Cloning and Functional Expression of Three Xylanase Genes from *Aspergillus fumigatus* in *Saccharomyces cerevisiae*

by
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SUMMARY

Lignocellulose, which is composed of cellulose, hemicellulose and lignin, is the main structural component of plant cell walls. Xylan is the main structural component of hemicellulose. Xylan is a complex heteropolysaccharide and, therefore, requires numerous synergistically acting enzymes for its complete hydrolysis. The focus of this study was on xylanases, which is a main chain cleaving enzyme required for xylan hydrolysis. Xylanases have numerous industrial applications and are commonly used in the biofuels, pulp and paper, food, animal feed and textile industries. Of particular interest is the use of xylanases in the biofuels industry due to the depletion of fossil fuels. A major bottleneck is, however, the low yield and high cost of the enzymatic hydrolysis process.

In this study, three different xylanase genes from *Aspergillus fumigatus*, isolated from a triticale compost heap, were cloned and expressed in *Saccharomyces cerevisiae*. This yeast is an attractive host for the expression of these heterologous proteins, since *A. fumigatus* is considered a human pathogen and would not be suited for large-scale enzyme production. The recombinant xylanases obtained in this study were functional after expression in the yeast host and yielded high levels of enzyme activity, ranging from 100 to 300 nkat/mg dry cell weight (DCW). Higher enzyme yields will reduce the overall cost of the enzymatic hydrolysis process, making these enzymes attractive to the biofuels industry. The recombinant xylanases obtained in this study were also free of other cellulases. This characteristic makes these enzymes attractive to the pulp and paper industry as cellulose fibres are required to remain intact.

Two of the recombinant xylanases, F10 and F11, were relatively stable at a temperature of 50°C with pH optima at pH 6, while the recombinant xylanase G1 only maintained half of its activity at this temperature and displayed pH optimum at pH 5. No synergistic effect was observed between the recombinant xylanases in this study. Future studies could investigate the synergistic interaction between these recombinant xylanases and other accessory enzymes used for the degradation of xylan, such as the esterases. Xylan hydrolysis levels could increase significantly due to a synergistic effect, which would further reduce the overall cost of the lignocellulose enzyme hydrolysis process.

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OPSOMMING

Lignosellulose, saamgestel uit sellulose, hemisellulose en lignien, vorm die hoof strukturele bestanddeel van plantselwande. Xilaan is die hoof strukturele komponent van hemisellulose. Xilaan is 'n komplekse hetero-polisakkaried en verskeie saamwerkende ensieme vir volledige hidroliese hiervan word benodig. Die fokus van hierdie studie is op xilinases, die hoof kettingbrekende-ensiem vir xilaan hidroliese. Xilinases het verskeie industriële toepassings onder meer in die biobrandstof-, papier en pulp-, voedsel-, dierevoeding- en tekstielindustrieë. Weens die uitputting van fossielbrandstofreserwes word xilinases in die biobrandstof industrie van groot waarde geag. Lae opbrengste en hoë kostes van die ensiemhidroliese proses bly egter 'n knelpunt.

In hierdie studie is drie verskillende xilinase gene vanuit 'n tritikale komposhoop *Aspergillus fumigatus* isolaat gekloneer en in *Saccharomyces cerevisiae* uitgedruk. Gis is 'n aanloklike gasheer vir die uitdrukking van hierdie heteroloë proteïne aangesien *A. fumigatus* as menspatogeen nie vir grootskaalse ensiemproduksie geskik is nie. Die rekombinante xilinases verkry in hierdie studie is funksioneel in die gis gasheer uitgedruk en hoë vlakke ensiemaktiwiteit is verkry, van 100 tot 300 nkat/mg droë sel massa (DSM). In die lig van hoër ensiemopbrengste wat die totale koste van die ensiem hidroliese proses verlaag, word die ensieme in hierdie studie aanloklik vir die biobrandstof industrie. Die rekombinante ensieme in hierdie studie verkry is ook vry van ander sellulases, 'n eienskap wat van waarde is vir die papier en pulp industrie waar die sellulose vesels intak moet bly.

Twee van die rekombinante xilinases, F10 en F11, was relatief stabiel by 'n temperatuur van 50°C met 'n pH optimum van pH 6, terwyl die rekombinante xilinase G1 slegs die helfte van sy aktiwitieit by hierdie temperatuur kon behou met 'n pH optimum van pH 5. Geen samewerkende effek kon tussen die drie rekombinante xilinases waargeneem word nie. Toekomstige studies kan die samewerkende effek tussen hierdie rekombinante xilinases en bykomstige ensieme betrokke by xilaanafbraak, soos byvoorbeeld die esterases, ondersoek. Xilaanhidroliese vlakke kan aansienlik as gevolg van hierdie samewerkende effek verhoog, wat die koste van ensiem hidroliese van lignosellulose verder kan verlaag.

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Contents

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CHAPTER 1:
GENERAL INTRODUCTION AND AIMS OF THE STUDY

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1.1 Introduction

Lignocellulose, the main structural element of plant cell walls, is comprised of three main polymers, namely cellulose, hemicellulose and lignin (Galbe and Zacchi 2002; Juhász et al. 2005; Kumar et al. 2008; Sánchez 2009). This study focuses on xylan, the most common hemicellulose and most abundant plant kingdom polymer, and the enzymes required for its hydrolysis. Plant xylans are a complex heteropolysaccharide, with a xylose backbone, which may be substituted with side chain branches such as arabinose, glucuronic acid, 4-*O*-methyl glucuronic acid, acetic acid, ferulic acid and *p*-coumaric acid (Biely 1985; Kulkarni et al. 1999; Subramaniyan and Prema 2002; Saha 2003; Collins et al. 2005). It is for this reason that a variety of synergistically acting enzymes are required for the complete hydrolysis of xylan (Biely 1985; Gilbert and Hazlewood 1993; Collins et al. 2005). Such enzymes include endo-1,4-β-xylanases, β-xylosidases and a several accessory enzymes such as α-glucuronidases (α-4-*O*-methyl glucuronosidases), α-L-arabinofuranosidases, *p*-coumaric acid esterases, acetylxylan esterases and ferulic acid esterases (Gilbert and Hazlewood 1993; Pérez et al. 2002; Subramaniyan and Prema 2002; Saha 2003; Shallom and Shoham 2003; Collins et al. 2005). The specific focus of this study was on xylanases (endo-1,4-β-xylanases).

Xylanases are of great interest in the pulp and paper, animal feed, food, textile and fuel industries (De Vries and Visser 2001; Polizeli et al. 2005). Of particular environmental importance is the use of xylanases in the bio bleaching of pulp as well as in the production of ethanol. Xylanases are used in the bio bleaching of pulps in order to reduce and/or eliminate the need for bleaching chemicals, such as chlorine, making this process more environmentally friendly (De Vries and Visser 2001). Fossil fuels, which humans currently depend on for energy generation, are rapidly becoming depleted (Sun and Cheng 2002). Burning fossil fuels is also harmful to the environment as it releases elevated levels of carbon dioxide into the atmosphere, contributing to air pollution and global warming (Galbe and Zacchi 2002; Rojo 2008;

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Li et al. 2009). The degradation of lignocellulosic biomass and the subsequent fermentation of the sugars into bioethanol is a promising environmentally friendly alternative energy source (Sun and Cheng 2002). Xylanases are used in the enzymatic hydrolysis process to hydrolyse xylan to reducing sugars (Polizeli et al. 2005). A major bottleneck of the enzymatic hydrolysis process is the low yield and high cost (Bhat and Bhat 1997; Sun and Cheng 2002; Rojo 2008). Therefore, it is necessary to improve the yield and reduce the cost to make the production of biofuels economically feasible on an industrial scale.

The large-scale production of industrial enzymes, such as xylanases, is a costly process with the cost of the substrate being a large contributing factor. Low-cost substrates, such as agricultural waste products, can be used to make the production of enzymes more economical (Bajaj and Abbass 2011). Most industrial processes are carried out at high temperatures and, therefore, thermostable enzymes are valuable to industries. Cellulase-free xylanases are of specific interest to the pulp and paper industry as the cellulose fibre is required to remain intact (Anthony et al. 2003; Abdel-Monem et al. 2012). *Aspergillus fumigatus* is characteristically found in soil and decaying organic matter, including compost heaps, where it plays a critical role in carbon and nitrogen recycling. *A. fumigatus* is one fungal species in which xylanase production, purification and characterisation have been studied in detail (Souza et al. 2012; Silva et al. 1999; Savitha et al. 2007; Peixoto-Nogueira et al. 2009; Anthony et al. 2003; Thiagarajan et al. 2006; Bajaj and Abbass 2011; Abdel-Monem et al. 2012). Many of these xylanases were produced on low-cost agricultural waste products and/or exhibited the above-mentioned properties.

However, *A. fumigatus* is a pathogenic organism and, therefore, it is advisable to clone and express the xylanase gene in a non-pathogenic host for the large-scale production and application of xylanases. Jeya and co-workers cloned and expressed a xylanase gene from *A. fumigatus* in the yeast, *Pichia Pastoris* (Jeya et al. 2009). *Saccharomyces cerevisiae* is also a popular host for the cloning and expression of

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xylanase genes. Numerous fungal xylanase genes have been expressed in this yeast (Ito et al. 1992; Crous et al. 1995; Pérez-González et al. 1996; Luttig et al. 1997; Li and Ljungdahl 1996; Ohta et al. 2001; Parachin et al. 2009; Chávez et al. 2002; La Grange et al. 1996). This study describes the heterologous expression of three different xylanase genes from *A. fumigatus* in *S. cerevisiae*.

1.2 Aims

The objective of this study was the cloning, expression and characterisation of three different xylanase genes from an *A. fumigatus,* isolated from a Triticale compost heap, in *S. cerevisiae*.

The specific aims of the study were as follows:

- Isolating mRNA and using a transcriptomic approach to obtain cDNA from *A. fumigatus*
- The successful cloning and sequencing of the xylanase genes
- Functional expression of the xylanase genes in *S. cerevisiae*
- Confirmation of protein size
- Determining enzyme activity of the functionally expressed xylanases
- Characterisation of the recombinant xylanases according to temperature, pH, substrate specificity and synergistic interactions

1.3 References

Abdel-Monem OA, El-Baz AF, Shetaia YM, El-Sabbagh SM (2012) Production and application of thermostable cellulase-free xylanase by *Aspergillus fumigatus* from agricultural wastes. Ind Biotechnol 8:152-161

Anthony T, Raj KC, Rajendran A, Gunasekaran P (2003) High molecular weight cellulase-free xylanase from alkali-tolerant *Aspergillus fumigatus* AR1. Enzyme Microb Tech 32:647-654

Bajaj BK, Abbass M (2011) Studies on an alkali-thermostable xylanase from *Aspergillus fumigatus* MA28. Biotech 1:161-171

Bhat MK, Bhat S (1997) Cellulose degrading enzymes and their potential industrial applications. Biotechnol Adv 15:583-620

Biely P (1985) Microbial xylanolytic systems. Trends Biotechnol 3:286-290

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Chávez R, Navarro C, Calderón I, Peirano A, Bull P, Eyzaguirre J (2002) Secretion of endoxylanase A from *Penicillium purpurogenum* by *Saccharomyces cerevisiae* transformed with genomic fungal DNA. FEMS Microbiol Lett 212:237-241

Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3-23

Crous JM, Pretorius IS, van Zyl WH (1995) Cloning and expression of an *Aspergillus kawachii* endo-1,4-β-xylanase gene in *Saccharomyces cerevisiaE*. curr Genet 28:467-473

De Vries RP, Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol Molec Biol Rev 65:497-522

Galbe M, Zacchi G (2002) A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 59:618-628

Gilbert HJ, Hazlewood GP (1993) Bacterial cellulases and xylanases. J Gen Microbiol 139:187-194

Ito K, Ikemasu T, Ishikawa T (1992) Cloning and sequencing of the xynA gene encoding xylanase A of *Aspergillus kawachii*. Biosci Biotech Biochem 56:906-912

Jeya M, Thiagarajan S, Lee J-K, Gunasekaran P (2009) Cloning and expression of GH11 xylanase gene from *Aspergillus fumigatus* MKU1 in *Pichia pastoris*. J Biosci Bioeng 108:24-29

Juhász T, Szengyel Z, Réczey K, Siika-Aho M, Viikari L (2005) Characterisation of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem 40:3519-3525

Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411-456

Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 35:377-391

La Grange DC, Pretorius IS, van Zyl WH (1996) Expression of a *Trichoderma reesei* β-xylanase gene (xyn2) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 62:1036-1044

Li X-L, Ljungdahl LG (1996) Expression of *Aureobasidium pullulans* xynA in, and secretion of the xylanase from, *Saccharomyces cerevisiae*. Appl Environ Microbiol 62:209-213

Li X-L, Yang H, Roy B, Wang D, Yue W, Jiang L, Park EY, Miao Y (2009) The most stirring technology in future: cellulase enzyme and biomass utilization. Afr J Biotechnol 8:2418-2422

Luttig M, Pretorius IS, van Zyl WH (1997) Cloning of two β-xylanase –encoding genes from *Aspergillus niger* and their expression in *Saccharomyces cerevisiae*. Biotechnol Lett 19:411-415

Ohta K, Moriyama S, Tanaka H, Shige T, Akimoto H (2001) Purification and characterisation of an acidophilic xylanase from *Aureobasidium pullulans var. melanigenum* and sequence analysis of the encoding gene. J Biosci Bioeng 92:262-270

Parachin NS, Siqueira S, de Faria FP, Torres FAG, de Moraes LMP (2009) Xylanases from *Cryptococcus flavus* isolate I-11: Enzymatic profile, isolation and heterologous expression of CfXYN1 in *Saccharomyces cerevisiae*. J Mol Catal B Enzym 59:52-57

Peixoto-Nogueira SC, Michelin M, Betini JHA, Jorge JA, Terenzi HF, Polizeli MLTM (2009) Production of xylanase by Aspergilli using alternate carbon sources: application of the crude extract on cellulose pulp bio bleaching. J Ind Microbiol Biotechnol 36:149-155

Pérez J, Muñoz-Dorado J, de la Rubia T, Martinez J (2002) Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int J Microbiol 5:53-63

Stellenbosch University http://scholar.sun.ac.za

Pérez-González JA, De Graaff LH, Visser J, Ramón D (1996) Molecular cloning and expression in *Saccharomyces cerevisiae* of two *Aspergillus nidulans* xylanase genes. Appl Environ Microbiol 62:2179-2182

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577-591

Rojo F (2008) Biofuels from microbes: a comprehensive view. J Microbial Biotech 1:208-210

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279-291

Sánchez C (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 27:185-194

Savitha S, Sadhasivam S, Swaminathan K (2007) Application of *Aspergillus fumigatus* xylanase for quality improvement of waste paper pulp. Bull Environ Contam Toxicol 78:217-221

Shallom D, Shoham Y (2003) Microbial hemicellulase*S. c*urr Opin Microbiol 6:219-228

Silva CHC, Puls J, de Sousa MV, Filho EXF (1999) Purification and characterisation of a low molecular weight xylanase from solid-state cultures of *Aspergillus fumigatus* fresenius. Rev Microbiol 30:114-119

Souza DT, Bispo ASR, Bon EPS, Coelho RRR, N*Asc*imento RP (2012) Production of thermophilic endo-β-1,4-xylanases by *Aspergillus fumigatus* FBSPE-05 using agro-industrial by-products. Appl Biochem Biotechnol 166:1575-1585

Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology and application. Crit Rev Biotechnol 22:33-46

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1-11

Thiagarajan S, Jeya M, Gunasekaran P (2006) Purification and characterisation of an endoxylanase from solid state culture of alkalitolerant *Aspergillus fumigatus* MKU1. Indian J Biotechnol 5:351-356

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CHAPTER 2:

LITERATURE REVIEW

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2.1 The Components of Lignocellulose

Lignocellulose is composed of cellulose, hemicellulose and lignin and it forms the main structural component of all plants (Galbe and Zacchi 2002; Juhász et al. 2005; Kumar et al. 2008; Sánchez 2009). These individual components are discussed in detail below.

2.1.1 Cellulose

Cellulose is the most abundant organic molecule on Earth and is the main constituent of the primary, secondary and tertiary cell walls of plants (O'Sullivan 1997; Hildén and Johansson 2004). Cellulose is also found in bacteria, fungi, algae and animals (O'Sullivan 1997). Cellulose constitutes 35-50% of lignocellulosic biomass (Saha 2003). Cellulose is an unbranched, fibrous, insoluble, crystalline homopolysaccharide which is composed of up to 15 000 repetitive D-glucose units that are linked by β-1,4-glucosidic bonds (O'Sullivan 1997; Hildén and Johansson 2004; Kumar et al. 2008). Adjacent cellulose chains are coupled by hydrogen bonds and Van der Waal's forces to form a microfibril. The cellulose fibre is composed of microfibrils which are grouped together and covered by hemicellulose and lignin (Pérez et al. 2002). The orientation of the microfibrils differs for the different cell wall levels. The primary cell wall is not very ordered and consists of cellulose chains running in all directions. The secondary cell wall, on the other hand, is ordered and the cellulose chains are grouped in parallel microfibrils. The tertiary cell wall consists of less cellulose as it is mainly composed of xylan (O'Sullivan 1997). Naturally occurring cellulose is 40-90% crystalline and the remainder is known as amorphous (Hildén and Johansson 2004). Amorphous cellulose is composed of non-organised cellulose chains and it is more susceptible to enzymatic degradation than crystalline cellulose (Pérez et al. 2002).

2.1.2 Hemicellulose

Hemicellulose is the second most abundantly occurring polysaccharide in nature. This polysaccharide constitutes about 20-35% of lignocellulosic biomass, making it

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the second largest component of lignocellulose (Kulkarni et al. 1999; Saha 2003). Hemicellulose comprises the structural component in cell walls of plants and is a storage polymer in seeds (Bastawde 1992). In comparison to cellulose, hemicelluloses are low molecular weight polymers (± 20 000 Da) (Bastawde 1992; Pérez et al. 2002). Another distinguishing feature of hemicellulose from cellulose is that the former is not chemically homogeneous. Instead, it is heterogeneous polymers of pentoses, hexoses and sugar acids. Pentoses include D-xylose and L-arabinose and hexoses include D-mannose, D-glucose and D-galactose (Bastawde 1992; Pérez et al. 2002; Saha 2003; Kumar et al. 2008). Sugar acids include 4-*O*-methyl-glucuronic, D-glucuronic and D-galacturonic acids. The hemicellulolytic sugars are linked together by β-1,4-glycosidic bonds and in some instances by β-1,3-glycosidic bonds (Pérez et al. 2002). The main heteropolymers of hemicellulose includes xylan, mannan, glucan, galactan and arabinan (Bastawde 1992; Juhász et al. 2005; Kumar et al. 2008). The monomeric unit of xylan is D-xylose and traces of L-arabinose. Mannan is made up of D-mannose units, galactan is made up of D-galactose and arabinan is composed of L-arabinose (Bastawde 1992). Hardwood hemicelluloses are mainly composed of glucuronoxylan, whereas softwood hemicellulose is mainly composed of glucomannan (Pérez et al. 2002; Saha 2003).

2.1.3 Lignin

Lignin is the third largest heterogeneous polymer of lignocellulose and comprises 10-25% of lignocellulosic biomass (Saha 2003; Kumar et al. 2008; Sánchez 2009). Cell walls contain lignin which provides them with structural support (Pérez et al. 2002). Lignin is a complex polyphenolic structure as it is a highly branched macromolecule with different types of aromatic acids (Juhász et al. 2005). Lignin is an amorphous, non-water soluble heteropolymer which consists of phenylpropane units which are joined together by various linkages. Lignin is joined to both cellulose and hemicellulose and forms a barrier, preventing lignocellulolytic enzymes from penetrating the interior of lignocellulose (Pérez et al. 2002; Sánchez 2009). Therefore, lignin protects the lignocellulose by providing resistance against cellulose- and

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hemicellulose-degrading microorganisms and oxidative stress (Pérez et al. 2002; Juhász et al. 2005). Lignin is thus the most recalcitrant of all the lignocellulolytic components to degradation (Dashtban et al. 2009).

2.2 The Hydrolysis of Lignocellulose

The structure of lignocellulosic biomass is complex and generally resistant to enzymatic hydrolysis. Therefore, it is necessary to pretreat lignocellulose materials prior to hydrolysis in order to degrade its intact structure (Galbe and Zacchi 2002; Saha 2003). Figure 2.1 depicts how preteatment degrades the intact structure of lignocellulose.

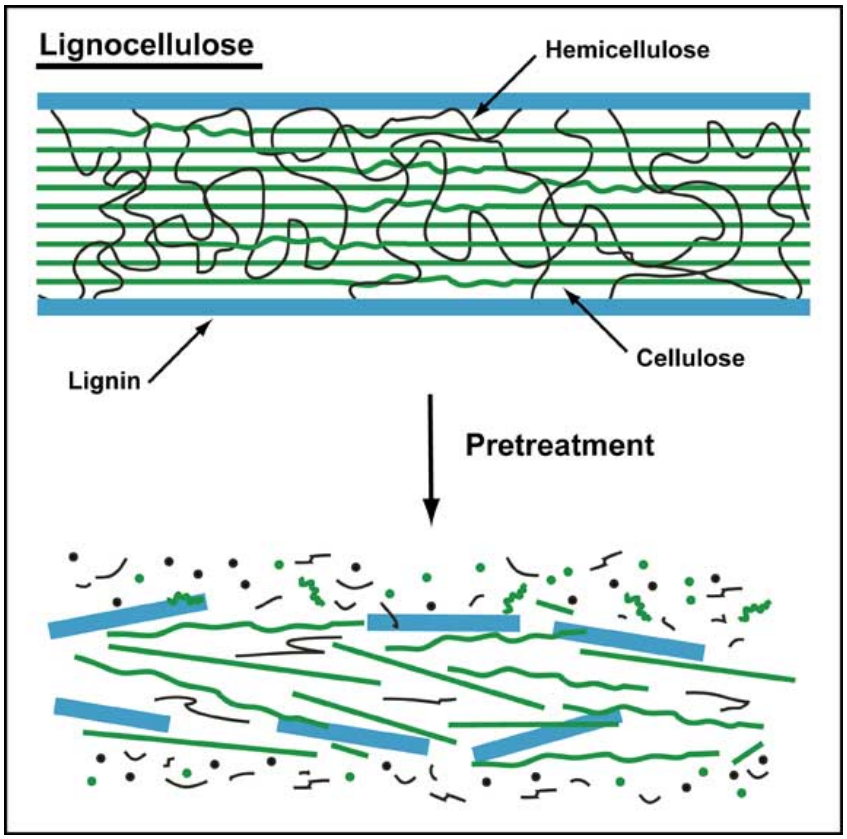

Figure 2.1: The structure of lignocellulose before and after pretreatment. Prior to pretreatment the cellulose, hemicellulose and lignin are intertwined, making the cellulose and hemicellulose components inaccessible for enzymatic hydrolysis. Pretreatment therefore, degrades the intact structure of lignocellulose, making the cellulose and hemicellulose accessible for enzymatic hydrolysis (http://dc102.4shared.com/doc/I-vF3M9S/preview.html).

Cellulose and hemicellulose consists of polymeric chains of sugar molecules which can be hydrolysed to monomeric sugars (Galbe and Zacchi 2002). Lignin, on the other hand, does not contain any sugars and, therefore, does not undergo hydrolysis

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(Kumar et al. 2008). The hydrolysis of cellulose/hemicellulose is catalyzed by cellulase/hemicellulase enzymes, resulting in soluble monomeric sugars such as hexoses and pentoses. These hydrolytic enzymes are produced by lignocellulolytic degrading microorganisms, such as bacteria and fungi (Sun and Cheng 2002; Kumar et al. 2008; Dashtban et al. 2009).

The focus of this literature review is on the hydrolysis of a component of hemicellulose. Both hemicellulases and cellulases are glycoside hydrolases (GHs) (EC 3.2.1) (Shallom and Shoham 2003; Bastawde 1992). However, some hemicellulases are carbohydrate esterases (CEs) responsible for the hydrolysis of ester linkages of acetate or ferulic acid side groups. The aerobic fungal genera *Trichoderma* and *Aspergillus* are renowned for the secretion of hemicellulases (Shallom and Shoham 2003). Several different enzymes are required to degrade hemicellulose as it is a more heterogeneous polymer than cellulose (Saha 2003; Juhász et al. 2005). The two main hemicellulose backbones are xylan and mannan, which are degraded by xylanase and mannanase enzymes, respectively (Juhász et al. 2005). Examples of such enzymes include endo-1,4,-β-xylanase, α-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase, and β-mannanase (Sun and Cheng 2002; Kumar et al. 2008). The xylo/manno-oligosaccharides are further degraded by β-xylosidases and β-mannosidases, respectively (Juhász et al. 2005). A more detailed discussion of xylan and xylanases is discussed below.

2.3 Xylan and Xylan-degrading Enzymes

Xylan as the main component of hemicellulose is positioned at the boundary between the lignin and cellulose. This structural polysaccharide is responsible for fiber cohesion and plant cell wall integrity. Xylan forms part of hardwoods in angiosperms, softwoods in gymnosperms and also forms part of annual plants (Collins et al. 2005). Xylan accounts for 20-35% of the total dry weight in hardwood and annual plants, whereas xylan only accounts for about 8% of the total dry weight of softwood (Haltrich et al. 1996). Xylanases are the enzymes which are responsible

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for the hydrolysis of xylan into sugars. The focus of this literature review will be on xylan and the enzymes which degrades this polysaccharide.

2.3.1 Structure of Xylan

Xylan is a complex, highly branched heteropolysaccharide. The homopolymeric backbone chain of xylan consists of xylopyranose units which are linked by β-1,4-bonds. Xylan can be substituted with side chain branches such as arabinose, glucuronic acid, 4-*O*-methyl glucuronic acid, acetic acid, ferulic acid and *p*-coumaric acid (Biely 1985; Kulkarni et al. 1999; Subramaniyan and Prema 2002; Saha 2003; Collins et al. 2005). These branches vary depending on the specific xylan source, such as softwood xylan, hardwood xylan, grasses and cereals (Biely 1985; Saha 2003; Collins et al. 2005). Hardwoods are composed of O-acetly-4-*O*-methylglucuronoxylans, whereas, softwoods are composed of arabino-4-*O*-methyl glucuronoxylans (Gilbert and Hazlewood 1993; Kulkarni et al. 1999; Subramaniyan and Prema 2002). Hardwood xylan is, therefore, substituted by 4-*O*-methyl glucuronic acid and acetic acid. Softwood xylan, on the other hand, is substituted by 4-*O*-methyl glucuronic acid and α-*O*-arabinofuranoside units (Biely 1985). Xylan has various degrees of polymerisation. Hardwood xylan has a higher degree of polymerisation (150-200 β-xylopyranose residues) than softwood xylan (70-130 β-xylopyranose residues) (Kulkarni et al. 1999). Xylan may be unsubstituted, and is then referred to as linear homoxylan or it may be substituted and is referred to as arabinoxylan, glucuronoxylan and glucuronoarabinoxylan (Biely 1985; Kulkarni et al. 1999; Saha 2003; Collins et al. 2005). Examples of linear unsubstitiuted xylan are found in tobacco, esparto grass and some marine algae (Kulkarni et al. 1999). Xylan is more accessible to enzymatic hydrolysis than cellulose as it does not form tightly packed crystalline structures (Gilbert and Hazlewood 1993). Due to the heterogeneous and complex structure of xylan, a large variety of synergistically acting enzymes are required for its complete hydrolysis (Biely 1985; Gilbert and Hazlewood 1993; Collins et al. 2005).

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2.3.2 Xylanases

Xylanases were only recognised by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961. Xylanases are O-glycoside hydrolases and were assigned the enzyme code EC 3.2.1.x. This widespread group of hemicellulolytic enzymes hydrolyse the 1,4-β-D-xylosidic bonds in xylan to produce xylose, which is a primary carbon source involved in cell metabolism. Most xylanases are excreted extracellularly i.e. into the surrounding medium (Collins et al. 2005). The cooperative action of a variety of xylanases is necessary for the complete hydrolysis of xylan, including endo-1,4-β-xylanases, β-xylosidases and a few accessory enzymes. Examples of accessory enzymes which hydrolyze substituted xylans by removing side chain groups include α-L-arabinofuranosidases, α-glucuronidases (α-4-*O*-methyl glucuronosidases), acetylxylan esterases, ferulic acid esterases and *p*-coumaric acid esterases. Figure 2.2 is a schematic representation of the structure of xylan and the enzymes required for its hydrolysis. Endo-1,4-β-D-xylanases (EC 3.2.1.8) randomly hydrolyse the β-1,4-xylosidic bonds of the xylan backbone to produce xylo-oligosaccharides. β-D-xylosidases (EC 3.2.1.37) then hydrolyse the non-reducing ends of xylobiose and short chain xylooligosaccharides to xylose (Gilbert and Hazlewood 1993; Pérez et al. 2002; Subramaniyan and Prema 2002; Saha 2003; Shallom and Shoham 2003; Collins et al. 2005). B-xylosidases are generally cell-bound, larger than endoxylanases and not as commonly occurring as endoxylanases (Pérez et al. 2002). α-L-arabinofuranosidases (EC 3.2.1.55) hydrolyse the α-arabinofuranose substituents of the xylan backbone. α-D-glucuronidases (EC 3.2.1.139) hydrolyse the α-1,2-glycosidic bond of 4-*O*-methyl glucuronic acid substituents from the xylan backbone. Esterases remove the acetic and phenolic acids which are bound to xylan. Acetylxylan esterase (EC 3.1.1.72) hydrolyses the acetyl-ester bonds on xylose to acetic acid. Ferulic acid esterase (EC 3.1.1.73) hydrolyses the feruloyl-ester bonds between arabinose side chain residues and ferulic acid which crosslinks xylan to lignin. *p*-coumaric acid esterase (EC 3.1.1.73) hydrolyses the *p*-coumaryl ester bonds of arabinose side chain residues to

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p-coumaric acid (Subramaniyan and Prema 2002; Saha 2003; Shallom and Shoham 2003). These esterases also aid in removing lignin as they cleave the ester bonds between lignin and hemicellulose (Subramaniyan and Prema 2002).

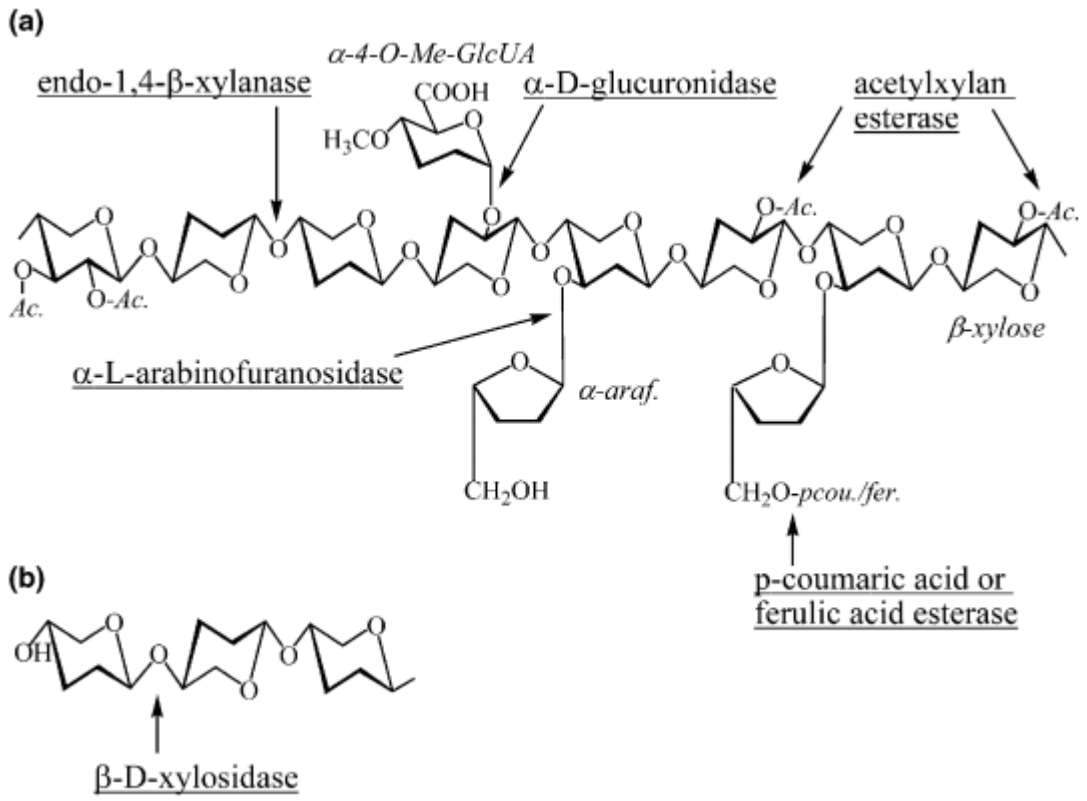

Figure 2.2: A schematic representation of the (a) structure of xylan and the enzymes required for its hydrolysis and (b) hydrolysis of the xylooligosaccharides, which are the products of xylan hydrolysis, by β-D-xylosidase (Collins et al. 2005).

2.3.3.1 Occurrence of Xylanases

Xylanases are widely distributed and are mainly produced by microorganisms. Xylanases are found in both prokaryotes and eukaryotes. Higher eukaryotes such as algae, protozoa, crustaceans, arthropods (insects), gastropods (snails) and germinating plant seeds have also been known to produce xylanases (Bastawde 1992; Polizeli et al. 2005). Complete xylanolytic enzyme systems are widespread among microorganisms which have diverse and widespread ecological niches. These xylanolytic microorganisms are mainly found in environments that are rich in

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degrading plant material, as well as in the rumen of ruminants (Kulkarni et al. 1999; Collins et al. 2005).

Aspergillus is one of the fungal genera which are renowned for their xylanase production (Polizeli et al. 2005; De Vries and Visser 2001). Examples of *Aspergillus* species which produce xylanases include, among others, *A. aculeatus* (Fujimoto et al. 1995), *A. awamori* (Kormelink et al. 1993), *A. flavipes* (Sherief 1990), *A. foetidus* (Bailey et al. 1991), *A. fumigatus* (Silva et al. 1999), *A. kawachii* (Ito et al. 1992), *A. nidulans* (Pérez-Gonzalez et al. 1996), *A. niger* (Luttig et al. 1997) , *A. oryzae* (Bailey et al. 1991), *A. terreus* (Gawande and Kamat 1999), *A. sojae* (Kimura et al. 1995), *A. sydowii* (Ghosh and Nanda 1994) and *A. tubigensis* (De Graaff et al. 1994).

2.3.3.2 Multiple Forms of Xylanases

Heteroxylans have a complex structure and, therefore, not all of the xylosidic bonds of xylan are equally accessible to xylanolytic enzymes. Therefore, multiple xylanases, each with specialised functions, are required for the hydrolysis of xylan (Wong et al. 1988). Many microorganisms produce multiple xylanases (Biely 1985; Wong et al. 1988; Biely et al. 1997). These may differ in physicochemical properties such as molecular mass and isoelectric points, structures, specific activities, yields and specificities. This would increase the efficiency and extent of hydrolysis as well as the diversity and complexity of the xylanolytic enzymes (Biely et al. 1997; Collins et al. 2005). Examples of such organisms include *Aspergillus niger*, which produces fifteen extracellular xylanases, and *Trichoderma viride*, which produces thirteen extracellular xylanases (Biely et al. 1985). There are several possibilities for the occurrence of multiple xylanases. One such possibility is differential processing of mRNA. Extracellular xylanases are often post-translationally modified through glycosylation, self-aggregation and proteolytic digestion. However, some multiple xylanases from a microorganism may be distinct gene products. Multiple xylanases can also result from different alleles of the same gene, referred to as allozymes (Biely 1985; Wong et al. 1988; Polizeli et al. 2005). Multiple xylanases of a microorganism which are distinct

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gene products presumably each have specific properties, which are necessary for the functioning of the xylanolytic system of the microorganism. It is possible that these distinct xylanolytic functions are evolutionarily conserved among microbial xylanolytic systems. Xylanase multiplicity has been most extensively studied in *Aspergillus, Trichoderma, Bacillus, Clostridium* and *Streptomyces* species (Wong et al. 1988).

2.3.3.3 Classification of Xylanases

Glycosyl hydrolases (GHs) are widespread groups of carbohydrate-active enzymes which are present in almost all organisms. These enzymes are involved in the hydrolysis and biosynthesis of glycosidic bonds between carbohydrates. The IUBMB enzyme nomenclature system, which used Enzyme Commision (EC) numbers, was used to classify glycosyl hydrolases (EC 3.2.1.x) according to their substrate specificity (Henrissat and Coutinho 2001). The first 3 digits (EC 3.2.1) indicate that these enzymes hydrolyse O-glycosyl linkages. The last number is the variable which indicates the substrate (Henrissat 1991). However, xylan has a heterogenous and complex structure, resulting in different xylanases with varying specific activities, primary sequences and three dimentional structures. Therefore, substrate specificity alone cannot be used to accurately classify xylanases (Collins et al. 2005). Wong et al. (1988) categorized xylanases into two groups based on physicochemical properties: those with low molecular weight (<30 kDa) and high pI and those with high molecular weight (>30 kDa) and low pI (Wong et al. 1988). However, not all xylanases, in particular fungal xylanases, can be classified by this system. Therefore, there was a need for a new system in order to accurately classify xylanases. A complete classification system for all glycosyl hydrolases was implemented, which is based on amino acid sequence similarities. This classification system is based on primary structure comparisons of the catalytic domains only and it groups enzymes in families of related sequences (Henrissat 1991). This sequence-based system has become the standard for classification of glycosyl hydrolases. Unlike the IUBMB enzyme nomenclature (the traditional EC classification), this classification system

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reflects the structural features of glycosyl hydrolases, with the members of the same family having similar 3-D structures (Henrissat and Coutinho 2001). If a family contains enzymes with different substrate specificities, the family is called "polyspecific" (Bourne and Henrissat 2001; Henrissat and Coutinho 2001). About one-third of glycosyl hydrolase families are polyspecific (Henrissat and Coutinho 2001). In 1991, there were 35 glycosyl hydrolase families (Henrissat 1991). Currently there are 130 glycosyl hydrolase families. A regularly updated list of all of the families of glycosyl hydrolases can be found on the carbohydrate-active enzymes (CAZy) server (http://www.cazy.org/Glycoside-Hydrolases.html).

According to the sequence-based classification system, xylanases can be grouped into two different glycosyl hydrolase families, family 10 (formerly known as F) and family 11 (formerly known as G) (Henrissat 1991). Interestingly, enzymes with xylanase activity have also been found in GH families 5, 7, 8, and 43. The sequences classified in these families contain distinct catalytic domains with endo-1,4-β-xylanase activity. Therefore, the current belief that enzymes which display xylanase activity are restricted to GH families 10 and 11 should be expanded, in order to include families 5, 7, 8 and 43. The different xylanase families differ in their physico-chemical properties, protein structure, modes of action and substrate specificities (Collins et al. 2005). For the purpose of this literature review, the focus will be on families 10 and 11 xylanases as they are the two main xylanase families.

Glycosyl Hydrolase family 10 consists of endo-1,4-β-xylanases (EC 3.2.1.8), endo-1,3-β-xylanases (EC 3.2.1.32) and cellobiohydrolases (EC 3.2.1.91) (Henrissat 1991). Endo-1,4-β-xylanases are the major enzymes belonging to this GH family. Substrate specificity studies have discovered that endo-1,4-β-xylanases of GH family 10 may not be entirely specific for xylan as they may also be active on cellulosic substrates (Gilkes et al. 1991). Family 10 endoxylanases have several catalytic activities, which display compatibility with β-xylosidases. This family of xylanases can hydrolyse aryl β-glycosides of xylobiose and xylotriose at the aglyconic bond. Family

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10 xylanases can also hydrolyse aryl β-D-cellobiosides at the agluconic bond. Glycosyl hydrolase family 10 consists of acidic high molecular mass (>30 kDa) endoxylanases (Biely et al. 1997).

Glycosyl Hydrolase family 11 consists solely of endo-1,4-β-xylanases (EC 3.2.1.8) (Henrissat 1991). Family 11 xylanases are "true xylanases" as they are only active on substrates which contain D-xylose. The products of family 11 xylanases can be further hydrolysed by family 10 xylanases. Family 11 xylanases have the same capability as family 10 in that they can hydrolyse aryl β-glycosides of xylobiose and xylotriose at the aglyconic bond. However, this family of enzymes differs from family 10 in that it is inactive on aryl cellobiosides. Glycosyl hydrolase family 11 consists of basic low molecular mass endoxylanases (Biely et al. 1997).

2.3.3.4 Structure of Xylanases

Glycosyl hydrolases are modular in structure and consist of catalytic and non-catalytic or ancillary domains (Bourne and Henrissat 2001). Non-catalytic domains of xylanases include carbohydrate binding modules (CBMs), such as cellulose- and xylan- binding domains, as well as dockerin domains and thermostabilising domains (Collins et al. 2005). Most hemicellulolytic enzymes contain a catalytic domain and one or more substrate binding domains (Kulkarni et al. 1999; Subramaniyan and Prema 2002). Family 10 and 11 xylanases have different tertiary structures which results in differences in catalytic activities between the two xylanase families (Biely et al. 1997).

The structure of the catalytic domain of family 10 xylanases is a cylindrical $(\alpha/\beta)_8$ barrel, which resembles a salad bowl, with the catalytic site positioned near the C-terminus of the barrel (Biely et al. 1997; Subramaniyan and Prema 2002). Figure 2.3 is a schematic representation of a family 10 xylanase which is mainly composed of $(\alpha/\beta)_8$ barrels. Family 10 xylanases have a relatively high catalytic efficiency for the degradation of shorter linear β-1,4-xylooligosaccharides which illustrates that they

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have small substrate binding sites (Biely et al. 1997). The catalytic site of this family generally has 4-5 substrate binding sites for xylose residues (Biely et al. 1997).

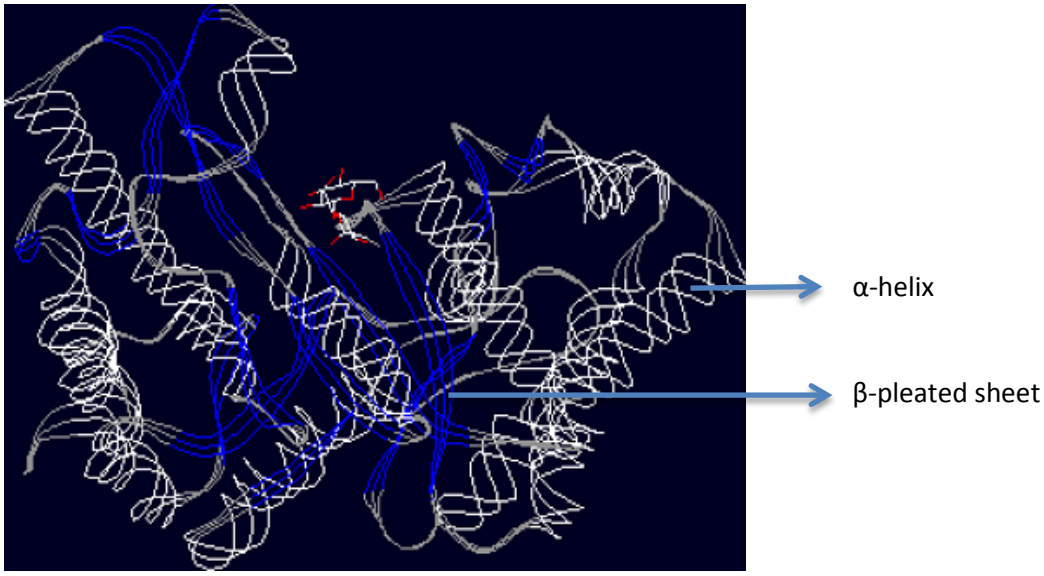

Figure 2.3: A schematic representation of a family 10 xylanase which is mainly composed of $(\alpha/\beta)_8$ barrels (Liu et al. 2004).

Family 11 xylanases are smaller and well-packed polypeptides which have a β-jelly roll structure, with catalytic domains mainly consisting of β-pleated sheets which are formed into a two-layered trough which surrounds the catalytic site (Törrönen et al. 1994; Törrönen and Rouvinen 1995; Krengel and Dijkstra 1996; Biely et al. 1997). Figure 2.4 is a schematic representation of a family 11 xylanase which is mainly composed of β-pleated sheets. Family 11 xylanases are most active on long chain xylo-oligosaccharides and, therefore, they have larger substrate binding sites. The catalytic groups in the cleft support about seven substrate binding sites for xylose residues. Due to the fact that family 10 xylanases do not have such deep clefts for substrate binding sites as family 11 xylanases, it can be deduced that family 10 have lower substrate specificity than family 11 xylanases. Family 10 xylanases, therefore, have greater catalytic versatility than family 11 xylanases (Biely et al. 1997).

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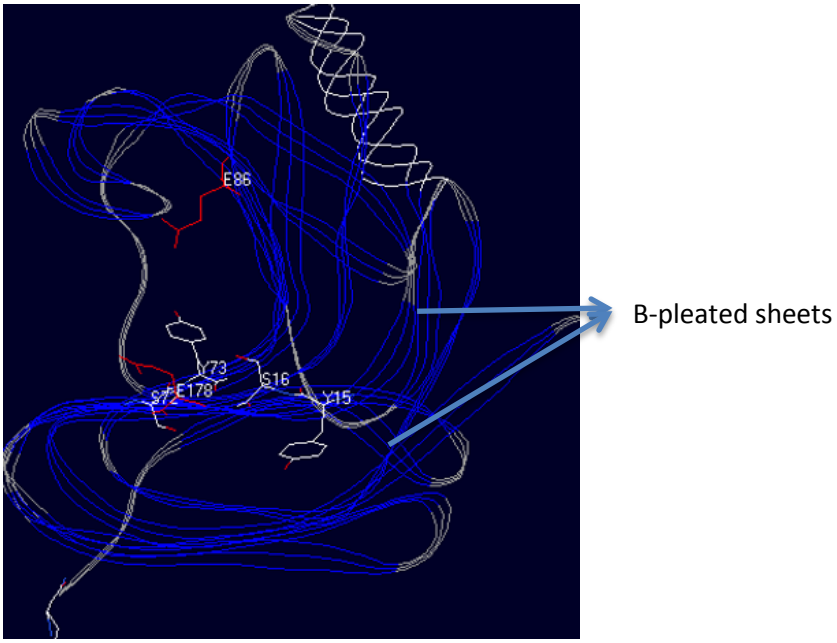

Figure 2.4: A schematic representation of a family 11 xylanase which is mainly composed of β-pleated sheets (Liu et al. 2004).

CBMs are substrate binding domains which bind the soluble enzyme with the insoluble polysaccharide, which increases the rate of catalysis. However, CBMs are not essential for hydrolysis (Subramaniyan and Prema 2002). The classification of CBMs into different families is based on comparing the primary structure to previously characterised sequences (Bourne and Henrissat 2001; Subramaniyan and Prema 2002). CBMs are small in size. CBMs mostly fold as sandwiched β-sheets due to their relatively small size (Bourne and Henrissat 2001). Substrate binding domains are more commonly found in F10 xylanases than in F11 xylanases (Subramaniyan and Prema 2002).

2.3.3.5 Applications of Xylanases

Xylanases have numerous industrial applications such as in the pulp and paper, animal feed, food, textile and fuel industries. In the pulp and paper industry, cellulase-free xylanases are used in bleaching of pulps. The cellulose fibres remain intact and the amount of bleaching chemicals, such as chlorine, is decreased. This decreases the cost of chemicals and is more environmentally friendly than chlorine (De Vries and Visser 2001). Xylanases are used in animal feed as they break down

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arabinoxylans in the feed, improving the digestion of nutrients by the animals (Polizeli et al. 2005). In the food industry, xylanases can be used in the making of bread, wine, beer, juices and artificial sweeteners. Xylanses solubilize the arabinoxylan component of bread dough, increasing the bread volume and quality (De Vries and Visser 2001). Xylanases can contribute to a more pronounced aroma in wine and can be used to clarify beer and juices by hydrolysing arabinoxylan to xylooligosaccharides (Polizeli et al. 2005). Xylose can be fermented by yeasts to xylitol which is an artificial non-carcinogenic sweetener which is suitable for diabetics and obese people (Polizeli et al. 2005). Xylanases are also employed by the textile industry to process plant fibres such as hessian or linen. It is important that the xylanase is free of cellulases during this process (Polizeli et al. 2005). Hydrolysis products of xylan can be used to produce fuel ethanol (Polizeli et al. 2005). The production of biofuels is a major focus of research due to the shortage of fossil fuels and therefore, biofuels will be discussed in detail.

Atmospheric carbon is captured by plants during photosynthesis and made into crude oil and coal (Dashtban et al. 2009). Humans currently depend on these fossil fuels for energy generation. However, the world population has grown over the past century and many countries have become industrialised leading to an increase in energy consumption (Sun and Cheng 2002; Li et al. 2009). This ever-increasing energy demand will continue to increase as economic growth rises. Hydrocarbon based fossil fuels are non-renewable and are, therefore, not a sustainable energy source (Rojo 2008). Fossil fuel supplies are, therefore, rapidly decreasing and cannot meet the increase in energy demand. Crude oil prices will, as a result, continue to rapidly increase (Coughlan 1992; Sun and Cheng 2002; Kumar et al. 2008; Rojo 2008). This would have devastating effects as the economy of many countries, such as the United States, relies on oil (Sun and Cheng 2002). In the early 1970s, the Organisation of the Petroleum Exporting Countries (OPEC) decreased oil production, resulting in a large increase in oil prices. Over the past 150 years, atmospheric CO_2 levels have rised from 280 ppm to 365 ppm (Galbe and Zacchi 2002). Burning fossil

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fuels releases elevated levels of carbon dioxide into the atmosphere, resulting in increased temperatures worldwide. This increases the global greenhouse effect and contributes to global warming (Galbe and Zacchi 2002; Rojo 2008; Li et al. 2009). The United States is responsible for the largest amount of emitted CO_2 (Galbe and Zacchi 2002). Increasing crude oil prices, the depletion of energy supplies, global warming and air pollution are growing economic and environmental concerns (Dashtban et al. 2009; Li et al. 2009). Therefore, it is of utmost importance to search for alternative energy sources which are sustainable, regenerative, cheap and ecologically friendly (Sun and Cheng 2002; Kumar et al. 2008; Rojo 2008; Dashtban et al. 2009).

Bioconversion of plant biomass is a promising alternative energy source as it is considered the most abundant and renewable biomaterial on Earth (Dashtban et al. 2009). Much research is being pumped into the field of biotechnology to investigate the conversion of biomass into fuels that are economical and can compete with current crude oil prices. Burning of biomass-derived compounds does not contribute to global warming as it releases CO_2 to the atmosphere that has already been fixed by photosynthesis (Rojo 2008). In addition to the low levels of CO_2 which are released into the atmosphere, combustion of bioethanol also releases low levels of non-combusted hydrocarbons, carbon monoxide, nitrogen oxides and exhaust volatile organic compounds (Galbe and Zacchi 2002). Fuel ethanol which is produced from biomass is, therefore, the cleanest liquid fuel which can replace fossil fuels (Li et al. 2009).

From as early as the 1980s, fuel ethanol derived from corn was used in gasoline fuels which contain as much as 10% volume of ethanol (Sun and Cheng 2002). New cars can use fuel-ethanol mixtures of 20% ethanol without adjusting the car engines. Some of the new car engines are now even able to run on pure ethanol. Flexible-fueled vehicles use an ethanol blend E85, which consists of 85% ethanol and 15% gasoline. This ethanol blend can be used independently or in combination with gasoline and will significantly decrease the use of petroleum and greenhouse gas

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emissions (Galbe and Zacchi 2002). The United States of America and Brazil are responsible for about 90% of global ethanol fuel production (Dashtban et al. 2009). Starch-based crops such as corn are generally used to produce ethanol in the USA, whereas sugar cane juice is generally used for ethanol production in Brazil (Wheals et al. 1999; Sánchez 2009). However, both corn and sugars are valuable food sources and due to the world's current food crisis, human food and fuel ethanol compete with each other (Sun and Cheng 2002; Dashtban et al. 2009; Li et al. 2009). Ethanol is also still a relatively costly energy source compared to fossil fuels as the raw material (sugar cane or maize) accounts for 40-70% of the total production cost (Claassen et al. 1999). Therefore, it became obvious that an alternative solution needs to be found.

Lignocellulosic biomass is the most abundantly occurring and renewable energy source in the biosphere, as it accounts for about 50% of the world's biomass (Claassen et al. 1999). Lignocellulosic wastes are produced by numerous industries, including agriculture, forestry, pulp and paper, food, municipal solid waste and animal waste (Pérez et al. 2002; Kim and Dale 2004; Wen et al. 2004; Dashtban et al. 2009). Lignocellulose materials, therefore, include crop residues such as sugarcane waste, corncobs, corn stems, palm, husk, rice straw, wheat straw, sunflower stalks, sunflower hulls and water-hyacinth, as well as forestry and paper industry waste, such as wood chips, sawdust and paper sludge streams. Grasses, municipal waste and solid animal waste are also deemed as potential bioethanol feedstocks (Sun and Cheng 2002; Kumar et al. 2008; Li et al. 2009). These agricultural, industrial and forestry lignocellulosic materials are being burned or wasted, despite their potential value. Instead, this lignocellulosic waste can be used as raw materials for ethanol production (Wheals et al. 1999; Sun and Cheng 2002; Kumar et al. 2008; Dashtban et al. 2009). This would meet many of the current energy and feedstock demands as well as provide numerous jobs and result in a large profit (Coughlan 1992). Therefore, the use of lignocellulosic wastes from numerous industries in the production of ethanol has been the current focus of research. This process does not compete with

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the limited amount of agricultural land that is necessary for food and feed production and is aimed to lower the production cost (Kumar et al. 2008; Dashtban et al. 2009). The conversion of lignocellulosic biomass to ethanol requires two processes, the hydrolysis of the cellulose and hemicellulose components of lignocellulose to reducing monomeric sugars and the subsequent fermentation of these reducing sugars to ethanol (Galbe and Zacchi 2002; Pérez et al. 2002; Sun and Cheng 2002). The hydrolysis of cellulose/hemicellulose is catalyzed by cellulase/hemicellulase enzymes, which are produced by lignocellulolytic degrading microorganisms, such as bacteria and fungi and the fermentation is carried out by yeast (Sun and Cheng 2002; Kumar et al. 2008; Dashtban et al. 2009). Ethanol production from lignocellulosic waste materials has the potential to replace 40% of the US gasoline (Wheals et al. 1999). The world's leading operating plant for the production of bioethanol from lignocellulosic waste is the Iogen Corporation in Canada. This plant uses as much as 40 tons of wheat, barley straw and oats every day to produce as much as 3 million litres of ethanol per year (Hahn-Hagerdal et al. 2006).

A bottleneck of the biofuel production process lies in the initial step of converting biomass into sugars (Dashtban et al. 2009). The main challenges currently facing the enzymatic hydrolysis process are the low yield and high cost (Bhat and Bhat 1997; Sun and Cheng 2002; Rojo 2008). The cost of converting lignocellulosic biomass to sugars for fermentation into bioethanol using enzymes is still too high to be carried out on an industrial scale (Galbe and Zacchi 2002).

Large-scale production of industrial enzymes is a costly process and the cost of the substrate is a large contributing factor to the overall economy of the production process. The use of low-cost substrates, such as agricultural waste products, contributes greatly to the economical feasibility of enzyme production (Bajaj and Abbass 2011). Most industrial applications use processes which are carried out at high temperatures. Therefore, industries generally require enzymes which are thermostable. The pulp and paper industry specifically requires xylanases which are

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free of cellulases so that the cellulose fibres remain undamaged (Anthony et al. 2003; Abdel-Monem et al. 2012). Alkali-tolerant enzymes are also attractive to the pulp and paper industry (Anthony et al. 2003; Bajaj and Abbass 2011).

2.4 Current Status of Xylanase Production in *A. fumigatus*

Numerous strains of *A. fumigatus* have been isolated and shown to produce xylanases which display some of the above-mentioned characteristics (i.e. thermostability, alkali-tolerance and absence of cellulases) and/or have been shown to produce xylanases on agricultural waste products, making them attractive to industries. A discussion of the current status of xylanase production in *A. fumigatus* is discussed below.

A. fumigatus FBSPE-05 produced thermophillic endoxylanase by solid state fermentation when using agro-industrial byproducts such as sugarcane bagasse, brewer's spent grain and wheat bran (Souza et al. 2012). This endoxylanase displayed maximum activity at 60°C and pH 6.0, suggesting that it is a thermophillic endoxylanase, and it remained stable at this temperature over a 1 hour incubation period (Souza et al. 2012).

A xylanase was purified and characterised from solid-state cultures of *A. fumigatus* Fresenius, which was isolated from a hot fountain in Brazil. The solid-state medium consisted of the agricultural waste product, wheat bran. The purified xylanase displayed optimal activity at 55°C and pH 5.5. This enzyme therefore had an acidic pH optimum and it was also thermostable. The purified xylanase was specific to xylan, with no detectable cellulase activity (Silva et al. 1999).

A. fumigatus was isolated from garden soil in India and the xylanase was purified and characterised. The optimum pH of the xylanase was pH 6.0. The optimum temperature was 60°C and this xylanase was a highly thermostable enzyme. This xylanase was used to improve the quality of waste paper pulp, which reduces the use of chemical bleaching agents (Savitha et al. 2007).

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A. fumigatus RP04, which was isolated from soil and decomposing leaves in Brazil, produced high levels of xylanase on agricultural wastes such as corncob and wheat bran. The optimal temperature was 70°C. The pH optimum was pH 5.0-5.5, indicating an acidic xylanase. However, the pH stability was at a more alkaline pH 6.0-8.0. No cellulase activity was detected, making this a cellulase-free xylanase. The application of this xylanase was demonstrated on cellulose pulp biobleaching (Peixoto-Nogueira et al. 2009).

Most fungi produce xylanases at acidic growth pH. However, there are a few alkali-tolerant fungi which produce cellulase-free xylanases, making them attractive to the pulp and paper industry. *A. fumigatus* AR1 which was isolated from a paper mill effluent was found to be alkali-tolerant and produce cellulase-free xylanase. Xylanase production was optimal at acidic pH 5, with a significant amount of xylanase being produced at alkaline pH 9.0, indicating the alkali-tolerance of this enzyme. Low-cost agricultural substrates supported xylanase production of this *A. fumigatus* isolate, with maximum xylanase levels being produced on rice straw (Anthony et al. 2003).

An endoxylanase was also purified from solid-state culture of alkali-tolerant *A. fumigatus* MKU1, which was isolated from a paper mill effluent sample. This strain of *A. fumigatus* produced xylanases when grown on wheat bran as a substrate under alkaline growth conditions of pH 9.0. High levels of endoxylanase activity were detected, with only trace amounts of cellulolytic activity, making this enzyme attractive to industry (Thiagarajan et al. 2006).

An alkalitolerant *A. fumigatus* strain MA28 was isolated from alkaline hot soils in India and found to produce cellulase-free xylanase which was thermo-stable and alkali-stable when grown on various agricultural wastes. Wheat bran was established to be the best inducer of xylanase activity when compared to the other agricultural waste products which were used in the study. An optimum temperature and pH of

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50°C and pH 8 was observed for xylanase activity, respectively. The xylanase also displayed activity at 60-70°C and at alkaline pH 8.0-9.0 (Bajaj and Abbass 2011).

A. fumigatus was isolated from soil samples in Egypt and was found to produce thermostable cellulase-free xylanase. The optimum temperature for the xylanase was 55-60°C. This enzyme displayed maximum activity at an alkaline pH 9.0. Different agricultural wastes, such as wheat bran, corn cobs, wheat straw, rice straw etc. were investigated as substrates for enzyme production, with wheat bran being the best substrate for the production of xylanases by this *A. fumigatus* isolate (Abdel-Monem et al. 2012).

All of the above-mentioned studies involved the purification and characterisation of xylanases from *A. fumigatus*. However, this fungal species is a pathogenic organism and therefore, for the large-scale production of xylanases, it is preferable to clone and express the xylanase gene in another non-pathogenic host. The only study published to date on the cloning and expression of a xylanase gene from *A. fumigatus* in another host is the study by Jeya and co-workers. They cloned and expressed a xylanase gene, *xynf11a*, from *A. fumigatus* MKU1 in *Pichia pastoris* under control of the AOX1 promoter. The recombinant xylanase showed high levels of xylanase activity, with maximum activity at pH 6.0 and 60°C. Substrate specificity studies were performed with this recombinant xylanase and it was shown to have no cellulase activity. This property makes this recombinant xylanase promising for bleaching of pulp in the pulp and paper industry (Jeya et al. 2009).

2.5 Cloning and Expression of Xylanase Genes in *S. cerevisiae*

The traditional yeast *S. cerevisiae* ferments glucose to ethanol but is incapable of fermenting other sugars, such as xylose and arabinose, to ethanol (Saha 2003). Despite the fact that this yeast cannot utilize or degrade xylan (La Grange et al. 1996), it has been found to be an attractive host for the expression of heterologous proteins, such as xylanases (Romanos et al. 1992). There are numerous characteristics that this yeast displays which explains this phenomenon. *S. cerevisiae* is a unicellular

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fungus which displays efficient post-translational processing such as glycosylation, protein folding, proteolysis etc. *S. cerevisiae* also only secretes a few proteins, enabling easier purification of the expressed heterologous proteins (Romanos et al. 1992, La Grange et al. 1996; Ahmed et al. 2009). A variety of relatively cheap culture media can be used to cultivate *S. c*erevisiae, eliminating the need for xylan to induce the production of xylanases (La Grange et al. 1996). The absence of contaminating cellulases also makes this host ideal for xylanase production, particularly in the pulp and paper industry (La Grange et al. 1996; Ahmed et al. 2009). *S. cerevisiae* also has complete GRAS (generally regarded as safe) status, allowing it to be used in the food industry (La Grange et al. 1996). *S. cerevisiae* is a well-established industrial microorganism and is, therefore, suitable for the industrial production of xylanases at a low cost (Ahmed et al. 2009). Industrial scale fermentation technology is well established for *S. cerevisiae*, making this organism suitable for large scale fermentation (La Grange et al. 1996). This yeast also has a high fermentation rate and high ethanol tolerance (Dashtban et al. 2009).

Consolidated bioprocessing (CBP) is a single step process for the enzymatic hydrolysis of lignocellulosic biomass and the fermentation of the resulting sugars to ethanol. This process can be used to greatly reduce the overall cost as microorganisms can be developed which are able to hydrolyse the substrate as well as ferment the hydrolytic sugars to end-product (Lynd et al. 2002). *S. cerevisiae* is unable to produce lignocellulolytic enzymes. However, lignocellulolytic enzymes can be heterologously expressed in this yeast host. The heterologous production of xylanase genes in *S. cerevisiae* is an example of CBP as this yeast host can ferment the resulting sugars to ethanol. Numerous fungal xylanase genes have been expressed in the yeast *S. cerevisiae*. Table 2.1 is a summary of xylanase genes from fungal origin expressed in *S. cerevisiae*.

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Table 2.1: Fungal xylanase genes expressed in *S. cerevisiae*

Fungal Organism	**Gene**	**Reference**
Aspergillus kawachii	*xynA*	Ito et al. 1992b
Aspergillus kawachii	*xyn3*	Crous et al. 1995
Aspergillus nidulans	*xlnA, xlnB*	Pérez-González et al. 1996
Aspergillus niger	*xyn4, xyn5*	Luttig et al. 1997
Aureobasidium pullulans	*xynA*	Li and Ljungdahl 1996
Aureobasidium pullulans var. *melanigenum*	*xyn1*	Ohta et al. 2001
Cryptococcus flavus	*Cfxyn1*	Parachin et al. 2009
Penicillium purpurogenum	*xynA*	Chávez et al. 2002
Trichoderma reesei	*xyn2*	La Grange et al. 1996

2.6 References

Abdel-Monem OA, El-Baz AF, Shetaia YM, El-Sabbagh SM (2012) Production and application of thermostable cellulase-free xylanase by *Aspergillus fumigatus* from agricultural wastes. Ind Biotechnol 8:152-161

Ahmed S, Riaz S, Jamil A (2009) Molecular cloning of fungal xylanases: an overview. Appl Microbiol Biotechnol 84:19-35

Anthony T, Raj KC, Rajendran A, Gunasekaran P (2003) High molecular weight cellulase-free xylanase from alkali-tolerant *Aspergillus fumigatus* AR1. Enzyme Microb Tech 32:647-654

Bailey MJ, Puls J, Poutanen K (1991) Purification and properties of two xylanases from *Aspergillus oryzae*. Biotechnol Appl Biochem 13:380-389

Bajaj BK, Abbass M (2011) Studies on an alkali-thermostable xylanase from *Aspergillus fumigatus* MA28. Biotech 1:161-171

Bastawde KB (1992) Xylan structure, microbial xylanases, and their mode of action. World J Microbiol Biotechnol 8:353-368

Bhat MK, Bhat S (1997) Cellulose degrading enzymes and their potential industrial applications. Biotechnol Adv 15:583-620

Biely P (1985) Microbial xylanolytic systems. Trends Biotechnol 3:286-290

Biely P, Markovič O, Mislovičová D (1985) Sensitive detection of endo-1,4-β-glucanases and endo-1,4-β-xylanases in gels. Anal Biochem 144:147-151

Biely P, Vršanská M, Tenkanen M, Kluepfel D (1997) Endo-β-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57:151-166

Stellenbosch University http://scholar.sun.ac.za

Bourne Y, Henrissat B (2001) Glycoside hydrolases and glycosyltransferases: families and functional module*S. c*urr Opin Struc Biol 11:593-600

Chávez R, Navarro C, Calderón I, Peirano A, Bull P, Eyzaguirre J (2002) Secretion of endoxylanase A from *Penicillium purpurogenum* by *Saccharomyces cerevisiae* transformed with genomic fungal DNA. FEMS Microbiol Lett 212:237-241

Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, De Vries SS, Weusthuis RA (1999) Utilisation of biomass for the supply of energy carriers. Appl Microbiol Biotechnol 52:741-755

Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3-23

Coughlan MP (1992) Enzymic hydrolysis of cellulose: an overview. Bioresour Technol 39:107-115

Crous JM, Pretorius IS, van Zyl WH (1995) Cloning and expression of an *Aspergillus kawachii* endo-1,4-β-xylanase gene in *Saccharomyces cerevisiaE. c*urr Genet 28:467-473

Dashtban M, Schraft H, Qin W (2009) Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. Int J Biol Sci 5:578-595

De Graaff LH, van den Broeck HC, van Ooijen AJ, Visser J (1994) Regulation of the xylanase-encoding xlnA gene of *Aspergillus tubigensis*. Mol Microbiol 12:479-490

De Vries RP, Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol Molec Biol Rev 65:497-522

Fujimoto H, Ooi T, Wang SL, Takizawa T, Hidaka H, Murao S, Arai M (1995) Purification and properties of three xylanases from *Aspergillus aculeatus*. Biosci Biotechnol Biochem 59:538-540

Galbe M, Zacchi G (2002) A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 59:618-628

Gawande PV, Kamat MY (1999) Production of *Aspergillus* xylanase by lignocellulosic waste fermentation and its application. J Appl Microbiol 87:511-519

Ghosh M, Nanda G (1994) Purification and some properties of a xylanase from *Aspergillus sydowii* MG49. Appl Environ Microbiol 60:4620-4623

Gilbert HJ, Hazlewood GP (1993) Bacterial cellulases and xylanases. J Gen Microbiol 139:187-194

Gilkes NR, Claeyssens M, Aebersold R, Henrissat B, Meinke A, Morrison HD, Kilburn DG, Warren RAJ, Miller RC (1991) Structural and functional relationships in two families of β-1,4-glycanases. Eur J Biochem 202:367-377

Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G (2006) Bio-ethanol – the fuel of tomorrow from the residues of today. Trends Biotechnol 24:549-556

Haltrich D, Nidetzky B, Kulbe KD, Steiner W, Zupancic S (1996) Production of fungal xylanases. Bioresour Technol 58:137-161

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280:309-316

Henrissat B, Coutinho PM (2001) Classification of glycoside hydrolases and glycosyltransferases from hyperthermophiles. Method Enzymol 330:183-201

Hildén L, Johansson G (2004) Recent developments on cellulases and carbohydrate-binding modules with cellulose affinity. Biotechnol Lett 26:1683-1693

Ito K, Ikemasu T, Ishikawa T (1992b) Cloning and sequencing of the xynA gene encoding xylanase A of *Aspergillus kawachii*. Biosci Biotech Biochem 56:906-912

Stellenbosch University http://scholar.sun.ac.za

Ito K, Ogasawara H, Sugimoto T, Ishikawa T (1992) Purification and properties of acid stable xylanases from *Aspergillus kawachii*. Biosci Biotechnol Biochem 56:547-550

Jeya M, Thiagarajan S, Lee J-K, Gunasekaran P (2009) Cloning and expression of GH11 xylanase gene from *Aspergillus fumigatus* MKU1 in *Pichia pastoris*. J Biosci Bioeng 108:24-29

Juhász, T, Szengyel Z, Réczey K, Siika-Aho M, Viikari L (2005) Characterisation of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem 40:3519-3525

Kim S, Dale BE (2004) Global potential bioethanol production from wasted crops and crop residues. Biomass Bioenergy 26:361-375

Kimura I, Sasahara H, Tajima S (1995) Purification and characterisation of two xylanases and an arabinofuranosidase from *Aspergillus sojae*. J Ferment Bioeng 80:334-339

Kormelink FJM, Searle-Van Leeuwen MJF, Wood TM, Voragen AGJ (1993) Purification and characterisation of three endo-(1,4)-β-xylanases and one β-xylosidase from *Aspergillus awamori*. J Biotechnol 27:249-265

Krengel U, Dijkstra BW (1996) Three-dimensional structure of endo-1,4-β-xylanase I from *Aspergillus niger*: molecular basis for its low pH optimum. J Mol Biol 263:70-78

Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411-456

Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 35:377-391

La Grange DC, Pretorius IS, van Zyl WH (1996) Expression of a *Trichoderma reesei* β-xylanase gene (xyn2) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 62:1036-1044

Li X-L, Ljungdahl LG (1996) Expression of *Aureobasidium pullulans* xynA in, and secretion of the xylanase from*, Saccharomyces cerevisiae*. Appl Environ Microbiol 62:209-213

Li X-L, Yang H, Roy B, Wang D, Yue W, Jiang L, Park EY, Miao Y (2009) The most stirring technology in future: cellulase enzyme and biomass utilization. Afr J Biotechnol 8:2418-2422

Liu L, Zhang J, Chen B, Shao W (2004) Principle component analysis in F/10 and G/11 xylanase. Biochem Biophys Res Commun 322:277-280

Luttig M, Pretorius IS, van Zyl WH (1997) Cloning of two β-xylanase –encoding genes from *Aspergillus niger* and their expression in *Saccharomyces cerevisiae*. Biotechnol Lett 19:411-415

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol R 66:506-577

Ohta K, Moriyama S, Tanaka H, Shige T, Akimoto H (2001) Purification and characterisation of an acidophilic xylanase from *Aureobasidium pullulans var. melanigenum* and sequence analysis of the encoding gene. J Biosci Bioeng 92:262-270

O'Sullivan AC (1997) Cellulose: the structure slowly unravel*S. c*ellulose 4:173-207

Parachin NS, Siqueira S, de Faria FP, Torres FAG, de Moraes LMP (2009) Xylanases from *Cryptococcus flavus* isolate I-11: Enzymatic profile, isolation and heterologous expression of CfXYN1 in *Saccharomyces cerevisiae*. J Mol Catal B Enzym 59:52-57

Peixoto-Nogueira SC, Michelin M, Betini JHA, Jorge JA, Terenzi HF, Polizeli MLTM (2009) Production of xylanase by Aspergilli using alternate carbon sources: application of the crude extract on cellulose pulp biobleaching. J Ind Microbiol Biotechnol 36:149-155

Pérez J, Muñoz-Dorado J, de la Rubia T, Martinez J (2002) Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int J Microbiol 5:53-63

Stellenbosch University http://scholar.sun.ac.za

Pérez-González JA, De Graaff LH, Visser J, Ramón D (1996) Molecular cloning and expression in *Saccharomyces cerevisiae* of two *Aspergillus nidulans* xylanase genes. Appl Environ Microbiol 62:2179-2182

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577-591

Rojo F (2008) Biofuels from microbes: a comprehensive view. J Microbial Biotech 1:208-210

Romanos MA, Scorer CA, Clare JJ (1992) Foreign gene expression in yeast: a review. Yeast 8:423-488

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279-291

Sánchez C (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 27:185-194

Savitha S, Sadhasivam S, Swaminathan K (2007) Application of *Aspergillus fumigatus* xylanase for quality improvement of waste paper pulp. Bull Environ Contam Toxicol 78:217-221

Shallom D, Shoham Y (2003) Microbial hemicellulase*S. c*urr Opin Microbiol 6:219-228

Sherief AA (1990) Separation and some properties of an endo-1,4-beta-D-xylanase from *Aspergillus flavipes*. Acta Microbiol Hung 37:301-306

Silva CHC, Puls J, de Sousa MV, Filho EXF (1999) Purification and characterisation of a low molecular weight xylanase from solid-state cultures of *Aspergillus fumigatus* Fresenius. Rev Microbiol 30:114-119

Souza DT, Bispo ASR, Bon EPS, Coelho RRR, N*Asc*imento RP (2012) Production of thermophilic endo-β-1,4-xylanases by *Aspergillus fumigatus* FBSPE-05 using agro-industrial by-products. Appl Biochem Biotechnol 166:1575-1585

Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology and application. Crit Rev Biotechnol 22:33-46

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1-11

Thiagarajan S, Jeya M, Gunasekaran P (2006) Purification and characterisation of an endoxylanase from solid state culture of alkalitolerant *Aspergillus fumigatus* MKU1. Indian J Biotechnol 5:351-356

Törrönen A, Harkki A, Rouvinen J (1994) Three-dimensional structure of endo-1,4-β-xylanase II from *Trichoderma reesei*: two conformational states in the active site. EMBO J 13:2493-2501

Törrönen A, Rouvinen J (1995) Structural comparison of two major endo-1,4-xylanases from *Trichoderma reesei*. Biochemistry 34:847-856

Wen Z, Liao W, Chen S (2004) Hydrolysis of animal manure lignocellulosics for reducing sugar production. Bioresour Technol 91:31-39

Wheals AE, Basso LC, Alves DMG, Amorim HV (1999) Fuel ethanol after 25 years. Trends Biotechnol 17:482-487

Wong KKY, Tan LUL, Saddler JN (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev 52:305-317

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CHAPTER 3:

A TRANSCRIPTOMIC APPROACH FOR CLONING AND EXPRESSION OF THREE XYLANASE GENES FROM *A. FUMIGATUS* IN *S. CEREVISIAE*

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3.1 Abstract

Xylan is a complex heteropolysaccharide which consists of xylopyranose residues which are linked by β-1,4-bonds. The focus of this study was on xylanases, the enzymes which are required to degrade xylan. First-strand cDNA was synthesized by reverse transcription from total RNA extracted from *A. fumigatus*. Three xylanase genes, *xynf10a* (F10), *xynf11a* (F11) and *xynG1* (G1), were amplified by PCR and cloned into the multiple cloning site of the shuttle vector, pDLG125, using the *Pac*I and *Asc*I restriction enzyme sites. The cloned products were then expressed in *S. cerevisiae* under control of the *ENO*1 promoter and terminator. The molecular weight of the proteins was determined with SDS-PAGE analysis and enzyme activity was confirmed with zymograms. Enzyme activity and the growth of the yeast were monitored over time. The recombinant xylanases were then characterised according to temperature, pH, substrate specificity and synergistic interactions. Nucleotide sequence analysis of the cDNA, containing the cloned xylanase gene products, revealed that F10, F11 and G1 contained a 978 bp, 687 bp and 666 bp open reading frame, respectively. The molecular mass of the recombinant xylanases F10, F11 and G1 was estimated by SDS-PAGE to be ~30 kDa, ~15 kDa and ~15 kDa, respectively. All three of the xylanases were functional after expression in the yeast host. F10 and G1 both displayed maximum xylanase activity of ~300 nkat/mg DCW, whereas F11 displayed maximum xylanase activity of only ~100 nkat/mg DCW. The optimum temperature range for F10 and G1 was between 50°C and 60°C and F11 had a temperature optimum of 60°C, with all of the xylanases being most stable at 50°C. The optimum pH of F10 was 6, F11 had an optimum pH range of between pH 4 and pH 6 and G1 had an optimal pH of 5. Of all of the different substrates used to detect different enzyme activities of the recombinants, all three of the xylanases were only able to hydrolyse xylan. This suggests that all three xylanases are endoxylanases or "true xylanases". There was no synergistic effect observed between the different xylanases. These xylanases are promising for use in various industries as high enzyme

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production levels were obtained and activity was observed over a wide range of temperatures and pH. A major bottleneck of the production of biofuels is the low yield and high cost of the enzymatic hydrolysis process. Higher production levels significantly reduces the cost of the enzymatic hydrolysis process making many industrial processes more economically feasible. No cellulase activity was detected for these xylanases, making them particularly valuable for the biobleaching of pulps in the paper industry.

3.2 Introduction

Approximately one third of all renewable organic carbon on Earth is xylan. This polysaccharide is, therefore, the second most abundant polysaccharide in nature, with cellulose being the most abundant. Xylan is a major structural polysaccharide in plant cells and is a complex, highly branched heteropolysaccharide. The xylan backbone consists of xylopyranose residues which are linked by β-1,4-bonds. This backbone can be substituted by side chain branches such as arabinose, glucuronic acid, 4-*O*-methyl glucuronic acid, acetic acid, ferulic acid and *p*-coumaric acid. Therefore, xylan requires numerous synergistically acting enzymes for its complete hydrolysis (Biely 1985; Subramaniyan and Prema 2002; Gilbert and Hazlewood 1993; Collins et al. 2005). Examples of such enzymes includes endo-1,4-β-xylanases, β-xylosidases and accessory enzymes such as α-L-arabinofuranosidases, α-glucuronidases (α-4-*O*-methyl glucuronosidases), acetylxylan esterases, ferulic acid esterases and *p*-coumaric acid esterases. Endo-1,4-β-D-xylanases randomly hydrolyse the β-1,4-xylosidic bonds of the xylan backbone, producing xylobiose and short chain xylo-oligosaccharides. The non-reducing ends of these molecules are then further hydrolysed by β-D-xylosidases to xylose (Gilbert and Hazlewood 1993; Pérez et al. 2002; Subramaniyan and Prema 2002; Saha 2003; Shallom and Shoham 2003; Collins et al. 2005).

Xylanases are generally classified into 2 different glycosyl hydrolase (GH) families, namely families 10 and 11 (Henrissat 1991). Acidic high molecular mass (>30 kDa)

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xylanases are characteristic of GH family 10, whereas basic low molecular mass xylanases are characteristic of GH family 11 (Biely et al. 1997). GH family 10 consists of endo-1,4-β-xylanases, endo-1,3-β-xylanases and cellobiohydrolases, whereas GH family 11 are "true xylanases" as they only consist of endo-1,4-β-xylanases (Henrissat 1991). In this study, three xylanase genes were isolated from *A. fumigatus*, one belonging to GH family 10 and two belonging to GH family 11.

Xylanases have numerous industrial applications, such as in the pulp and paper, biofuels, animal feed, food and textile industries (De Vries and Visser 2001; Polizeli et al. 2005; Kulkarni et al. 1999). The industry of interest in this study is the biofuels industry due to the world's current energy crisis as fossil fuel supplies are rapidly becoming depleted. Fossil fuels are also harmful to the environment due to the high levels of carbon dioxide that is emitted. Xylanases can be used in the biofuels industry as they catalyse the hydrolysis of xylan to reducing sugars (xylose), which can then be fermented to ethanol (Subramaniyan and Prema 2002; Galbe and Zacchi 2002; Pérez et al. 2002; Sun and Cheng 2002). The yeast, *S. cerevisiae*, is unable to ferment xylose to ethanol. *S. cerevisiae* is, however, an ideal host organism for the heterologous expression of xylanase genes, enabling this recombinant yeast to ferment xylose to ethanol. *S. cerevisiae* is a well-established industrial microorganism which has complete GRAS (generally regarded as safe) status as well as a high fermentation rate and ethanol tolerance (La Grange et al. 1996; Dashtban et al. 2009). These characteristics make *S. cerevisiae* a popular host organism for the heterologous expression of xylanase genes. Numerous xylanase genes from the fungal genus *Aspergillus* have been cloned and expressed in *S. cerevisiae* (Crous et al. 1995; Pérez-González et al. 1996; Luttig et al. 1997).

A major bottleneck of the biofuels production process is the low yield and high cost of the enzymatic hydrolysis process (Bhat and Bhat 1997; Sun and Cheng 2002; Rojo 2008). The cost can be greatly reduced by obtaining higher production levels of enzymes. Therefore the xylanase genes from *A. fumigatus*, and their enzyme

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production levels, were evaluated in this study. Three xylanase genes from *A. fumigatus* were expressed in *S. cerevisiae*, the levels of enzyme activity were determined and the enzymes were characterised according to temperature, pH, substrate specificity and synergistic interactions.

3.3 Materials and Methods

3.3.1 Microbial Strains and Plasmids

The different microbial strains and plasmids that were used in this study are summarised in Table 3.1, along with their genotypes and source or reference.

Table 3.1: Microbial strains and plasmids used in this study

Strains or Plasmids	**Genotype**	**Source/Reference**
Strains:		
Aspergillus fumigatus Trit15F1	Wild Type	This study
Escherichia coli DH5α	*F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1*	Invitrogen
Saccharomyces cerevisiae NI-C-D4	*MATα, trp1, ura3, pep4*	Wang et al. 2001
Plasmids:		
pJET1.2/blunt		Fermentas
pDLG125	*Bla URA3 ENO1*$_{PT}$	W.H. van Zyl laboratory, Department of Microbiology, Stellenbosch University

3.3.2 Media and Cultivation

All chemicals used in this study were of analytical grade.

During the initial isolation of *A. fumigatus*, this fungus was cultivated in synthetic complete (SC) media [0.176% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium

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sulphate and 1.5% (w/v) bacteriological agar] with the addition of 1% (w/v) Birchwood xylan (Sigma-Aldrich).

For the isolation of total RNA, *A. fumigatus* was cultivated in Czapek-Dox Broth [0.3% (w/v) sodium nitrate, 0.1% (w/v) dipotassium phosphate, 0.05% (w/v) magnesium sulphate, 0.05% (w/v) potassium chloride and 0,001% (w/v) ferrous sulphate], supplemented with 0,16% (w/v) Yeast Nitrogen Base (YNB), 0.2% (w/v) glucose and 2% (w/v) Beechwood xylan (Sigma-Aldrich). *A. fumigatus* was inoculated to a final concentration of $1X10^6$ spores/ml and cultured in 1-L Erlenmeyer flasks containing 200 ml media. The cultures were then incubated at 30°C on a rotary shaker at 100 rpm for 3, 6 and 9 days.

E. coli DH5α was cultivated in Luria-Bertani (LB) broth at 37°C on a rotary shaker. The LB broth was supplemented with 100 µg/ml ampicillin in order to select for ampicillin resistant transformants. *E. coli* cultures were also grown on Luria-Bertani agar plates and incubated at 37°C.

S. cerevisiae NI-C-D4 was cultivated in yeast extract peptone dextrose (YPD) broth when used for the transformation of the recombinant plasmids. The recombinant plasmids expressed in *S. cerevisiae* NI-C-D4 were cultivated in selective double-strength buffered synthetic complete (SC) medium, pH 6.0 [1.34% (w/v) yeast nitrogen base without amino acids but containing ammonium sulphate (Difco), 2% (w/v) glucose, 0.26% (w/v) amino acid pool lacking uracil, 2% (w/v) succinic acid, 1.2% (w/v) sodium hydroxide]. The *S. cerevisiae* transformants were cultured in 125 ml Erlenmeyer flasks containing 25 ml medium and incubated at 30°C on a rotary shaker at 200 rpm. The yeast strain was also grown on SC^{-URA} plates containing 2% agar and incubated at 30°C.

3.3.3 Isolation, Screening and Preliminary Identification of *A. fumigatus*

Fungi with xylanolytic activities were isolated from a triticale compost heap at the Welgevallen organic farm at Wechmars Hof, Stellenbosch. For the isolation of these fungi, one gram of compost material was suspended in 10 ml physiological salt

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solution containing 0.85% sodium chloride. This suspension was vortexed for 1 minute, followed by a serial dilution (10^{-1} to 10^{-5}) which was plated onto SC media with the addition of 1% (w/v) Birchwood xylan (Sigma-Aldrich). The Birchwood xylan present in the SC media is the selective component for xylanase detection. The plates were incubated at 30°C to allow for growth. Pure cultures were then obtained and maintained on malt extract agar plates or slants and stored at 4°C.

Trit15F1 was one of the fungal isolates which showed growth on the SC media which contained Birchwood xylan. This isolate was, therefore, screened for xylanase activity using plate assays. Trit15F1 was grown on SC media with the addition of 0.04% Remazol Brilliant Blue (RBB) xylan and 0.2% glucose, which was incubated at 30°C for 2 days. Plate assays are not quantitative; however, zone diameters were compared to that of the industrially significant *Trichoderma reesei* RutC30 strain, which was used as a positive control in the plate assay. The fungal isolate Trit15F1 was selected due to its zone diameter being comparable to that of the positive control.

In order to quantify the xylanase activity, the fungal isolate Trit15F1 was cultivated in 50 ml SC medium containing 1% triticale as the sole carbon source to ensure sufficient enzyme induction. Trit15F1 was also cultivated in SC medium containing 1% potato dextrose. Cultures were incubated in 250 ml Erlenmeyer flasks at 30°C on a rotary shaker at 200 rpm for 7 days. Samples were taken after days 3, 5 and 7 and extracellular endoxylanase activity was determined using the reducing sugar assay (Bailey et al 1992).

In order to preliminary identify Trit15F1, this fungal isolate was cultivated in liquid SC media containing 2% glucose at 30°C on a rotary shaker at 100 rpm for 5 days. Biomass was then harvested by filtration and genomic DNA was isolated with the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research), according to the manufacturer's instructions. Trit15F1 was putatively identified by amplifying the internal transcribed spacer regions (ITS1 and ITS2) of the nuclear ribosomal RNA gene using the primers ITS1 and ITS4 and the isolated genomic DNA template (White

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et al. 1990). The 25 µl PCR reaction mix contained 100 ng genomic DNA, 0.2 µM of each primer, 10 µM deoxynucleotides and 1 x buffer (WhiteSci). The PCR reaction was carried out in a GeneAmp PCR System 2400 (Perkin Elmer) and the PCR conditions were as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 minute and extension at 72°C for 1 minute; followed by final extension at 72°C for 7 minutes. The reaction mixture was separated on a 0.8% agarose gel at 80 V followed by ethidium bromide staining and visualised using UV light. The ±600 bp amplicon was excised and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA), according to the manufacturer's instructions. This amplicon was then cloned using the InsTAclone™ PCR cloning Kit (Fermentas), according to the manufacturer's instructions. DNA sequence determination was carried out at the Central Analytic Facility, Stellenbosch University. The sequences were analysed using DNAMAN software (version 4.13, Lynnon BioSoft) and subjected to similarity search by NCBI BLAST (Basic Local Alignment Tool).

3.3.4 RNA Isolation and First Strand cDNA Synthesis

Total RNA was extracted from *A. fumigatus* using a manual method. The fungal mycelia were harvested after days 3, 6 and 9 of growth using a Whatman™ glass microfiber filter and filter apparatus under vacuum. The samples were pooled and the fungal biomass was then ground with a mortar and pestle using liquid nitrogen in order to break open the cell walls. In order to inhibit RNases and eliminate proteins, 400 µl STE buffer (0.25 M Tris-HCl pH 7.2, 0.1 M NaCl and 10 mM EDTA) and 400 µl phenol:chloroform:isoamylalcohol(PCI) (25:24:1) was added to 0.3 g of the ground mycelia. This was then incubated on ice for 10 minutes. The reaction mixture was then centrifuged at 13000 rpm for 5 minutes at 4°C and the supernatant was harvested and transferred to a fresh eppendorf tube. To the supernatant, 1 volume of PCI was added followed by centrifugation. This PCI step was repeated until the supernatant was clear. Hereafter, 1 volume of chloroform:isoamylalcohol(CI) (24:1) was added to the clear supernatant and centrifuged at 13000 rpm for 5 minutes at 4°C. The supernatant was transferred to a fresh eppendorf tube. In order to

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precipitate the total RNA, 1/50 volumes 5 M sodium chloride and 2 volumes 100% ethanol was added to the harvested supernatant and incubated at -80°C for 1 hour. The samples were then centrifuged at 13000 rpm for 10 minutes at 4°C, washed with 70% ethanol, dried and the pellet was resuspended in 50 µl DEPC-treated nuclease-free water.

This RNA isolation protocol resulted in the isolation of total RNA as well as genomic DNA. Therefore, for the selective isolation of RNA, the samples were then precipitated at -80°C overnight with 3 M lithium chloride (Manning 1991). Lithium chloride precipitation aids in the removal of genomic DNA and the smaller tRNA. In order to ensure complete elimination of the contaminating genomic DNA prior to cDNA synthesis, the samples were DNase treated with the TURBO DNA-*free*™ Kit (Ambion®). To 10 µl RNA, 0.1 volumes 10X TURBO DNase Buffer and 1 µl TURBO DNase were added. This suspension was mixed gently and then incubated at 37°C for 30 minutes. After incubation, 2 µl of resuspended DNase Inactivation Reagent was added. This suspension was then mixed well and incubated for 5 minutes at 25°C, with occasional mixing. This was followed by centrifugation at 10 000 x g for 1.5 minutes in order to pellet the genomic DNA. The resultant RNA that was harvested in the supernatant was free of contaminating genomic DNA. This kit subsequently removes the DNase and divalent cations from the RNA samples so that the RNA is suitable for cDNA synthesis.

The RNA then served as a template for first strand cDNA synthesis. First-strand cDNA was synthesized with the RevertAid™ Reverse Transcriptase kit (Fermentas). For the synthesis of first-strand cDNA, 2 µl of 10 µM gene-specific forward primer was added to 10.5 µl of template RNA, mixed gently and incubated at 65°C for 5 minutes in order to eliminate any secondary structures. The reaction was then cooled on ice. This was followed by the addition of 4 µl 5x Reaction Buffer, 0.5 µl RiboLock™ RNase Inhibitor, 2 µl 10 mM dNTP mix and 1 µl RevertAid™ Reverse Transcriptase. The reaction was then mixed gently and incubated at 42°C for 60 minutes. This resulted

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in first-strand cDNA which was then used as a template in the synthesis of second strand cDNA.

3.3.5 Second Strand cDNA Synthesis (PCR Amplification)

A search of NCBI database (http://www.ncbi.nlm.nih.gov/; Sayers et al. 2009) revealed three different *A. fumigatus* endoxylanase sequences *xynf10a, xynf11a* and *xynG1*. Primers were designed and synthesized (Integrated DNA Technologies) based on these three *A. fumigatus* sequences. The primers, as well as their sequences, which were used in this study are listed in table 3.2. The restriction enzyme sites included in the primers are indicated in bold. The restriction enzyme site for *Pac*I is TTAATTAA and the restriction enzyme site for *Asc*I is GGCGCGCC. For the purpose of this study, the xylanase genes obtained from these gene-specific primers will be referred to as F10, F11 and G1. The primers which were used for the sequencing of the xylanase genes (pJET1.2F and pJET1.2R), as well as the primers used for the initial identification of the *A. fumigatus* isolate (ITS1 and ITS4), have also been included in table 3.2.

Table 3.2: Primers, as well as their respective sequences, which were used in this study

Primer	Sequence (5' – 3')	Length (nt)
Xynf10aF	GACT**TTAATTAA**ATGGTCGTCCTCAGCAAGCTCGTCAG	38
Xynf10aR	GACT**GGCGCGCC**TCAGAGAGCAGCAATGATGGC	33
Xynf11aF	GACT**TTAATTAA**ATGGTTTCTTTCTCCTACCTGCTGCTGG	40
Xynf11aR	GACT**GGCGCGCC**CTAGTAGACAGTGATGGAAGC	33
XynG1F	GACT**TTAATTAA**ATGGTCTCATTCTCTTCTCTCGTTCTCGC	41
XynG1R	GACT**GGCGCGCC**CTAAGAAACAGTGATAGTAGC	33
pJET1.2F	CGACTCACTATAGGGAGAGCGGC	23
pJET1.2R	AAGAACATCGATTTTCCATGGCAG	24
ITS1	TCCGTAGGTGAACCTTGCGG	20
ITS4	TCCTCCGCTTATTGATATGC	20

The restriction enzyme sites included in the primers are indicated in bold.

Second-strand cDNA was synthesized according to the Phusion® High Fidelity PCR Master Mix (Finnzymes) protocol. The PCR reaction mixture was as follows: 2 µl first-strand cDNA, 18 µl milliQ water, 2.5 µl 10 µM forward primer, 2.5 µl 10 µM

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reverse primer and 25 µl 2X Phusion Master Mix. Phusion is a high fidelity DNA polymerase which generates long templates with high accuracy and speed, making this enzyme ideal for cloning. PCR was carried out in a MultiGene thermal cycler (Labnet International, Inc.). The PCR cycling conditions were as follows: initial denaturation at 98°C for 30 seconds, denaturation at 98°C for 10 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds, followed by final extension at 72°C for 10 minutes. The denaturation, annealing and extension steps were repeated for 30 cycles.

The resultant PCR products were loaded on a 0.8% agarose gel. After electrophoresis, the gel was visualised and the relevant bands were excised and purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research), according to the manufacturer's instructions. The purified DNA was then ready for subsequent cloning steps.

3.3.6 Cloning of the Xylanase Genes

Purified PCR products were ligated into the multiple cloning site of the pJET1.2/blunt cloning vector with the CloneJET™ PCR Cloning Kit (Fermentas). The ligation reaction was as follows: 10 µl 2X Reaction Buffer, 3 µl purified PCR product, 1 µl pJET1.2/blunt cloning vector, 5 µl nuclease free water and 1 µl T4 DNA Ligase. This ligation reaction was incubated at 22°C for 10 minutes. The recombinant plasmids were then chemically transformed into *E. coli* DH5α with heat shock. The frozen chemically competent cells of *E. coli* DH5α were thawed on ice for 15 minutes. The 20 µl ligation reaction was then added to 80 µl of the competent cells and incubated on ice for 30 minutes. The cells were heat shocked at 37°C for 10 minutes. The transformation reaction (100 µl) was plated out on Luria-Bertani ampicillin agar plates and incubated at 37°C overnight. Transformants were obtained and plasmid DNA was isolated with the High Pure Plasmid Isolation kit (Roche), according to the manufacturer's instructions. The plasmid DNA was then digested with the restriction enzyme *Bgl*II (Fermentas) in order to confirm the presence and size of the genes. Two clones of

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each gene were sent for sequencing. Both F11 and G1 were sequenced at the Central Analytical Facility (CAF) at Stellenbosch University. The larger gene, F10, was sent to Inqaba Biotechnologies for sequencing. The primers which were used for all sequencing reactions were pJET1.2F and pJET1.2R and the complete open reading frames were obtained.

For expression of the xylanase genes in *S. cerevisiae*, the genes were subcloned into the episomal shuttle vector pDLG125, which has the *Pac*I and *Asc*I cloning sites. For the preparation of the vector pDLG125, plasmid DNA was isolated from overnight cultures with the High Pure Plasmid Isolation kit (Roche), according to the manufacturer's instructions. The vector pDLG125 was then digested with *Pac*I and *Asc*I (New England Biolabs). The digested plasmid DNA was then separated on a 0.8% agarose gel and the relevant 6000 bp band, representative of the pDLG125 backbone, was excised and purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). The linearised pDLG125 backbone vector was then ready for subcloning of the xylanase genes from the pJET1.2/blunt cloning vector.

For subcloning, the pJET1.2/blunt cloning vectors containing the xylanase genes were digested with *Pac*I and *Asc*I and the xylanase genes were gel purified, as described above. The xylanase genes were then ligated into the multiple cloning site of pDLG125. These genes were, therefore, under control of the inducible *ENO* promoter and terminator. T4 DNA Ligase was used for the ligation reaction which was incubated at 16°C for 5 hours. This was followed by chemical transformation into *E. coli* DH5α.

Both positive and negative controls were included in all subsequent experiments. The positive control was the pDLG125 backbone plasmid with a *Trichoderma reesei* endoxylanase gene (*xyn2*) (La Grange et al. 1996). The positive control was also subcloned so that it was under exactly the same backbone plasmid, pDLG125, as the xylanase genes obtained in this study. The negative control was the pDLG125 backbone plasmid without a xylanase gene.

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3.3.7 Transformation of *S. cerevisiae*

The recombinant plasmids were transformed into *S. cerevisiae* NI-C-D4 by a heat shock method that uses lithium acetate, Salm sperm DNA and PEG-4000. A colony of *S. cerevisiae* NI-C-D4 was inoculated into 5 ml YPD broth and grown at 30°C overnight. The cells (1.5 ml) were centrifuged at 8000 rpm for 10 seconds. The supernatant was discarded and the yeast cells were washed with 1 ml distilled water and centrifuged at 8000 rpm for 5 seconds. The cells were then resuspended in 100 µl 1X TEL Buffer and the reaction was incubated at 30°C for 20 minutes. This was followed by the addition of 10 µl 10 mg/ml Salm sperm DNA and 10 µg plasmid DNA which was incubated at 30°C for 20 minutes. After this incubation period, 560 µl 50% PEG-4000 and 140 µl 5X TEL Buffer (12.75 g LiOAc, 2.5 ml 0.5 M EDTA and 2.5 ml 1 M Tris made up to 250 ml) was added, resuspended and incubated at 30°C for 20 minutes. The reaction was then heat shocked at 42°C for 15 minutes followed by centrifugation for 10 seconds. The cells were washed with 1 ml distilled water, followed by another 10 second centrifugation step. The cells were then resuspended in 100 µl of milliQ water and the transformation reactions were plated out onto SC^{-}_{URA} agar plates supplemented with 2% glucose and 0.1% RBB-xylan in order to screen for xylanase activity. The results obtained were compared to that of the positive and negative controls transformations.

3.3.8 SDS-PAGE and Zymogram Analysis

Extracellular protein fractions were isolated by harvesting supernatant of the recombinant proteins that were cultivated in selective double strength buffered SC^{-}_{URA} medium. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out by using the method described by Laemmli (1970). The proteins were separated on a 10% (v/v) SDS-PAGE gel. To 20 µl of each protein sample, 7 µl loading buffer [60 mM Tris-HCl (pH 6.8), 25% (w/v) glycerol, 2% (w/v) SDS, 14 mM β-Mercaptoethanol and 0.1% (w/v) Bromophenol Blue] was added and boiled for 3 minutes at 100°C. The boiled protein samples were then loaded onto the 10% SDS-PAGE gel and electrophoresis was carried out at 200 V. Upon completion of electrophoresis, the gel

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was silver stained. The gel was incubated overnight in fixing solution [50% (v/v) methanol, 10% (v/v) acetic acid, 100 mM ammonium acetate], washed with water and incubated in 0.005% sodium thiosulphate for 20 minutes. The gel was then incubated with 0,1% silver nitrate for 20 minutes, washed with distilled water and then incubated in developer [0.036% (w/v) formaldehyde, 2% (w/v) sodium carbonate] until bands became visible. Hereafter, the gel was incubated in 50 mM EDTA for 60 minutes, washed with distilled water and then photographed. The PageRuler^{TM} unstained protein ladder (Fermentas) was used for protein size determination.

For zymogram analysis to detect xylanase activity, 10% SDS-polyacrylamide gels containing 0.2% (w/v) Beechwood xylan were used. The supernatant of the protein samples was concentrated 3X through the Vivaspin 15 polyethersulfone columns (10 000 MWCO). To 20 µl of the concentrated supernatant, 7 µl β-Mercaptoethanol-free loading buffer [125 mM Tris-HCl (pH 6.8), 15% (w/v) sucrose, 2.5% (w/v) SDS and 0.02% (w/v) bromophenol blue] was added. The resuspended protein samples were incubated at 80°C for 3 minutes and then loaded onto the gels. Electrophoresis was carried out at 200 V. The PageRuler^{TM} prestained protein ladder (Fermentas) was used to determine the protein size. The proteins were renatured after electrophoresis by incubating the gels in 0.1 M sodium acetate buffer (pH 5) containing 1% (v/v) deionized Triton-X-100 for 2 hours at room temperature. The buffer was replaced every 30 minutes during the 2 hour incubation period. The gels were then incubated in fresh 0.1 M sodium acetate/Triton X-100 buffer at 50°C for 2 hours. The gels were then stained with 0.1% (w/v) Congo Red for 15 minutes and destained with 1 M sodium chloride for 1 hour in order to visualise protein bands with xylanase activity.

3.3.9 Enzyme Activity Assays

Growth curves were carried out along with enzyme assays in order to determine if the recombinant xylanase genes had an effect on the growth of the yeast and to determine when the enzyme was produced. The experiment was done in triplicate in

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125 ml Erlenmeyer flasks containing 25 ml SC^{-URA} media and inoculated to OD_{600nm} 0.1 from overnight precultures. The flasks were incubated on a rotary shaker at 200 rpm at 30°C. Cultures were left to grow for 8 hours during their lag phase. Thereafter, samples were taken every 2 hours during the logarithmic phase of growth and OD values at 600nm were measured. The experiment was conducted for 120 hours. Enzyme activity was measured along with the growth curve every 12 hours. Xylanase activity was assayed according to Bailey and co-workers (Bailey et al. 1992). Appropriate dilutions of the supernatant were made in 50 mM citrate buffer (pH 5.0) with 1% Beechwood xylan (Roth) as the substrate. The reaction was incubated at 50°C for 5 minutes. The amount of released reducing sugars was determined by the addition of dinitrosalicylic acid (DNS). The absorbance was measured at 540 nm. One nanokatal (nkat) of enzyme activity is defined as the amount of enzyme required to catalyse the conversion of 1 nmole of substrate per second under the experimental conditions. Enzyme activity was normalised with dry cell weight.

3.3.10 Characterisation of the Recombinant Xylanases

The temperature optimum of the recombinant xylanases was determined by measuring the relative activity at different temperatures ranging from 20°C to 90°C. The activity was assayed as described above, at the respective temperatures, for 5 minutes. The maximum activity was expressed as 100% and was used as a reference to determine the relative activity of the different reactions. The pH optimum was also determined by measuring the relative activity over a wide range of pH, using 50 mM citrate (pH 3.0), 50 mM phosphate-citrate (pH 4.0 to 7.0) and 50 mM phosphate (pH 8.0) buffers. Thermostability of the xylanases was determined by incubating the enzymes at the optimal temperature, as well as 10°C above and below this optimum for 60 minutes. The activity was assayed at the optimal temperature and pH for 5 minutes, as described above, and relative activity was determined.

Substrate specificity was determined for the recombinant xylanases. A range of substrates was used for the enzyme assays, including Beechwood xylan, oats spelts

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xylan, tobacco stalks xylan, carboxymethyl cellulose (CMC), *p*-nitrophenyl-β-D-xylanopyranoside (*p*NPX), *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) and locust bean gum (Sigma-Aldrich). Beechwood xylan, oats spelts xylan and tobacco stalks xylan were used to detect xylanase activity. CMC was the substrate used to detect endoglucanase activity. The substrates *p*NPX and *p*NPG were used to determine β-xylosidase and β-glucosidase activities, respectively. Locust bean gum was used to detect mannanase activity. All reactions were conducted in triplicate. Xylanase, endoglucanase and mannanase activity was assayed as previously described and the released reducing sugars were determined with 3,5-dinitrosalicylic acid (Bailey et al. 1992). For β-xylosidase and β-glucosidase activities, 10 µl supernatant was added to 1 µl 0.005 M substrate (*p*NPX or *p*NPG, dissolved in dimethylformamide) and 89 µl 0.05 M citrate buffer (pH 5). This reaction was incubated at 50°C for 10 minutes. The reaction was stopped by adding 1 M sodium carbonate. The *p*NP released from the substrates was detected at an absorbance of 405 nm. Protein concentrations were determined with the DC Protein Assay kit (Bio-Rad) which is based on the method described by Lowry and co-workers (Lowry et al. 1951). Enzyme activity was expressed as nkat/mg protein.

Synergistic assays were performed in order to determine if there was a synergistic effect between the different xylanases. Equal concentrations of proteins were used in this experiment, as determined by the DC Protein Assay kit (Bio-Rad). Hereafter the synergistic and additive effect of different combinations of xylanases was tested. Xylanase activity was assayed in triplicate as previously described (Bailey et al. 1992) and expressed as nkat/mg protein. The four different combinations used in this study were as follows: F10 and F11, F10 and G1, F11 and G1 and F10 and F11 and G1. When 2 different combinations of enzymes were used, then half the volume of each supernatant was added to the synergistic assay i.e. for F10 and F11 half volume F10 and half volume F11 was used. When 3 different combinations of enzymes were used, then a third of the volume of each supernatant was added to the synergistic assay i.e. for F10 and F11 and G1 a third volume F10, a third volume F11 and a third

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volume G1 was used. In order to determine the additive activities of the individual supernatants so that it could be compared to the synergistic activities, each enzyme was added to a volume of citrate buffer. When 2 different combinations of enzymes were used, then half the volume of the supernatant and half the volume of the citrate buffer were added i.e. to determine the additive activity of F10 and F11, half volume of F10 and half volume of citrate buffer was used for the assay and the activity was determined. Half volume of F11 and half volume of citrate buffer were also used and the activity was determined. These activities were then added together to determine the additive activity of F10 and F11. When 3 different combinations of enzymes were used, then a third of the volume of the supernatant and two-thirds of the volume of citrate buffer was added i.e. to determine the additive activity of F10 and F11 and G1, a third volume of F10 and two-thirds volume of citrate buffer was used for the assay and the activity was determined. A third volume of F11 and two-thirds volume of citrate buffer was used for the assay and the activity was determined. A third volume of G1 and two-thirds volume of citrate buffer was used for the assay and the activity was determined. These three activities were then added together to determine the additive activity of F10, F11 and G1. The synergistic activities were compared to the additive activities in order to determine if the enzymes have a significant synergistic effect or if the obtained activity is just a result of the additive effect of the individual enzyme's activities.

3.4 Results

3.4.1 Initial Enzyme Characterisation and Preliminary Identification of *A. fumigatus*

As depicted in Figure 3.1, the fungal isolate *A. fumigatus* Trit15F1, displayed significantly high xylanase activity after the 7 day period when grown on triticale as the sole carbon source. The xylanase activity obtained by this fungal isolate was significantly higher than that obtained by *T. reesei* RutC30, when grown on both triticale and potato dextrose.

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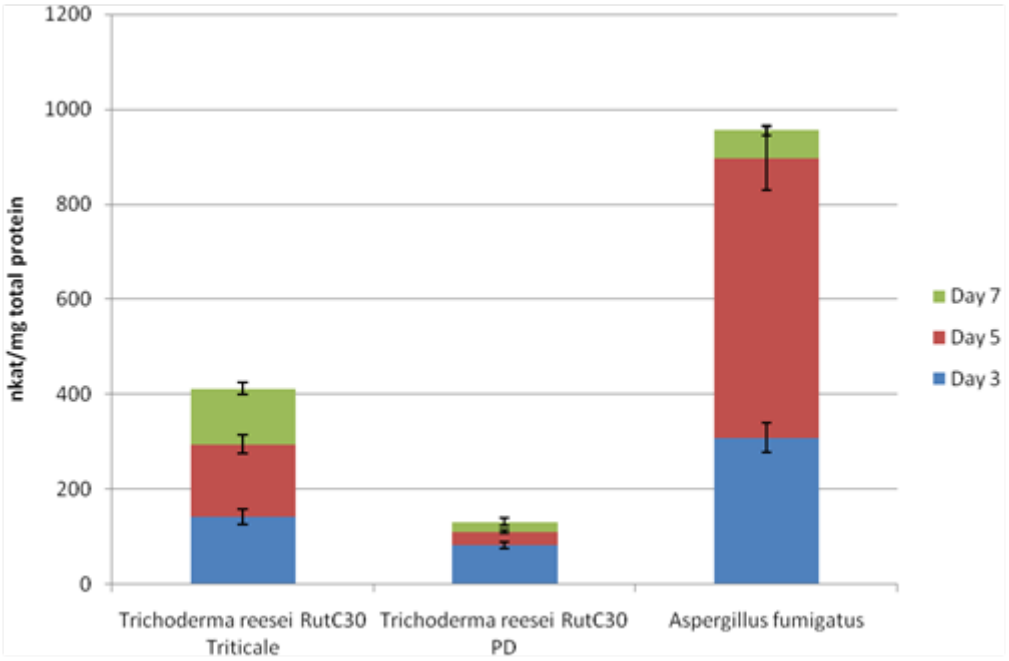

Figure 3.1: Xylanase activity, expressed as nkat/mg protein, of the fungal isolate *A. fumigatus* Trit15F1 when cultivated on 1% triticale bran for 7 days. The measured activity was compared to that of *T. reesei* RutC30 cultivated on both 1% triticale bran and potato dextrose (PD).

The ±600 bp of the amplified ITS region was used for peliminary identification and showed 98% homology to the available internal transcribed spacer region sequence of *A. fumigatus*. The fungal isolate Trit15F1 was therefore, preliminarily identified as *A. fumigatus*.

3.4.2 cDNA Cloning of the Xylanase Genes in *E. coli*

Three xylanases genes, F10, F11 and G1, were successfully cloned by ligating the purified, intron-free cDNA PCR products into the multiple cloning site of the pJET1.2/blunt cloning vector. The recombinant plasmids were transformed into *E. coli* and the clones were sequenced.

Figure 3.2 - 3.4 represents the nucleotide and deduced amino acid sequences of the cDNA clones of the xylanase genes F10, F11 and G1. Genomic DNA of the xylanase genes were also obtained and sequenced in order to make a comparison with their corresponding cDNA sequences to determine the size and position of the introns in the open reading frames of the genomic sequences. Figure 3.5 – 3.7 represents the

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alignment of the cDNA sequence (top strand) and the genomic sequence (bottom strand) for F10, F11 and G1. The position of the introns in the genomic sequences of the xylanase genes are highlighted in yellow. Figure 3.8 – 3.10 represents the alignment of the amino acids of the cDNA sequences (top strand) and the amino acid sequences that are available on NCBI (bottom strand) which were used to design the gene specific primers used in this study. This alignment indicates the percentage identity of our obtained gene sequences with the gene sequences that are already published in the NCBI database. Table 3.3 is a summary of all of the sequencing results of the three different xylanase genes, F10, F11 and G1 as depicted in figures 3.2 – 3.10.

Table 3.3: A summary of the sequencing results of both the cDNA and genomic DNA of the xylanase genes F10, F11 and G1 (aa = amino acids)

	F10	**F11**	**G1**
Length of cDNA	978 bp (325 aa)	687 bp (228 aa)	666 bp (221 aa)
Molecular Weight of cDNA	35 163 Da	24 449 Da	23 766 Da
Length of Genomic DNA	1400 bp	739 bp	712 bp
Number of Introns in Genomic DNA	8	1	1
Length/Length Range of Introns	49-57 bp	52 bp	46 bp
% Homology to aa Sequences on NCBI	100%	100%	99.55% (1 aa change). Accession no.: ADM47839

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1          ATGGTCGTCCTCAGCAAGCTCGTCAGCAGCATTYTCTTTGTCTCCCTGGTTTCGGCGGGC
1           M  V  V  L  S  K  L  V  S  S  I  X  F  V  S  L  V  S  A  G

61         GTGATCGAAGAACGCCAGGCAGCCAGCATCAACCAGGCGTTTACCTCCCATGGCAAGAAG
21          V  I  E  E  R  Q  A  A  S  I  N  Q  A  F  T  S  H  G  K  K

121        TACTTTGGCACCGCCAGTGACCAAGCTCTGCTCCAGAAGTCGCAGAATGAGGCCATTGTG
41          Y  F  G  T  A  S  D  Q  A  L  L  Q  K  S  Q  N  E  A  I  V

181        CGCAAAGACTTTGGCCAGCTGACGCCGGAGAATAGCATGAAGTGGGATGCGACTGAGCCA
61          R  K  D  F  G  Q  L  T  P  E  N  S  M  K  W  D  A  T  E  P

241        TCGCAAGGAAGATTCAACTTCGCTGGTGCTGATTTCCTGGTCAACTATGCAAAACAGAAT
81          S  Q  G  R  F  N  F  A  G  A  D  F  L  V  N  Y  A  K  Q  N

301        GGCAAGAAGGTCCGCGGACACACCTTAGTCTGGCACTCCCAACTCCCGTCCTGGGTGTCG
101         G  K  K  V  R  G  H  T  L  V  W  H  S  Q  L  P  S  W  V  S

361        GCTATCAGCGACAAAAACACCCTGACCTCGGTGCTGAAGAACCACATCACCACCGTCATG
121         A  I  S  D  K  N  T  L  T  S  V  L  K  N  H  I  T  T  V  M

421        ACCCGGTACAAGGGCCAGATCTACGCCTGGGACGTCGTCAACGAGATCTTCAACGAGGAC
141         T  R  Y  K  G  Q  I  Y  A  W  D  V  V  N  E  I  F  N  E  D

481        GGCTCCCTCCGCGACAGCGTCTTCTCCCGCGTGCTGGGCGAGGACTTTGTGCGGATTGCC
161         G  S  L  R  D  S  V  F  S  R  V  L  G  E  D  F  V  R  I  A

541        TTCGAGACGGCGCGCTCTGTGGATCCCTCGGCGAAGCTGTACATCAACGATTACAATCTC
181         F  E  T  A  R  S  V  D  P  S  A  K  L  Y  I  N  D  Y  N  L

601        GACTCGGCTAGCTATGGCAAAACCCAGGGGATGGTGAGATATGTCAAGAAGTGGCTGGCT
201         D  S  A  S  Y  G  K  T  Q  G  M  V  R  Y  V  K  K  W  L  A

661        GCGGGCATTCCTATCGATGGAATCGGCACTCAAACCCACCTTGGTGCGGGTGCTTCGTCC
221         A  G  I  P  I  D  G  I  G  T  Q  T  H  L  G  A  G  A  S  S

721        AGCGTCAAAGGAGCATTGACTGCTCTTGCGTCTTCCGGCGTCTCTGAGGTCGCCATTACC
241         S  V  K  G  A  L  T  A  L  A  S  S  G  V  S  E  V  A  I  T

781        GAGCTGGATATCGCGGGTGCGAGCTCCCAGGACTACGTCAATGTCGTCAAGGCATGCCTG
261         E  L  D  I  A  G  A  S  S  Q  D  Y  V  N  V  V  K  A  C  L

841        GATGTCCCCAAGTGTGTGGGAATCACCGTCTGGGGGGGTGTCGGACAGGGACTCGTGGCGC
281         D  V  P  K  C  V  G  I  T  V  W  G  V  S  D  R  D  S  W  R

901        TCCGGCTCGTCTCCGCTGCTGTTCGACAGCAACTACCAGCCCAAGGCGGCGTATAATGCC
301         S  G  S  S  P  L  L  F  D  S  N  Y  Q  P  K  A  A  Y  N  A

961        ATCATTGCTGCTCTCTGA
321         I  I  A  A  L  *
```

Figure 3.2 This figure represents the cDNA nucleotide and deduced amino acid sequence of the xylanase gene F10

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```
1          ATGGTTTCTTTCTCCTACCTGCTGCTGGCGTGCTCCGCCATTGGAGCTCTGGCTGCCCCC
1           M  V  S  F  S  Y  L  L  L  A  C  S  A  I  G  A  L  A  A  P

61         GTCGAACCCGAGACCACCTCGTTCAATGAGACTGCTCTTCATGAGTTCGCTGAGCGCGCC
21          V  E  P  E  T  T  S  F  N  E  T  A  L  H  E  F  A  E  R  A

121        GGCACCCCAAGCTCCACCGGCTGGAACAACGGCTACTACTACTCCTTCTGGACTGATGGC
41          G  T  P  S  S  T  G  W  N  N  G  Y  Y  Y  S  F  W  T  D  G

181        GGCGGCGACGTGACCTACACCAATGGCGCCGGTGGCTCGTACTCCGTCAACTGGAGGAAC
61          G  G  D  V  T  Y  T  N  G  A  G  G  S  Y  S  V  N  W  R  N

241        GTGGGCAACTTTGTCGGTGGAAAGGGCTGGAACCCTGGAAGCGCTAGAACCATCAACTAC
81          V  G  N  F  V  G  G  K  G  W  N  P  G  S  A  R  T  I  N  Y

301        GGAGGCAGCTTCAACCCCAGCGGCAATGGCTACCTGGCTGTCTACGGCTGGACCACCAAC
101         G  G  S  F  N  P  S  G  N  G  Y  L  A  V  Y  G  W  T  T  N

361        CCCTTGATTGAGTACTACGTTGTTGAGTCGTATGGTACATACAACCCCGGCAGCGGCGGT
121         P  L  I  E  Y  Y  V  V  E  S  Y  G  T  Y  N  P  G  S  G  G

421        ACCTTCAGGGGCACTGTCAACACCGACGGTGGCACTTACAACATCTACACGGCCGTTCGC
141         T  F  R  G  T  V  N  T  D  G  G  T  Y  N  I  Y  T  A  V  R

481        TACAATGCTCCCTCCATCGAAGGCACCAAGACCTTCACCCAGTACTGGTCTGTGCGCACC
161         Y  N  A  P  S  I  E  G  T  K  T  F  T  Q  Y  W  S  V  R  T

541        TCCAAGCGTACCGGCGGCACTGTCACCATGGCCAACCACTTCAACGCCTGGAGCAGACTG
181         S  K  R  T  G  G  T  V  T  M  A  N  H  F  N  A  W  S  R  L

601        GGCATGAACCTGGGAACTCACAACTACCAGATTGTCGCCACTGAGGGTTACCAGAGCAGC
201         G  M  N  L  G  T  H  N  Y  Q  I  V  A  T  E  G  Y  Q  S  S

661        GGATCTGCTTCCATCACTGTCTACTAG
221         G  S  A  S  I  T  V  Y  *
```

Figure 3.3: This figure represents the cDNA nucleotide and deduced amino acid sequence of the xylanase gene F11

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```
1          ATGGTCTCATTCTCTTCTCTCGTTCTCGCTGCCTCCACCGTTGCTGGCGTGCTAGCTACA
1           M  V  S  F  S  S  L  V  L  A  A  S  T  V  A  G  V  L  A  T

61         CCCGGCTCGGAGCAATACGTTGAGCTAGCCAAGCGGCAGCTCACCAGCTCTCAGACTGGC
21          P  G  S  E  Q  Y  V  E  L  A  K  R  Q  L  T  S  S  Q  T  G

121        ACGAATAACGGCTACTACTACTCCTTCTGGACCGACGGCGGCGGCCAGGTGACCTACACC
41          T  N  N  G  Y  Y  Y  S  F  W  T  D  G  G  G  Q  V  T  Y  T

181        AACGGCAATGGCGGCCAGTATCAGGTCGACTGGAACAACTGCGGCAACTTTGTTGCTGGG
61          N  G  N  G  G  Q  Y  Q  V  D  W  N  N  C  G  N  F  V  A  G

241        AAGGGCTGGAACCCGGCCAGCGAGAAAGCGGTCACCTACAGCGGCTCCTGGCAGACCAGC
81          K  G  W  N  P  A  S  E  K  A  V  T  Y  S  G  S  W  Q  T  S

301        GGAAACGGCTACCTCTCCGTGTACGGCTGGACGACCAGTCCGCTGGTCGAATTCTACATC
101         G  N  G  Y  L  S  V  Y  G  W  T  T  S  P  L  V  E  F  Y  I

361        GTGGAGAGTTACGGCTCCTATGACCCCTCCACGGGAGCCACCCATCTCGGCACCGTCGAG
121         V  E  S  Y  G  S  Y  D  P  S  T  G  A  T  H  L  G  T  V  E

421        AGCGACGGGGCCACGTACAACCTCTACAAGACGACGCGGACGAATGCGCCGTCCATCCAG
141         S  D  G  A  T  Y  N  L  Y  K  T  T  R  T  N  A  P  S  I  Q

481        GGCACGGCTACTTTTGACCAGTACTGGTCGGTTCGGACTTCGCACCGGCAGAGTGGAACT
161         G  T  A  T  F  D  Q  Y  W  S  V  R  T  S  H  R  Q  S  G  T

541        GTGACGACGAAGAACCACTTTGATGCGTGGAGAAATGCGGGTCTGCAATTGGGGAACTTT
181         V  T  T  K  N  H  F  D  A  W  R  N  A  G  L  Q  L  G  N  F

601        GACTATATGATTGTTGCGACGGAGGGGTACCAGAGCAGCGGCTCTGCTACTATCACTGTT
201         D  Y  M  I  V  A  T  E  G  Y  Q  S  S  G  S  A  T  I  T  V

661        TCTTAG
221         S  *
```

Figure 3.4: This figure represents the cDNA nucleotide and deduced amino acid sequence of the xylanase gene G1

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```
ATG GTC GTC CTC AGC AAG CTC GTC AGC AGC ATT CTC TTT GTC TCC CTG GTT TCG GCG
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GGC GTG ATC GAA GAA CGC CAG GCA GCC AGC ATC AAC CAG GCG TTT ACC TCC CAT GGC
GGC GTG ATC GAA GAA CGC CAG GCA GCC AGC ATC AAC CAG GCG TTT ACC TCC CAT GGC

AAG AAG TAC TTT GGC ACC GCC AGT GAC CAA GCT CTG CTC CAG AAG TCG CAG AAT GAG
AAG AAG TAC TTT GGC ACC GCC AGT GAC CAA GCT CTG CTC CAG AAG TCG CAG AAT GAG

GCC ATT GTG CGC AAA GAC TTT GGC CAG CTG ACG CCG GAG AAT AGC ATG AAG TGG GAT
GCC ATT GTG CGC AAA GAC TTT GGC CAG CTG ACG CCG GAG AAT AGC ATG AAG TGG GAT

GCG ACT GAG C ..................................................... CA
GCG ACT GAG C GTAGGTCTCTCGGCCACTGTGGCTGACGTTAACTTGTTGACATGACTGTCTGTGTAG CA

TCG CAA GGA AGA TTC AAC TTC GCT GGT GCT GAT TTC CTG GT ..................
TCG CAA GGA AGA TTC AAC TTC GCT GGT GCT GAT TTC CTG GT ATGCAATCTGCTCATCTCGG

............................. C AAC TAT GCA AAA CAG AAT GGC AAG AAG GTC
TCGAGCTCCTGCTGAAGGACAATAAATAGGT C AAC TAT GCA AAA CAG AAT GGC AAG AAG GTC

CGC GGA CAC ACC TTA G ..................................................
CGC GGA CAC ACC TTA G GTATTCATGCGCCCTCACGGCATTTCGAGGATACAGCCAAGCTGACAGTGTAG

TC TGG CAC TCC CAA CTC CCG TCC TGG GTG TCG GCT ATC AGC GAC AAA AAC ACC CTG
TC TGG CAC TCC CAA CTC CCG TCC TGG GTG TCG GCT ATC AGC GAC AAA AAC ACC CTG

ACC TCG GTG CTG AAG AAC CAC ATC ACC ACC GTC ATG ACC CGG TAC AAG GGC CAG ATC
ACC TCG GTG CTG AAG AAC CAC ATC ACC ACC GTC ATG ACC CGG TAC AAG GGC CAG ATC

TAC GCC TGG G ..................................................... AC GTC
TAC GCC TGG G TATTTTGCCCTCTATCCCACACAATGCCAGCCCCAGCTAATAGCTGCAAAGG AC GTC

GTC AAC GAG ATC TTC AAC GAG GAC GGC TCC CTC CGC GAC AGC GTC TTC TCC CGC GTG
GTC AAC GAG ATC TTC AAC GAG GAC GGC TCC CTC CGC GAC AGC GTC TTC TCC CGC GTG

CTG GGC GAG GAC TTT GTG CGG ATT GCC TTC GAG ACG GCG CGC TCT GTG GAT CCC TCG
CTG GGC GAG GAC TTT GTG CGG ATT GCC TTC GAG ACG GCG CGC TCT GTG GAT CCC TCG

GCG AAG CTG TAC ATC AAC GAT TAC AA ..................................
GCG AAG CTG TAC ATC AAC GAT TAC AA GTAAGCTTGTGGTTTTGTCGAGAGATGTACTCCGTCCTGG

.................T CTC GAC TCG GCT AGC TAT GGC AAA ACC CAG GGG ATG GTG AGA
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........................................... C ACT CAA ACC CAC CTT GGT GCG
GCGGAGCTGTGTGTGATGATTGTACGCTGACTCTTCCTGAAGG C ACT CAA ACC CAC CTT GGT GCG

GGT GCT TCG TCC AGC GTC AAA GGA .........................................
GGT GCT TCG TCC AGC GTC AAA GGA TAAGTCTCCTTGGTTTTCTTGCCTACGTAACGCTGACCCCCCG

...... GCA TTG ACT GCT CTT GCG TCT TCC GGC GTC TCT GAG GTC GCC ATT ACC GAG
TGTACA GCA TTG ACT GCT CTT GCG TCT TCC GGC GTC TCT GAG GTC GCC ATT ACC GAG

CTG GAT ATC GCG GGT GCG AGC TCC CAG GAC TAC GTC AAT GT ..................
CTG GAT ATC GCG GGT GCG AGC TCC CAG GAC TAC GTC AAT GT ATGTCTCCTGATTGCCAGTG

............................. C GTC AAG GCA TGC CTG GAT GTC CCC AAG TGT
GCAGGGTCATCGATACTAATAGAAACAGGT C GTC AAG GCA TGC CTG GAT GTC CCC AAG TGT
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GTG GGA ATC ACC GTC TGG GGG GTG TCG GAC AGG GAC TCG TGG CGC TCC GGC TCG TCT
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CCG CTG CTG TTC GAC AGC AAC TAC CAG CCC AAG GCG GCG TAT AAT GCC ATC ATT GCT
CCG CTG CTG TTC GAC AGC AAC TAC CAG CCC AAG GCG GCG TAT AAT GCC ATC ATT GCT

GCT CTC TGA
GCT CTC TGA
```

Figure 3.5: Alignment of cDNA (top strand) and genomic (bottom strand) sequences of the xylanase gene F10. The position and length of the introns in the genomic DNA are highlighted in yellow.

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```
ATG GTT TCT TTC TCC TAC CTG CTG CTG GCG TGC TCC GCC ATT GGA GCT CTG GCT GCC
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CCC GTC GAA CCC GAG ACC ACC TCG TTC AAT GAG ACT GCT CTT CAT GAG TTC GCT GAG
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CGC GCC GGC ACC CCA AGC TCC ACC GGC TGG AAC AAC GGC TAC TAC TAC TCC TTC TGG
CGC GCC GGC ACC CCA AGC TCC ACC GGC TGG AAC AAC GGC TAC TAC TAC TCC TTC TGG

ACT GAT GGC GGC GGC GAC GTG ACC TAC ACC AAT GGC GCC GGT GGC TCG TAC TCC GTC
ACT GAT GGC GGC GGC GAC GTG ACC TAC ACC AAT GGC GCC GGT GGC TCG TAC TCC GTC

AAC TGG AGG AAC GTG GGC AAC TTT GTC GGT GGA AAG GGC TGG AAC CCT GGA AGC GC
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.................................................... T AGA ACC ATC AAC TAC
TAGGTACCGAGCTTTGTCAACGTCGGATGTGCAGACCTGTGGCTGACAGAAG T AGA ACC ATC AAC TAC

GGA GGC AGC TTC AAC CCC AGC GGC AAT GGC TAC CTG GCT GTC TAC GGC TGG ACC ACC
GGA GGC AGC TTC AAC CCC AGC GGC AAT GGC TAC CTG GCT GTC TAC GGC TGG ACC ACC

AAC CCC TTG ATT GAG TAC TAC GTT GTT GAG TCG TAT GGT ACA TAC AAC CCC GGC AGC
AAC CCC TTG ATT GAG TAC TAC GTT GTT GAG TCG TAT GGT ACA TAC AAC CCC GGC AGC

GGC GGT ACC TTC AGG GGC ACT GTC AAC ACC GAC GGT GGC ACT TAC AAC ATC TAC ACG
GGC GGT ACC TTC AGG GGC ACT GTC AAC ACC GAC GGT GGC ACT TAC AAC ATC TAC ACG

GCC GTT CGC TAC AAT GCT CCC TCC ATC GAA GGC ACC AAG ACC TTC ACC CAG TAC TGG
GCC GTT CGC TAC AAT GCT CCC TCC ATC GAA GGC ACC AAG ACC TTC ACC CAG TAC TGG

TCT GTG CGC ACC TCC AAG CGT ACC GGC GGC ACT GTC ACC ATG GCC AAC CAC TTC AAC
TCT GTG CGC ACC TCC AAG CGT ACC GGC GGC ACT GTC ACC ATG GCC AAC CAC TTC AAC

GCC TGG AGC AGA CTG GGC ATG AAC CTG GGA ACT CAC AAC TAC CAG ATT GTC GCC ACT
GCC TGG AGC AGA CTG GGC ATG AAC CTG GGA ACT CAC AAC TAC CAG ATT GTC GCC ACT

GAG GGT TAC CAG AGC AGC GGA TCT GCT TCC ATC ACT GTC TAC TAG
GAG GGT TAC CAG AGC AGC GGA TCT GCT TCC ATC ACT GTC TAC TAG
```

Figure 3.6: Alignment of cDNA (top strand) and genomic (bottom strand) sequences of the xylanase gene F11. The position and length of the intron in the genomic DNA is highlighted in yellow.

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```
ATG GTC TCA TTC TCT TCT CTC GTT CTC GCT GCC TCC ACC GTT GCT GGC GTG CTA GCT
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ACA CCC GGC TCG GAG CAA TAC GTT GAG CTA GCC AAG CGG CAG CTC ACC AGC TCT CAG
ACA CCC GGC TCG GAG CAA TAC GTT GAG CTA GCC AAG CGG CAG CTC ACC AGC TCT CAG

ACT GGC ACG AAT AAC GGC TAC TAC TAC TCC TTC TGG ACC GAC GGC GGC GGC CAG GTG
ACT GGC ACG AAT AAC GGC TAC TAC TAC TCC TTC TGG ACC GAC GGC GGC GGC CAG GTG

ACC TAC ACC AAC GGC AAT GGC GGC CAG TAT CAG GTC GAC TGG AAC AAC TGC GGC AAC
ACC TAC ACC AAC GGC AAT GGC GGC CAG TAT CAG GTC GAC TGG AAC AAC TGC GGC AAC

TTT GTT GCT GGG AAG GGC TGG AAC CCG GCC AGC GAG AA ......................
TTT GTT GCT GGG AAG GGC TGG AAC CCG GCC AGC GAG AA GTATGCGTCCTCTCCCTGCTTGTT

...................... A GCG GTC ACC TAC AGC GGC TCC TGG CAG ACC AGC GGA
AGGTTCAAGCTAATGGATTCAG A GCG GTC ACC TAC AGC GGC TCC TGG CAG ACC AGC GGA

AAC GGC TAC CTC TCC GTG TAC GGC TGG ACG ACC AGT CCG CTG GTC GAA TTC TAC ATC
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ATC CAG GGC ACG GCT ACT TTT GAC CAG TAC TGG TCG GTT CGG ACT TCG CAC CGG CAG
ATC CAG GGC ACG GCT ACT TTT GAC CAG TAC TGG TCG GTT CGG ACT TCG CAC CGG CAG

AGT GGA ACT GTG ACG ACG AAG AAC CAC TTT GAT GCG TGG AGA AAT GCG GGT CTG CAA
AGT GGA ACT GTG ACG ACG AAG AAC CAC TTT GAT GCG TGG AGA AAT GCG GGT CTG CAA

TTG GGG AAC TTT GAC TAT ATG ATT GTT GCG ACG GAG GGG TAC CAG AGC AGC GGC TCT
TTG GGG AAC TTT GAC TAT ATG ATT GTT GCG ACG GAG GGG TAC CAG AGC AGC GGC TCT

GCT ACT ATC ACT GTT TCT TAG
GCT ACT ATC ACT GTT TCT TAG
```

Figure 3.7: Alignment of cDNA (top strand) and genomic (bottom strand) sequences of the xylanase gene G1. The position and length of the intron in the genomic DNA is highlighted in yellow.

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Figure 3.8: Alignment of the cDNA amino acid sequence (top strand) and the available amino acid sequence on NCBI (bottom strand) for the xylanase gene F10 (accession number for the available sequence on NCBI is ABN48479). The native signal peptide is indicated by the blue arrow.

Figure 3.9: Alignment of the cDNA amino acid sequence (top strand) and the available amino acid sequence on NCBI (bottom strand) for the xylanase gene F11 (accession number for the available sequence on NCBI is ABN48478). The native signal peptide is indicated by the blue arrow.

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Figure 3.10: Alignment of the cDNA amino acid sequence (top strand) and the available amino acid sequence on NCBI (bottom strand) for the xylanase gene G1 (accession number for the available sequence on NCBI is ADM47839). The amino acid change is highlighted in red with the native signal peptide indicated by the blue arrow.

3.4.3 Expression of the Recombinant Xylanases in *S. cerevisiae*

For expression of the recombinant xylanase genes in *S. cerevisiae*, the genes were subcloned from pJET1.2/blunt into pDLG125 which contains the *ENO*1 promoter and terminator. The cloned xylanase genes were, therefore, under control of the strong constitutive *ENO*1 promoter and terminator. The recombinant plasmids were transformed into *S. cerevisiae* NI-C-D4. The yeast colonies were transferred onto selective SC medium which contained RBB-xylan in order to determine the ability of the recombinant yeasts to degrade xylan (Fig. 3.11). All three xylanase genes showed clearing zones on the RBB-xylan plates. The positive control, a *Trichoderma reesei* endoxylanase gene (*xyn2*), also showed clearing zones, as was expected. For comparative purposes, the positive control was under the same backbone plasmid (pDLG125) as the three xylanase genes in this study and was also transformed in *S. cerevisiae* NI-C-D4. The negative control contained the pDLG125 backbone plasmid (i.e. no xylanase gene). As expected, the negative control did not show a clearing zone. Clearing zones obtained by all three xylanase genes, F10, F11 and G1, as well as the positive control, indicates the degradation of RBB xylan. Therefore, all of the recombinant xylanase genes, as well as the positive control, are functional xylanases, due to their ability to hydrolyse xylan.

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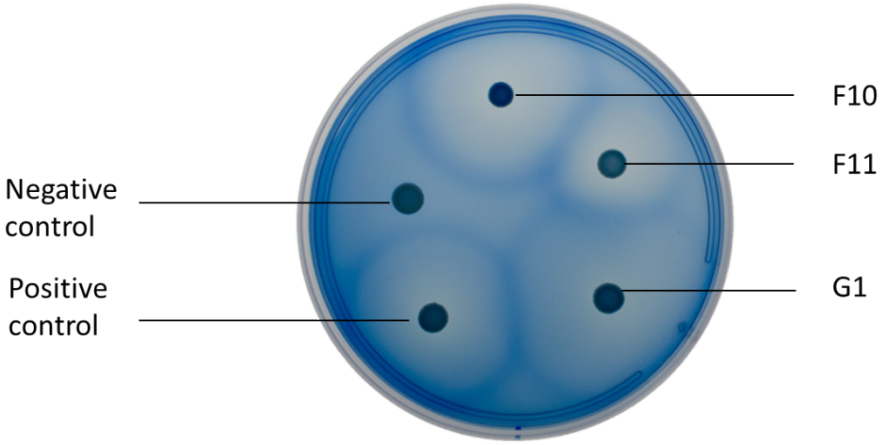

Figure 3.11: Yeast colonies of xylanase genes F10, F11 and G1, as well as the positive and negative controls. The corresponding zones around the yeast colonies indicate xylanase activity.

3.4.4 SDS-PAGE and Zymogram Analysis of the Expressed Proteins

SDS-PAGE analysis was used to determine the sizes of the proteins F10, F11 and G1. As depicted in Fig. 3.12, F10 had a pronounced protein band of ~ 30 kDa and both F11 and G1 had a protein band of ~15 kDa, while F11 also showed a pronounced smear of between 80 and 120 kDa. The positive control had a protein band of ~21 kDa. Table 3.4 displays the expected sizes and the obtained sizes of the different proteins. An interesting phenomenon was observed for the SDS-PAGE gel analysis. The protein bands on the SDS-PAGE gel were smaller for all three xylanases (F10, F11 and G1) than their expected sizes. For F11 and G1, the obtained proteins were almost half of their expected size as determined from the amino acid sequences. The positive control was, however, the same size as was expected. Repetition of the SDS-PAGE gel yielded identical results.

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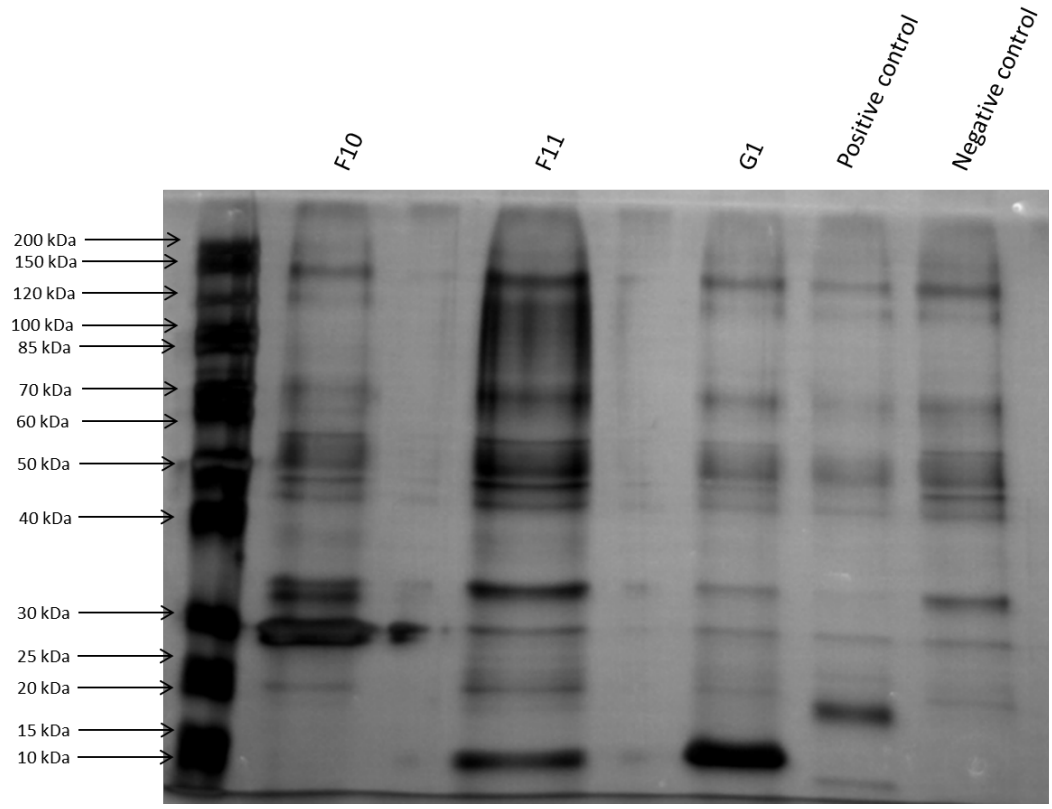

Figure 3.12: SDS-PAGE gel of the xylanase proteins F10, F11 and G1 expressed in *S. cerevisiae*, as well as the positive and negative controls. The PageRuler™ unstained protein ladder (Fermentas) was used for size determination.

Table 3.4: Expected and obtained sizes of the different proteins.

Proteins	**Expected Size**	**Obtained Size**
F10	35 kDa	~30 kDa
F11	24 kDa	~15 kDa + 85-120kDa
G1	24 kDa	~15 kDa
Positive Control	21 kDa	~21 kDa

Zymogram analysis of SDS-PAGE gels confirms if the proteins were active as well as the protein sizes (Fig. 3.13). Although protein sizes were smaller than expected, zymogram analysis showed that enzymes were still active. F10 yielded a strong activity band at ~30 kDa which corresponds to the protein band size on the

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SDS-PAGE gel. However, there are also less intense other activity bands on this gel. This could be due to dimers or trimers or different glycosylated forms of the protein. F11 did not show an activity band at the size range of ~15 kDa as depicted on the SDS-PAGE gel. Instead this F11 protein activity was present as a smear of hyperglycosylated protein larger than 100 kDa. G1 displayed activity throughout the path of the protein. This could also be due to hyperglycosylation. The positive control displayed a distinct activity band at the expected size of ~21 kDa. The positive control, similarly to F11, also showed a smear of hyperglycosylated protein larger than 100 kDa. The negative control showed no band of activity, as is expected.

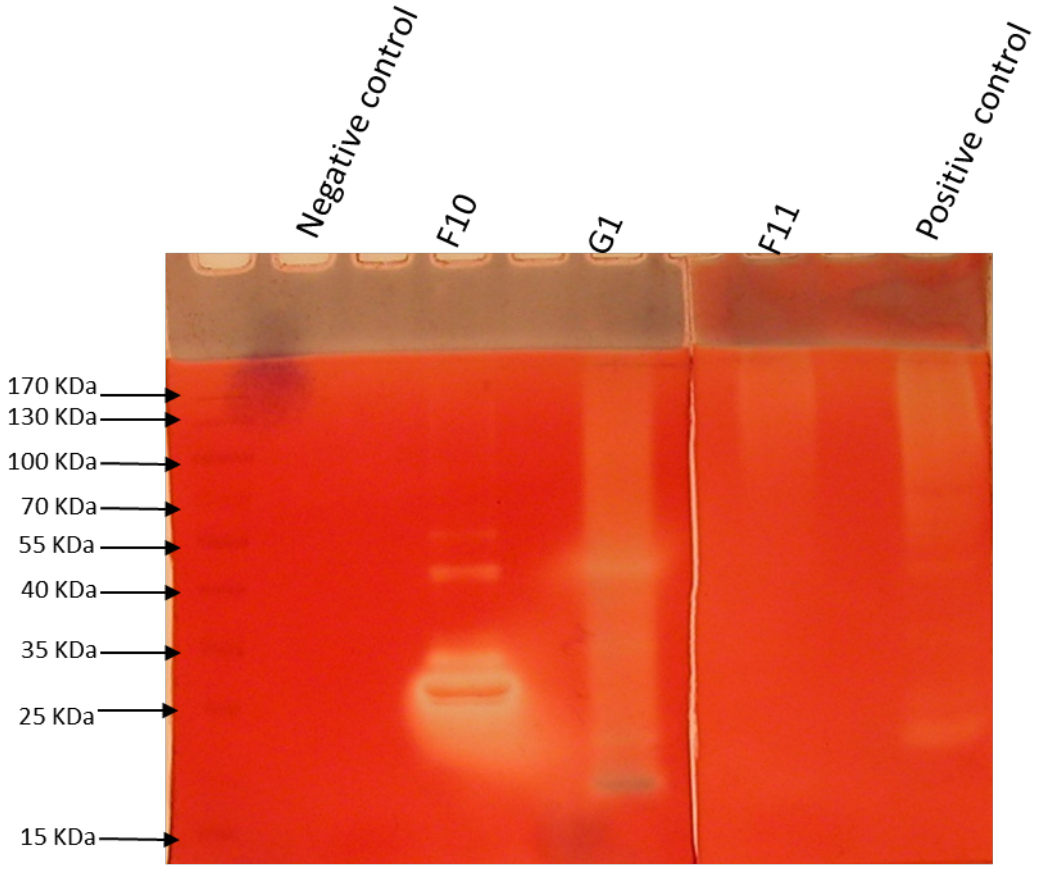

Figure 3.13: Zymogram analysis of F10, F11, G1 and the positive and negative controls.

3.4.5 Endoxylanase Activity of the Recombinant Xylanases

Xylanase activity was studied, along with the growth of the yeast transformants, under shake flask conditions over a 120 hour time period. Yeasts expressing both F10

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and F11 had the same growth trend as the positive and negative controls, reaching a maximum optical density (OD_{600nm}) of just less than 4 after 120 hours (Fig. 3.14). Therefore, these recombinant genes did not have an effect on the growth of the yeast. The yeast expressing G1, on the other hand, had a significantly higher growth trend than the positive and negative controls, reaching a maximum optical density (OD_{600nm}) of just more than 6 after 120 hours. The OD values were therefore ~1.5X higher than the OD values for the other recombinant yeasts and controls. The yeast expressing G1 started to show significantly higher growth than the controls after 20 hours already. Therefore, G1 was the only recombinant gene which had an effect on the growth of the yeast. For all three genes, as well as the positive and negative controls, the yeasts displayed maximum optical density after ~ 28 hours, whereafter they entered stationary phase. After 48 hours, a sharp increase in growth was observed in all of the yeasts. This phenomenon was assumed to be due to the production of ethanol which the yeast used as an energy source.

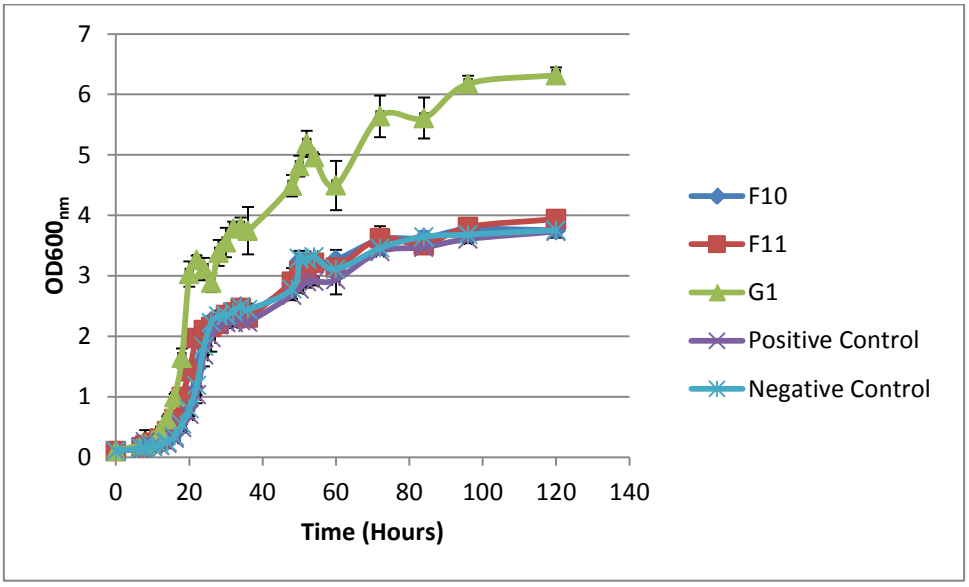

Figure 3.14: Growth curves of the recombinant yeasts, expressing xylanases F10, F11 and G1 as well as the positive and negative controls over 120 hours in order to determine if the recombinant xylanases had an effect on the growth of the yeast.

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Endoxylanase activity was monitored along with the growth curve over the 120 hour period and expressed in nkat/mg dry cell weight (DCW) (Fig. 3.15). Maximum xylanase activity was detected after about 84 hours of growth for all of the genes and the positive control. The negative control, which did not contain a xylanase gene, displayed no endoxylanase activity, as was expected. F10 and G1 had similar xylanase activity levels, reaching maximum activity of ~300 nkat/mg DCW. The endoxylanase activity profiles of F11 and the positive control were similar, with maximum xylanase activity of ~100 nkat/mg DCW. Therefore, 2 of the recombinant genes (F10 and G1) produced significantly higher endoxylanase activity compared to the positive control.

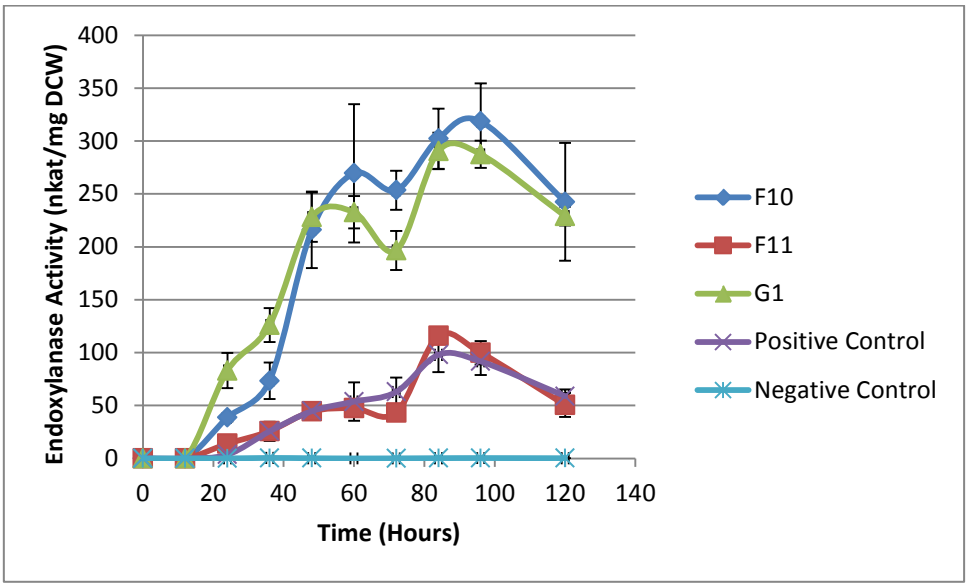

Figure 3.15: Endoxylanase activities of recombinant xylanases F10, F11 and G1, expressed in nkat/mg DCW, in comparison to the controls over a 120 hour time period.

3.4.6 Characterisation of the Recombinant Xylanases

The temperature optima of F10 and G1 were in the range of 50°C to 60°C. F11 had a distinct temperature optimum at 60°C (Fig. 3.16).

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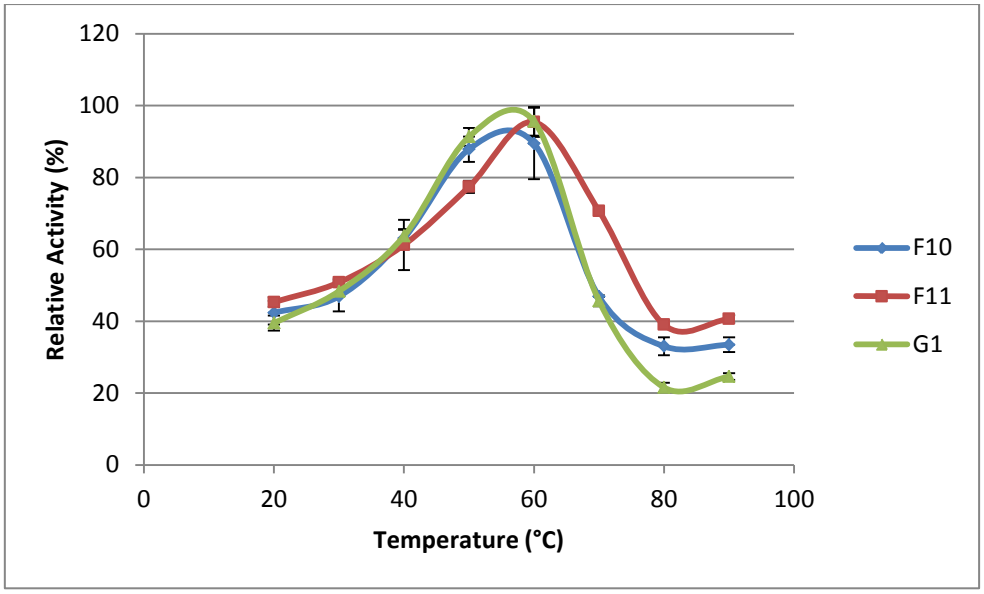

Figure 3.16: Temperature optima of the recombinant xylanases F10, F11 and G1 expressed as % relative activity.

Thermostability of the xylanases was determined at 50°C, 60°C and 70°C (Fig. 3.17). Two of the recombinant xylanases, F10 and F11, were more thermostable at 50°C and not at their temperature optimum of 60°C. F10 and F11 retained ~ 80% of its activity after the 60 minute incubation period at 50°C. The recombinant xylanase G1 was the least thermotolerant, retaining only ~ 50% of its activity after the 60 minute incubation period at 50°C.

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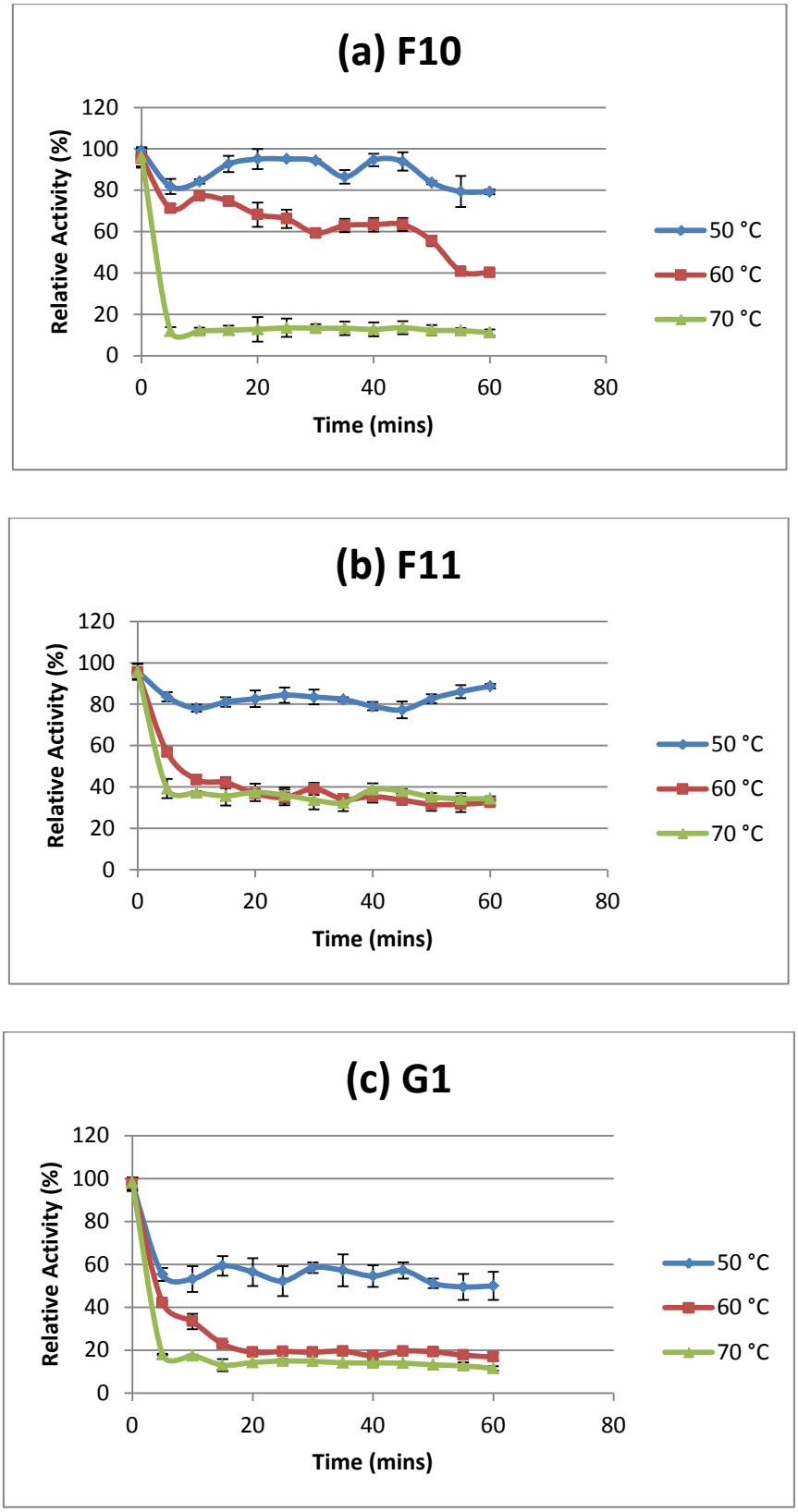

Figure 3.17: Thermostability of the recombinant xylanases (a) F10, (b) F11 and (c) G1 at 50°C, 60°C and 70°C over a 60 minute incubation period. Thermostability was expressed as % relative activity.

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The optimum pH of F10 was pH 6. F11 had a pH optima range between pH 4 and 6. G1 had an optimal pH of 5 (Fig. 3.18).

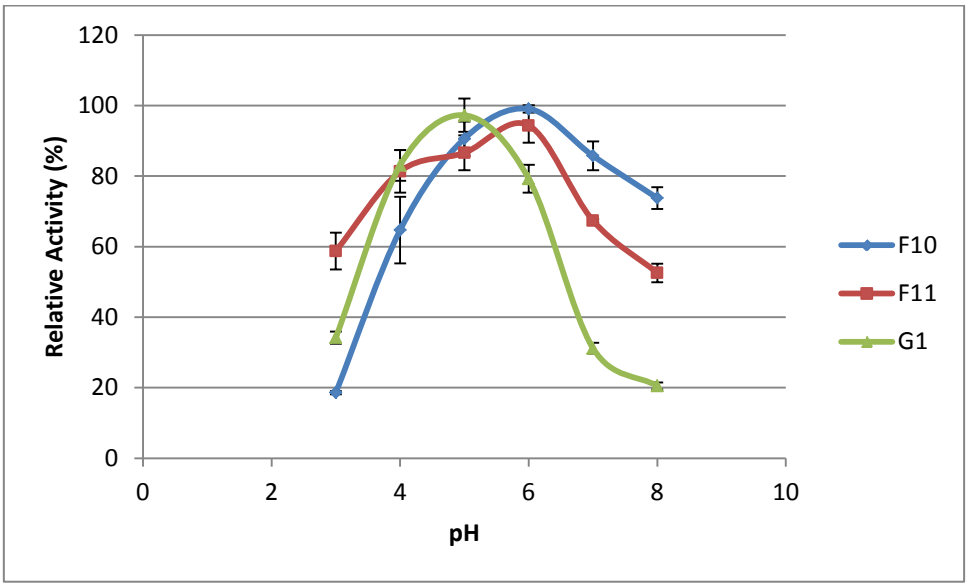

Figure 3.18: pH optima of recombinant xylanases F10, F11 and G1 expressed as % relative activity over the pH range 3-8.

All three xylanases did not have any detectable endoglucanase, β-xylosidase, β-glucosidase or mannanase activities when CMC, *p*NPX, *p*NPG and Locust Bean Gum was used as substrates, respectively (data not shown). Xylanase activity was, however detected on all three of the different xylan substrates used in this study i.e. Beechwood, Oats Spelts and Tobacco Stalks xylan (Table 3.5). For all three of the xylanases, the lowest enzyme activity was detected on Oats Spelts xylan. In both F10 and F11, the highest activity was detected on Tobacco Stalks xylan and the second highest activity was detected on Beechwood xylan. G1 displayed very similar activities on Tobacco Stalks and Beechwood xylan. All three of the xylan substrates supported significant xylanase activity.

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Table 3.5: Enzyme activity, expressed in nkat/mg protein, of F10, F11 and G1 on different xylan substrates.

Xylanases	**Beechwood xylan**	**Oats Spelts Xylan**	**Tobacco Stalks Xylan**
F10	1128.8 ± 109.7	1009.9 ± 105.1	1435.2 ± 234.8
F11	209.3 ± 19.1	189.1 ± 14.1	317.7 ± 35.9
G1	7537.3 ± 483.3	6263.0 ± 682.7	7676.5 ± 701.6

Table 3.6 represents the synergistic and additive activities of all three xylanases, expressed as nkat/mg protein. The synergistic activity is the activity of the combinations of enzymes used in the assay. The additive activity is the sum of the activities of the individual enzymes used in the assay. If the synergistic activity is significantly larger than the additive activity, then there is a synergistic effect between the enzymes. In all of the different combination scenarios, the synergistic activity was not significantly larger than the additive activity of each of the individual enzymes. Therefore, there was no synergistic effect between the three different xylanases.

Table 3.6: Synergistic and additive activities, expressed in nkat/mg protein, of various combinations of the enzymes

Xylanases	**Synergistic Activity**	**Additive Activity**
F10 and F11	9434.0 ± 1073.7	11613.3 ± 921.5
F10 and G1	27875.3 ± 2224.3	29300.6 ± 2596.2
F11 and G1	22910.9 ± 2036.6	22840.8 ± 1949.7
F10 and F11 and G1	23580.6 ± 314.5	21579.2 ± 2420.0

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3.5 Discussion

The fungal isolate Trit15F1 was isolated from a triticale compost heap and was found to display significantly high xylanase activity when compared to *T. reesei* RutC30. This fungal isolate was preliminarily identified as *A. fumigatus* based on the ITS region sequence. It should be noted that a preliminary identification of the fungal isolate was deemed sufficient for this study with no further detailed phylogenetic analysis or morphological characterisation of this isolate conducted as part of this study to confirm the isolate identity.

Three xylanase genes (F10, F11 and G1) isolated from *A. fumigatus* were cloned in *E. coli* and the resultant clones were sequenced. Sequencing results revealed that F10 was the largest gene, with F11 and G1 both being significantly smaller. F10 belongs to glycosyl hydrolase family 10, whereas both F11 and G1 belongs to glycosyl hydrolase family 11. GH family 10 xylanases consist of high molecular mass endoxylanases. GH family 11 xylanases, on the other hand, consists of low molecular mass endoxylanases (Biely et al. 1997). F10 had 8 introns, whereas both F11 and G1 contained only 1 intron. GH family 11 xylanases usually have 1 to 2 introns in their open reading frames. Examples of GH family 11 xylanases includes xyl2 of *Cochliobolus sativus* which contains 2 introns of 53 and 57 bp (Emami and Hack 2001), *Aureobasidium pullulans xynA* contains 1 intron of 59 bp (Li and Ljungdahl 1994) and *xyn8* from *Fusarium* sp. contains 2 introns of 56 and 55 bp (Xie et al. 2012). Family 10 xylanases usually contain more than 2 introns. Examples of the introns present in GH family 10 genes includes *Penicillium funiculosum* xylanase (*xynD*) which contains 3 introns (Furniss et al. 2005), *Aspergillus kawachii* xylanase (*xynA*) which contains 9 introns (Ito et al. 1992) and *Aspergillus usamii* xylanase (*AuXyn10A*) which also contains 9 introns (Wang et al. 2011). The DNA sequences were compared to the sequences available on the NCBI database. The amino acid sequences of clones F10 and F11 was 100% identical to endoxylanase amino acid sequences (accession number: EF375874.1 and EF375873.1, respectively) from *A. fumigatus* strain MKU1 on the NCBI database. The amino acid sequence of G1 was 99.55%

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identical to the sequence on NCBI database (accession number: GQ458016.1), differing by only one amino acid. Although all three of these xylanase gene sequences are publically available on NCBI, there is no published report on the functional characterisation of two of these xylanases (F10 and G1). F11 is the exception as it has been expressed in *Pichia pastoris* and the recombinant xylanase has been characterised (Jeya et al. 2009). Therefore, this study aimed to characterise all of the recombinant xylanases, F10, F11 and G1, expressed in the yeast host, *S. cerevisiae*.

Yeast can be easily genetically manipulated, require simple media to grow rapidly to a high cell density and are ideal eukaryotic host organisms for the production of high levels of secreted proteins (Romanos et al. 1992). *P. pastoris* and *S. cerevisiae* are the two most commonly used yeasts for the expression of recombinant proteins (Cereghino and Cregg 2000; Romanos et al. 1992). *S. cerevisiae* was the choice of yeast host for all three xylanase genes in this study. *S. cerevisiae* has several properties which makes it an attractive host for the expression of foreign proteins, such as xylanases. This yeast allows for post-translational processing such as glycosylation, protein folding and proteolysis. *S. cerevisiae* can be cultivated on relatively cheap media and does not need xylan to induce for xylanase production. *S. cerevisiae* is an ideal host for the production of xylanases as it cannot degrade cellulose or xylan. Therefore, xylanases produced by this yeast are pure as they do not contain contaminating cellulases, which is particularly important in the paper industry. *S. cerevisiae* only secretes a few proteins in low abundance. Therefore, a secreted heterologous protein will be most prominent in the medium. *S. cerevisiae* is a well-established industrial organism which has a high fermentation rate and ethanol tolerance, as well as complete GRAS status (Romanos et al. 1992, La Grange et al. 1996; Ahmed et al. 2009). Examples of xylanase genes from *Aspergillus* species which have already been expressed in *S. cerevisiae*, includes *Aspergillus kawachii xyn3* (Crous et al. 1995), *Aspergillus nidulans xlnA and xlnB* (Pérez-González et al. 1996)

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and *Aspergillus niger xyn4* and *xyn5* (Luttig et al. 1997). Prior to this study, *A. fumigatus* xylanase genes had not been expressed in *S. cerevisiae*.

The proteins were recombinantly expressed in *S. cerevisiae* with their native secretion signal. All of the xylanase genes obtained in this study were functional after being expressed in *S. cerevisiae* as they all had the ability to degrade xylan. The size of the proteins which were expressed in *S. cerevisiae* was determined with SDS-PAGE analysis. These proteins were found to be smaller than their expected size as determined from the amino acid sequences. A similar result was found by Shin and co-workers where a xylanase gene expressed in *Escherichia coli* DH5α was 34 kDa instead of the expected size of 59 kDa (Shin et al. 2002). A 52 kDa endo-β-1,4-glucanase was extracellularly produced as 33 kDa in *Bacillus megaterium* (Ahn et al. 1993). A 52 kDa endo-β-1,4-glucanase from *Bacillus subtilis* was expressed in *Escherichia coli* and reduced to 34.5 kDa (Hoon et al. 1995). It was suggested that these decreases in protein sizes could be due to proteolytic cleavage (Shin et al. 2002; Ahn et al. 1993; Hoon et al. 1995). Zymogram analysis yielded a strong activity band at ~ 30kDa for F10, which corresponds to the size of F10 on the SDS-PAGE gel. However, other activity bands were also visible on the zymogram which could be due to dimers, trimers or different glycosylated forms of the protein. F11 only showed a smear of hyperglycosylated protein larger than 100 kDa, while the 15 kDa band observed showed no endoxylanase activity. The positive control also had this hyperglycosylated protein smear on the top of the zymogram. However, the positive control also showed a distinct activity band at the expected size of ~21 kDa. G1 showed smears of activity throughout the path of the protein on the zymogram which leads us to believe that G1 is hyperglycosylated. These zymogram results indicating hyperglycosylation and possible dimers/trimers is another possible contributing fact to the proteins being smaller than their expected sizes.

The high level expression of a foreign gene can be a significant metabolic burden to the host cell. The growth rate can, therefore, be reduced and the efficiency of gene

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expression can be affected. The expression of some genes can be toxic to the yeast (Romanos et al. 1992). Growth curves revealed that G1 was the only recombinant gene which had an enhanced effect on the growth of the yeast. This recombinant yeast grew to a significantly higher cell density than the other recombinant yeasts, as well as the positive and negative control. Cultures were regularly examined microscopically for contamination and were reported as clear of contamination. Currently, an explanation for this interesting phenomenon of the recombinant yeast, G1, enhancing total biomass production has not been determined. Volumetrically, G1 had the highest endoxylanase activity. However, endoxylanase activity was normalised with dry cell weight (Fig. 3.15). Therefore, the normalised activity of G1 was lower than the volumetric activity due to the higher growth rate of G1. The normalised data showed that G1 and F10 had similar enzyme activity levels. Both of these genes had significantly higher endoxylanase activity levels than the positive control. The endoxylanase activity of F11 was in the same range as the positive control. All of these recombinant xylanase genes are incredibly promising in terms of enzyme production levels. The low yield and high cost of the enzymatic hydrolysis of lignocellulose to reducing sugars is the main challenge currently facing the use of lignocellulosic biomass in biofuels production (Bhat and Bhat 1997; Sun and Cheng 2002; Rojo 2008). All of the enzymes obtained in this study displayed significantly high enzyme activity and may be promising candidates for the production of biofuels.

F10 and G1 displayed displayed maximum endoxylanase activity between 50°C and 60°C. F11 displayed maximum xylanase activity at 60 °C. All three of the xylanases were most stable at 50°C, with F10 and F11 being the most thermostable xylanases. The same result for temperature optima and thermostability were obtained by La Grange and co-workers for the *Trichoderma reesei* β-xylanase gene (*xyn2*) (La Grange et al. 1996). When F11 was expressed in *Pichia pastoris*, Jeya and co-workers reported that this enzyme had an optimum temperature of 60°C and was also more stable at 50°C than 60°C (Jeya et al. 2009). The same results were observed for *Aspergillus*

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kawachii endoxylanase gene (*xyn3*) (Crous et al. 1995) as well as the xylanase (X-I) which was purified from *Aspergillus sojae* (Kimura et al. 1995). An incubation temperature of 50°C is the standard condition used for xylanase assays of mesophilic fungi (Bailey et al. 1992). The xylanases obtained in this study were relatively stable at the standard assay temperature of 50°C. However, because xylanases can become unstable over a long incubation period, the standard incubation time for endoxylanase assays is only 5 minutes (Bailey et al. 1992).

F10 displayed pH optima of 6, F11 displayed pH optima range of pH 4 to pH 6 and G1 displayed pH optima of 5. La Grange and co-workers reported that the *Trichoderma reesei xyn2* also has a pH optimum of 6 (La Grange et al. 1996). When F11 was expressed in *Pichia pastoris*, it also had a pH optimum of 6 (Jeya et al. 2009). The optimal pH of most fungal xylanases is between pH 4 and 6 (Fujimoto et al. 1995; Kimura et al. 1995; Luttig et al. 1997; Xie et al. 2012; Tanaka et al. 2005; Kimura et al. 2000; Tanaka et al. 2006). This explains why pH 5.3 was selected as the standard reaction condition for mesophilic fungal xylanase assays (Bailey et al. 1992). The pH optimas of 5 and 6 obtained in this study correspond to the pH optima of other xylanases from fungal origin which have already been characterised.

Substrate specificity studies revealed that all of the recombinant xylanases displayed highest specificity towards xylan, with no activity being detected on other substrates i.e. CMC, pNPX, pNPG and locust bean gum. Xylanase activity was detected on Beechwood, Oats Spelts and Tobacco Stalks xylan, with the lowest xylanase activity being detected on Oats Spelts xylan. Substrate specificity was also studied by Jeya and co-workers of F11 expressed in *Pichia Pastoris*. This enzyme was also only specific for xylan, and displayed no activity on pNPX and CMC. Interestingly, when F11 was expressed in *Pichia pastoris*, xylanase activity was higher on Oats Spelts xylan than Beechwood xylan (Jeya et al. 2009). This can be explained by the fact that different yeast backgrounds yield different results. This confirms the importance of expressing genes in different yeast hosts for various applications. F11 and G1 belong

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to GH family 11, which consists solely of endo-1,4-β-xylanases (Henrissat 1991) which are only active on xylan based substrates (Collins et al. 2005). Other examples of family 11 xylanases which are only active on xylan include a xylanase purified from *Acrophialophora nainiana* (Salles et al. 2000) and from *Fusarium oxysporum* (Christakopoulos et al. 1996). F10 on the other hand belongs to GH family 10. This family of enzymes consists of endo-1,4-β-xylanases, endo-1,3-β-xylanases and cellobiohydrolases (Henrissat 1991), of which endo-1,4-β-xylanases are the most dominant (Collins et al. 2005). Family 10 xylanases display lower substrate specificity than xylanases belonging to family 11 (Biely et al. 1997). Substrate specificity studies have revealed that GH family 10 endo-1,4-β-xylanases may also be active on cellulosic substrates (Gilkes et al. 1991). Therefore, unlike xylanases belonging to GH family 11, these enzymes may not be entirely specific for xylan. A GH family 10 xylanase was purified from *Aureobasidium pullulans* and found to hydrolyse xylan, with weak activity visible on pN*P*-β-D-cellobioside and pN*P*-β-D-xylopyranoside. This enzyme had no activity on carboxymethyl cellulose and pN*P*-α-L-arabinofuranoside (Tanaka et al. 2006). The recombinant enzymes obtained in this study were all only able to hydrolyse xylan, suggesting that they are all endoxylanases. The inability of these enzymes to degrade cellulose, as well as the fact that *S. cerevisiae* cannot degrade cellulose, is advantageous in the bleaching of pulps as purified cellulose is needed for this process (Tanaka et al. 2006; Jeya et al. 2009; La Grange et al. 1996). Therefore, the recombinant enzymes obtained in this study may be very valuable to the pulp and paper industry.

Filamentous fungi, like many microorganisms, produce multiple xylanases (Wong et al. 1988). Therefore, it was not surprising that three xylanases were obtained from *A. fumigatus* in this study. Three xylanases have also been purified from *Aspergillus aculeatus* (Fujimoto et al. 1995), *Aspergillus awamori* (Kormelink et al. 1993), *Aspergillus kawachii* (Ito et al. 1992) and *Fusarium oxysporum (*Ruiz-Roldán et al. 1999; Gómez-Gómez et al. 2001). Two xylanases were purified from *Aspergillus sojae* (Kimura et al. 1995) and *Aspergillus niger* (Luttig et al. 1997). Four xylanases have

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been identified in *Aureobasidium pullulans* (Li and Ljungdahl 1994). Synergistic assays were performed in this study in order to determine if there is any synergistic effect between these three xylanases that *A. fumigatus* produces. These results revealed that there is no significant synergy between the three xylanases. Therefore, the degradation of xylan is not enhanced by the combined effect of the different xylanases. The efficient degradation of polysaccharides requires synergistic interaction between the polysaccharide degrading enzymes (De Vries and Visser 2001). Xylan is a complex, highly branched heteropolysaccharide. Hardwood xylan is substituted by 4-*O*-methyl glucuronic acid and acetic acid, whereas softwood xylan is substituted by 4-*O*-methyl glucuronic acid and α-arabinofuranoside units (Biely 1985). Therefore, the synergistic interaction of xylan degradation is usually between a main-chain cleaving enzyme and one or more accessory enzymes (De Vries and Visser 2001). The xylanases obtained in this study are all main-chain cleaving enzymes and, therefore, the absence of synergy between these enzymes was not unusual. There are numerous examples of synergistic interactions between enzymes which degrade plant cell wall polysaccharides, such as xylan. Synergy has been reported between endo-(1,4)-β-xylanase, (1,4)-β-xylosidase, (1,4)-β-D-arabinoxylan arabinofuranohydrolase and an acetyl xylan esterase in the degradation of different xylans (Kormelink and Voragen 1993). These enzymes were all purified from *Aspergillus awamorii* (Kormelink et al. 1991; Kormelink et al. 1993), with the exception of acetyl xylan esterase which was purified from *Aspergillus niger* (Kormelink et al. 1993b). Bartolome and co-workers reported the synergistic effect between endoxylanases and feruloyl esterase, as the addition of endoxylanases increased the release of ferulic acid from xylan (Bartolome et al. 1995). There are numerous other studies on the synergistic effect of the enzymes involved in xylan degradation (De Vries et al. 2000; Gasparic et al. 1995; Vardakou et al. 2004; Faulds et al. 2006; Bachmann and McCarthy, 1991).

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3.6 Conclusion

Three xylanase genes from *A. fumigatus* were cloned and expressed in *S. cerevisiae* for the first time during this study. This is the first record of the functional characterisation of two of the xylanase genes. The xylanase genes were all found to be functional upon expression in the yeast host and proteins were detected on SDS-PAGE gels and zymograms. None of the genes had a toxic effect on the growth of the yeast. However, one of the genes seemed to have an enhanced effect on the yeast's growth. High enzyme activity levels, ~ 300 nkat/mg DCW (F10 and G1) and ~ 100 nkat/mg DCW (F11), were obtained in this study and two of the xylanase genes displayed significantly higher activity than the positive control. All three of the xylanases seemed to display activity over a wide range of temperatures and pH. The above mentioned favourable characteristics of these enzymes make them incredibly attractive to the biofuels industry. These xylanases are true endoxylanases as they are only able to hydrolyse xylan. The inability of these enzymes to degrade cellulose makes them extremely valuable to the pulp and paper industry. Future research could involve doing further synergistic studies by combining these xylanases with other xylan-degrading enzymes and accessory enzymes in order to see if xylan degradation is enhanced.

3.7 References

Ahmed S, Riaz S, Jamil A (2009) Molecular cloning of fungal xylanases: an overview. Appl Microbiol Biotechnol 84:19-35

Ahn DH, Kim H, *Pac*k MY (1993) Cleavage of *Bacillus subtilis* endo-β-1,4-glucanase by *B-megaterium* protease. Biotechnol Lett 15:127-132

Bachmann SL, McCarthy AJ (1991) Purification and cooperative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca*. Appl Environ Microbiol 57:2121-2130

Bailey MJ, Biely P, Poutanen K (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23:257-270

Bartolome B, Faulds CB, Tuohy M, Hazlewood GP, Gilbert HJ, Williamson G (1995) Influence of different xylanases on the activity of ferulic acid esterase on wheat bran. Biotechnol Appl Bioc 22:65-73

Bhat MK, Bhat S (1997) Cellulose degrading enzymes and their potential industrial applications. Biotechnol Adv 15:583-620

Biely P (1985) Microbial xylanolytic systems. Trends Biotechnol 3:286-290

Stellenbosch University http://scholar.sun.ac.za

Biely P, Vršanská M, Tenkanen M, Kluepfel D (1997) Endo-β-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57:151-166

Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24:45-66

Christakopoulos P, Nerinckx W, Kekos D, Macris B, Claeyssens M (1996) Purification and characterisation of two low molecular mass alkaline xylanases from *Fusarium oxysporum* F3. J Biotechnol 51:181-189

Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3-23

Crous JM, Pretorius IS, van Zyl WH (1995) Cloning and expression of an *Aspergillus kawachii* endo-1,4-β-xylanase gene in *Saccharomyces cerevisiaE. c*urr Genet 28:467-473

Dashtban M, Schraft H, Qin W (2009) Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. Int J Biol Sci 5:578-595

De Vries RP, Kester HCM, Poulsen CH, Benen JAE, Visser J (2000) Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharide*S. c*arb Res 327:401-410

De Vries RP, Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol Molec Biol Rev 65:497-522

Emami K, Hack E (2001) Characterisation of a xylanase gene from *Cochliobolus sativus* and its expression. Mycol Res 105:352-359

Faulds CB, Mandalari G, Lo Curto RB, Bisignano G, Christakopoulos P, Waldron KW (2006) Synergy between xylanases from glycoside hydrolase family 10 and family 11 and a feruloyl esterase in the release of phenolic acids from cereal arabinoxylan. Appl Microbiol Biotechnol 71:622-629

Fujimoto H, Ooi T, Wang S-L, Takizawa T, Hidaka H, Murao S, Arai M (1995) Purification and properties of three xylanases from *Aspergillus aculeatus*. Biosci Biotech Biochem 59:538-540

Furniss CSM, Williamson G, Kroon PA (2005) The substrate specificity and susceptibility to wheat inhibitor proteins of *Penicillium funiculosum* xylanases from a commercial enzyme preparation. J Sci Food Agric 85:574-582

Galbe M, Zacchi G (2002) A review of the production of ethanol from softwood. Appl Microbiol Biotechnol 59:618-628

Gasparic A, Martin J, Daniel AS, Flint HJ (1995) A xylan hydrolase gene cluster in *Prevotella ruminicola* $B_1$4: sequence relationships, synergistic interactions, and oxygen sensitivity of a novel enzyme with exoxylanase and beta-(1,4)-xylosidase activities. Appl Environ Microbiol 61:2958-2964

Gilbert HJ, Hazlewood GP (1993) Bacterial cellulases and xylanases. J Gen Microbiol 139:187-194

Gilkes NR, Claeyssens M, Aebersold R, Henrissat B, Meinke A, Morrison HD, Kilburn DG, Warren RAJ, Miller RC (1991) Structural and functional relationships in two families of β-1,4-glycanases. Eur J Biochem 202:367-377

Gómez-Gómez E, Roncero MIG, Pietro AD, Hera C (2001) Molecular characterisation of a novel endo-β-1,4-xylanase gene from the vascular wilt fungus *Fusarium oxysporum*. Curr Genet 40:268-275

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280:309-316

Stellenbosch University http://scholar.sun.ac.za

Hoon K, Kim SF, Ahn DH, Lee JH, *Pac*k MY (1995) Internal cleavage of *Bacillus subtilis* BSE616 endo-β-1,4-glucanase expressed in *Escherichia coli*. J Microbiol Biotechnol 5:26-30

Ito K, Ogasawara H, Sugimoto T, Ishikawa T (1992) Purification and properties of acid stable xylanases from *Aspergillus kawachii*. Biosci Biotechnol Biochem 56:547-550

Jeya M, Thiagarajan S, Lee J-K, Gunasekaran P (2009) Cloning and expression of GH11 xylanase gene from *Aspergillus fumigatus* MKU1 in *Pichia pastoris*. J Biosci Bioeng 108:24-29

Kimura I, Sasahara H, Tajima S (1995) Purification and characterisation of two xylanases and an arabinofuranosidase from *Aspergillus sojae*. J Ferment Bioeng 80:334-339

Kimura T, Suzuki H, Furuhashi H, Aburatani T, Morimoto K, Karita S, Sakka K, Ohmiya K (2000) Molecular cloning, overexpression, and purification of a major xylanase from *Aspergillus oryzae*. Biosci Biotechnol Biochem 64:2734-2738

Kormelink FJM, Lefebvre B, Strozyk F, Voragen AGJ (1993b) Purification and characterisation of an acetyl xylan esterase from *Aspergillus niger*. J Biotechnol 27:267-282

Kormelink FJM, Searle-Van Leeuwen MJF, Wood TM, Voragen AGJ (1991) Purification and characterisation of a (1,4)-β-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*. Appl Microbiol Biotechnol 35:753-758

Kormelink FJM, Searle-Van Leeuwen MJF, Wood TM, Voragen AGJ (1993) Purification and characterisation of three endo-(1,4)-β-xylanases and one β-xylosidase from *Aspergillus awamori*. J Biotechnol 27:249-265

Kormelink FJM, Voragen AGJ (1993) Degradation of different [(glucurono)arabino]xylans by a combination of purified xylan-degrading enzymes. Appl Microbiol Biotechnol 38:688-695

Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411-456

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

La Grange DC, Pretorius IS, van Zyl WH (1996) Expression of a *Trichoderma reesei* β-xylanase gene (*xyn2*) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 62:1036-1044

Li XL, Ljungdahl LG (1994) Cloning, sequencing, and regulation of a xylanase gene from the fungus *Aureobasidium pullulans* Y-2311-1. Appl Environ Microbiol 60:3160-3166

Lowry OH, Rosebrough NJ, Farr AR, Randall RJ (1951) Protein measurement with the Folin Phenol reagent.

Luttig M, Pretorius IS, van Zyl WH (1997) Cloning of two β-xylanase-encoding genes from *Aspergillus niger* and their expression in *Saccharomyces cerevisiae*. Biotechnol Lett 19:411-415

Manning K (1991) Isolation of nucleic acids from plants by differential solvent precipitation. Anal Biochem 195:45-50

Pérez J, Muñoz-Dorado J, de la Rubia T, Martinez J (2002) Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int J Microbiol 5:53-63

Pérez-González JA, De Graaff LH, Visser J, Ramón D (1996) Molecular cloning and expression in *Saccharomyces cerevisiae* of two *Aspergillus nidulans* xylanase genes. Appl Environ Microbiol 62:2179-2182

Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577-591

Rojo F (2008) Biofuels from microbes: a comprehensive view. J Microbial Biotech 1:208-210

Stellenbosch University http://scholar.sun.ac.za

Romanos MA, Scorer CA, Clare JJ (1992) Foreign gene expression in yeast: a review. Yeast 8:423-488

Ruiz-Roldán MC, Di Pietro A, Huertas-González MD, Roncero MI (1999) Two xylanase genes of the v*Asc*ular wilt pathogen *Fusarium oxysporum* are differentially expressed during infection of tomato plants. Mol Gen Genet 261:530-536

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279-291

Salles BC, Cunha RB, Fontes W, Sousa MV, Filho EXF (2000) Purification and characterisation of a new xylanase from *Acrophialophora nainiana*. J Biotechnol 81:199-204

Sayers EW, Barrett T, Benson DA, Bryant SH, Canese K, Chetvernin V, Church DM, DiCuccio M, Edgar R, Federhen S, Feolo M, Geer LY, Helmberg W, Kapustin Y, Landsman D, Lipman DJ, Madden TL, Maglott DR, Miller V, Mizrachi I, Ostell J, Pruitt KD, Schuler GD, Sequeira E, Sherry ST, Shumway M, Sirotkin K, Souvorov A, Starchenko G, Tatusova TA, Wagner L, Y*Asc*henko E, Ye J (2009). Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 2009 Jan;37(Database issue):D5-15. Epub 2008 Oct 21

Shallom D, Shoham Y (2003) Microbial hemicellulase*S. c*urr Opin Microbiol 6:219-228

Shin ES, Yang MJ, Jung KH, Kwon EJ, Jung JS, Park SK, Kim J, Yun HD, Kim H (2002) Influence of the transposition of the thermostabilizing domain of *Clostridium thermocellum* xylanase (XynX) on xylan binding and thermostabilization. Appl Environ Microbiol 68:3496-3501

Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology and application. Crit Rev Biotechnol 22:33-46

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1-11

Tanaka H, Muguruma M, Ohta K (2006) Purification and properties of a family-10 xylanase from *Aureobasidium pullulans* ATCC 20524 and characterization of the encoding gene. Appl Microbiol Biotechnol 70:202-211

Tanaka H, Nakamura T, Hayashi S, Ohta K (2005) Purification and properties of an extracellular endo-1,4-β-xylanase from *Penicillium citrinum* and characterisation of the encoding gene. J Biosci Bioeng 100:623-630

Vardakou M, Katapodis P, Topakas E, Kekos D, Macris BJ, Christakopoulos P (2004) Synergy between enzymes involved in the degradation of insoluble wheat flour arabinoxylan. Innov Food Sci Engin Technol 5:107-112

Wang, B.D., Chen, D.C., Kuo, T.T. (2001) Charaterization of a *S. cerevisiae* mutant with oversecreting phenotype. Appl Microbiol Biotechnol 55: 712-720

Wang J, Zhang H, Wu M, Tang C (2011) Cloning and sequence analysis of a novel xylanase gene, *Auxyn10A*, from *Aspergillus usamii*. Biotechnol Lett 33:1029-1038

Wong KKY, Tan LUL, Saddler JN (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev 52:305-317

Xie Z-L, Gao H-Y, Zhang Q, Wang H, Liu Y (2012) Cloning of a novel xylanase gene from a newly isolated *Fusarium* sp. Q7-31 and its expression in *Escherichia coli*. Braz J Microbiol:405-417

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CHAPTER 4:

GENERAL DISCUSSION AND CONCLUSIONS

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4.1 General Discussion

Three xylanase genes were isolated from *A. fumigatus* and cloned and expressed in *S. cerevisiae*. The recombinant enzymes were characterised according to temperature, pH, substrate specificity and synergistic interactions. This is the first documented study of the cloning, expression and characterisation of these three xylanase genes in *S. cerevisiae*. This yeast is an ideal host organism for the heterologous expression of xylanase genes. This organism has complete GRAS (generally regarded as safe) status and is a well-established industrial microorganism which has a high fermentation rate and ethanol tolerance (La Grange et al. 1996; Dashtban et al. 2009). These characteristics make this recombinant yeast particularly attractive to the biofuels industry.

All three of the xylanase genes obtained in this study were functional after being expressed in *S. cerevisiae*. High enzyme activity levels were obtained and proteins were detected on SDS-PAGE gels and zymograms. These enzymes were active over a wide range of temperature and pH and were only able to hydrolyse xylan. These characteristics make these enzymes ideal candidates for use in numerous industries, such as the biofuels and pulp and paper industries. There was no significant synergistic interaction between the three different xylanases, indicating that xylan degradation was not enhanced by the combined effect of the three xylanases. The xylanases obtained in this study were all endoxylanases, which are main-chain cleaving enzymes. Synergy during xylan degradation is usually observed between main-chain cleaving enzymes and one or more accessory enzymes (De Vries and Visser 2001). Therefore, the absence of synergistic interactions between the xylanases in this study was not unexpected. This leaves an opportunity for future research to investigate the synergistic interaction between these main-chain cleaving xylanases and other xylan-degrading enzymes and accessory enzymes.

The enzymatic hydrolysis of lignocellulosic biomass is currently not economically feasible due to low yield and high cost (Bhat and Bhat 1997; Sun and Cheng 2002;

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Rojo 2008). High levels of enzyme activity are, therefore, necessary in order to achieve higher product yields and reduce the cost of the enzymatic hydrolysis process. High levels of xylanase activity were observed in this study. This will result in a lower cost, making the enzymatic hydrolysis of lignocellulosic biomass economically feasible. Therefore, the xylanases obtained in this study are promising for numerous industrial applications.

4.2 Conclusions

The following conclusions can be made from this study:

- The cDNA copies of each gene was successfully cloned and sequencing results confirmed the presence of the xylanase genes
- All three xylanase genes isolated from *A. fumigatus* were functionally expressed in *S. cerevisiae*
- The molecular weight of the proteins as detected on SDS-PAGE gels was smaller than the expected size based on the amino acid sequences
- High levels of enzyme activity were obtained for all three xylanases and they were functional over a wide range of temperature and pH. These enzymes, therefore, have great potential in the biofuels industry
- The xylanases obtained in this study are all true endoxylanases as they are only able to hydrolyze xylan. This makes them incredibly attractive to the pulp and paper industry

4.3 References

Bhat MK, Bhat S (1997) Cellulose degrading enzymes and their potential industrial applications. Biotechnol Adv 15:583-620

Dashtban M, Schraft H, Qin W (2009) Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. Int J Biol Sci 5:578-595

De Vries RP, Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiol Molec Biol Rev 65:497-522

La Grange DC, Pretorius IS, van Zyl WH (1996) Expression of a *Trichoderma reesei* β-xylanase gene (*xyn2*) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 62:1036-1044

Rojo F (2008) Biofuels from microbes: a comprehensive view. J Microbial Biotech 1:208-210

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1-11