2001; Hoq *et al*., 1994; Prathumpai *et al*., 2004). Easily metabolisable sugars such as glucose, sucrose and in some cases xylose have been reported to repress the formation of xylanase (Hoq *et al*., 1994; Chandra and Chandra, 1995; de Souza *et al*., 2001; Prathumpai *et al*., 2004).

Fed-batch cultures have been used to minimise or completely avoid the phenomenon of catabolite repression. Fed-batch processes involve the feeding of concentrated complete medium or one or more nutrients to the culture at a rate that prevents the carbon source from reaching the threshold value for catabolite repression. This process also facilitates control of the specific growth rate and oxygen transfer rate, allowing high cell density cultivations (Spohr *et al*., 1998). There are, however, some disadvantages associated with fed-batch cultures, including high shear stress and poor mixing due to high viscosities caused by the high biomass concentrations that can be achieved as a result of this process (Olsvik *et al*., 1993; Mekagiansar *et al*., 1993).

The present study describes the influence of xylose concentration on xylanase production by *Aspergillus oryzae* using fed-batch culture with spent sulphite liquor concentrate (SSLc) as carbon substrate. The effect of increasing the $(NH_4)_2SO_4$ concentration on xylanase production was also noted.

5.3. Materials and methods

5.3.1. Fungal strain

The fungal strain used in the study was *Aspergillus oryzae* NRRL 3485 and was maintained on Sabouraud-dextrose agar (Biolab Diagnostics Ltd., Midrand, South Africa) slants and stored at 4 ^{o}C with subculturing at 12-week intervals.

5.3.2. Fed-batch cultivation

5.3.2.1. Culture conditions

Fed-batch cultures were carried out using a 15 l Biostat C bioreactor (B Braun Biotech International, Melsungen, Germany). The bioreactor was equipped with a pH electrode (Mettler Toledo, Halstead, UK) and a polarographic oxygen electrode (Mettler Toledo). An electronic balance (Mettler Toledo) upon which the feed reservoir was mounted and a peristaltic pump (Watson Marlow Ltd., Cornwall, UK) with silicone rubber tubing were used to pump the sterile medium into the bioreactor. The feed was initiated at the onset of automatic titration with acid for pH control of the culture, which was an indication that the culture had reached the end of the exponential growth phase and that the xylose concentration in the culture medium was at a very low level. A constant feed rate using different culture medium flow rates of 95, 130 and 195 g h^{-1} (corresponding to 1.83, 2.38 and 3.95 g xylose h^{-1}) was used to evaluate the effect of xylose on xylanase production in fed-batch culture. The DOT was maintained at or above 25 % of saturation by cascade control of the aeration rate between 0.05 to 8.0 std l min^{-1} and the stirrer speed between 400 and 800 rpm.

5.3.2.2. Fed-batch culture medium

The medium composition for the different fed-batch culture experiments was as shown in Table 5.1. Each fed-batch cultivation was initially started as a batch culture of 5 l. SSLc was used as carbon substrate and was diluted five-fold with distilled water for the feed and 40-fold for the batch culture medium. The first fed-batch culture was conducted using an $(NH_4)_2SO_4$ concentration of 5 g l^{-1} in the medium and 35 g l^{-1} in the feed. In subsequent experiments (Feeds 2, 3 and 4) the $(NH_4)_2SO_4$ concentration in the bioreactor medium was increased to 7.5 g l^{-1} and to 52.5 g l^{-1} in the feed (Table 5.1). All cultivations were carried out at 30 °C with the culture pH controlled at pH 7.5 by automatic titration with 5 N KOH and 5 N H_2SO_4.

5.3.3. Analyses

Xylanase and protease activity as well as biomass concentration were determined as described in Chapter 3. One unit of xylanase activity (U) was defined as the amount of enzyme that liberated 1 µmol of xylose equivalent per minute per ml of culture filtrate, whereas one unit of protease activity was defined as the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvette. The residual xylose concentration in fed-batch culture was determined using a HPLC Dionex system as described in Chapter 3.

Table 5.1. Medium composition and feed rates used to evaluate the effect of xylose concentration on xylanase production by *Aspergillus oryzae* in different fed-batch cultures, using SSLc diluted 40 and five-fold with distilled water in the batch culture medium and feed medium, respectively, as carbon feedstock.

Constituent	Batch culture	Feed 1 (130 g h^{-1})	Feed 2 (130 g h^{-1})	Feed 3 (195 g h^{-1})	Feed 4 (95 g h^{-1})
Yeast extract (g l^{-1})	10	70	70	70	70
Citric acid (g l^{-1})	0.25	1.75	1.75	1.75	1.75
$(NH_4)_2SO_4$ (g l^{-1})	5[a] / 7.5[b]	35	52.5	52.5	52.5
K_2HPO_4 (g l^{-1})	5	35	35	35	35
$MgSO_4.7H_2O$ (g l^{-1})	0.5	3.5	3.5	3.5	3.5
$CaCl_2.2H_2O$ (g l^{-1})	0.02	0.14	0.14	0.14	0.14
TES (ml l^{-1})	1	7	7	7	7
Xylose[c] (g l^{-1})	2.5	21.02	20.12	22.22	21.82

[a] Concentration used in conjunction with Feed 1 medium

[b] Concentration used in conjunction with Feed 2 to 4 media

[c] Average xylose concentration in SSLc after dilution and addition of medium components

TES Trace elements solution (du Preez and van der Walt, 1983)

5.4. Results

5.4.1. Effect of fed-batch culture

Figure 5.1 shows the fed-batch culture profile for *A. oryzae* with SSLc as carbon substrate using a constant feed rate of 130 g h^{-1}. The feed was initiated at 14 h which coincided with the onset of acid titration for pH control and a biomass concentration of 1.8 g l^{-1}, until 52 h of cultivation, at which time the feed reservoir was almost exhausted. Xylanase production, however, only began after 14 h of cultivation, at which time the residual xylose concentration in the medium had decreased from an initial value of 2.34 g l^{-1} to 0.17 g l^{-1}. The residual xylose concentration in the medium remained at a low level not exceeding 0.18 g l^{-1} throughout the cultivation period, with the extracellular xylanase activity increasing steadily from 15 U ml^{-1} at 14 h to a maximum of 210 U ml^{-1} at 46 h of cultivation, but dropping suddenly thereafter. A maximum volumetric rate of xylanase production of 7.14 U ml^{-1} h^{-1}

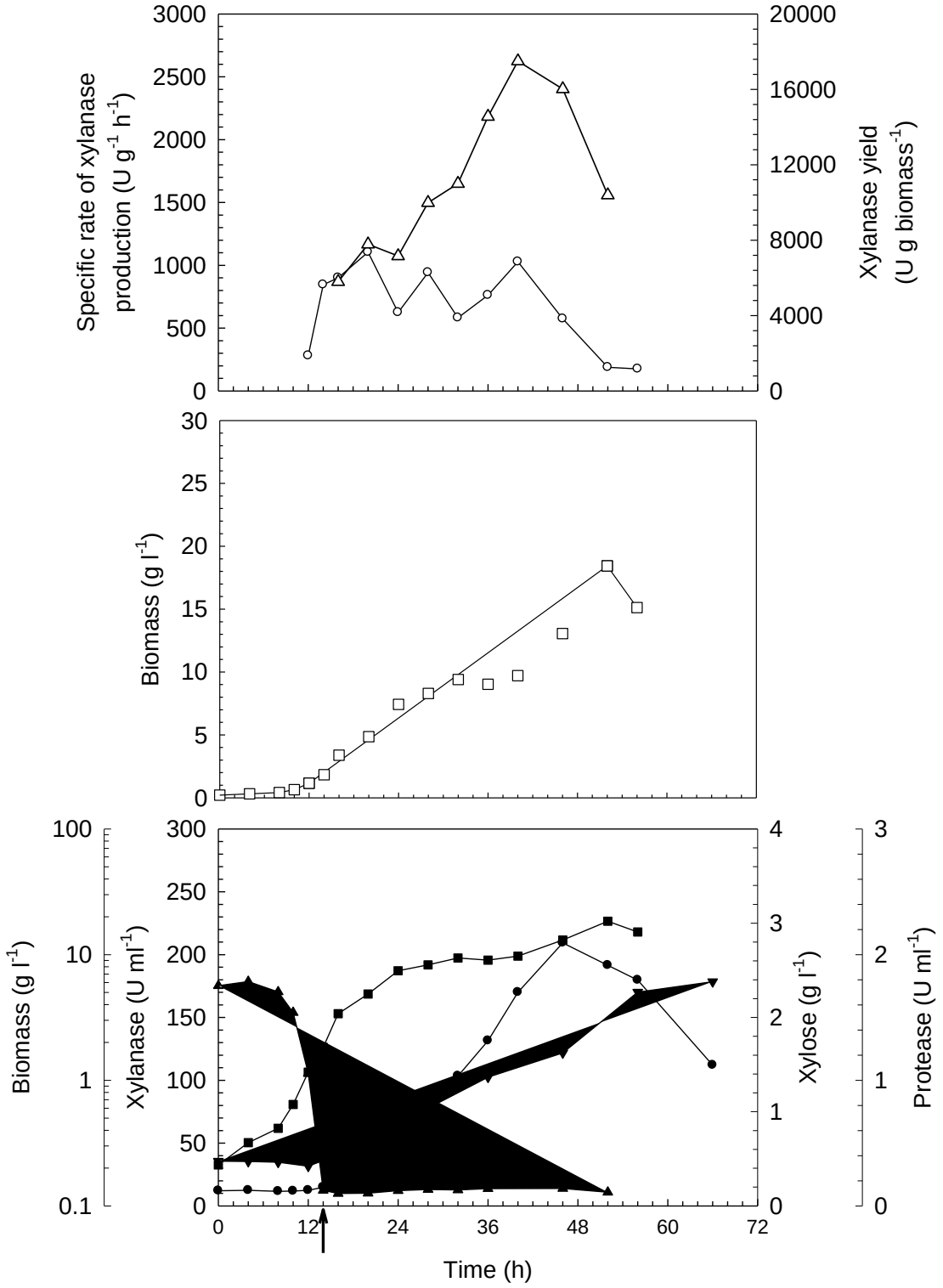

Figure 5.1. Fed-batch cultivation profile for *A. oryzae* NRRL 3485 at a constant feed rate of 130 g h^{-1} initiated at 14 h (indicated by arrow). Symbols: ● xylanase; ■ biomass; ▲ xylose; ▼ protease; ○ specific rate of xylanase production; ∆ xylanase yield; □ biomass.

was obtained with this feed rate and xylanase production lasted for a 32 h period. The specific rate of xylanase production peaked at 1 104 U h^{-1} at 20 h, whereas the xylanase yield reached 17 500 U g $biomass^{-1}$. The protease activity increased steadily after initiation of the feed to 1.78 U ml^{-1} at the end of the cultivation period, when a biomass concentration of 18.4 g l^{-1} was reached. The biomass concentration increased linearly, suggesting that rate of feed addition was growth rate-limiting.

5.4.1.1. Increased $(NH_4)_2SO_4$ concentration

The effect of increasing the $(NH_4)_2SO_4$ concentration from 5.0 g l^{-1} to 7.5 g l^{-1} in the batch culture medium and from 35.0 g l^{-1} to 52.5 g l^{-1} in the feed, as shown in Table 5.1, on xylanase production is shown in Figure 5.2. As in the previous fed-batch culture, a constant feed rate of 130 g h^{-1} was maintained and the onset of pH control by acid titration signalled the initiation of the feed. However, feeding only commenced at 16 h instead of 14 h as in the previous fed-batch experiment, at which time the residual xylose concentration had decreased to 0.34 g l^{-1} from an initial concentration of 3.2 g l^{-1}. By 19 h, the residual xylose concentration had decreased to 0.13 g l^{-1} and remained between 0.13 and 0.03 g l^{-1} until feeding was terminated. The biomass concentration, which was 1.10 g l^{-1} at the time of feed initiation, increased to a maximum of 26 g l^{-1} at 62 h. As in the previous fed-batch experiment, the biomass increased linearly during feeding. The xylanase activity increased steadily from 13 U ml^{-1} at 16 h to a maximum of 260 U ml^{-1} at 62 h, 4 h before termination of feeding. In this instance, xylanase production lasted up to 48 h and reached a higher volumetric activity than in the previous fed-batch culture. However, the maximum volumetric rate of xylanase production of 5.49 U ml^{-1} h^{-1} was lower than in the previous fed-batch culture. The specific rate of xylanase production gradually decreased from a high of 2 700 U g^{-1} h^{-1} at 14 h and the xylanase yield reached 15 300 U g $biomass^{-1}$ at 12 h. The protease activity increased steadily after initiation of the feed to 2.39 U ml^{-1} at the end of the cultivation period.

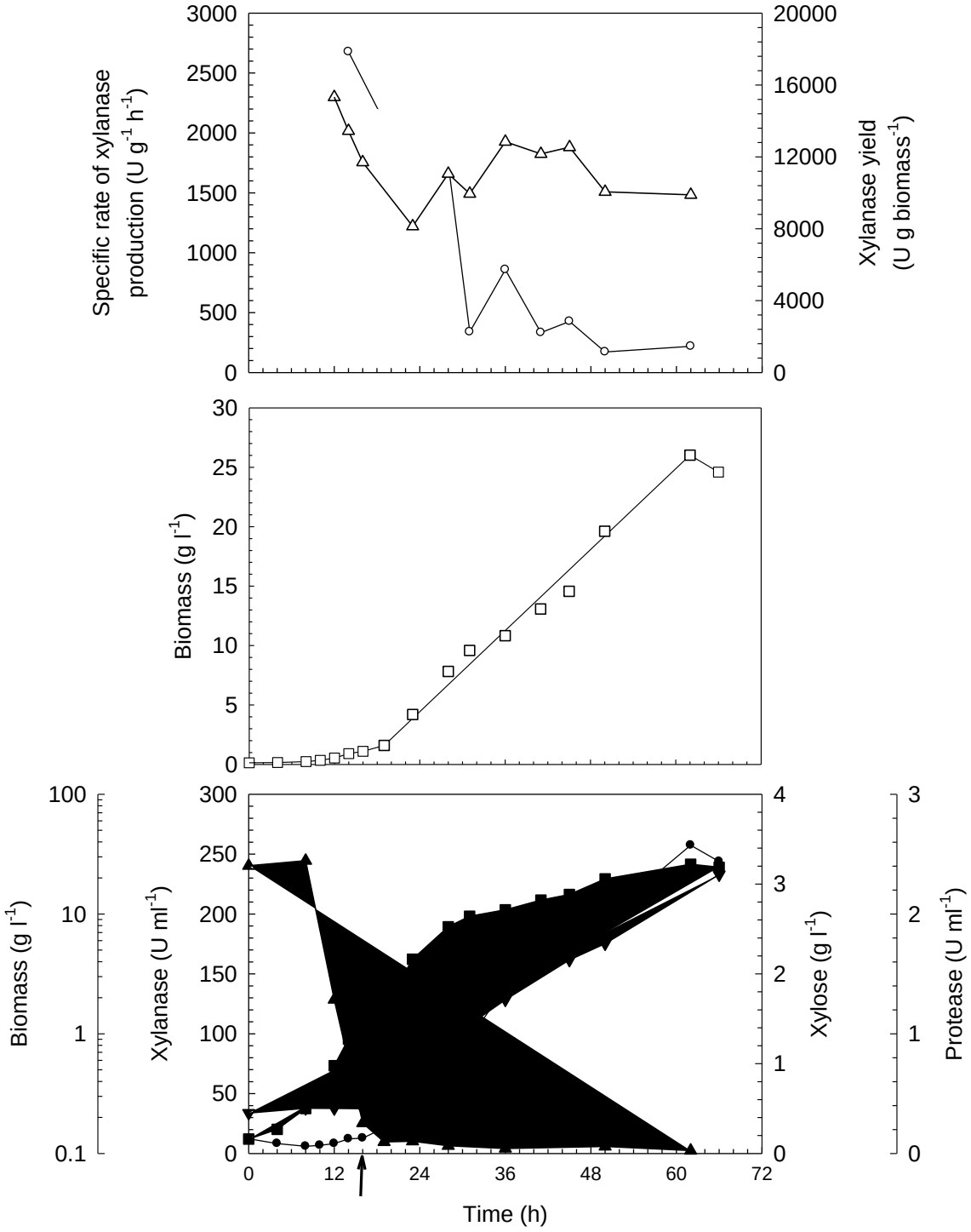

Figure 5.2. Fed-batch cultivation profile for *A. oryzae* NRRL 3485 with an increased $(NH_4)_2SO_4$ concentration and a constant feed rate of 130 g h^{-1} initiated at 16 h (indicated by arrow). Symbols: ● xylanase; ■ biomass; ▲ xylose; ▼ protease; ○ specific rate of xylanase production; Δ xylanase yield; □ biomass.

5.4.1.2. Increased constant feed rate

Using an increased $(NH_4)_2SO_4$ concentration (Table 5.1, Feed 3), the constant feed rate was increased from 130 g h^{-1} to 195 g h^{-1}. As in the previous fed-batch cultures, the onset of pH control by acid titration signalled the initiation of the feed. Feeding commenced at 16 h, at which time the residual xylose concentration in the medium had decreased from an initial value of 2.4 g l^{-1} to 0.33 g l^{-1} and remained between 0.27 and 0.23 g l^{-1} during feeding (Figure 5.3). The biomass concentration at time of feed initiation was 2.7 g l^{-1} and increased to a maximum of 20.1 g l^{-1} at 43 h. Despite the increased feed rate, the rate of feed addition was still growth rate-limiting, as was indicated by the linear increase in the biomass concentration. However, as a result of the increased feed rate, the feeding period as well as the xylanase production period was significantly reduced, with xylanase production occurring for 29 h. The xylanase activity increased steadily from 21 U ml^{-1} at 16 h to only 83 U ml^{-1} at 43 h, at which time the feed was terminated. A maximum volumetric rate of xylanase production of 2.38 U ml^{-1} h^{-1} was obtained and the specific rate of xylanase production peaked at only 638 U g^{-1} h^{-1} at 20 h. The xylanase yield reached 7 600 U g $biomass^{-1}$ at 12 h and the protease activity increased to 2.15 U ml^{-1} at the end of the cultivation period.

5.4.1.3. Decreased constant feed rate

Figure 5.4 shows the fed-batch cultivation profile for *A. oryzae* obtained using a reduced constant feed rate of 95 g h^{-1}. The feed was initiated at 14 h at which time the residual xylose concentration in the batch culture had decreased from an initial value of 2.94 g l^{-1} to 0.12 g l^{-1}. However, after 20 h until termination of the feed, the residual xylose concentration remained below 0.01 g l^{-1}. The biomass concentration was 1.5 g l^{-1} at the time of feed initiation and this increased to a maximum of 15.7 g l^{-1} at 42 h then decreased to 7.8 g l^{-1} at the time of feed termination at 72 h. Xylanase production commenced with the initiation of the feed, increasing from 16 U ml^{-1} to a maximum of 166 U ml^{-1} at 60 h. The maximum volumetric rate of xylanase production was 3.08 U ml^{-1} h^{-1}, whereas the specific rate of xylanase production reached a value of 2 400 U g^{-1} h^{-1} at 16 h. The xylanase yield reached 16 000 U g $biomass^{-1}$ at 30 h and the protease activity reached 2.31 U ml^{-1} at the end of the cultivation period.

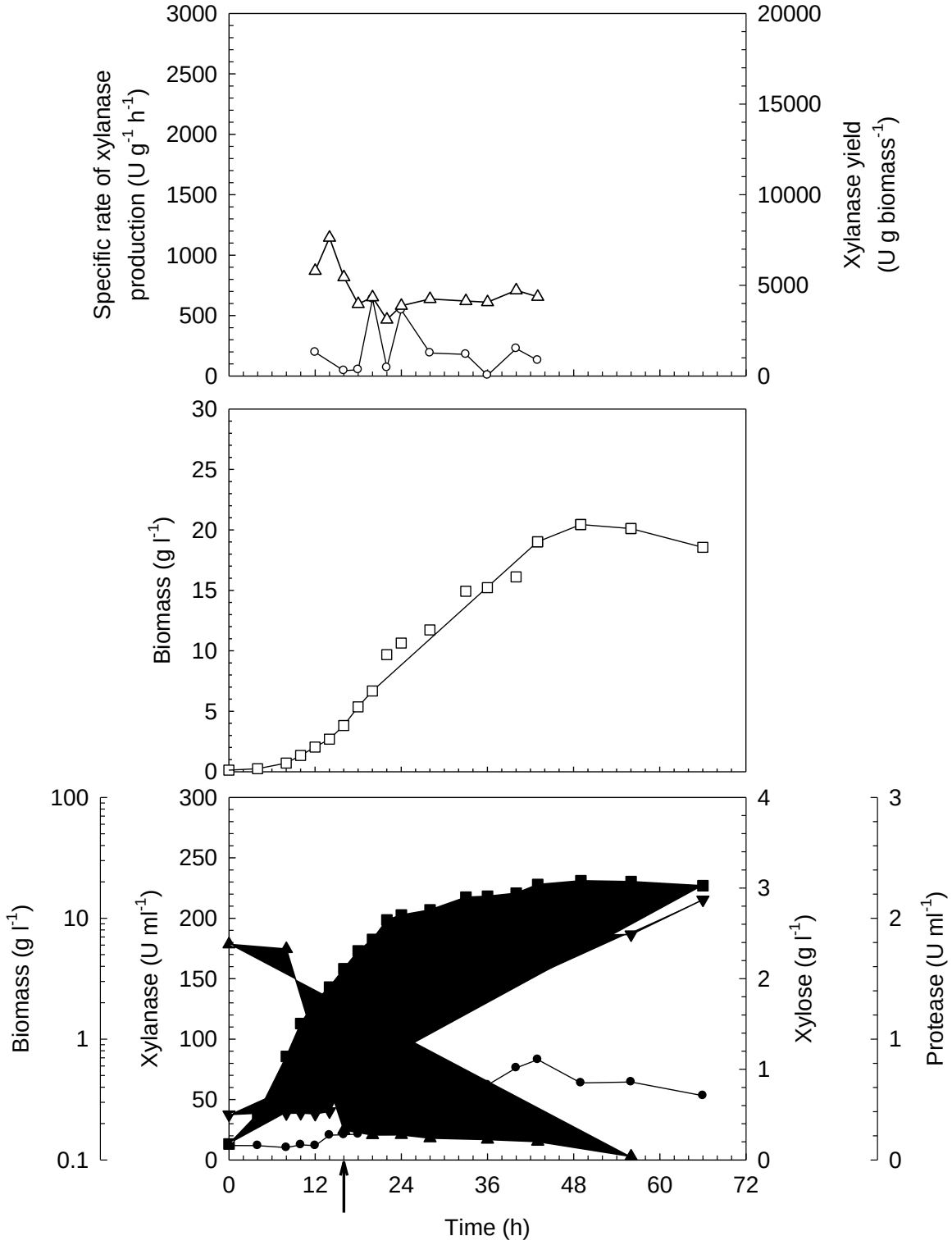

Figure 5.3. Fed-batch cultivation profile for *A. oryzae* NRRL 3485 at a constant feed rate of 195 g h^{-1} initiated at 16 h (indicated by arrow). Symbols: ● xylanase; ■ biomass; ▲ xylose; ▼ protease; ○ specific rate of xylanase production; Δ xylanase yield; □ biomass.

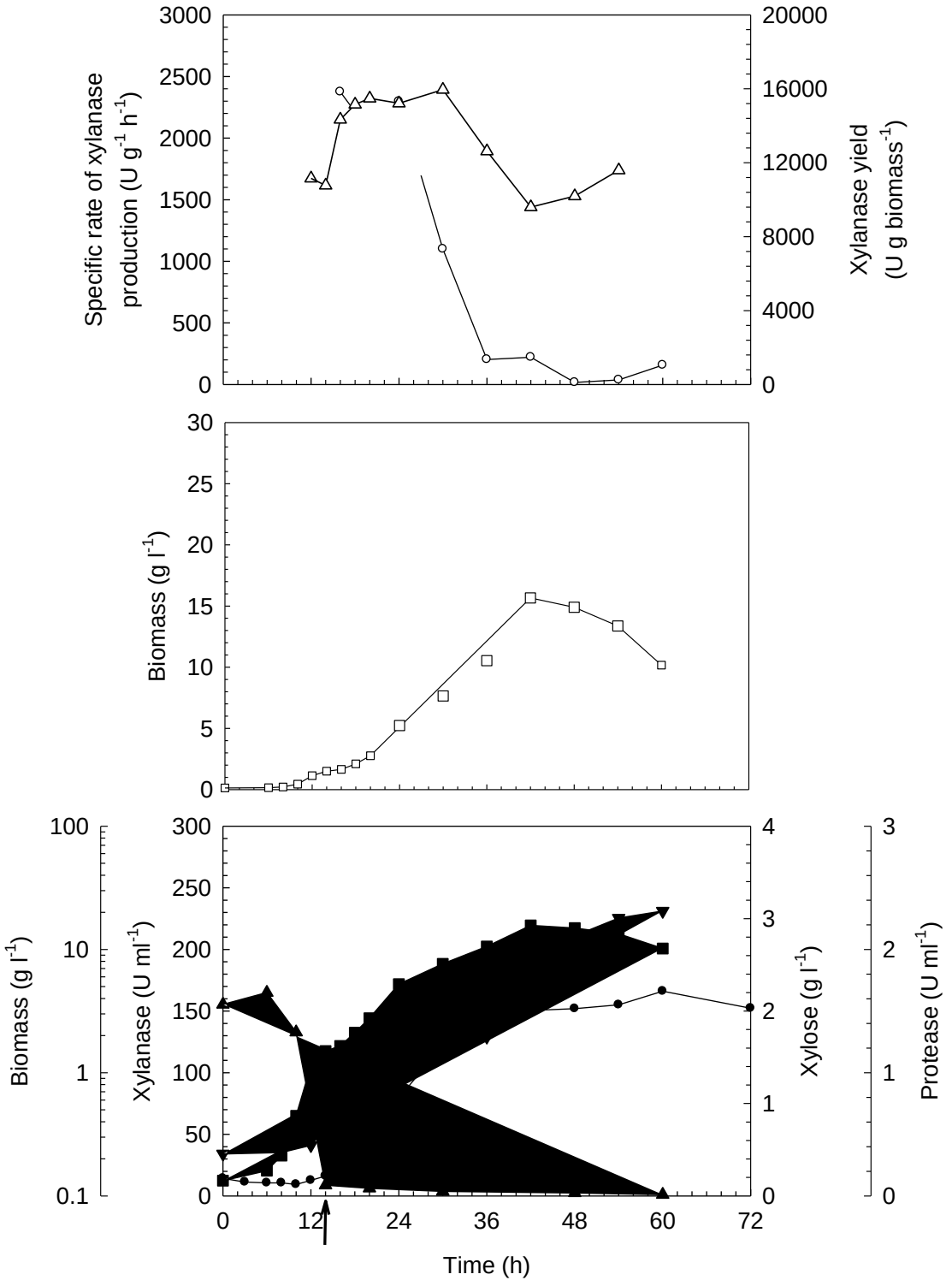

Figure 5.4. Fed-batch cultivation profile for *A. oryzae* NRRL 3485 at a constant feed rate of 95 g h^{-1} initiated at 14 h (indicated by arrow). Symbols: ● xylanase; ■ biomass; ▲ xylose; ▼ protease; ○ specific rate of xylanase production; ∆ xylanase yield; □ biomass.

5.4.2. Effect of fed-batch culture on growth parameters

The effects of $(NH_4)_2SO_4$ concentration and feed rate on the growth parameters of *A. oryzae* are summarised in Figure 5.5. Increasing the $(NH_4)_2SO_4$ concentration and maintaining a constant feed rate of 130 g h^{-1} (Feed 2) resulted in a high xylanase activity, specific rate of xylanase production, biomass concentration and biomass yield as well as a high protease activity. Values obtained for the biomass yield per g xylose were highest under these conditions and lowest using the feed rate of 95 g h^{-1}, with values of up to 1.81 and 0.55 g biomass g $xylose^{-1}$ obtained using the feed 2 and 4 regimens (Table 5.1), respectively. However, the xylanase yield per g biomass, as well as the maximum volumetric rate of xylanase production was higher when using the feed 1 regimen (Table 5.1) in which the medium contained a lower $(NH_4)_2SO_4$ concentration than the other fed-batch cultures. The residual xylose concentration at the termination of feed of all the fed-batch cultures was below 0.15 g l^{-1}.

5.5. Discussion

The current study demonstrated the effect of xylose concentration on xylanase production by *A. oryzae* in fed-batch culture. Xylose has been reported to have either an inducing or repressing effect on xylanase production, with its effects varying even within related species. Xylose was reported to act as inducer in *A. terreus* (Hrmová *et al.*, 1991), *A. awamori* (Siedenberg *et al.*, 1998) and *A. tubigensis* (de Graaff *et al.*, 1992), but as a repressor in *A. nidulans* (Piñaga *et al.*, 1994) and *A. oryzae* NRRL 3485 (Chapter 3). Previous studies (Chapters 3 and 4) suggested that xylose caused carbon catabolite repression of xylanase production and when the xylose concentration in the culture decreased to low levels the repression was alleviated with xylanase production occurring. Thus, by varying the feed flow rate it was possible to control the xylose concentration in the culture, thereby minimising the repressive effect of xylose. Xylanase production started when the xylose concentration in the culture decreased to a low level, resulting in carbon substrate-limited growth. The maximum volumetric rate of xylanase production as well as the length of the production phase was shown to be dependent on the feeding rate, which was varied in different experiments from 95 to 195 g medium h^{-1}. The highest xylanase yield was obtained at a feed rate of 130 g h^{-1}, with a higher feeding rate resulting in a decrease in the xylanase yield as well as a decrease in the period of xylanase production. These findings correlated with

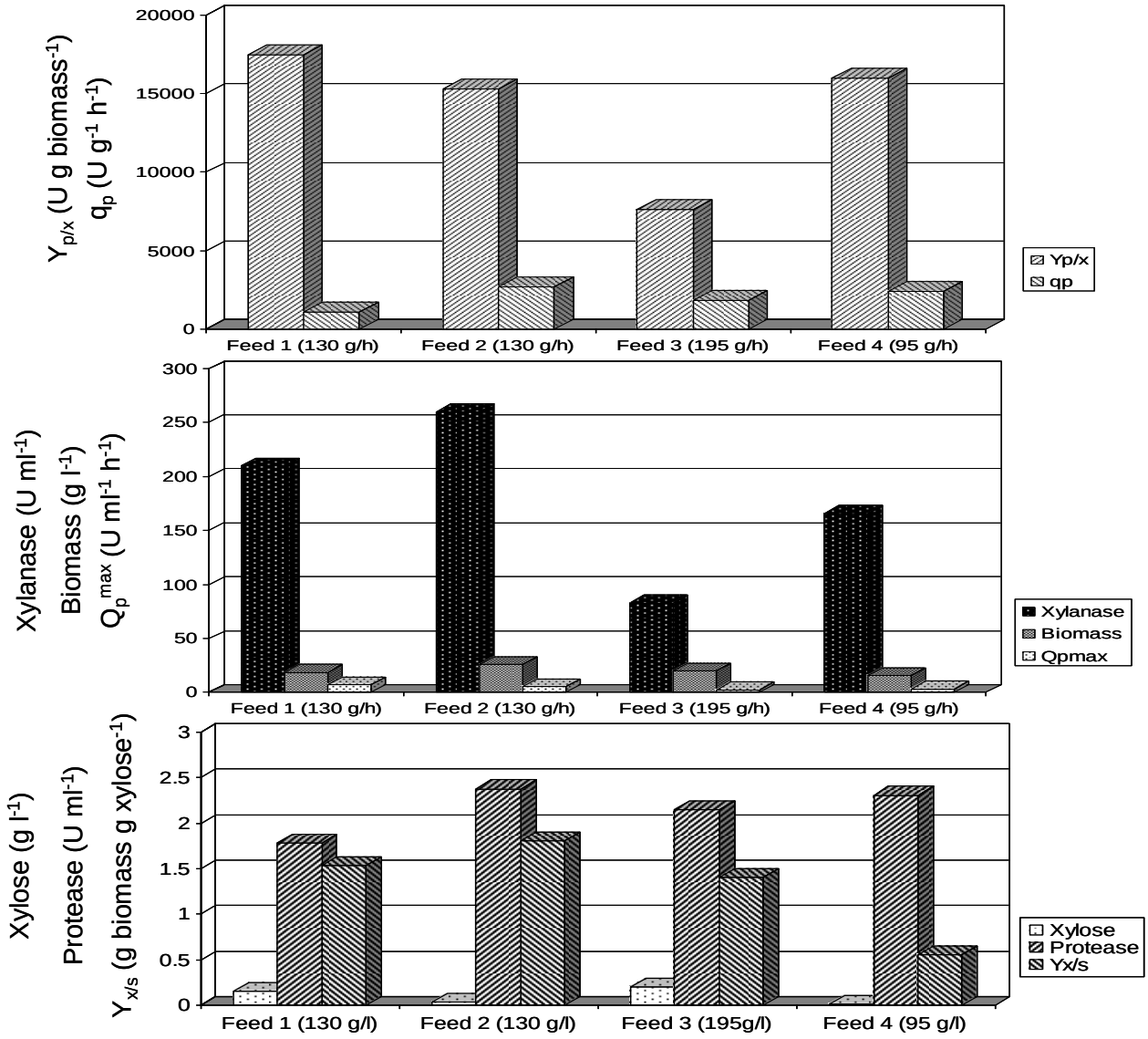

Figure 5.5. Effect of $(NH_4)_2SO_4$ concentration and feed rate on growth parameters of *A. oryzae* NRRL 3485 in fed-batch culture. The residual xylose concentration was determined at the termination of the feed. Q_p^{max}: Maximum volumetric rate of xylanase production; q_p: Specific rate of xylanase production; $Y_{x/s}$: Biomass yield coefficient; $Y_{p/x}$: Xylanase yield coefficient.

those of Samain *et al.* (1997), who evaluated a thermophilic *Bacillus* strain for xylanase production in glucose-limited fed-batch culture and reported that the rate of xylanase synthesis and also the length of the enzyme production phase were dependent on the feed rate.

The correlation between the growth rate and feed rate is also illustrated in Figures 5.1 to 5.4. The feed rate was shown to be growth rate-limiting, as was evident from the linear increase in biomass concentration of *A. oryzae* over the feeding period. Xylanase production ceased abruptly when the activity reached a value of 210 U ml^{-1} at 46 h (Figure 5.1). A similar phenomenon was reported by Samain *et al*. (1997) and this sudden decline in activity was attributed to nitrogen limitation. Increasing the ammonium concentration in the medium and feed and maintaining the feed rate at 130 g h^{-1} resulted in higher xylanase activities and prolonged xylanase production for up to 48 h. Similarly, increasing the ammonium concentration also resulted in a high biomass yield of up to 1.81 g biomass g $xylose^{-1}$. This abnormally high value may be attributed to the utilisation of non-sugar carbon present in the SSL, as was evident from the total organic carbon analyses performed on SSL-based shake flask cultures in Chapter 3.

The data presented in the current study conclusively showed that the use of fed-batch cultivation to maintain a low xylose concentration in the culture significantly enhanced xylanase production rates as well as xylanase yields. Nitrogen also played an important role in xylanase synthesis and increasing the $(NH_4)_2SO_4$ concentration resulted in even higher xylanase activities. These data with respect to nitrogen suggest that optimisation of the nitrogen source and concentration could enhance xylanase production even further.

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CHAPTER 6

THE EFFECT OF ULTRAFILTRATION AND OVERLIMING OF SPENT SULPHITE LIQUOR AS CARBON SUBSTRATE FOR XYLANASE PRODUCTION

Contents

6.1. Abstract

Lignocellulosic acid hydrolysates contain a variety of compounds that are inhibitory to microbial growth. The detoxification of such hydrolysates is, therefore, necessary for the removal of inhibitors. This study evaluated the effect of ultrafiltration and overliming of spent sulphite liquor concentrate (SSLc). Ultrafiltration of this effluent resulted in a significant decrease in the COD, in the true colour as well as in the total and suspended solids. The total sugar composition, acetic acid concentration and total polyphenol content, however, remained unchanged. Overliming, on the other hand, resulted in a significant loss of total sugars with moderate removal of total polyphenols as well as a slight decrease in the COD values. Overliming of the SSLc and ultrafiltered SSLc aliquots at 60 °C to pH 12 for a duration of 60 min resulted in large sugar losses of up to 79 and 67 %, respectively, concomitant with a slight increase in the total polyphenol content of up to 5 % and 6 %, respectively. The pretreatment of effluent aliquots had an adverse effect on xylanase production by *Aspergillus oryzae*. Xylanase activities of up to 56 and 129 U ml^{-1} were obtained with overlimed SSLc and ultrafiltered SSLc aliquots, respectively, which were significantly lower than obtained with untreated SSLc and $SSLc_F$ which yielded xylanase activities of up to 183 and 168 U ml^{-1}, respectively. Overlimed effluent aliquots gave low xylanase activities, low biomass concentrations as well as low xylanase yield coefficients. Although pretreatment of effluent removed potential inhibitors, overliming, and to a lesser extent ultrafiltration, also resulted in less xylanase production. Since xylanase production is known to be inducible, overliming of the effluent samples may also have removed or degraded the inducing compound/s together with potential inhibitory compounds.

6.2. Introduction

The separation of the cellulosic constituents of wood from the non-cellulosic fraction during the sulphite pulping process results in the generation of an effluent referred to as spent sulphite liquor (SSL). SSL from different mills may vary in composition as a result of the type of wood used as well as the degree of cooking required to produce the desired pulp quality (Holderby and Moggio, 1960). Considerable research was conducted on the utilisation of spent sulphite liquors from about 1950 to 1970 and this included evaporation and burning for the recovery of the fuel value and/or spent pulping chemicals, evaporation for the preparation of lignosulphonate compounds and microbial cultivation using the SSL sugars as

carbon feedstock for the production of valuable products such as yeast protein, ethanol and lactic acid (Holderby and Moggio, 1960; Pineault *et al.*, 1977; Šestáková, 1979).

In addition to fermentable sugars, acid hydrolysates such as SSL also contain a wide spectrum of degradation products that include aliphatic acids (acetic acid, formic acid and levilunic acid), furan compounds (furfural and hydroxymethylfurfural) as well as various phenolic compounds. Acetic acid is formed as a result of the deacetylation of hemicellulose, whereas formic and levulinic acids are formed from the degradation of furfural and hydroxymethylfurfural, respectively, which are a result of the degradation of monosaccharide pentose and hexose sugars (Ulbricht *et al*., 1984; Larsson *et al*., 1999). Hydrolysate components have been extensively studied (Nishikawa *et al.*, 1988; Ranatunga *et al.*, 1997; Larsson *et al.*, 1999) and the many individual inhibitory compounds found in lignocellulosic hydrolysates have been investigated. There is, however, no single compound that has emerged as the dominant inhibitor, although furfural was found to increase the toxicity of other compounds for yeasts (Palmqvist *et al.*, 1999).

Pretreatment of lignocellulosic hydrolysates is necessary for the removal of compounds that would potentially be inhibitory to the microbial utilisation of these hydrolysates. An ideal detoxification method should selectively remove microbial inhibitors without the removal of fermentable sugars from the hydrolysates. Several detoxification methods have been studied and developed, including evaporation, steam stripping, extraction with organic solvents, overliming, ion-exchange, sulphite treatment, activated carbon treatment, wood charcoal treatment and enzyme treatment as well as organism acclimatisation (Frazer and McCaskey, 1989; Lawford and Rousseau, 1992; Olsson *et al.*, 1995; Jönsson *et al.*, 1998; Larsson *et al.*, 1999; Martinez *et al.*, 2000; Miyafuji *et al.*, 2003). In most cases, however, the most economical method of detoxification has been overliming with solid $Ca(OH)_2$ and this has been reported to be an effective method of reducing the toxicity of various lignocellulosic hydrolysates (Lawford and Rousseau, 1992; Olsson and Hahn-Hägerdal, 1996; Larsson *et al.*, 1999; Martinez *et al.*, 2000).

Overliming is a well established protocol shown to significantly decrease the inhibitory effect of lignocellulosic hydrolysates from many different lignocellulosic materials (Olsson and Hahn-Hägerdal, 1996; Larsson *et al.*, 1999; Martinez *et al.*, 2000; Ranatunga *et al.*, 2000). The overliming process may remove inhibitory compounds by precipitation, chemically convert toxic compounds into non-toxic forms and/or add some substance to the hydrolysate

that may enhance the fermentation capacity of microorganisms (Ranatunga *et al*., 2000). Overliming is traditionally performed by the addition of alkali ($Ca(OH)_2$ or NaOH) to increase the pH up to 10, followed by adjustment to the suitable cultivation pH (Millati *et al.*, 2002). In a comparative study of six different detoxification methods, Larsson *et al.* (1999) showed that $Ca(OH)_2$ addition was the most effective treatment for spruce hydrolysates. Similarly, Martinez *et al.* (2000) found that $Ca(OH)_2$ addition to bagasse hemicellulose hydrolysates resulted in an increased fermentability of the hydrolysate by a yeast strain. In both instances, overliming resulted in the removal of furans and phenolics, but with no change in the acetic acid concentration. A major drawback of the overliming protocol is the concomitant removal of fermentable sugars with increasing $Ca(OH)_2$ addition (Larsson *et al.*, 1999; Martinez *et al.*, 2000; Millati *et al.*, 2002). Increased fermentability of lignocellulosic hydrolysates has also been achieved by combining other pretreatment methods with overliming. Ranatunga *et al.* (2000) found that overliming after continuous ion-exchange treatment of a dilute-acid hydrolysate resulted in an increased fermentability of the hydrolysate by a recombinant *Zymomonas mobilis* strain.

In the current study, the effect of ultrafiltration and overliming of spent sulphite liquor for use as carbon substrate in the production of xylanase by *Aspergillus oryzae* was investigated. The effects of overliming variables such as pH, time and temperature on the composition of SSL were also evaluated.

6.3. Materials and methods

6.3.1. Spent sulphite liquor

Spent sulphite liquor was supplied in a concentrated form from an acid sulphite pulp mill in South Africa, where it had been concentrated by a three-step evaporation process. This concentrate was designated SSLc. All pretreatment procedures (ultrafiltration and/or overliming) as well as cultivations for xylanase production were conducted using SSLc that had been diluted ten-fold with distilled water.

6.3.2. Ultrafiltration

A volume of 10 l SSLc was filtered through a cross-flow ultrafiltration system equipped with a Prep/Scale-TFF (SD031) cartridge (Millipore, Millipore Corporation, Bedford, MA, USA) with a cut-off molecular weight of 30 kDa. The resultant permeate of ca. 6.5 l was subsequently

collected and stored at 4 ºC until further evaluation and the retentate discarded. The stored effluent aliquot was designated $SSLc_F$.

6.3.3. Overliming

The overliming procedure involved increasing the pH of SSLc and $SSLc_F$ to pH 10 or 12 by the addition of $Ca(OH)_2$ to 500 ml effluent aliquots and stirring vigorously for 0, 30 and 60 min, respectively, at 25 or 60 ºC, which were maintained using a temperature-controlled water bath (Labotec (Pty) Ltd, Midrand, South Africa). Subsequently, the aliquots were filtered using Whatman No. 3 filter papers (Whatman International, Maidstone, UK), adjusted to pH 6 with 2 N H_2SO_4 and refiltered. These aliquots were used as carbon substrate for xylanase production or stored at -20 ºC until further analyses. Overlimed SSLc and $SSLc_F$ aliquots were designated $SSLc_1$ and $SSLc_{F1}$, respectively.

6.3.4. Fungal strain and culture conditions

Aspergillus oryzae NRRL 3485 was used in shake flask cultures for the evaluation of xylanase production with the respective effluent samples. The strain was maintained on Sabouraud-dextrose agar slants (Biolab Diagnostics, Midrand, South Africa) and stored at 4 ºC with subculturing at 12-week intervals.

The SSLc, $SSLc_F$, $SSLc_1$ and $SSLc_{F1}$ aliquots were used as respective carbon sources for the evaluation of xylanase production, with the following nutrients added (l^{-1}): citric acid, 0.25 g; $(NH_4)_2SO_4$, 5 g; K_2HPO_4, 5 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.02 g; yeast extract, 10 g and 1 ml of a trace element solution (du Preez and van der Walt, 1983). All media were adjusted to pH 6 prior to autoclaving. The inoculum as well as shake flask cultures were prepared as previously described in Chapter 3.

6.3.5. Analytical procedures

The pretreated as well as untreated SSLc effluent aliquots were analysed for sugar composition, acetic acid concentration, COD, total polyphenols as well as total and suspended solids according to the procedures described in Chapter 3. The true colour of the SSLc and $SSLc_F$ were spectrophotometrically determined using method 8025 described in the HACH DR/2000 spectrophotometer handbook (Anon., 1997).

Xylanase activity was determined as described in Chapter 3 using the dinitrosalicylic acid (DNS) assay with birchwood xylan (Sigma) as substrate (Bailey *et al.*, 1992), whereas fungal

biomass was gravimetrically determined after centrifugation of duplicate 5-ml aliquots of the respective culture samples, washing twice with equal volumes of distilled water and drying at 105 ºC.

6.3.6. Statistical analysis

Analysis of variance (ANOVA) was conducted to determine whether significant differences existed between treatments (NCSS, 2004). The interaction between overliming duration (reaction time) and pH was further investigated by means of the Tukey-Kramer multiple comparison test ($\alpha = 0.05$) (NCSS, 2004)

6.4. Results

6.4.1. Effect of pretreatment variables on SSLc properties

Results of ANOVA conducted to determine the effect of pretreatment variables such as ultrafiltration, overliming time (reaction time), pH and temperature on the composition and chemical properties of SSLc are shown in Table 6.1. The overliming time, pH, as well as temperature were found to have a significant effect on the total sugar content as well as on the total polyphenol content of the SSLc samples. The COD values, however, were significantly affected by the overliming pH and temperature ($p \leq 0.001$) as well as by ultrafiltration. The acetic acid concentration, however, was unaffected by these pretreatment variables.

Table 6.1. Analysis of variance for the effect of various treatments and on the composition and chemical properties of SSLc.

Treatment	Measurements			
	Total sugar content	**Acetic acid content**	**Total polyphenol content**	**COD**
Reaction time	$p \leq 0.001$	NS	$p \leq 0.001$	NS
pH	$p \leq 0.001$	NS	$p \leq 0.01$	$p \leq 0.001$
Temperature	$p \leq 0.001$	NS	$p \leq 0.001$	$p \leq 0.001$
Ultrafiltration	$p \leq 0.05$	NS	NS	$p \leq 0.001$

NS Not significant

6.4.2. Ultrafiltration of SSLc

The effect of ultrafiltration on the chemical composition of the SSLc is shown in Table 6.2. The results showed that ultrafiltration resulted in a decrease in COD, true colour, total and suspended solids as well as total sugars, whereas the concentration of acetic acid as well as total polyphenols remained essentially unchanged. The COD decreased more than 20-fold and the colour by more than ten-fold, with the total solids decreasing by more than 15-fold.

Table 6.2. Comparison of the composition and chemical properties of SSLc and $SSLc_F$. Mean values of duplicate determinations are shown.

Property	**SSLc**	**$SSLc_F$**
Total sugars (g l^{-1})	10.6	10.1
Acetic acid (g l^{-1})	3.14	2.97
COD (mg l^{-1})	7.5×10^5	3.4×10^4
Total polyphenols (g l^{-1})	10.7	11.1
True colour (PCU)	2.2×10^4	1.9×10^3
Total solids (g l^{-1})	68.9	4.2
Suspended solids (g l^{-1})	1.3	0.3

COD Chemical oxygen demand

PCU Platinum-cobalt unit (one colour unit is equivalent to 1 mg platinum l^{-1} as chloroplatinate ion).

SSLc Spent sulphite liquor concentrate

$SSLc_F$ Spent sulphite liquor concentrate after ultrafiltration

6.4.3. Overliming of SSLc

The composition and chemical properties of the SSLc after different overliming treatments at 25 and 60 ºC are shown in Tables 6.3 and 6.4. In comparison to the untreated SSLc, aliquots overlimed at 25 °C to pH 12 for a period of 30 or 60 min showed a significant loss of total sugars as well as a significant decrease in the total polyphenol content. Under these conditions, up to a 27 and 45 % loss of total sugars, and a 19 and 27 % decrease in total polyphenols occurred, respectively. Overliming at 60 °C under similar conditions resulted in an even greater loss of the total sugars of up to 37 and 79 %, respectively. However, overliming for 30 min did not decrease the total polyphenols content significantly and increasing the duration of overliming to 60 min resulted in a significant increase in the total

Table 6.3. Composition and chemical properties (mean values of duplicate experiments) of SSLc after different overliming treatments at pH 10 or 12 and at 25 °C for 0, 30 and 60 min.

Property	Untreated	pH 10			pH 12		
		0 min	30 min	60 min	0 min	30 min	60 min
Total sugars (g l^{-1})	10.6 ± 0.6^{cde}	10.1 ± 0.4^{cde}	9.2 ± 0.4^{bd}	9.1 ± 0.3^{bc}	9.6 ± 0.6^{be}	7.7 ± 0.4^{ab}	5.8 ± 0.6^{a}
Acetic acid (g l^{-1})	3.1 ±0.1 ^{a}	2.8 ± 0.2 ^{a}	2.9 ±0.1 ^{a}	3.1 ± 0.1 ^{a}	2.9 ± 0.2 ^{a}	2.9 ±0.2 ^{a}	3.1 ± 0.2 ^{a}
Total polyphenols (g l^{-1})	11.1 ± 0.1^{d}	10.7 ± 0.2^{cd}	10.1 ± 0.4^{c}	9.8 ± 0.3^{bc}	10.0 ± 0.1^{c}	9.0 ± 0.1^{ab}	8.1 ± 0.1^{a}
COD (mg l^{-1})	754 500 ± 10 607^{b}	416 000 ± 9 900^{a}	363 500 ± 64 347^{a}	370 000 ± 2 828^{a}	333 500 ± 707^{a}	333 000 ± 2 828^{a}	342 500 ± 4 950^{a}

Table 6.4. Composition and chemical properties (mean values of duplicate experiments) of SSLc after different overliming treatments at pH 10 or 12 and at 60 °C for 0, 30 and 60 min.

Property	Untreated	pH 10			pH 12		
		0 min	30 min	60 min	0 min	30 min	60 min
Total sugars (g l^{-1})	10.6 ± 0.6^{c}	9.1 ± 0.5^{c}	8.8 ± 0.4^{c}	8.7 ± 0.6^{c}	9.2 ± 0.4^{c}	6.4 ± 0.6^{b}	2.2 ± 0.6^{a}
Acetic acid (g l^{-1})	3.1 ± 0.1^{a}	3.0 ± 0.1 ^{a}	2.8 ± 0.1 ^{a}	2.9 ± 0.1 ^{a}	3.0 ± 0.1 ^{a}	3.0 ± 0.1 ^{a}	2.8 ± 0.2 ^{a}
Total polyphenols (g l^{-1})	11.1 ± 0.1^{c}	10.3 ± 0.1^{ab}	9.7 ± 0.2^{a}	10.3 ± 0.2^{b}	10.1 ± 0.1^{b}	10.6 ± 0.1^{bc}	11.2 ± 0.2^{d}
COD (mg l^{-1})	754 500 ± 10 607^{c}	455 500 ± 23 335^{b}	445 500 ± 9 192^{b}	450 000 ± 18 385^{b}	364 500 ± 17 678^{a}	333 000 ± 16 971^{a}	392 500 ± 14 849^{ab}

COD Chemical oxygen demand

Means values with the same superscript letter(s) in the same row do not differ significantly ($p \leq 0.001$)

polyphenol content of up to 6 %. Although overliming resulted in a significant decrease in the COD values in comparison to the untreated SSLc samples, there was no significant difference within the $SSLc_1$ samples overlimed at 25 °C. However, when the overliming pH was increased from 10 to pH 12 at 60 °C the COD values of samples decreased significantly. The acetic acid concentration remained essentially unchanged throughout all the different overliming treatments.

6.4.4. Overliming of SSLcF

Results showing the chemical properties of the $SSLc_F$ after subjecting to different overliming treatments at 25 and 60 ºC are shown in Tables 6.5 and 6.6. As was observed with the $SSLc_1$ samples, overliming of the $SSLc_F$ samples resulted in a significant loss of total sugars, where up to a 45 % loss of total sugars resulted from overliming at 25 °C to pH 12 for 60 min and an even greater loss of up to 67 % when samples were overlimed at 60 °C under similar conditions. Similarly, a significant decrease of up to 27 % in the total polyphenol content occurred with overliming at 25 °C to pH 12 for 60 min, but increased significantly when aliquots were subjected to overliming at 60 °C to pH 12 for 60 min (Tables 6.4 and 6.5). These overliming conditions also resulted in a significant decrease in the COD values, whereas the effects of overliming the $SSLc_{F1}$ samples at 25 °C were variable. As was the case with the $SSLc_1$ samples, the acetic acid concentration remained essentially unchanged throughout the different overliming treatments.

6.4.5. Xylanase production with SSLc and SSLcF

Cultivation profiles of *A. oryzae* showing xylanase production with SSLc and $SSLc_F$ as carbon substrates are shown in Figure 6.1. The SSLc yielded a higher xylanase activity of 183 U ml^{-1}, whereas 168 U ml^{-1} (mean values) was obtained with $SSLc_F$ as carbon substrate. Xylanase production with both substrates only commenced after a 24 h lag phase and peaked after three days of cultivation, dropping considerably thereafter (Figure 6.1). The biomass concentrations, however, were similar in both culture media and up to 19.1 and 19.8 g l^{-1} were obtained, respectively. A xylanase yield of up to 9 600 U g $biomass^{-1}$ was obtained in the SSLc medium, whereas a yield of up to 8 500 U g $biomass^{-1}$ was obtained with $SSLc_F$ as carbon substrate. This suggested that xylanase production was more efficient with the SSLc.

Table 6.5. Composition and chemical properties (mean values of duplicate experiments) of $SSLc_F$ after different overliming treatments at pH 10 or 12 and at 25 °C for 0, 30 and 60 min.

Property	Untreated	pH 10			pH 12		
		0 min	30 min	60 min	0 min	30 min	60 min
Total sugars (g l^{-1})	10.1 ± 0.8^{b}	10.0 ± 0.8^{b}	9.6 ± 0.5^{b}	9.5 ± 0.1^{b}	9.7 ± 0.2^{b}	8.2 ± 0.3^{b}	5.9 ± 0.6^{a}
Acetic acid (g l^{-1})	3.0 ± 0.1^{a}	2.9 ± 0.1^{a}	2.8 ± 0.2^{a}	3.0 ± 0.1^{a}	2.9 ± 0.2^{a}	2.7 ± 0.1^{a}	2.9 ± 0.3^{a}
Total polyphenols (g l^{-1})	11.1 ± 0.1^{d}	10.7 ± 0.2^{cd}	10.1 ± 0.4^{c}	9.8 ± 0.3^{bc}	10.0 ± 0.1^{c}	9.0 ± 0.1^{ab}	8.1 ± 0.1^{a}
COD (mg l^{-1})	$33\,850 \pm 212^{ab}$	$37\,550 \pm 636^{c}$	$34\,550 \pm 1\,202^{b}$	$38\,700 \pm 566^{c}$	$31\,750 \pm 778^{a}$	$32\,800 \pm 141^{ab}$	$31\,300 \pm 566^{a}$

Table 6.6. Composition and chemical properties (mean values of duplicate experiments) of $SSLc_F$ after different overliming treatments at pH 10 or 12 and at 60 °C for 0, 30 and 60 min.

Property	Untreated	pH 10			pH 12		
		0 min	30 min	60 min	0 min	30 min	60 min
Total sugars (g l^{-1})	10.1 ± 0.8^{b}	9.5 ± 0.3^{b}	8.8 ± 0.5^{b}	9.0 ± 0.4^{b}	9.3 ± 0.4^{b}	6.5 ± 0.6^{a}	3.3 ± 0.3^{a}
Acetic acid (g l^{-1})	3.0 ± 0.1^{a}	3.0 ± 0.2^{a}	3.0 ± 0.1^{a}	2.8 ± 0.3^{a}	2.9 ± 0.1^{a}	2.8 ± 0.2^{a}	3.1 ± 0.1^{a}
Total polyphenols (g l^{-1})	11.1 ± 0.1^{c}	10.3 ± 0.1^{ab}	9.8 ± 0.2^{a}	10.5 ± 0.2^{b}	10.4 ± 0.1^{b}	10.8 ± 0.1^{bc}	11.8 ± 0.2^{d}
COD (mg l^{-1})	$33\,850 \pm 212^{cd}$	$31\,050 \pm 495^{c}$	$31\,000 \pm 566^{c}$	$34\,950 \pm 1\,344^{d}$	$20\,300 \pm 283^{a}$	$23\,600 \pm 566^{b}$	$26\,600 \pm 1\,131^{b}$

COD Chemical oxygen demand

Means values with the same superscript letter(s) in the same row do not differ significantly ($p \leq 0.001$)

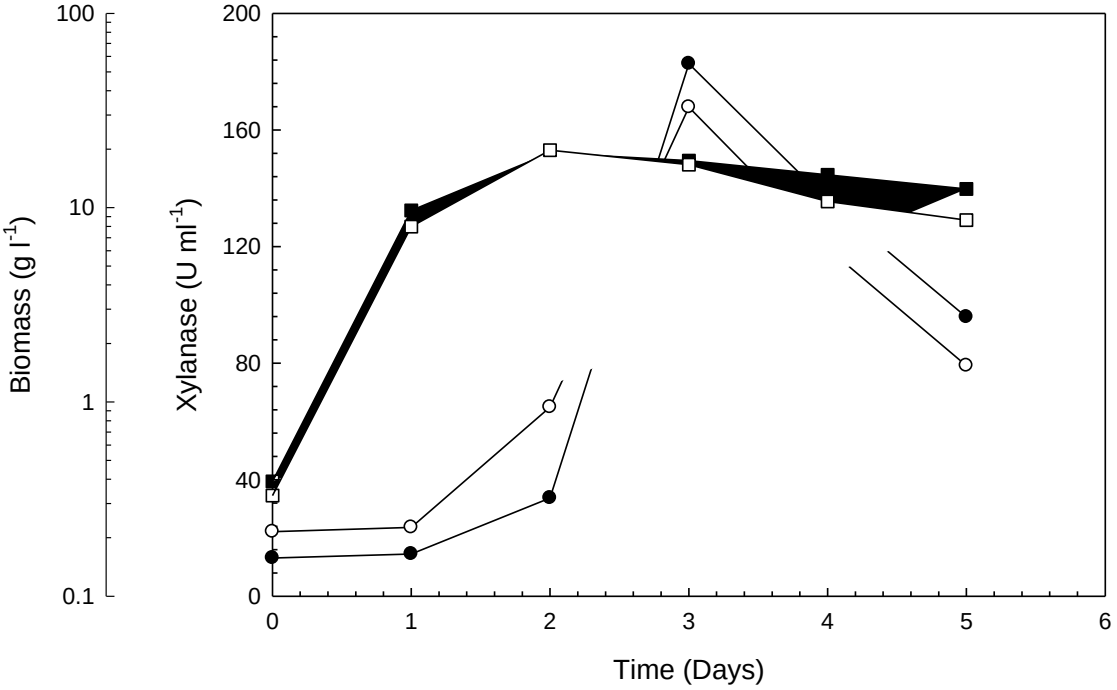

Figure 6.1. Cultivation profiles of *A. oryzae* NRRL 3485 with SSLc (solid symbols) and $SSLc_F$ (open symbols) as the respective carbon substrates in shake flask cultures at 30 ºC. Symbols: ●, o xylanase activity; ■, □ biomass concentration. The mean values of triplicate experiments in shake flasks are shown.

6.4.6. Effect of pretreatment variables on growth characteristics

ANOVA analysis to determine the effect of pretreatment variables on xylanase production, biomass concentration and xylanase yield coefficients with SSLc as carbon substrate are shown in Table 6.7. All the treatment variables had a significant impact on the above growth characteristics, with the exception of overliming temperature, which showed no significant effect on biomass.

Table 6.7. Analysis of variance for the effect of various treatments on the growth characteristics of *A. oryzae* with SSLc as carbon substrate.

Treatment	Measurements		
	Xylanase activity	**Biomass**	**$Y_{p/x}$**
Reaction time	$p \leq 0.05$	$p \leq 0.001$	$p \leq 0.05$
pH	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.05$
Temperature	$p \leq 0.001$	NS	$p \leq 0.001$
Ultrafiltration	$p \leq 0.001$	$p \leq 0.001$	$p \leq 0.001$

$Y_{p/x}$ Xylanase yield coefficient

NS Not significant

6.4.7. Xylanase production using overlimed effluent aliquots

Results of the cultivation of *A. oryzae* using $SSLc_1$ and $SSLc_{F1}$ as carbon substrates are shown in Tables 6.8 to 6.11. Lower xylanase activities were obtained with both $SSLc_1$ and $SSLc_{F1}$ effluent aliquots than with SSLc. Xylanase activities obtained with $SSLc_1$ as carbon substrate did not differ significantly, despite the different overliming treatments (Tables 6.8 and 6.9). However, with $SSLc_{F1}$ as carbon substrate, the xylanase activities obtained using aliquots that had been overlimed to pH 10 at 25 or 60 °C were significantly higher than with aliquots overlimed to pH 12 (Tables 6.10 and 6.11). Xylanase activities obtained using $SSLc_{F1}$ were also generally higher than those obtained using $SSLc_1$ samples. Biomass concentrations obtained with $SSLc_1$ were significantly lower than with SSLc, with the aliquots overlimed to pH 12 at 25 or 60 °C for 60 min yielding the lowest biomass concentrations (Tables 6.8 and 6.9). Similarly, low biomass concentrations were obtained with $SSLc_{F1}$ aliquots treated at 25 °C. However, there was no significant difference between the biomass concentrations obtained with aliquots overlimed to pH 10 at 60 °C and with SSLc, whereas overliming to pH 12 at 60 °C yielded significantly lower biomass concentrations (Tables 6.10 and 6.11). The xylanase yield obtained with $SSLc_1$ samples followed a similar trend as the xylanase activities, whereas no significant difference in the xylanase yield coefficients was found when using $SSLc_{F1}$ aliquots.

Table 6.8. Xylanase production by *A. oryzae* (mean values of duplicate experiments) with $SSLc_1$ aliquots that had been subjected to different overliming treatments at pH 10 or 12 and at 25 °C for 0, 30 and 60 min as carbon substrates.

Property	**Untreated**	**pH 10**			**pH 12**		
		0 min	30 min	60 min	0 min	30 min	60 min
Xylanase (U ml^{-1})	182.9 ± 9.5^{b}	48.3 ± 5.9^{a}	46.2 ± 6.6^{a}	56.1 ± 3.8^{a}	28.1 ± 2.7^{a}	28.1 ± 3.3^{a}	39.4 ± 5.0^{a}
Biomass (g l^{-1})	19.1 ± 0.8^{d}	12.3 ± 0.7^{c}	9.9 ± 0.5^{c}	9.6 ± 1.1^{c}	11.5 ± 2.1^{bc}	9.4 ± 0.9^{ab}	5.4 ± 1.1^{a}
$Y_{p/x}$ (U g $biomass^{-1}$)	9 751.5 ± 74.2^{b}	3 947.0 ± 709.9^{a}	4 679.0 ± 439.8^{a}	5 907.5 ± 1 093.9^{a}	2 472.5 ± 208.6^{a}	2 999.5 ± 57.3 ^{a}	7 408.0 ± 530.3^{a}

Table 6.9. Xylanase production by *A. oryzae* (mean values of duplicate experiments) with $SSLc_1$ aliquots that had been subjected to different overliming treatments at pH 10 or 12 and at 60 °C for 0, 30 and 60 min as carbon substrates.

Property	**Untreated**	**pH 10**			**pH 12**		
		0 min	30 min	60 min	0 min	30 min	60 min
Xylanase (U ml^{-1})	182.9 ± 9.5^{b}	29.5 ± 4.0^{a}	27.1 ± 1.0^{a}	32.0 ± 2.5^{a}	22.0 ± 0.7^{a}	18.8 ± 1.6^{a}	23.2 ± 10.0^{a}
Biomass (g l^{-1})	19.1 ± 0.8^{d}	11.1 ± 1.1^{c}	10.4 ± 0.1^{c}	9.9 ± 1.0^{c}	8.9 ± 0.6^{bc}	6.4 ± 0.3^{ab}	4.8 ± 0.5^{a}
$Y_{p/x}$ (U g $biomass^{-1}$)	9 751.5 ± 74.2^{b}	2 695.0 ± 623.7^{a}	2 618.0 ± 77.8^{a}	3 236.0 ± 66.5^{a}	2 495.5 ± 259.5^{a}	2 935.0 ± 113.1^{a}	5 010.5 ± 2 621.2^{a}

$Y_{p/x}$ Xylanase yield coefficient, U g $biomass^{-1}$

Means values with the same superscript letter(s) in the same row do not differ significantly ($p \leq 0.01$)

Table 6.10. Xylanase production by *A. oryzae* (mean values of duplicate experiments) with $SSLc_{FI}$ aliquots that had been subjected to different overliming treatments at pH 10 or 12 and at 25 °C for 0, 30 and 60 min as carbon substrates.

Property	**Untreated**	**pH 10**			**pH 12**		
		0 min	30 min	60 min	0 min	30 min	60 min
Xylanase (U ml^{-1})	167.8 ± 3.7^{d}	129.4 ± 2.6^{c}	117.7 ± 1.2^{c}	111.5 ± 9.9^{c}	70.4 ± 10.3^{b}	48.7 ± 12.9^{ab}	21.0 ± 7.7^{a}
Biomass (g l^{-1})	19.8 ± 0.7^{d}	15.9 ± 0.7^{cd}	14.7 ± 1.4^{c}	14.9 ± 1.9^{c}	13.6 ± 0.7^{c}	9.0 ± 0.4^{b}	4.4 ± 0.5^{a}
$Y_{p/x}$ (U g $biomass^{-1}$)	$8\,477 \pm 117.4^{a}$	$8\,146.5 \pm 526.8^{a}$	$8\,037.0 \pm 691.6^{a}$	$7\,614.5 \pm 1\,645.4^{a}$	$5\,203.0 \pm 1\,029.5^{a}$	$5\,468.5 \pm 1\,662.4^{a}$	$4\,949.0 \pm 2\,334.9^{a}$

Table 6.11. Xylanase production by *A. oryzae* (mean values of duplicate experiments) with $SSLc_{FI}$ aliquots that had been subjected to different overliming treatments at pH 10 or 12 and at 60 °C for 0, 30 and 60 min as carbon substrates.

Property	**Untreated**	**pH 10**			**pH 12**		
		0 min	30 min	60 min	0 min	30 min	60 min
Xylanase (U ml^{-1})	167.8 ± 3.7^{d}	82.4 ± 0.4^{bc}	106.8 ± 13.4^{c}	101.3 ± 1.6^{c}	53.5 ± 14.5^{ab}	42.2 ± 15.8^{a}	22.6 ± 7.5^{a}
Biomass (g l^{-1})	19.8 ± 0.7^{c}	16.5 ± 1.3^{c}	17.8 ± 1.0^{c}	17.3 ± 0.4^{c}	10.1 ± 1.1^{b}	7.9 ± 0.4^{ab}	5.2 ± 0.7^{a}
$Y_{p/x}$ (U g $biomass^{-1}$)	$8\,477.0 \pm 117.4^{a}$	$5\,009.5 \pm 412.2^{a}$	$6\,030.0 \pm 1\,090.4^{a}$	$5\,872.5 \pm 30.4^{a}$	$5\,244.5 \pm 847.8^{a}$	$5\,329.5 \pm 1\,768.5^{a}$	$4\,485.5 \pm 2\,051.3^{a}$

$Y_{p/x}$ Xylanase yield coefficient, U g $biomass^{-1}$

Means values with the same superscript letter(s) in the same row do not differ significantly ($p \leq 0.01$)

6.5. Discussion

In the pulp and paper industry, membrane separation technology or ultrafiltration has the ability to separate high molecular-weight lignin from low molecular-weight cooking chemicals and sugars (Wallberg *et al.*, 2003) and the most well-known application is in the recovery of lignosulphonate from spent sulphite liquor. Ultrafiltration is especially important if the cooking waste liquor is to be recycled and this process has been found to be feasible and economically attractive in simulation studies previously conducted by Kirkman *et al*. (1986). The significant decrease in COD, colour, total and suspended solids in the SSLc after ultrafiltration ($SSLc_F$) was a clear indication of the efficiency of removal of lignolytic material by this process. One of the major problems with the application of this process appears to be membrane fouling, which frequently results in a rapid drop in permeate flux and consequently leads to poor productivity (Urbantas and Tay, 1986). However, due to the small scale of this study, this problem was not encountered.

Amongst the many methods reported to detoxify lignocellulosic hydrolysates, the treatment of hydrolysates with solid calcium hydroxide (overliming) is one of the most economical method of detoxification and has been reported as an effective method of reducing the toxicity of various hydrolysates (Ranatunga *et al.*, 2000). The effect of overliming and the significance of overliming parameters such as the overliming pH, duration as well as the temperature in the removal of potential inhibitors was clearly demonstrated in this study. The analysis of variance further highlighted the effects of the various pretreatment variables on the composition and chemical properties of the effluent aliquots. An increase in the overliming pH at 25 °C resulted in greater removal of total polyphenols and a decrease in COD values with a concomitant loss of total sugars in both $SSLc_1$ and $SSLc_{F1}$ samples. The overliming temperature had a significant impact on the loss of total sugars, with considerably more sugars being lost at the higher temperature. The dramatic loss of sugars has been reported as one of the major drawbacks of the overliming procedure (Ranatunga *et al.,* 2000; Nigam, 2002). Similarly, Millati *et al.* (2002) and Purwadi *et al.* (2004) reported substantial sugar losses with the overliming of dilute acid hydrolysates. Millati *et al.* (2002) found, however, that the degradation of sugars was accompanied by the appearance of lactic acid. Purwadi *et al.* (2004), on the other hand, did not determine lactic acid in the dilute acid hydrolysates but found that with the disappearance of glucose there was a concomitant increase in the mannose concentration and this was attributed to the epimerisation of glucose in the presence of Ca^{2+}.

Overliming for a longer period at 25 ºC resulted in greater amounts of total polyphenols being removed from the effluent aliquots, indicating that the detoxification occurred gradually over time. However, this was not the case when effluent aliquots were treated by overliming at 60 °C, where instead of a decrease there was a slight but significant increase in the total polyphenol content. Others have also reported that overliming of dilute acid hydrolysates resulted in an increase in the phenolic content (Millati *et al.*, 2002; Nilvebrant *et al.*, 2003; Purwadi *et al.*, 2004). This increase in phenolic content in dilute acid lignocellulosic hydrolysates was attributed to fragmentation of soluble aromatic oligomers in the hydrolysate (Nilvebrant *et al.*, 2003).

Acetic acid is known to inhibit microbial growth and its effect is dependent on the strain, the concentration of the undissociated form as well as the cultivation pH (van Zyl *et al.*, 1991; Taherzadeh *et al.*, 1997). The acetic acid concentration in the SSLc was, however, previously shown to have little or no inhibitory effect on the *Aspergilus oryzae* strain at a cultivation pH of above 5.0 (Chapter 4). Therefore, the non-removal of acetic acid after overliming was of little consequence due to its relatively low concentration in the effluent as used here.

The general perception is that detoxification of lignocellulosic hydrolysates by overliming increased the fermentability of the hydrolysate as well as the rates of product formation and product yield. However, most of the research that had been conducted using dilute acid hydrolysates focused on ethanol production (Nigam, 2002; Millati *et al.*, 2002; Larsson *et al.*, 1999; Martinez *et al.*, 2000; Ranatunga *et al.*, 2000; Purwadi *et al.*, 2004). Martinez *et al.* (2000) found that overliming of a bagasse hemicellulose hydrolysate at 60 ºC was more effective than at 25 ºC in reducing toxicity and this resulted in higher ethanol yields. Similarly, Purwadi *et al.* (2004) found that the detoxification of dilute acid hydrolysates at a high temperature yielded high ethanol yields and resulted in a better fermentability of the hydrolysates. Results obtained in this study with regard to xylanase production did not follow suit, however, and this could be attributed to the removal or degradation of an inducer, which at this juncture is unknown, by the overliming procedure. Significantly lower xylanase activities as well as xylanase yields were obtained with overlimed SSLc and $SSLc_F$ aliquots compared to the untreated samples. The observation that the xylanase yield, in terms of enzyme activity per g biomass, was also decreased by the pretreatment procedures suggested that the decreased xylanase production was not merely due to the lower biomass concentration, but that pretreatment adversely affected its induction.

Results presented in this study conclusively show that all the pretreatment variables such as ultrafiltration, overliming time, pH and temperature played an important role in the effective removal of potential inhibitors in the SSLc and $SSLc_F$ effluent fractions. Ultrafiltration of the SSLc significantly reduced the COD as well as colour of the effluent. A major drawback with the overliming process was the loss of hydrolysate sugars, as clearly highlighted in the current study. An ideal pretreatment method should selectively remove inhibitors whilst maintaining the fermentable sugars in the hydrolysate. It is, therefore, necessary to optimise the process to remove inhibitors with minimal loss of sugars. It is, however, obvious that using pretreated SSLc as carbon substrate had an adverse effect on the production of xylanase by *Aspergillus oryzae*. Since the complete composition and chemical properties of the SSLc are not entirely known, its treatment by overliming may have resulted in the removal of one or more compounds that may have acted as inducers for xylanase production. Pretreatment of SSLc by ultrafiltration and/or overliming for use as carbon feedstock for xylanase production by the *Aspergillus oryzae* strain would, therefore, not serve to enhance xylanase production.

6.6. References

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Apart from being one of the most important industries in the world, the pulp and paper industry is also one of the major polluters, discharging a wide variety of gaseous, liquid and solid wastes into the environment. Most of the waste water is generated by the chemical pulping process where up to 200 m^3 effluent $tonne^{-1}$ of pulp is produced, which contains wood debris and soluble wood materials (Cecen *et al*., 1992). The most toxic substances are generated by the pulp bleaching process where chemicals such as chlorine are used in the brightening of the pulp (Pokhrel and Viraraghavan, 2004). These waste waters from the pulping and bleaching processes, if discharged untreated, can cause considerable damage to receiving waters since they have a high biochemical/chemical oxygen demand and contain chlorinated compounds, suspended solids, lignin and lignin derivatives, tannins, resin acids, fatty acids, etc. (Ali and Sreekrishnan, 2001). This has led to a growing concern about the potential adverse effects of these waste water pollutants on aquatic biota and general public health through the contamination of drinking water supplies as well as recreational waters, resulting in stricter environmental protection regulations being formulated and enforced.

Remedial action taken to reduce the pollution load from the pulp and paper industries has resulted in the emergence of various innovative measures in the different stages of the pulp and paper making process. In the pulping stage, processes for extended delignification to remove as much residual lignin as is practically possible without compromising pulp yield include extended cooking, oxygen delignification, ozone delignification and biopulping. Extended delignification would significantly reduce the amount of bleaching chemicals required to attain the target brightness. New and cleaner technologies developed to replace the use of chlorine in the bleaching stage include elemental chlorine free bleaching (ECF), totally chlorine-free bleaching (TCF) and enzymatic bleaching, also known as biobleaching, such as the application of xylanases (Ali and Sreekrishnan, 2001).

One of the most important biotechnological applications of cellulase-free xylanases (ß-1,4-endoxylanase, EC 3.2.1.8) is in the pulp and paper industry where they are employed as pretreatment agents in kraft bleaching processes at mill scale. Application of xylanases in this industry has been shown not only to result in a reduction in the cost of bleaching chemicals as a result of reduced chemical consumption, but also reduces the environmental problems caused by the use of chlorine (Viikari, *et al*., 1986, 1987; Bim and Franco, 2000; Christov and Prior, 1996, 1997). The pretreatment of kraft pulp with xylanases has been shown to reduce the requirement of oxidising chemicals by up to 20 to 40 % (Viikari *et al*.,

1986, 1987; Vicuna *et al*., 1997). One important prerequisite for xylanases that are to be applied in the pulp and paper industry is that they should be completely free of cellulase activity (Subramaniyan and Prema, 2000), as the presence of cellulase activity may have detrimental implications in terms of cellulose loss and reduced pulp quality. Also, xylanases that are active and stable at high temperatures and alkaline pH values are desirable as the pretreatment with xylanases in mills normally takes place at high temperatures and alkaline pH in brown stock high-density storage tanks.

Xylanases are produced by a wide range of microorganisms including bacteria, yeasts and filamentous fungi (Haltrich *et al*., 1996; Sunna and Antranikian, 1997; Subramaniyan and Prema, 2002) and can be induced by xylan hydrolysis products such as xylobiose, xylotriose and xylose (Piñaga *et al*., 1994; Purkarthofer and Steiner, 1995; Zhao *et al*., 1997) and repressed by easily metabolisable sugars such as glucose and also in some cases xylose (Hoq *et al*., 1994; Chandra and Chandra, 1995; Kadowaki *et al*., 1997). One of the limiting factors in the large-scale production and application of xylanases is the cost of the carbon substrate, which should not only act as carbon and energy source but also provide the necessary inducing compounds for xylanase synthesis. The use of purified substrates such as xylan for large-scale production is not economically sound; hence alternative substrates have been sought and evaluated. Lignocellulosic substrates such as wheat bran, corn cobs, sugar cane bagasse, rice straw, hay and barley husks have been evaluated as alternative and inexpensive carbon substrates for xylanase production (Haltrich *et al*., 1996; Nascimento *et al*., 2002; Xiong *et al*., 2004). The utilisation of these substrates may, however, require prior pretreatment for increased availability of the substrate for microbial utilisation and, in cases where the carbon substrate is a lignocellulosic hydrolysate, for the removal of potential inhibitors (Biswas *et al*., 1988; Pham *et al*., 1998; Shah and Madamwar, 2005).

Besides the selection of an appropriate carbon substrate, culture conditions also play a significant role in influencing xylanase production. Factors such as cultivation pH, temperature, aeration and agitation rates have been reported to have a significant effect on xylanase production (Royer and Nakas, 1989; Smith and Wood, 1991; Purkarthofer *et al*., 1993; Gomes *et al*., 1994; Singh *et al*., 2000).

One of the most significant findings of the current study was that the SSL served as carbon substrate as well as induced xylanase production in the fungal strains evaluated in this

investigation. To put these results in perspective: Christov *et al*. (1999) evaluated several fungal strains for xylanase production in shake flask cultures using bleach plant effluent as carbon substrate and reported maximum activities of only 6 U ml^{-1}. Similarly, Adsul *et al*. (2004), using shake flask cultures, evaluated the production of xylanase by *P. janthinellum* and *T. viride* using polysaccharides from bagasse and reported maximal xylanase activities of 130 and 80 U ml^{-1}, respectively. These latter values are considerably lower than the xylanase activities reported in this thesis of 172 and 173 U ml^{-1} obtained with shake flask cultures of *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 grown on SSL as carbon feedstock. Furthermore, the xylanase activities obtained with *A. oryzae* NRRL 3485 using fed-batch culture ranks amongst the highest xylanase activities reported for *Aspergillus* strains grown on lignocellulosic substrates. The fact that SSL is an abundantly available cheap carbon substrate and that the *A. oryzae* strain was not only able to grow in the SSL, but also produced relatively high xylanase activities, makes these findings even more significant. A comparison of xylanase activities produced by some fungal strains using lignocellulosic materials as carbon substrates is shown in Table 7.1. Xylanase production by *Aspergilli* is widespread, although the xylanase activities produced are considerably lower than those produced by fungal strains such as *Thermomyces* and *Trichoderma*, where activities have been shown to reach as high as 3000 U ml^{-1} (Table 7.1).

Another interesting finding of the current study was the apparent utilisation of the non-sugar carbon component in the SSL by *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157, which resulted in very high biomass yields when calculated on the basis of only the sugars present in the SSL. Evidence of the utilisation of the non-sugar component of the SSL was provided by the determination of the total organic carbon content. This finding was not surprising, since it has been reported and demonstrated that *Aspergilli* are able to degrade and utilise phenolic and polysaccharide components found in a mixture of soluble lignocarbohydrate complexes extracted from various lignocellulosic substrates, reactive textile dyes from textile mill discharges as well as waste water from olive-oil mills (Duarte and Costa-Ferreira, 1994; Sumathi and Manju, 2000; Hoyos *et al*., 2002).

Shake flask cultures of *Aspergillus oryzae* NRRL 3485 and *Aspergillus phoenicis* ATCC 13157 yielded higher xylanase activities with SSL than with xylan as carbon substrate. However, as a result of the high biomass concentrations obtained when using the SSL-based medium due to the apparent utilisation of the non-sugar carbon component as additional carbon substrate, the xylanase yield per g biomass was, in fact, lower than with

Table 7.1. comparison of xylanase activities produced by some fungal strains using various lignocellulosic materials as carbon substrates.

Strain	Carbon substrate	Xylanase (DNS assay, U ml^{-1})	Cultivation time (h)	Reference
Aspergillus oryzae NRRL 3485	SSL	260	66	Current study
Aspergillus fumigatus AR1	Rice straw	30	84	Anthony *et al*., 2003
Aspergillus oryzae VTT-D-85248	Steamed lignocellulose	198	96	Bailey and Viikari, 1993
A. foetidus ATCC 14916	Corn cobs	547	96	Christov *et al*., 1999
Aspergillus niger NRRL 3536	Bleach plant effluent	6	96	Christov *et al*., 1999
Aspergillus ochraceus	Wheat bran	14	144	Biswas *et al*., 1988
Aspergillus fumigatus DTT-D-82195	Solka floc	150	160	Bailey and Poutanen, 1989
Thermomyces lanuginosus DSM 5826	Corn cobs	1950	118	Purkarthofer *et al*., 1993
Trichoderma reesei	Lactose + Solka floc	3350	240	Haapala *et al*., 1994

SSL Spent sulphite liquor

the xylan-based medium. This meant, therefore, that xylan was still the more potent inducer when taking into account the xylanase produced per gram biomass. Because one of the major costs of xylanase production is the cost of the substrate, the ability of the SSL to serve as carbon substrate as well as inducer for the production of xylanase is, nevertheless, an important finding as this could lead to reduced enzyme production costs.

The phenomenon of xylanase multiplicity, which has been extensively researched and discussed (Biely *et al*., 1985; Wong *et al*., 1988; Kormelink *et al*., 1993), was also observed with the two *Aspergillus* strains, with *A. oryzae* NRRL 3485 exhibiting three and *A. phoenicis* ATCC 13157 two xylanases, respectively. The relatively high pH and temperature optima of the *A. oryzae* xylanase preparation prompted further investigations pertaining to the production of xylanase by this strain.

In characterising the crude xylanase extracts of *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157, it was found that the xylanase preparation from *A. oryzae* exhibited unusual pH and temperature optima not typical to fungal xylanases, whereas that of *A. phoenicis* exhibited typical characteristics. The xylanase preparation obtained from *A. oryzae* was stable over a wide pH range with a pH optimum of 6.5 and a temperature optimum of 65 °C. Enzyme characteristics that render xylanases attractive for practical process conditions in the pulp and paper industry include stability and activity at high temperatures and alkaline pH values, since the substrate in the mills is at temperatures of 55 to 70 °C and at an alkaline pH (Viikari *et al*., 1994; Beg *et al*., 2001; Collins *et al*., 2005). Thus, xylanases that would be functional under these conditions would obviate the need for a cooling step and adjustment of the substrate pH to values suitable for the xylanase, thereby making the biobleaching process a more economically viable process.

Another significant finding that emanated from this study was the ability of the *A. oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 crude xylanase preparations obtained with SSL as carbon substrate to enhance the bleachability of the oxygen-delignified soda aq eucalyptus pulp. The finding that the two *Aspergillus* strains produced cellulase-free xylanases rendered these enzymes suitable for their potential application in biobleaching. To obtain the desired bleaching effect, the resulting xylanase preparation should be completely free of any cellulase activity (Subramaniyan and Prema, 2000). The presence of cellulase activity in xylanase preparations may have serious economic implications arising from the loss of cellulose, degradation of pulp quality and increase in effluent treatment

costs. Thus, the application of cellulase-xylanase preparations from *A. oryzae and A. phoenicis* in biobleaching without the need for purification and with SSL as carbon substrate would serve to enhance the economic viability of biobleaching technology.

Batch cultures of *Aspergillus oryzae* NRRL 3485 conducted at different cultivation pH values with SSL as carbon substrate showed that the cultivation pH was an important parameter for xylanase production. It was found that the xylanase activity increased with increasing cultivation pH, reaching a maximum at pH 7.5. Low xylanase activities were obtained below or above this pH value. Also, at a low cultivation pH of 4.0, the inhibitory effect of acetic acid in the SSL was profound, resulting in a prolonged growth lag phase as well as a decreased xylanase production. A high cultivation pH value of 7.5, therefore, not only favoured xylanase production by *A. oryzae* but also minimised the inhibitory effect of the acetic acid in the SSL. The agitation rate, however, which has been shown to affect the production of xylanase by some fungal strains (Purkarthofer *et al*., 1993; Hoq *et al*., 1994; Singh *et al*., 2000), did not have any adverse effect on the production of xylanase. The impeller tip velocity between the ranges of 1.56 to 3.12 m s^{-1}, corresponding to stirrer speeds of 400 to 800 rpm, had no effect on the production of xylanase by the *A. oryzae* strain. Although only the effects of cultivation pH and agitation rates were investigated, it is probable that other cultivation parameters such as the cultivation temperature and dissolved oxygen tension may affect the production of xylanase by *A. oryzae*. Therefore, in order to optimise xylanase production, these factors need to be investigated and their effects determined.

During the initial stages of the study, results suggested that xylose, the main sugar constituent in the SSL, caused carbon catabolite repression of xylanase production and that the repression was alleviated only when the xylose concentration in the culture decreased to low levels. Although xylose as sole carbon source did not act as inducer for xylanase production in *A. oryzae* (Chapter 3, Table 3.2), it is the main sugar constituent of the SSL and high xylanase activities were obtained when using SSL as carbon substrate. This suggested that the SSL contained uncharacterised compound/s that acted as inducer/s for xylanase production. Therefore, to minimise the repressive effect of xylose, fed-batch cultures of *A. oryzae* were conducted using a continuous feeding strategy and different feed flow rates. High xylanase activities were indeed obtained in fed-batch culture using a xylose feed rate of 2.38 g xylose h^{-1} and even higher xylanase activities when the $(NH_4)_2SO_4$ concentration was increased by a factor of 1.5 in both the culture medium and feed medium.

Table 7.2. Comparison of maximum xylanase activities obtained with *A. oryzae* NRRL 3485 using different culture methods with spent sulphite liquor as carbon substrate.

	Method of cultivation		
	Shake flask	Bioreactor batch	Fed-batch
Xylanase activity (U ml^{-1})	172	199	260

Although shake flask cultures of *A. oryzae* NRRL 3485 with SSL as carbon substrate yielded high xylanase activities, higher xylanase activities were obtained bioreactor batch cultures that were further improved by fed-batch cultivation (Table 7.2). However, despite having been able to enhance xylanase production by using fed-batch culture, enzyme production has in no way yet been optimised, as other feeding rates and regimens, such as using exponential feed rates, were not explored. One important fact that arose from the fed-batch work was evidence that xylose was indeed a repressor of xylanase production and that this repressive effect could be alleviated by using this type of cultivation system to maintain a very low residual xylose concentration in the culture.

Most lignocellulosic hydrolysates require pretreatment to remove potential inhibitors prior to their utilisation by microorganisms. SSL can also be considered a wood hydrolysate and, therefore, some compounds in the waste water may be inhibitory to microbial growth. Ultrafiltration and overliming pretreatment procedures were carried out on SSL aliquots and the effects of these pretreatment procedures on xylanase production evaluated. The results clearly showed that overliming of the SSL at a high pH (pH 12) and temperature (60 °C) led to a significant loss of total sugars and that overliming was detrimental to xylanase production by *A. oryzae*. In terms of xylanase activities, ultrafiltration of the SSL resulted in slightly lower xylanase activities than with untreated SSL aliquots. Although pretreatment of the SSL did not serve to enhance xylanase production, the results obtained strongly suggested that the inducing compound/s may have been removed together with potentially inhibitory compounds.

Although high xylanase activities were obtained with SSL as carbon substrate, there is still room for improvement. Avenues open to explore include cultivation parameters such as optimising the cultivation temperature and dissolved oxygen tension, optimising the medium

composition and evaluating the possibility of eliminating supplementation of the medium with yeast extract, as this could render xylanase production impractical on industrial scale. Also, optimisation of the fed-batch cultivation process could further improve xylanase production using SSL as carbon substrate. The observation that pretreatment of the SSL was not a prerequisite makes the process of xylanase production with SSL as carbon substrate even more attractive.

This investigation demonstrated that it was feasible to use an effluent from the pulp and paper industry as carbon feedstock for the production of xylanases. The finding that relatively high xylanase activities could be obtained with SSL as carbon substrate was even more meaningful after demonstrating that these crude xylanase preparations enhanced pulp brightness and reduced bleaching chemical consumption by a significant margin. This discovery is of considerable potential benefit to the aforesaid industry, offering a cheap and abundant carbon source for the production of enzymes for use in biobleaching, thereby reducing costs as well as minimising hazardous environmental pollution.

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Summary

The application of xylanolytic enzymes in the pulp and paper industry is considered one of the most important new large-scale biotechnological applications of enzymes. Incorporation of these enzymes in the bleaching process has resulted in the decreased use of chlorine-based chemicals as well as the production of less hazardous waste waters. One of the factors limiting the widespread commercial application of xylanases is the cost of production, which may be decreased by the use of lignocellulosic materials as inducer as well as carbon substrate. The main objective of this study was to evaluate spent sulphite liquor (SSL) that had been concentrated five-fold at a South African pulp mill as an alternative inexpensive carbon feedstock for xylanase production by strains of *Aspergillus* and *Gliocladium*.

These fungal strains were evaluated for xylanase production with SSL, xylan and D-xylose as carbon substrates initially in shake flask cultures. *Aspergillus oryzae* NRRL 3485 and *A. phoenicis* ATCC 13157 yielded higher xylanase activities with SSL than with xylan as carbon substrate, although xylan induced a higher xylanase activity per g cell mass. Xylose induced little to no xylanase activity in the fungal strains evaluated. Apart from the sugars in the SSL, the above *Aspergillus* strains also utilised a non-sugar component in the SSL as additional carbon substrate, as indicated by total organic carbon analysis. The utilisation of the non-sugar component in the SSL resulted in high biomass concentrations which gave high biomass yield coefficients if calculated in terms of only the sugars present in the SSL.

Characterisation of the crude xylanase preparations of *A. oryzae* and *A. phoenicis* indicated three and two xylanase isozymes with molecular weights of approximately 32, 22, 19 and 25 and 21 kDa, respectively. The *A. oryzae* xylanase preparation exhibited unusually high pH and temperature optima of 6.5 and 65 °C, respectively, therefore being better suited for biobleaching applications in the pulp and paper industry, whereas the properties of the xylanase preparation from *A. phoenicis* were typical of fungal xylanases.

In bioreactor cultures of *A. oryzae* using SSL as carbon substrate, the cultivation pH had a significant effect on xylanase production, with pH 7.5 resulting in the highest xylanase activity. The agitation rate in the range of 400 to 800 rpm had no effect on xylanase production by *Aspergillus oryzae* NRRL 3485. Xylose repression of xylanase production was alleviated in fed-batch culture operating at a constant feed rate of 2.38 g xylose h^{-1}, where a xylanase activity of up to 210 U ml^{-1} was reached. Xylanase production was further

increased up to 260 U ml^{-1} by increasing the $(NH_4)_2SO_4$ concentration in the medium. These values rank amongst the highest xylanase activities reported for *Aspergillus* strains.

Pretreatment of the SSL using ultrafiltration and overliming resulted in decreased xylanase activities. Although both pretreatment procedures resulted in the removal of compounds inhibitory to microbial growth, they may have also resulted in the removal of the inducing compound/s, hence the low xylanase activities obtained with the pretreated SSL.

SSL, a waste water of the pulp and paper industry, therefore could serve both as carbon substrate and inducer for xylanase production, yielding high xylanase activities as well as biomass concentrations. Furthermore, these xylanase preparations were cellulase-free and in laboratory trials with hardwood pulp their application decreased the usage of chlorine bleaching chemicals by 20 to 30% without sacrificing brightness, thus demonstrating their potential for application as biobleaching agents in the pulp and paper industry.

Keywords: Spent sulphite liquor, Pulp and paper, Xylanase, Xylan, Xylose, Fungus, *Aspergillus*, Biobleaching.

Opsomming

Die gebruik van xilanolitiese ensieme in die pulp- en papiernywerheid word as een van die mees belangrike nuwe grootskaalse biotegnologiese toepassings van ensieme beskou. Die inkorporering van hierdie ensieme in die verbleikingsproses het die verminderde gebruik van chloorgebaseerde chemikalieë asook die produksie van minder-gevaarlike afvalwater tot gevolg gehad. Een van die faktore wat die algemene kommersiële toepassing van xilanases beperk, is die produksiekoste, wat verlaag kan word deur die gebruik van lignosellulose-stowwe as induseerder sowel as koolstofsubstraat. Die hoofdoel van hierdie studie was om die sulfietafvalwater (SAW) wat vyfvoudig deur 'n Suid-Afrikaanse pulpmeule gekonsentreer is, as 'n alternatiewe goedkoop koolstofbron vir xilanaseproduksie deur stamme van *Aspergillus* en *Gliocladium* te evalueer.

Hierdie fungusstamme is ten opsigte van xilanaseproduksie met SAW, xilaan en D-xilose as koolstofsubstrate aanvanklik in skudfleskulture geëvalueer. *Aspergillus oryzae* NRRL 3485 en *A. phoenicis* ATCC 13157 het hoër xilanase-aktiwiteite met SAW as met xilaan as koolstofsubstraat gelewer, alhoewel xilaan 'n hoër xilanase-aktiwiteit per g selmassa geïnduseer het. Xilose het min tot geen xilanase-aktiwiteit in die fungusstamme wat geëvalueer is, geïnduseer. Buiten die suikers in die SAW het die bg. *Aspergillus* stamme ook 'n nie-suiker komponent in die SAW as addisionele koolstofsubstraat benut, soos wat deur totale organiese koolstofanalise aangedui is. Die benutting van die nie-suiker komponent van die SAW het hoë biomassa-konsentrasies tot gevolg gehad, wat hoë biomassa-opbrengskoëffisiente gegee het indien dit in terme van slegs die suikers wat in die SAW teenwoordig was, bereken is.

Karakterisering van die onsuiwer xilanase preparate van *oryzae* and *A. phoenicis* het drie en twee xilanase-isosieme met molekulêrgewigte van ongeveer 32, 22, 19 en 25 en 21 kDa, onderskeidelik, aangedui. Die *A. oryzae* xylanase preparaat het ongewoon hoë pH- en temperatuuroptima van onderskeidelik 6.5 en 65 °C getoon, en was derhalwe beter geskik vir bioverbleikingstoepassings in die pulp- en papiernywerheid, terwyl die eienskappe van die xilanase preparaat van *A. phoenicis* tiperend van fungus-xilanases was.

In bioreaktorkulture van *A. oryzae* met SAW as koolstofsubstraat het die kwekings-pH 'n beduidende effek op xilanaseproduksie gehad, en pH 7.5 het die hoogste xilanase-aktiwiteit tot gevolg gehad. Die roersnelheid in die bestek van 400 tot 800 opm het geen effek op xilanaseproduksie deur *Aspergillus oryzae* NRRL 3485 gehad nie. Xilose-repressie van

xilanaseproduksie is verminder deur gebruik te maak van 'n gevoerde lotkultuur ("fed-batch culture") wat teen 'n konstante voersnelheid van 2.38 g xylose h^{-1} bedryf is, waarin 'n xilanase-aktiwiteit van tot 210 E ml^{-1} bereik is. Xylanase-produksie is nog verder tot 260 E ml^{-1} verhoog deur die $(NH_4)_2SO_4$ konsentrasie in die medium te vermeerder. Hierdie waardes is van die hoogste xilanase-waardes wat nog vir *Aspergillus* stamme gerapporteer is.

Voorafbehandelilng van die SAW deur ultrafiltrasie en oorneutralisasie het verlaagde xilanase-aktiwiteite tot gevolg gehad. Alhoewel albei vooraf-behandelingsprosedures die verwydering van verbindings wat inhiberend vir mikrobiese groei was, tot gevolg gehad het, het hulle dalk ook die verwydering van die induserende verbinding/s meegebring; derhalwe die lae xilanase-aktiwiteite wat met die voorafbehandelde SAW verkry is.

SAW, 'n afvalwater van die pulp- en papiernywerheid, kon dus as beide 'n koolstofsubstraat en induseerder vir xilanaseproduksie dien, wat hoë xilanase-aktiwiteite asook biomassakonsentrasies gelewer het. Verder, die xilanase preparate was sellulase-vry en in laboratoriumproewe met hardehoutpulp het hul aanwending die verbruik van chloor-verbleikingschemikalieë met 20 tot 30% verminder sonder om helderheid te benadeel, wat dus hul potensiaal vir aanwending as bioverbleikingsagense in die pulp- en papiernywerheid gedemonstreer het.

Sleutelwoorde: Sulfietafvalwater, Pulp en papier, Xilanase, Xilaan, Xilose, Fungus, *Aspergillus*, Bioverbleiking.