Genetic engineering and evaluation of *Aspergillus niger* for heterologous polysaccharase production

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

S.H. Rose

SUMMARY

Cellulose and hemicellulose represents the two most abundant groups of renewable polysaccharides known to man. Apart from their presence in plant material, they also contribute to a significant portion of inexpensive readily available material, such as wastes and byproducts from forestry / agricultural origin. The chemical composition of plant material varies, but the biomass content consists of approximately 75% carbohydrate polymers (cellulose and hemicellulose) and 25% lignin.

The enzymes required for the degradation of cellulose and hemicellulose are collectively called cellulases and hemicellulases. These enzymes have a broad spectrum of industrial applications including the production of fuel ethanol through fermentations, reducing the amount of chlorine required for bleaching in the pulp and paper industry, increasing dough volume in the baking industry, improving digestion and nutritional value of animal feed, increasing clarification and enhancing the filterability of wine, beer and fruit juice, etc. Therefore, a large potential market exists for cellulases and hemicellulases provided their production is economical and the product, authentic.

Aspergilli occur in a wide variety of habitats including soil, stored food and feed products and decaying vegetation. The advantages for using *A. niger* as host for heterologous enzyme production include good protein secretion, industrial fermentation technology dating as far back as 1919, being a non-pathogenic fungus with GRAS status, no special substrate or cultivation requirements, FDA approval of numerous enzymes (homologous and heterologous) produced, etc.

In this study an *Aspergillus* expression vector was constructed using the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_p) of *A. niger* and the glucoamylase terminator ($glaA_T$) of *Aspergillus awamori*. The cDNA copies of the *egl* and *xyn2* genes of *Trichoderma reesei, cbh1-4* of *Phanerochaete chrysosporium, manI* of *Aspergillus aculeatus* and *xyn3* of *Aspergillus kawachii* were introduced into the expression vector, respectively. All the plasmids were co-transformed with plasmid p3SR2 to *A. niger* and transformants selected for stable plasmid integration into the genome of the host. The recombinant enzymes EgI, Xyn2, Cbh1-4, ManI and XynC were successfully expressed and secreted at activity levels of 2300, 8000, 500, 6000 and

900 nkat/ml, respectively. The enzymes were produced as functional entities and were subsequently characterized. The EgI, Xyn2 and ManI were evaluated as feed additives for the possible use in the animal feed industry. Improved biomass gain was observed with *in vivo* studies on poultry.

With the possible mass production of heterologous enzymes in mind, a simple medium had to be devised for their inexpensive production. Molasses medium (available from the South African sugar industry) was therefore evaluated and the cultivation conditions optimized for it's possible use as cultivation substrate for *A. niger*. The evaluation was done on the grounds of EgI and Xyn2 activity produced which was monitored over time.

This study highlighted the possible use of *A. niger* for the heterologous production of enzymes, the use of industrial substrate for cultivation and paved the way for the high level expression of industrially important genes at low cost and a positive environmental impact.

OPSOMMING

Sellulose en hemisellulose verteenwoordig die twee vollopste herwinbare polisakkariede bekend. Behalwe vir hul teenwoordigheid in plantmateriaal, dra hulle ook by tot 'n beduidende fraksie van goedkoop, maklik bekombare materiaal soos afval- en byprodukte van bosbou / landbou oorsprong. Soos te verwagte, variëer die chemiese samestelling van die plantmateriaal, maar die biomassa-inhoud bestaan uit naastenby 25% lignien en 75% koolhidraatpolimere (sellulose and hemicellulose).

Die ensieme benodig vir die afbraak van sellulose en hemisellulose staan gesamentlik as sellulases en hemisellulases bekend. Hierdie ensieme het 'n breë spektrum van industriële toepassings insluitende die produksie van brandstofalkohol d.m.v. fermentasies, vermindering in die hoeveelheid chloor benodig vir die bleikproses in die pulp-en-papier industrie, toename in deegvolume in die bakkersindustrie, verbetering van verteerbaarheid en verhoging van voedingswaarde van dierevoer, toename in verheldering en verbeterde filtreerbaarheid van wyn, bier en vrugtesap, ens. Dus bestaan daar 'n groot potensiële mark vir sellulases en hemisellulases, mits hul produksie ekonomies en die produk outentiek is.

Aspergilli kom in 'n wye verskeidenheid van omgewings voor, insluitende grond, gestoorde voedsel- en voerprodukte asook ontbindende plante materiaal. Die voordele vir die gebruik van *A. niger* as gasheer vir heteroloë ensiemproduksie sluit in 'n goeie proteïen produseerder, industriële fermentasietegnologie dateer sover terug as 1919, 'n nie-patogeniese fungus met GRAS-status, benodig geen spesiale substrate of kwekingskondisies nie, FDA goedkeuring vir 'n groot aantal ensieme (homoloog sowel as heteroloog) wat reeds geproduseer word, ens.

In hierdie studie is 'n *Aspergillus* uitdrukkingsvektor gekonstrueer deur van die konstitutiewe gliseraldehied-3-fosfaat dehidrogenase promoter (gpd_P) van *A. niger* en die glukoamilase termineerder ($glaA_T$) van *Aspergillus awamori* gebruik te maak. Die cDNA kopiee van die die *egI* en *xyn2* van *Trichoderma reesei*, *cbh1-4* van *Phanerochaete chryso*sporium, *manI* van *Aspergillus aculeatus* en die *xynC* van *Aspergillus kawachii* was onderskeidelik na die uitdrukkingsplasmied oorgedra. Alle plasmiede is gesamentlik met die p3SR2 plasmied na *A. niger* getransformeer en vir stabiele integrasie in die

gasheergenoom geselekteer. Die rekombinante ensieme EgI, Xyn2, Cbh1-4, ManI en Xyn3 is suksesvol uitgedruk en teen aktiviteitsvlakke van 2300, 8000, 500, 6000 en 900 nkat/ml, onderskeidelik uitgeskei. Die ensieme is as funksionele entiteite geproduseer en vervolgens gekaraktiriseer. Die EgI, Xyn2 en ManI is as voertoevoegings vir die moontlike gebruik in die dierevoerindustrie geëvalueer. Verbeterde biomassa toename is in die *in vivo* studie op pluimvee waargeneem.

Met die moontlikheid van grootskaalse heteroloë ensiemproduksie in gedagte, moes 'n eenvoudige substraat vir hul goedkoop produksie gevind word. Molasse medium (verkrygbaar vanaf die Suid Afrikaanse suiker industrie) was derhalwe geëvalueer en die kwekingskondisies geoptimiseer vir die moontlike gebruik as kwekingssubstraat vir *A. niger*. Vir die evaluasie is die EgI en Xyn2 aktiwiteite onder verskillende toestande geproduseer en oor tyd gemonitor.

Hierdie studie beklemtoon die moontlike gebruik van *A. niger* vir heteroloë produksie van ensieme, die gebruik van industriële substrate as kwekingsmedium en baan die weg vir ekonomiese, hoëvlakuitdrukking van industrieelbelangrike ensieme met 'n positiewe implikasie op die omgewing.

Dedicated to my husband

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PREFACE

This dissertation is presented as a compilation of several chapters and one manuscript. Each chapter is introduced separately and is written in the style of the journal to which the manuscript was submitted for publication.

Chapter 3   Rose SH and WH van Zyl (2002) Constitutive expression of the *Trichoderma reesei* β-1,4-xylanase gene (*xyn2*) and the β-1,4-endoglucanase gene (*egI*) in *Aspergillus niger* in molasses and defined glucose media. Applied Microbiology and Biotechnology 58:461-468

Chapter 4   Rose SH and WH van Zyl (2003) The impact of cultivation conditions and strain properties on fungal growth and recombinant β-xylanase II production by *Aspergillus niger*.   Submitted for publication in Biotechnology and Bioengineering.

Chapter 5   Rose SH and WH van Zyl (2003) Constitutive expression of the *Phanerochaete chrysosporium* β-1,4-cellobiohydrolase gene (*cbh1-4*), the *Aspergillus aculeatus* β-1,4-endomannanase gene (*man1*) and the *Aspergillus kawachii* β-1,4-endoxylanase (*xynC*) in *Aspergillus niger*. In preparation for publication in Applied Microbiology and Biotechnology.

Chapter 6   Rose SH, F Crots, LG Eckermans and WH van Zyl (2003) Investigation of the application of enzyme technology for poultry rearing. In preparation for publication in Poultry Science.

Appendix   **I:** A method for providing a recombinant strain producing lignocellulosic hydrolases. DrG Ref. 599514

II: A method for providing a yeast and fungal strain to produce β mannanase and for producing coffee extracts. DrG Ref. 607114

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3. **Constitutive expression of a *Trichoderma reesei* β-1,4-endoxylanase gene (*xyn2*) and the β-1,4-endoglycanase gene (*egI*) in *Aspergillus niger* using the constitutive *gpd* promoter**

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1. Introduction

Lignocellulose is plant material containing varying amounts of cellulose, hemicellulose, pectin and lignin (Aristidou and Penttilä 2000). Plant biomass in general contains about 25 - 60% (w/w) cellulose, 10 - 35% (w/w) hemicellulose and 25% (w/w) lignin. Cellulose is a high molecular weight, linear polysaccharide consisting of β-1,4-linked glucose units, while hemicellulose is of low molecular weight with a wide variation in both structure and composition. Mannose, xylose and galactose are the sugars that represent the building blocks of the backbone chains of the different hemicellulose species. Lignin is a heterogeneous polyphenolic polymer consisting of phenyl propane units (mainly *p*-coumaryl, *p*-coniferyl and *p*-sinapyl alcohols) connected to each other in a random fashion.

Cellulose represents the largest form of fixed carbon in nature, with hemicellulose and pectin representing the second and third most abundant polysaccharides present in primary plant cell walls (Himmel et al. 1999). The secondary cell walls also contain these polysaccharides, but is strengthened through covalently cross-linked polymeric lignin. The pectin is mainly present in the middle lamellae connecting the individual plant cells. Present strategies seek the efficient bioconversion of lignocellulose to biocommodity products, using lignocellulosic waste produced by various industries as inexpensive feedstock.

Aspergillus species are versatile organisms with the ability to grow on inexpensive readily available material such as agricultural residues (corn stalks and wheat straw), wood residues (unharvested dead and diseased trees), specifically grown crops (poplar, sugar cane, sorghum, etc.) and waste streams (municipal solid waste, recycled paper, bagasse, etc.) (Aristidou and Penttilä 2000). The chemical composition of the different agricultural by-products varies, but approximately 75% carbohydrate polymers are available for hydrolysis (chemical or enzymatic) to fermentable sugars. Thus, the use of inexpensive media for cultivation, together with *Aspergillus niger*'s GRAS status, it's long history in the food industry, the significant contribution to the production of

antibiotics, etc. makes *Aspergillus* the ideal host for the economic production of industrially important enzymes.

1.1 Aims of the study

cDNA copies of the *egI* and *xyn2* of *Trichoderma reesei,* the *cbh1-4* of *Phanerochaete chrysosporium*, the *manI* of *Aspergillus aculeatus* and the *xynC* of *Aspergillus kawachii* have previously been cloned and expressed in *Saccharomyces cerevisiae* (Setati et al. 2001, Van Rensburg et al. 1998 and 1996, La Grange et al. 1996, Crous et al. 1995, Penttilä et al. 1987). Of the five genes mentioned only the *egI* has been successfully expressed in *Aspergillus oryzae* using the TAKA amylase promoter (Takashima et al. 1998a). To date, all genes encoding polysaccharide degrading enzymes, have been expressed under the transcriptional control of an inducible promoter with *A. oryzae* being used almost exclusively as host (Takashima et al. 1999a and b, 1998a and b, Wahleithner et al. 1996).

The aim of this study was the genetic manipulation of *Aspergillus niger* to synthesise and secrete authentic industrially important enzymes using the following strategy:

1) the isolation of the constitutive glyceraldehyde-3-phosphate-dehydrogenase (*gpd*) promoter from the genome of *Aspergillus niger*;

2) the construction of an *A. niger* expression cassette containing the *gpd* promoter and glucoamylase (*glaA*) terminator derived from *A. awamori*;

3) the heterologous expression of the β-1,4-endoglucanase (*egI*) and the β-1,4-endoxylanase (*xyn2*) from *Trichoderma reesei,* the β-1,4-cellobiohydrolase (*cbh1-4*) of *Phanerochaete chrysosporium*, the β-1,4-mannanase (*manI*) of *A. aculeatus* and the β-1,4-endoxylanase (*xynC*) of *A. kawachii* in *A. niger*;

4) the characterisation of the heterologous enzymes, EgI, Xyn2, Cbh1-4, ManI and Xyn3, produced by *A. niger*;

5) evaluating the use of molasses and the optimisation thereof, as an economical substrate for the cultivation of *A. niger* and the production of heterologous enzymes and

6) the *in vivo* evaluating the effect of EgI, Xyn2 and ManI as feed additives, on the growth rate and feed consumption of broiler chickens.

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CHAPTER 2

LITERATURE REVIEW

LIGNOCELLULOSE

2. LIGNOCELLULOSE

2.1 Cellulose

2.1.1 Structure

Cellulose is predominantly present in the cell walls of all plants, representing the largest form of fixed carbon in nature. The primary cell wall is 1 – 3 μm thick and consists of about 9 – 25% cellulose (Salisbury and Ross 1985). Thirty to 40 pairs of unbranched cellulose molecules are combined to form a microfibril, a long cylindrical fibre of about 3.5 nm. Microfibrils behave like crystals due to the parallel arrangement of the cellulose strands. The microfibrils are laid down roughly at right angles to the long axis of the cells, slipping past each other as the cells elongate. The cellulose strands are embedded in a matrix of materials including pectic substances, lignin and hemicelluloses, which are chemically much more complex. Secondary cell walls are much thicker than primary cell walls, consisting of 41 – 45% (w/w) cellulose, about 30% (w/w) hemicellulose and 22 -28% (w/w) lignin. The latter serves to resist compression as well as changes in cell shape. Cellulose, hemicellulose and lignin are collectively known as lignocellulose.

Cellulose is usually found in close association with hemicellulose and lignin, providing tensile strength to cell walls. The cellulose strands consist of β-1,4 linked β-D-glucopyranose units (Figure 1), with the glucose residue rotated by 180° relative to its neighbour, making cellobiose the basic repeating unit (Aristidou and Penttilä 2000, Eriksson et al. 1990). The size of the molecule (which is the number of repeating glucose units also known as the DP, Degree of Polymerisation) can vary from 30 to 10000 with the cellulose chain being insoluble (Schülein 2000). However, the simplicity of the repeating cellobiose unit is deceptive and not indicative of the complex arrangement at the fibril, fibre or wood level. The rigid structure of cellulose is responsible for the natural resistance to biological degradation (Linder and Teeri 1997). This is due to the tendency of the chains to form long crystals, which is stabilised by intermolecular forces. In general, these highly ordered crystalline regions are interspersed by more disordered amorphous regions, with only a limited number of algae and bacteria capable of
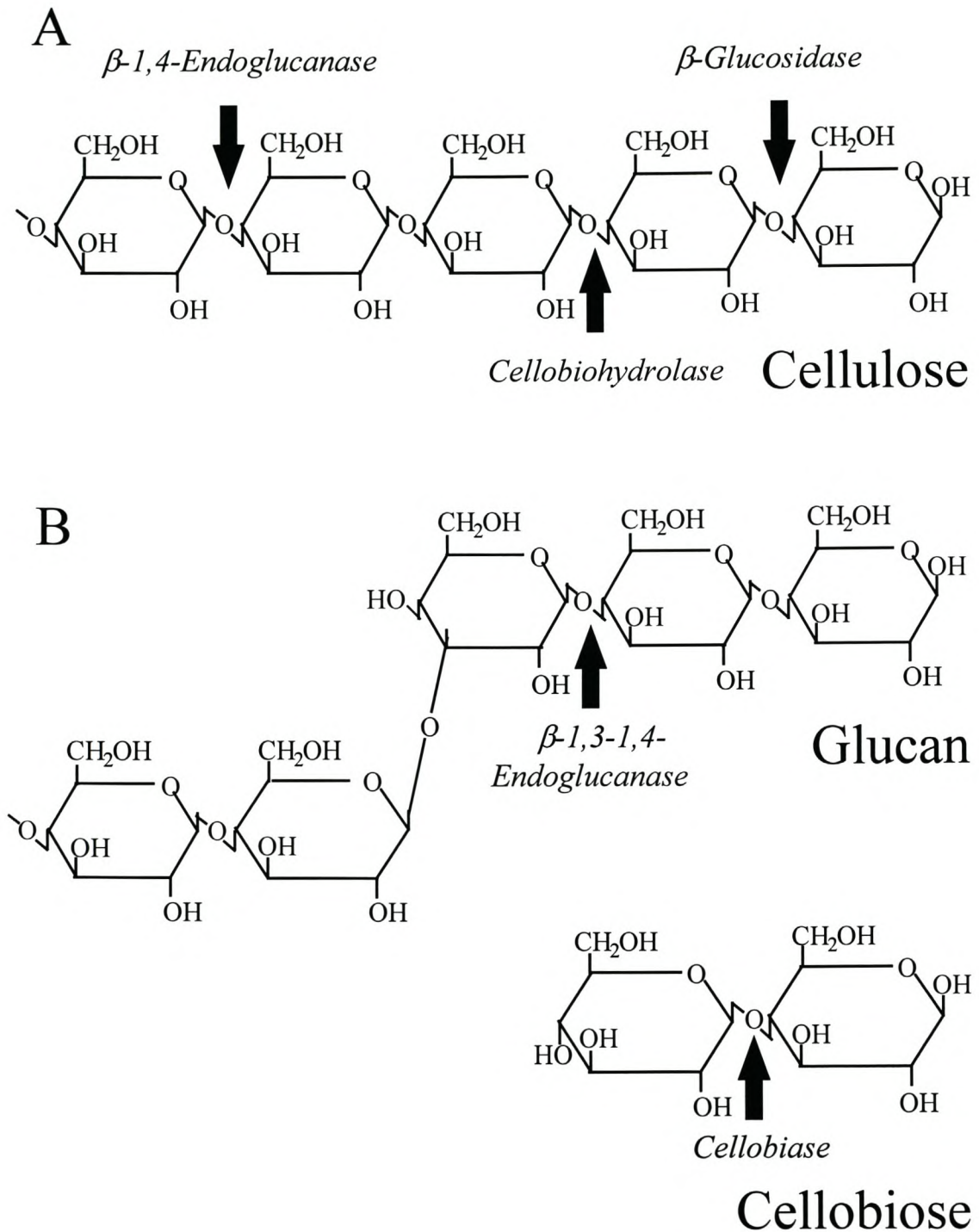
Figure 1. A schematic representation of a hypothetical (A) cellulose and (B) glucan chain and the various sites of attack by the enzymes involved in the degradation.

producing cellulose of high crystalline perfection (Schülein 2000). Crystalinity and DP of the cellulose varies depending on the source of origin as well as the physical treatments involved in the purification and drying process.

2.1.2 Degradation

The conversion of native cellulose requires the synergistic action of at least three different enzymes, collectively known as cellulases (Aristidou and Penttilä 2000). These include a β-1,4-endoglucanase (β-1,4-glucan glucanohydrolase EC 3.2.1.4), a β-1,4 exoglucanase (cellobiohydrolase EC 3.2.1.91 and exo-β-1,4-glucohydrolase EC 3.2.1.74) and a β-glucosidase (β-D-glucoside glucohydrolase EC 3.2.1.21). Depending on the host, these cellulases may be glycosylated and may exist in multiple forms. The endoglucanases act randomly to hydrolyse the amorphous cellulose and soluble cellulose derivatives with little release of reducing sugars. The cellobiohydrolases attack the crystalline as well as amorphous cellulose from the reducing or non-reducing end leaving cellobiose as end product. Amorphous cellulose is rapidly degraded to cellobiose, whereas the hydrolysis of crystalline cellulose is much slower. Although crystalline cellulose degradation is largely dependent on the action of the cellobiohydrolases, it also requires synergism with the endoglucanases for efficient hydrolysis (Mansfield et al. 1999). Crystallinity might therefor influence degradation when synergism is lacking due to an incomplete cellulase system.

The β-glucosidases complete the hydrolysis by removing glucose residues from the non-reducing end of short oligosaccharides (with a DP of two to five) (Aristidou and Penttilä 2000). Cellobiases are part of the β-glucosidase family, but only attack cellobiose resulting in the production of glucose as end product. Technically, cellobiases are not considered part of the cellulases since they do not act directly on cellulose (Nevalainen et al. 1988).

2.2 Hemicelluloses

2.2.1 Structure

A typical primary cell wall may contain 25 – 50% hemicelluloses (Salisbury and Ross 1985). Hemicelluloses are low molecular weight heteropolysaccharides (DP < 200) with a wide variation in structure and composition (Aristidou and Penttilä 2000). Unlike cellulose, hemicellulose has a random amorphous structure with little strength. It forms a branching, molecular network filled with water (Salisbury and Ross 1985).

Hemicelluloses are generally situated between the lignin and the cellulose fibres in plant cell walls (Beg et al. 2001). The hemicellulose seems to be interspersed with and covalently linked to the lignin, while producing a coat around the underlying bundles of cellulose, *via* hydrogen bonding. The relationship between the cellulose, hemicellulose and lignin may be important in maintaining the integrity of the cellulose *in situ* and in protecting the fibres against degradation. Hemicelluloses are usually hydrogen bound to cellulose and other hemicelluloses, playing a role in stabilising the cell wall matrix and renders the cell wall insoluble in water (Himmel et al. 1999).

Commonly occurring hemicelluloses are xylan, arabinoxylan, glucomannan, galactomannan, with D-xylose, L-arabinose, D-mannose and D-galactose representing the principle building blocks (Beg et al. 2001). Xylan is the most abundant hemicellulose, constituting up to 35% of the dry weight of plants. It consists of β-1,4-linked β-D-xylopyranose units with substituents such as *O*-acetyl, α-L-arabinofuranosyl, α-1,2-linked glucuronic acid and 4-*O*-methylglucuronic acid residues attached to the backbone (De Vries et al. 2000, Kulkarni et al. 1999). See Figure 2 for more details regarding xylan structure. Xylan in hardwood is present as *O*-acetyl-4-*O*-methyl glucuronoxylan and in softwood as 4-*O*-methyl glucuronoxylan. Xylans may be partly acetylated depending on the origin. The feruloyl groups present, may play a role in cross linking lignin and xylan chains. The side chains determine the solubility, physical conformation and association with other hemicellulases; greatly influencing the extent of enzymatic cleavage (Kulkarni et al. 1999).

Homoxylans (exclusively xylosyl residues, no substituents) are rare, but have been isolated from esparto grass and tobacco stalks (Beg et al. 2001). Xylans with a

β-1,3 backbone have been reported in some marine algae while mixed links of the β-1,3 and β,1-4 variety are found in some seaweed.

β-D-Endoxylanase

β-Xylosidase
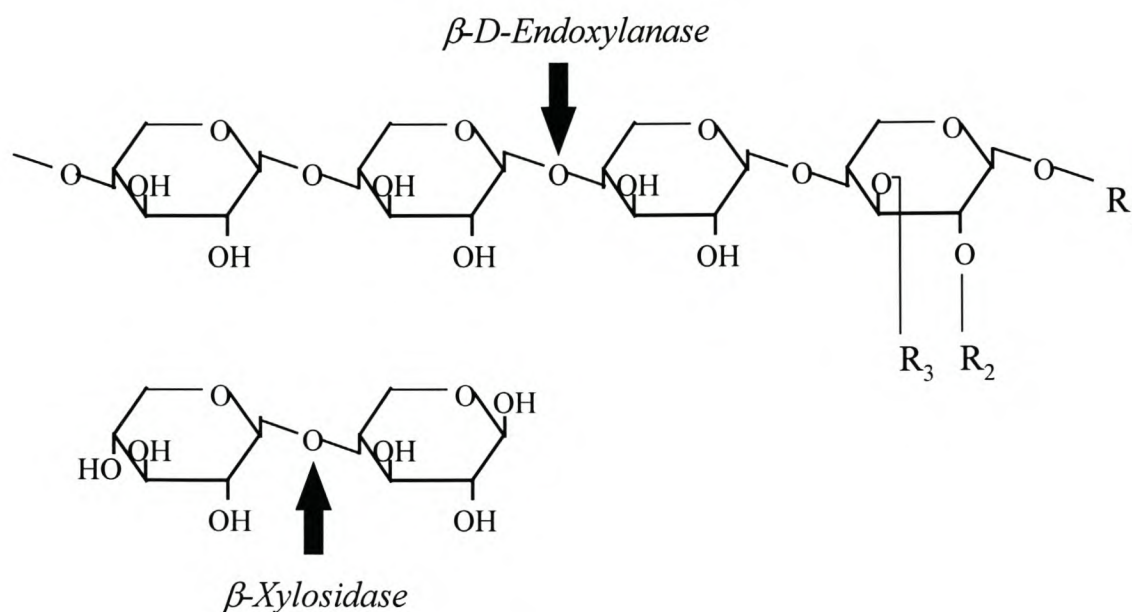
R$_1$: p-Coumaric acid

α-D-Glucuronic acid

α-D-Arabinofuranose

R$_2$: Acetyl group

α-D-Glucuronopyranose [C$_\alpha$ lignin],

4-O-Methyl-α-D-Glucuranopyranose [C$_\alpha$-lignin],

α-L-Arabinofuranose [C$_\alpha$ and C$_8$ lignin, Ferulic acid, Acetyl group, p-Coumaric acid]

Cα lignin

R$_3$: α-L-Arabinofuranose [C$_\alpha$ and C$_8$ lignin, Furelic acid, Acetyl group, p-Coumaric acid]

β-D-Galactopyranosyl (1-5) α-L-Arabinofuranose

β-D- Xylopyranosyl (1-2) α-L-Arabinofuranose

α-L-Arabinofuranose (1-2, 1-3, 1-2,3 Arabinofuranose)n

β-D-Galactopyranosyl (1-4) D-Xylopyranosyl (1-2) α-L-Arabinofuranosyl

Figure 2. A schematic representation of a hypothetical strand of xylan and the sites of attack by some of the enzymes involved in the degradation of the backbone chain (Kulkarni et al. 1999).

2.2.2 Degradation

Endo-β-1,4-xylanases [EC 3.2.1.8] produce oligosaccharides through random cleavage of the backbone chain, while β-1,4-xylosidases [3.2.1.37] act principally on xylan oligosaccharides producing xylose as product (Jeffries 1994). Some endoxylanases prefer straight chain substrates, while others are able to accommodate side chains or branching. Main chain substituents are liberated by their corresponding glycosidases (such as α-L-arabinofuranosidases [EC 3.2.1.55] liberating α-L-arabinofuranosyl and α-D-glucuronidases [EC 3.2.1.139] releasing 4-*O*-methyl-D-glucuronosyl and D-glucuronosyl residues) and their corresponding esterases liberating acetic acid, *p*-coumaric acid and ferulic acid residues.

2.3 Hydrolases

2.3.1 Classification

The hydrolase proteins and genes were previously randomly named and given the appropriate designation according to the IUB-MB enzyme nomenclature (Henrissat et al. 1998). This classification was based on the enzyme's substrate specificity and occasionally on the enzyme's molecular mechanism, but did not reflect the structural features of the enzyme. Therefor, a classification was proposed where the hydrolases were classified according to their amino acid sequence similarities (Henrissat et al. 1998). This classification reflects (1) the structural features of the enzyme, rather than substrate specificity, (2) helps to reveal the evolutionary relationship between enzymes, (3) provides a convenient tool to derive mechanistic information from the protein sequence. However, acceptance of this new classification system will require the changing of the acronyms for all the β-1,4-glycanases described to date. Because the fold of the proteins are more conserved than the sequence, some of the families can be grouped in clans when (1) new sequences are found to be related to more than one family, (2) when the sensitivity of sequence comparison methods is increased, or (3) structural determinations demonstrate the resemblance between members of different families. At present eleven clans have been identified and named clans GH-A to GH-K,

with the GH referring to Glycoside Hydrolase. The web site http://afmb.cnrs-mrs.fr provides complete information regarding the different families and clans.

2.3.2 Domain structure and function

Hydrolases are often composed of various combinations of functional domains such as catalytic domains, cellulose binding domains, reiterated domains, thermostabilising domains, linkers connecting domains and domains with yet unknown function (Black et al. 1997, Henrissat 1992). The relative position of the domains (N- or C-terminal end of the protein) seems unimportant. Most enzymes contain one catalytic domain, but bifunctional enzymes are not uncommon.

2.3.2.1 Catalytic domain

Glycosidases are classified as inverting enzymes when the stereochemistry of the linkage at the anomeric centre is inverted in the product (Kulkarni et al. 1999, Mansfield et al. 1999, Warren 1998). In the case of cellulose, which contains β-1,4 linkages, cleaving by inversion would yield α-(poly)sugars as end-product. The enzyme is retaining when the stereochemistry of the linkage at the anomeric centre is retained in the product, thus in the case of cellulose, cleaving would yield β-(poly)sugars as end-product. Both mechanisms involve two catalytic carboxyl groups on the enzyme. Figure 3 illustrates the difference between the two mechanisms. In a single displacement mechanism (used by inverting enzymes) water attacks the anomeric centre directly displacing the released group in an acid/base catalysed process. The double displacement mechanism employed by retaining enzymes involves a covalent glycosyl-enzyme intermediate (Kulkarni et al. 1999, Birsan et al. 1998, Davies 1998, Kleywegt et al. 1997). This involves the attack of a nucleophile at the anomeric centre with general acid-catalysed displacement of the leaving group, leaving a covalent glycosyl-enzyme acylal intermediate. Water attacks the anomeric centre of the intermediate in the general base catalysed process to yield the product and release the enzyme in its original state. Retaining enzymes can transglycosylate and can also be used to synthesise glycosides under appropriate conditions. These mechanisms were first proposed in 1953; further investigations have

Inverting mechanism

Retaining mechanism
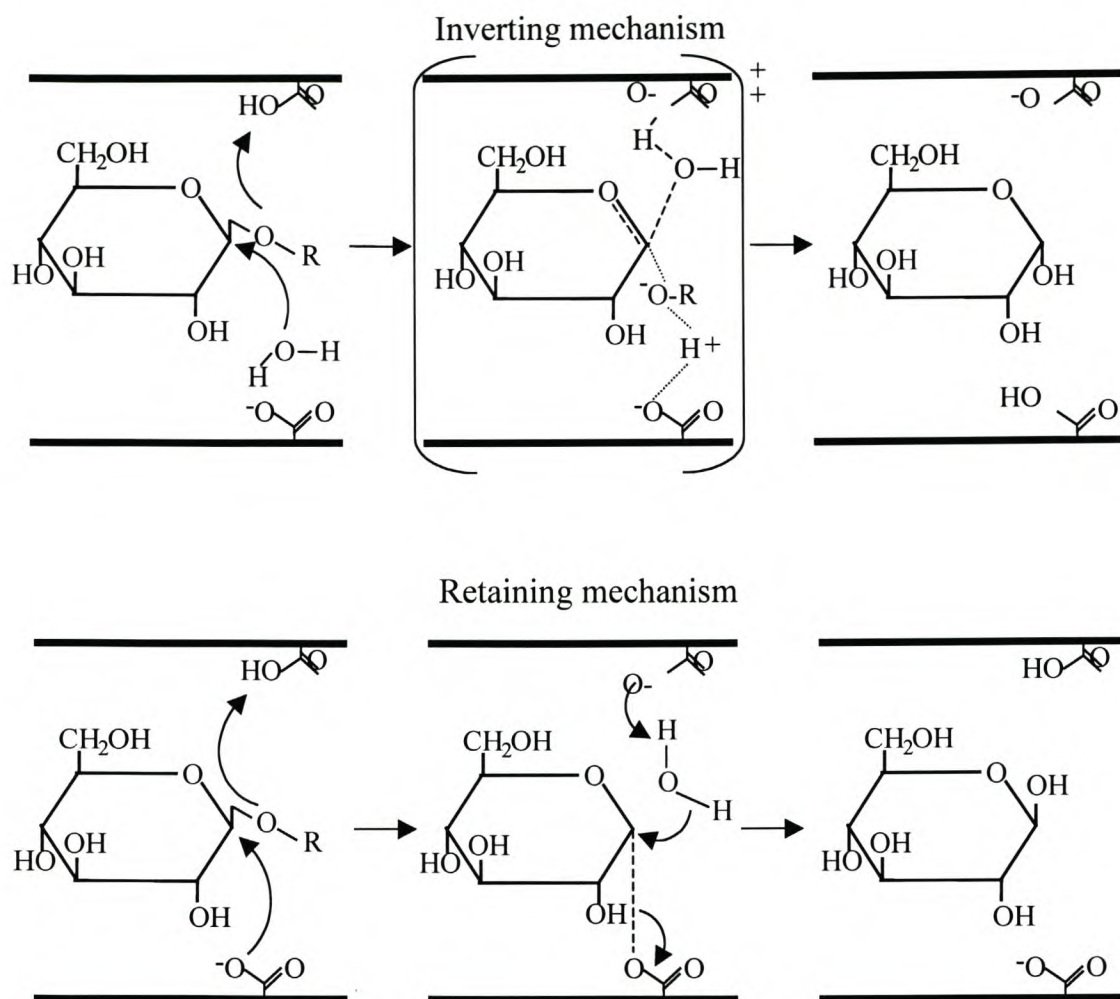
Figure 3. Mechanisms of (A) inverting and (B) retaining glycosides (Whithers 2001).

failed to provide any convincing evidence against such mechanisms (Kulkarni et al. 1999).

The three dimensional structures of the cellulases display a variety of topologies including β-sheets, β/α-barrels and α-helical proteins (Schülein 2000). However, the active site of the exoglucanases generally has a tunnel shaped structure whereas that of the endoglucanases has an open cleft shape (Schülein 2000, Sinnott 1998, Teeri et al. 1998, Warren et al. 1998, Kleywegt et al. 1997). In the case of the exoglucanases a single glucan strand enters the tunnel from one end and threaded through the tunnel, followed by cleavage at the far end of the tunnel, releasing cellobiose units. The loops

inside the active site of the cellobiohydrolases function by trapping the chain inside the tunnel, preventing it from re-adhering to the cellulose crystal (Schülein 2000). This measure allows for a "processive" degradation of the substrate.

Endoglucanases apparently lack the long peptide loops in the active site and thus have more accessible active sites allowing them to cleave bonds in the middle of the glucan chain. The ability of cellobiohydrolases exhibiting different degrees of endoglucanase activity might be ascribed to the presence of fewer loops, loops that are shorter or enzymes that are able to undergo conformational changes to open or close their active sites (Warren 1998). "Breathing" of active site loops can therefor enable the cellobiohydrolases to exhibit occasionally low levels of endoglucanase activity. Some endoglucanases also appear to hydrolyse the substrate progressively from the initial site of attack (Wilson et al. 1998). Therefor, it would be more accurate to say that some enzymes are preferentially exo-acting and others are preferentially endo-acting (Warren 1998). This makes sense seeing that hydrolysis would be more efficient when the cellobiohydrolases did not always have to depend on the available substrate end, but could create ends themselves. Furthermore, it would be easier for the cellobiohydrolases to find the substrate ends created by the endoglucanases if the endoglucanases removed a few sugar residues at the initial site of attack. Taking these facts into consideration it might therefor make more sense to classify the hydrolase enzymes according to their structure as has been suggested by Henrissat et al. (1998), rather than by their mode of action.

2.3.2.2 Carbohydrate binding modules (CBMs)

The hydrolysis of all polysaccharides requires the adsorption of the enzyme to the substrate. Enzymes bind to their substrates by means of several interactions such as electrostatic, hydrophobic, charge-transfer interactions of hydrogen bonding (Tenkanen et al. 1995). Temperature, pH and salt concentration of the environment are the main parameters affecting the enzyme-substrate interactions. Increasing the ionic strength of the environment decreased the strength of the ionic interaction, while strengthening the hydrophobic interactions. (With hydrophobic interactions the effect is ion specific.) This phenomenon is also observed with an increase in temperature. Increasing the pH,

however, weakens the hydrophobic interactions (Tenkanen et al. 1995). The pH of the environment effects the electrostatic interactions by affecting the charge of the protein and the sorbent at the given pH.

Some enzymes contain specific binding sites as part of their structure. With the aid of these binding sites the enzymes can recognise and bind to their substrates. In enzymes such as cellulases, the adsorption is facilitated by a carbohydrate binding module (CBM) formally known as a cellulose binding domain (CBD), which is connected to a catalytic domain (Teeri et al. 1998). CBMs are in general small compact domains with a flat binding surface which depends on three (in the case of fungal CBMs) aromatic amino acids for relatively tight binding of the enzyme to the cellulose. Although CBMs bind to cellulose with a high association constant and sometimes irreversibly, they also show surface diffusion and redistribution (Schwarz 2001). In cellulases the CBM serves the role of increasing the local concentration of the cellulase enzymes at the cellulose surface and might assists the catalytic function of the enzyme by liberating cellulose chains from the surface of cellulose crystal through a non-hydrolytic mechanism (Boraston et al. 1998, Teeri et al. 1998). This liberation of the glucan chain requires energy, which in theory should be supplied by the hydrolysis reaction (Sinnott 1998).

CBMs differ in their affinities and substrate specificities, with some binding to the amorphous and others to the crystalline cellulose region. Even the CBMs binding to crystalline cellulose differ in their specificity and seem to recognise non-overlapping sites (Schwarz 2001). These binding sites include plane crystal surfaces, edges, slight irregularities on the crystal surface, different crystal forms (such as I_α or I_β cellulose) etc. In the case of cellulosomes (a high molecular weight, multi-cellulase enzyme complex responsible for cellulose degradation) the cellulosomal enzymes are non-covalently bound to the cellulosome integrating protein, which carries the CBM. Therefor a more versatile CBM may be required for a cellulosome, because the CBM has to enhance the activity of the whole cellulase complex system (Carrard et al. 2000). The CBMs of cellulosomes, exhibit a lower affinity for crystalline cellulose with a more general binding behaviour for a broad range of sites on the cellulose crystal (Schwarz 2001). In

the case of unassociated enzymes, the CBMs may be more specialised in order to direct the catalytic domain towards the substrate of preference (Carrard et al. 2000).

CBMs are not restricted to cellulases, nor does all enzymes require a binding domain (Teeri et al. 1998). However, removal of the CBM generally reduces the hydrolytic efficiency of the enzyme on crystalline cellulose, but not on amorphous cellulose (Linder et al. 1999, Boraston et al. 1998, Teeri et al. 1998). The addition of a CBM to an enzyme increases it's activity as well as it's synergistic interaction with other enzymes (Linder and Teeri 1997).

The role of the CBM in hemicellulases remains to be elusive. The CBM might be responsible for mediating contact between the enzyme and the plant cell wall (Black et al. 1997). Seeing that cellulose is more abundant than hemicellulose, it makes sense for the enzyme to first attach to the cellulose, "knowing" the hemicellulose would be in the immediate vicinity. Strongly adsorbed cellulases inhibit flocculation and sedimentation of cellulose, a property ascribed to the presence of the CBM (Linder and Teeri 1997). By covering the surface the CBM prevents re-aggregation of the cellulose crystals thereby keeping the substrate "open" and facilitating mass transfer. That would also result in a greater surface area and an increase in the available substrate.

Some organisms have been shown to produce proteases which specifically remove CBMs (Linder and Teeri 1997). Thus, it has been suggested that the presence or the absence of a CBM could be controlled by differential splicing. Cellulases with CBM are required in the early stages of cellulose degradation when most of the substrate is still insoluble. Later, when the substrate has been largely solubilised into oligosaccharides, enzymes operating in the liquid phase may be preferred and brought about by means of specific proteolysis of the CBM.

2.3.2.3 Linker regions and protease resistance

The glycosylated linker region joins the CBM and the catalytic domains, or multiple catalytic domains to one another. The linker is susceptible to proteases and is often rich in proline, glycine and hydroxyl containing amino acids (Black et al. 1997). The linker should be sufficiently long and flexible to allow efficient orientation and separation for independent folding of individual domains (Carrard et al. 2000). Apart from spatial

separation the role of the linker seems unclear. If the CBM restrict substrate availability by anchoring the enzyme on a fixed location, the presence of the linker may then increase the number of available glycosydic bonds to the enzyme's active site, by conferring flexibility to the structure of the enzyme (Carrard et al. 2000, Linder and Teeri 1997). This would especially be beneficial to the hemicellulases that would otherwise be attached to the cellulose and thus out of reach of the intended substrate.

Protease resistance seems to be directly related to thermotolerance, but glycosylated enzymes seem to be more resistant than their unglycosylated counterparts (Fontes et al. 1995). Enzymes have also been reported to be resistant to proteolytic attack in the presence of their appropriate substrate, probably due to the conformational changes that result in a much tighter tertiary structure that is more resistant, or through the steric hindrance the substrate provides which protects susceptible peptide bonds against protease action.

2.4 Limiting factors in wood degradation

The depletion of fossil fuels intensified studies on the conversion of lignocellulose to fuel ethanol. Enzymatic hydrolysis of lignocellulose is preferred to acid hydrolysis mainly because of the higher yield in fermentable sugars. However, the interaction of the enzymes with the various substrates are affected by the lignin content, degree of polymerisation, physiochemical properties of the substrate (such as crystallinity) and surface area (Mansfield et al. 1999). The factors involved in the slow enzymatic hydrolysis of lignocellulose are still unclear. Pure substrates are usually used in most of the research, therefor associations with other components were seldom investigated.

Lignin has a significant effect on cellulose degradation since it irreversibly adsorb cellulases and most probably other hydrolases as well (Mansfield et al. 1999). The adsorption could be the most important factor determining the effectiveness of the degradation process. Lignin also plays a role in preventing fibre swelling, thus preventing accessibility of the cellulose and hemicelluloses to the hydrolytic enzymes. Therefor the removal of lignin, makes the cellulose more accessible by increasing the surface area. However, pre-treatment can also effectively alter the structure of the original cellulose,

rendering it more susceptible to degradation. By increasing the surface area though pore formation, the accessibility of the cellulase and hemicellulase enzymes will obviously increase. Since lignin and hemicellulose occupy smaller spaces in softwoods than in hard woods, their removal from soft woods results in smaller pore volume than is the case with hardwoods, resulting in a less efficient hydrolysis than would be the case for hardwoods. The removal of lignin results in (1) an increase in pore volume (surface area), (2) increases the substrate's susceptibility to hydrolysis and (3) increases in the levels of enzyme adsorption to the various polysaccharides.

The purpose of pre-treatment is mainly to remove the lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity of the materials (Sun and Cheng 2002). Pre-treatment requires the (1) formation of sugars or the ability to form sugars by enzymatic hydrolysis, (2) avoid the degradation or loss of carbohydrate, (3) avoid the formation of by-products inhibitory to the subsequent hydrolysis and fermentation process and (4) should be cost-effective. Pre-treatment can involve either physical, physico-chemical, chemical or biological processes. Pre-treated lignocellulose is hydrolysed faster than the untreated counterpart regardless of the method used.

When lignocellulose is degraded, a significant amount of cellulosic material remain in a recalcitrant, insoluble state (Mansfield et al. 1999). It has been established that beyond a definite molecular weight range, cellulose is recalcitrant to hydrolysis and degradation is limited. It is unclear whether the degree of polymerisation is the limiting factor acting alone or in association with other factors. Crystallinity has originally been thought to play a major role in limiting the hydrolysis of cellulose. A significant amount of research has shown that when all other factors were the same, the degree of crystallinity of the substrate has no effect on the hydrolysis process (Mansfield et al. 1999).

2.5 *Aspergillus* as heterologous host

2.5.1 Introduction

Aspergilli occur in a wide variety of habitats including soil, stored food and feed products and decaying vegetation (Schuster et al. 2002, Kozakiewicz and Smith 1994). They are particularly abundant in tropical and subtropical regions and are able to thrive in conditions of low water activity and high temperatures. *Aspergillus niger* in particular,

can grow on substrates with a water activity as low as 0.74 and in temperatures ranging from 9 - 60°C (optimum 17 - 42°C). Aspergilli can be kept at subzero temperatures due to their tolerance of low temperatures and their ability to survive the stress imposed by the exposure. *A. niger* has the ability to grow in substrates ranging from pH 1.5 - 9.8 (Schuster et al. 2002), although spore germination requires a pH range of 5 - 7 (Kozakiewicz and Smith 1994).

Heterologous gene expression has predominantly been applied in strains from *Aspergillus* and *Trichoderma* mainly because gene isolation and genetic manipulation have been successfully achieved with these genera (Archer et al. 1994). The objectives were the production of high yields of authentic target proteins and their simple purification, preferably from the spent medium rather than the biomass. The advantages for using *A. niger* as host include the following:

- naturally a good protein secretor (Gouka et al. 1997, Archer et al. 1994),
- no inclusion bodies are formed (Gouka et al. 1997, Archer et al. 1994),
- industrial technology in submerged and solid state fermentation dating as far back as 1919 (Schuster et al. 2002),
- is a non-pathogenic fungus* with GRAS status (Schuster et al. 2002),
- efficient post-translational modification occurs (Smart 1994),
- no special substrate requirements are needed (Smart 1994),
- FDA approval of numerous enzymes (homologous and heterologous) expressed (Schuster et al. 2002),
- a wide range of markers and promoters are available (Archer et al. 1994, Davies 1994, Smart 1994, Turner 1993 and 1994),
- stable genomic integrations can be obtained (Mainwaring et al. 1999).

* *A. niger* is generally regarded as a safe organism, although hyper sensitivity have been observed in rare cases (Schuster et al. 2002). In cases where patients have a history of severe illness or is undergoing immuno-suppressive treatments, *A. niger* can colonise the human body as an opportunistic invader. Ear infections (otomycosis) are common in the tropics, but can easily be treated and is usually preceded by mechanical damage of the skin barrier.

Some disadvantages of using *A. niger* as host for heterologous protein expression include:

- a limited range of largely unsophisticated vectors (Archer et al. 1994),
- low DNA transformation frequency obtained (Archer et al. 1994),
- often the genetic background is poorly characterised (Archer et al. 1994),
- potent proteases are produced (Van den Hombergh et al. 1997),
- difficulty in controlling the pH of the culture (Van den Hombergh et al. 1997),
- no clear relationship exist between copy number and gene expression levels (Van Gorcom et al. 1994),
- variety of natural proteins secreted makes purification of a single product difficult,
- difficulty in estimating biomass of mycelial growth (Miller et al. 1998, Lamar et al. 1995) and
- the lack of true stable episomal plasmids (Turner 1993).

2.5.2 Transformation

The main reason for the development of *Aspergillus* expression-secretion systems was the desire to make use of the exceptional protein secretion capacity exhibited by this genus (Davies 1994). The well established and safe use of *A. niger* and *Aspergillus oryzae* in a variety of industrial processes, as well as the well characterised genetic system of *Aspergillus nidulans* paved the way for the development of an *Aspergillus* expression system. This fungal system is cheaper to use than animal or insect cell systems, while outperforming the traditional use of the *Saccharomyces cerevisiae* system for protein secretion. Protein yields of up to 30 g/l can be achieved in *Aspergillus* systems (Davies 1994).

Aspergillus has proved to be able to produce therapeutically important proteins at levels comparing to industrial enzymes. No significant differences between yields from various *Aspergillus* host species have been observed. Fungal strains specialised for particularly high levels of one extracellular protein, do not necessarily provide a higher yield of another heterologous protein (Davies 1994). There is therefor nothing special about the use of *A. niger* or *A. oryzae* for expression, despite the myth that these species

provide a higher yield of heterologous protein. However, a number of specialised high level secretion strains are now available for *A. niger* which are not yet available for *A. oryzae* or *A. nidulans*.

Several methods exist for the DNA transformation of fungi including electroporation and whole mycelial transformation by means of lithium acetate. Electroporation is largely ineffective without the prior removal of the cell wall (Davies 1994). This, however, defeats the objective of convenience and time saving. The DNA transformation of whole mycelia requires the biolistic or shotgun approach where the mycelia are penetrated by DNA-coated tungsten balls. This method, however, is not readily assessable for most laboratories.

2.5.2.1 Protoplast transformation

Removal of the cell wall (Turner and Ballance 1985) still remains the most successful method for DNA transformation to *Aspergillus*. This process of protoplast formation and regeneration was perfected for *A. nidulans* in 1985 (Peberdy and Ferenzy 1985). The commercially available lyzing enzyme mix (marketed by Sigma as Novozyme 234), is in most cases successfully used for the cell wall removal. A number of inorganic salts may be used as osmotic stabiliser for the protoplasts produced. However, unlike with yeast, the use of sugars and sugar alcohols for this purpose, results in a poor yield of protoplasts. Before DNA uptake can take place, the access mycelial debris is removed, leaving only the protoplasts. This can be achieved either by means of centrifugation or by filtering the protoplasts through myracloth. Protoplasts generated from germinating conidia are more homogeneous in nature than those produced from mycelia. They tend to be uninucleate, but end up loosing that feature upon the addition of polyethylene glycol (PEG), due to the high frequency of fusion that occurs (Peberdy and Ferenzy 1985).

2.5.2.2 DNA uptake

DNA uptake is stimulated by the addition of PEG and calcium ions. Increasing the PEG concentration to 60% generally leads to an increase in the frequency of transformation (Turner and Ballance 1985). However, exposure to PEG should be reduced to the

absolute minimum due to it's toxic effects. Regeneration takes place on the selective plates and does not require a separate incubation in liquid media. In the transformation of *pyr*G auxotrophs to prototrophy, the agar concentration of the overlay can make a significant difference in the transformation frequency. When using the *amd*S marker and acetamide for selection, the overlay should be avoided since it is problematic for the utilisation of the nitrogen source present in the agar.

2.5.2.3 DNA targeting

At present, chromosomal integration is the only way in which stable transformants can be obtained (Turner 1993, Turner and Ballance 1985). Integration can take place through homologous recombination (Type I) at the site of the host gene locus. Type II transformation refers to integration at random, probably due to fortuitous occurrence of some homology in another part of the host genome. Type III transformation is a rare, double cross-over event which leads to replacement of a host gene (normally a mutated gene) by the wild type gene on the plasmid, without the integration of the rest of the plasmid. Linearisation of the vector before DNA transformation increases the targeting of integration, but has little effect on the transformation frequency. However, the integrations in *A. niger* are not always stable (Mainwaring et al. 1999). This clearly emphasises the importance of preserving adequate supplies of stock cultures and avoid sub-culturing as much as possible. The incubation time of fed batch cultures therefor has to be limited when the strain being cultivated is not genetically stable.

Autonomously replicating extra chromosomal vectors are currently under construction, but the elements are unstable and the rate at which the plasmid is lost, remains high (Davies 1994). Although autonomously replicating sequences (ARS) do not provide sufficient basis for stable plasmid maintenance, they do increase DNA transformation frequencies (Monero et al. 1994). ARS have received little attention in the past, probably due to the underestimation of the importance of episomal plasmids. Stable episomal plasmids can open a whole new field of study (including promoter studies, comparing gene expression levels, effect of gene copy number, genomic libraries with easy plasmid recovery, etc.), provided the plasmids maintain stable copy numbers through generations.

2.5.3 Problems associated with the expression of foreign gene products in *A. niger*

2.5.3.1 Increasing production levels

Production of foreign proteins in fungi, was generally detected at levels compared to those obtained in other expression systems. However, the levels were much lower than those obtained for the production of native fungal proteins (Punt et al. 2002). Thus, several strategies have been developed for improving the levels of foreign gene expression. Yet, it does not always prove necessary to make expression constructs for a specific species since successful cross species expression between filamentous fungal species is not uncommon (Davies 1994). The native promoter region is sometimes recognised and functions as it does in the native host.

2.5.3.2 Copy number

The level of protein secretion is often (but not in all cases) related to the copy number of the gene (Takashima et al. 1998a, Davies 1994). This implies that several transformants need to be screened in order to identify a transformant that produces high levels of a desired protein. Yet, it seems that the position of integration plays a much greater role in determining the production level of the protein. In some studies the copy number shows a linear relationship to product yield, although with a high variance. At a high copy number there is no good correlation between copy number and foreign gene expression levels (Van Gorcom et al. 1994). Transformants containing a high copy number often produce much less protein than the theoretical calculated level. High copy number integrations present new problems since plasmid integration takes place at random, knocking out other genes in the process. Increasing the copy number of the desired gene will therefore only be beneficial once stable episomal, multicopy plasmids have been developed.

Increased levels of secretion related chaperones such as the ER-chaperone BipA, protein disulphide isomerase PoliA, polyl-peptidyl isomerase CypB and calnexin ClxA, should in theory improve protein yields (Punt et al. 2002). To date, only limited success has been achieved with an increase in chaperone concentration.

2.5.3.3 Protein carriers / fusion proteins

Fusion of the heterologous protein to a well-secreted product of *Aspergillus* (also known as the carrier) often results in a fusion protein secreted at higher yield (Gouka et al. 1997). It is desired to use the entire *Aspergillus* protein, not just a portion to facilitate secretion. The aim in construction of such a fusion protein is to separate the carrier from the heterologous protein by a linker region in the expectation that the folding of the two 'domains' would be independent (unhampered) (Archer et al. 1994). Correct folding of the domains is likely to aid passage through the secretory pathway. A means of cleaving the heterologous protein from the carrier protein, such as a KEX2-like endoproteolytic cleavage site, is not always required for proper processing (Punt et al. 2002) as was previously believed (Archer et al. 1994). The fusion approach is not always successful, but remains the best option in attempts to increase the levels of foreign protein production.

2.5.3.4 Cultivation conditions

Well optimised fermentation processes should provide yields of 5-10 times greater than that obtained by shake flasks (Davies 1994). Secretion occurs at the growing hyphal tip through vesicles that fuse with the plasma membrane (Peberdy 1994). The vesicles form the last step of the intracellular secretory pathway that begins at the endoplasmic reticulum and proceeds through the Golgi system, carrying the extracellular proteins. Therefore, increasing the level of hyphal branching leads to an increase in secretion levels (Redkar et al. 1998).

It is evidently clear that solid substrate fermentations (SSF) is more productive than submerged fermentations (SmF). SSF pectinases produced by *A. niger* is less affected by catabolic repression than SmF (Acuña-Argüelles et al. 1995, Solis-Pereira et al. 1993). The production and productivities were also higher with the enzymes showing no sign of substrate inhibition when produced by SSF, which was not the case for SmF. The alpha-amylase production by *Bacillus licheniforms* was less affected by catabolite repression and by final product concentration when produced by means of SSF.

With SSF the enzymes have a broader pH range due to their slower denaturing at extreme pH values. The optimal temperature values for enzyme activities were higher with the enzymes being more thermostable when the enzymes were produce by SSF (Acuña-Argüelles et al. 1995, Deschamp and Huet 1985). In SSF sugar consumption started at 16 h of culture growth in such an aggressive way that at 24 h more than 90% of the initial sugar was consumed (Solis-Pereira et al. 1993). In SmF, 90% of sugar consumption was attained only after 96 h of growth. Thus SSF provides a system with higher productivity due to larger enzyme yields and shorter fermentation times.

With SSF less water is used, eliminating the problem with bacterial contamination, the conditions under which the fungus grows resembles that of nature, less solvent is required in the case where the product has to be extracted, less moisture needs to be removed if the products needs to be dried and in most cases the waste can be used for animal feed (Berovic and Ostroversnik 1997). The investment in a simple plant, machinery, equipment, erection and commissioning is economically beneficial. However, the disadvantage to SSF is the relatively limited possibilities for measurements and effective process control.

It is clear that several factors (not all related to cultivation conditions) need to be taken into consideration when expression levels is less than what was to be expected. A combination of host strain selection, strain development, molecular approaches, cultivation conditions and specific heterologous protein requirements (such as the addition of Cu^{2+} or haemoglobin to the medium required for the production of some laccases) is necessary to achieve attractive protein yields.

2.5.3.5 Proteases (specifically *A. niger*)

The problems associated with the low production levels appear not to be at the level of transcription, but rather occur within the secretory pathway or after secretion. The main culprit is the proteolytic degradation caused by the host proteases (Van den Hombergh et al. 1997). *A. niger* produces a wide range of proteolytic activities, including intra and extracellular proteases with the acid proteases accounting for the major extracellular proteolytic activities.

The genes involved in global regulations are the *cre*A, *are*A and *pac*C (Van den Hombergh et al. 1997). The *cre*A gene encodes a negative regulator protein that mediates carbon catabolite repression. The *are*A and *pac*C are involved in nitrogen metabolite repression and pH regulation, respectively. The *are*A gene encodes an activator protein required for alleviation of nitrogen metabolite repression. The pH regulator protein, PACC, is only active at an alkaline pH and acts as an activator of the alkaline target genes and a repressor of acid target genes. PACC is rendered inactive in an acidic environment. The promoter regions of two protease encoding genes were studied and several recognition sites were found for all three global regulators.

A. niger produces nine proteases (Table 1). Extracellular proteases are assumed to play a role in the nitrogen metabolism of the fungus (Van den Hombergh et al. 1997). PEPA and PEPB constitute 84% and 6% of the extracellular acidic proteolytic activity, whereas PEPE is responsible for 68% of the intracellular protease activity. Furthermore, the disruption of the *pep*E gene results in a decrease in intracellular serine endopeptidase and serine carboxypeptidase activity. This phenomenon might imply that PEPE is involved in the cascade activation of peptide activities in the vacuole in a similar way to that found in yeast. PEPE activates pro-PEPE as well as pro-PEPC to active PEPE and PEPC. PEPC then activates pro-CRY to form active CRY.

Table 1: The classification of the proteases produced by *A. niger*

Classification	Type	Protease Name	Localisation	Activity
aspartyl	pepsin	PEPA	extracellular	endoprotease
	pepsin	PEPE	intracellular	endoprotease
	nonpepsin	PEPB	extracellular	endoprotease
	subtilisin	PEPC	intracellular	endoprotease
	subtilisin	PEPD	extracellular	endoprotease
serine	serine carboxy*	PEPF	intracellular	exoprotease
	serine carboxy*	PEPG	extracellular	exoprotease
	serine carboxy*	CRY	intracellular	exoprotease
metallo	thermolysin	PEPH	extracellular	exoprotease

* serine carboxypeptidase

Intracellular proteases are most probably involved in non-specific protein degradation and turnover or in the activation of precursors of several vacuolar enzymes (Van den Hombergh et al. 1997). They are expressed at high levels under all growth conditions tested, with extremely limited growth related variations. They can be regarded as constitutively produced proteases, which do not respond to global regulatory mechanisms. When *A. niger* was cultivated in media supplemented with an easily metabolised nitrogen source (such as ammonia), it produced a limited amount of both *pep*A and *pep*B transcripts (Jarai and Buxton 1994). Cultivation without ammonia showed a dramatic increase in transcription levels. Interestingly, replacing the ammonia with urea (believed to be a less favoured nitrogen source) resulted in an even further drop in transcript levels, whereas the opposite effect would be expected. It has been suggested that urea might involve some additional regulatory mechanism in addition to it's nitrogen metabolite repression. Also, *pep*A and *pep*B showed low levels of expression when cultivation take place in the presence of an easily metabolised carbon source. The genes are strongly derepressed when grown without a carbon source, suggesting that the genes are regulated by carbon catabolite repression. Glycerol represses *pep*B, but seems to derepress *pep*A. Thus, although both genes are regulated in a similar fashion, the extent to which their expression is repressed by the individual carbon sources is not identical.

Induction by proteins plays a secondary role in the regulation of extracellular proteases (Jarai and Buxton 1994). Data indicate that proteins (BSA) act as an inducer of *pep*A and *pep*B when a less favoured carbon source is used, regardless of the nitrogen source. The transcription levels of *pep*A and *pep*B are elevated with growth in acidic conditions, whereas under alkaline conditions the transcripts were present only at low levels or even undetected in the absence of a carbon or nitrogen source.

Disruption of the protease genes results in an increase in heterologous protein, but normally requires the disruption of more than one gene since protein degradation involves several different proteases (Jarai and Buxton 1994). Disruption of the *pep*A, *pep*B and *pep*E genes leaves 16% extracellular, 94% extracellular and 32% intracellular activity remaining (Van den Homberg et al. 1997). Disruption of several protease genes, however, results in growth defects. Even in the best protease-deficient strains, the residual levels of protease activity can still result in serious losses in protein yield.

Down regulation of the protease expression is an alternative strategy with significant potential, especially when the genetic manipulation of the host needs to be limited. All extracellular proteases produced by *A. niger* can be affected by the use of specific carbon and nitrogen sources, as well as by the presence of an inhibitor and the pH of the media (Van den Hombergh et al. 1997). Metabolic repression appears to be the major regulatory factor and suggests that induction by proteins may only play a secondary role in the regulation of extracellular proteases.

2.5.3.6 Acid production

Previous studies suggest that a hierarchy exist among the different regulatory phenomena (Jarai and Buxton 1994). The role pH regulation plays is higher in the hierarchy than carbon or nitrogen metabolite repression or the induction by an available protein source. Since pH plays such an important role in the elevated levels of the proteases and the difficulty of buffering the growth media, it is important to know how to control the acid production of *A. niger*. Acids produced by *Aspergillus* can be classified into two groups, i.e. those derived from sugars by simple oxidation (gluconic, kojic acid) and those which are related to the tricarboxylic acid intermediates (citric, cis-itaconic, malic, oxalic and epoxy-succinic acid) (Kubicek et al. 1994). Citric acid is the most common acid produced and results in a rapid decrease in extracellular pH. Among the various parameters influencing the accumulation of citric acid, the type and concentration of the sugars used as carbon source, most severely determines the extent of acid production. Sugars which are rapidly taken up and metabolised (sucrose, mannose, glucose and maltose) provide high yields and a high rate of acid accumulation. An increase in sugar concentration leads to an increase in the intracellular concentration of fructose-2,6-bisphosphate, which results in the induction of citric acid accumulation (Kubicek et al. 1994). A deficiency in manganese, zinc, iron and nitrogen also results in an increase in citric acid production (Guitiérrez-Rojas et al. 1995).

The production of glycerol precedes the production of citric acid when the fungus is cultivated in > 300 g/l glucose (Gutiérrez-Rojas et al. 1995). The glycerol is produced to act as osmotic stabiliser to combat the osmotic stress resulting from the high sugar levels. The glycerol also inhibits the mitochondrial NADP$^+$ dependent isocitrate

dehydrogenase, which leads to citric acid accumulation. A glucose concentration of 10 g/l is sufficient to induce continued citric acid production even after depletion of the glycerol in the media.

Distinct differences in growth behaviour were also observed at different pH values (Punt et al. 2002). The fungal morphology changes from large pellets to small pellets with an increase in pH. Small pellets are favoured over large pellets because of the higher ratio of active mycelium (fungal tips on the out side of the pellet) to inactive mycelium (on the inside). Therefore, neutral pH benefits expression levels due to a less active proteolytic system and / or better fungal morphology.

2.5.3.7 Quantification of biomass

No definitive method exists to quantify fungal biomass in soil and turbid media. Direct microscopic techniques are laborious and tend to underestimate the amount of fungi, while calculations with pure culture derived conversion factors tend to overestimate fungal biomass (Miller et al. 1998). Several methods have been investigated for the determination of fungal biomass including standard plate counts (Atkinson et al. 1995), chitin and glucosamine analysis (Bossuyt et al. 1996, Wittenberg et al. 1989, Roberts et al. 1987, Matcham et al. 1985), activity assays such as cellulase activities (Miller et al. 1998), etc. The principal source of error with chitin analysis is the tendency of the chitin content of mycelium to vary with age and the presence of extraneous hexosamines within the substrates (such as plant material), insects present in soil, etc., which cause interference with the assays (Matcham et al. 1985). Determining the ergosterol level seems to be a more sensitive method especially during the initial stages of colonisation. Schnürer (1993) compared and quantified fungal growth in terms of radial growth rate, colony forming units (CFU), ergosterol levels and microscopical determinations of hyphal length and spore number. Changes in ergosterol levels were found to be closely related to changes in hyphal lengths. Using ATP content and reverse transcriptase (mRNA is used as template for the PCR reaction) as determinant have the additional advantage that only biologically active biomass is measured, thus no interference from substrates or dead mycelia (Lamar et al. 1995, Liewen and Marth 1985). These, however, are expensive techniques if used on routine basis.

2.5.4 Heterologous expression of polysaccharases in *Aspergillus*

Despite the numerous problems associated with expression in *Aspergillus*, a large number of genes have successfully been expressed in *Aspergillus*, some even of commercial value. Table 2 only summarises the lignin and polysaccharide degrading enzymes heterologously produced in *Aspergillus* spp. A summary of non-polysaccharase extracellular proteins (heterologous and homologous) produced in various fungi has been compiled by Verdoes et al. (1995). *Aspergillus* genes expressed in another *Aspergillus* species, was not considered as truly heterologous expression (for the purpose of this study), and was therefore omitted from Table 2.

Table 2: A summary of the lignin, cellulose and hemicellulose degrading enzymes heterologously produced by different species of the genus *Aspergillus*

Donor	Genes	Host	Promoter[1]	Reference
Laccases				
Caldariomyces fumago		*A. niger*	*gla*	Conesa et al. 2001, 2002
Coprinus cinereus	*lcc1*	*A. oryzae* *A. awamori*	α-amylase	Yaver et al. 1999, Conesa et al. 2002
Myceliophthora thermophila	*MtL*	*A. oryzae*	α-amylase	Berka et al.1997
Phanerochaete chrysosporium	*mnp1*	*A. oryzae* *A. niger*	TAKA *gla*	Stewart et al. 1996, Conesa et al. 2000
P. chrysosporium	*LiP H8*	*A. niger*	NOS *gla*	Aifa et al. 1999, Conesa et al. 2000
Pleurotus eryngii	*mnpl2*	*A. nidulans*	*alcA*	Ruiz-Dueñas et al. 1999
P. eryngii	*aao*	*A. nidulans*	*alcA*	Varela et al. 2001
Rhizoctonia solani	*lcc1, lcc2, lcc4*	*A. oryzae*	TAKA	Wahleithner et al. 1996
Schizophyllum commune		*A. sojae*	Tannase	Hatamoto et al.1999
Trametes villosa	*lcc1, lcc2*	*A. oryzae*	TAKA	Xu et al. 1999, Yaver et al. 1996

Table 2: A summary of the lignin, cellulose and hemicellulose degrading enzymes heterologously produced by different species of the genus *Aspergillus* (continue)

Donor	Genes	Host	Promoter[1]	Reference
Cellulases				
Humicola grisea	*eg2*	*A. oryzae*	TAKA	Takashima et al. 1997
H. grisea	*egl3, egl4*	*A. oryzae*	*amyB*	Takashima et al. 1999a
H. grisea	*bgl4*	*A. oryzae*	*amyB*	Takashima et al. 1999b
H. grisea	*exol, cbh2*	*A. oryzae*	TAKA	Takashima et al. 1998b
H. grisea	*egl, cbh-1*	*A. oryzae*	*amyB*	Takashima et al. 1996
Humicola insolens	CMC1-5	*A. oryzae*	TAKA	Dalbøge and Heldt-Hansen 1994
P. chrysosporium	*cbh1-4*	*A. niger*	*gpd*	Rose and Van Zyl 2003
Trichoderma reesei	*bgl2*	*A. oryzae*	*amyB*	Takashima et al. 1999b
T. reesei	*cbh1, cbh2, egl, eg3, eg5, bgl1*	*A. oryzae*	TAKA	Takashima et al. 1998a
T. reesei	*egl*	*A. niger*	*gpd*	Rose and Van Zyl 2002
Xylanases				
H. insolens	*xyl1-3*	*A. oryzae*	TAKA	Dalbøøge and Heldt-Hansen 1994
T. reesei	*xyn2*	*A. niger*	*gpd*	Rose and Van Zyl 2002

I All promoters (with the exception of the gpd promoter) used in the expression of polysaccharases were inducible, NOS: nopaline synthase, plant promoter

- **Laccases**

The chloroperoxidase gene (*cpo*) of *Caldariomyces fumago* was successfully expressed in *A. niger* under the transcriptional control of the *gla* promoter (Conesa et al. 2001). The specific activity (of 47U/nmol) and pH optimum (of 2.75) of the recombinant enzyme was similar to that of the native equivalent. Similarly, significant amounts of lignin peroxidase (from *Coprinus cinereus*) was expressed in *A. oryzae* and *A. awamori* (Conesa et al. 2002). While the production levels in *A. oryzae* was increased by the addition of extra heme to the culture medium, it had no effect on the expression levels in *A. awamori*. Yaver et al. (1999) expressed the cDNA of *lcc1* from *Coprinus cinereus* in

A. oryzae by means of the α-amylase promoter. The yield obtained was 20 times higher (8 – 13.5 mg/l) than that obtained from fermentations with *C. cinereus* itself.

The laccase gene, *lcc1* (cDNA), was cloned from the thermophilic fungus, *Myceliophthora thermophila* and expressed in *A. oryzae* (Berka et al. 1997). *Lcc1* was cloned under the transcriptional control of the TAKA α-amylase promoter and terminator sequences native to *A. oryzae* and yielded levels of 1 U/ml. The recombinant laccase has a pH optimum of 6.5 with syringaldazine as substrate and pH 2.7 with ABTS as substrate. This scenario is consistent with the hypothesis that electron transfer kinetics are more important than substrate binding in determining pH activity profile of laccases. Enzyme yield was 11-19 mg/l, with the recombinant enzyme retaining more than 95% of it's activity after incubation at 60°C for 20 minutes.

Two peroxidases, MnPL1 and MnPL2, produced by *Pleurotus eryngii* have previously been cloned (Ruiz-Dueñas et al. 1999). MnPL2 was expressed in *A. nidulans* under the regulatory control of the *alcA* inducible promoter and *trpC* terminator. The native and heterologous enzymes expressed similar properties such as size and levels of activity. The aryl alcohol oxidase, *aao*, of the same species was also successfully expressed in *A. nidulans* at levels of up to 500 mU/ml (Varela et al. 2001). The recombinant enzymes showed the same molecular mass and catalytic properties as that of the native protein.

Aifa et al. (1999) reported the expression of a lignin peroxidase, LiP H8, from *Phanerochaete chrysosporium* in *A. niger*. The plant promoter, nopaline synthase (NOS), was used to regulate expression. The activity obtained by the transformants were weak (1.12 nkat/mg), probably due to the use of an inefficient promoter. Expression under the control of the *gla* promoter yielded no activity due to incorrect processing (Conesa et al. 2000). The manganese peroxidase (*mnp1*) of the same organism was expressed by means of the TAKA amylase promoter in *A. oryzae* (Stewart et al. 1996). The enzyme was produced in active form, but at levels similar to that of the parental host. Production in *A. niger* also proved successful with the enzyme yield increasing 10 fold with the addition of hemoglobin to the medium (Conesa et al. 2000).

Wahleithner et al. (1996) reported the cloning of four laccases of the plant pathogenic fungus, *Rhizoctonia solani* (Wahleithner et al. 1996). Three of the laccases

(cDNA of *lcc1, lcc2* and *lcc3*) have been cloned and expressed in *A. oryzae* under the transcriptional control of the TAKA α-amylase promoter and terminator sequences. The laccases expressed optimum activity at pH 6 - 7 with syringaldazine as substrate and a pH optimum of less than 4 when ABTS was used. Syringaldazine is a phenolic compound whose oxidation involves the release of 2 protons, while ABTS is a non-phenolic benzothiazoline whose oxidation involves no proton gain or loss. The pH induced changes in laccase activity and might only affect the pH optima relative to substrates whose oxidation requires the gain or loss of protons.

The lacAL gene coding for the laccase of *Schizophyllum commune* was also expressed in *Aspergillus sojae* under the control of the tannase promoter and terminator sequences of *A. oryzae*, expressing activity levels of up to 774 U/ml (Hatamoto et al. 1999). The enzymes were produced with the transformants cultured in media containing tannic acid.

Two laccases, *lcc1* and *lcc2* (cDNA) were cloned from *Trametes villosa* and expressed in *A. oryzae* using the TAKA α-amylase promoter and terminator sequences of *A. oryzae* (Yaver et al. 1996). The optimum activity for the laccases was pH 5 - 5.5 with syringaldazine and pH 2.7 with ABTS. The *lcc1* was mutated by site directed mutagenesis and again expressed in *A. oryzae* (Xu et al. 1999). The F463M mutation resulted in a 5- and 38-fold increase in K_m for syringaldazine and ABTS, respectively.

- **Endoglucanase**

The thermostable endoglucanases (*egl2*, *egl3* and *egl4*) from *Humicola grisea* was successfully cloned and expressed in *A. oryzae* (Takashima et al. 1999a, Takashima et al. 1997). The genes were placed under the transcriptional control of the *amy*B (TAKA α-amylase) promoter and expressed at high levels with growth on maltose as carbon source. The optimum temperature of EGL2 was 75°C with more than 80% residual activity left after heating at 75°C for ten minutes. A levels of 32 U/mg was obtained for EGL2. The *egl3* and *egl4* genes yielded enzymes which preferred CMC (21 and 8 U/mg, respectively) to avicel as substrate (Takashima et al. 1999a). The EGL4CBD fusion protein consisting of the EGL4 catalytic domain and the EGL3 C-terminal region was also successfully produced in *Aspergillus*.

The genomic copies of the *egI*, *eg III* and *egV* endoglucanase genes from *T. reesei* have been cloned and expressed in *A. oryzae* (Takashima et al. 1998a). Transcription was regulated by the TAKA α-promoter with high levels of expression observed during growth on maltose as sole carbon source. Specific activities of 60, 30 and 9 U/mg was obtained for EGI, EGIII and EGV, respectively. EGI showed a broad range of substrate specificities and high levels of activities towards RBB-glucan, CMC, PNPC (*p*-nitrophenyl-β-D-cellobioside) and avicel.

The cDNA copy of *egI* have also been expressed in *A. niger* (as part of this study) under the transcriptional control of the *gpd* promoter (Rose and Van Zyl 2002). Levels of 2300 and 1400 nkat/ml (137.97 and 83.98 U) were obtained in shake flasks using glucose and molasses medium respectively.

- ## Cellobiohydrolases

The genomic copies of the *cbhI* and *cbhII* cellobiohydrolase genes from *T. reesei* were successfully cloned and expressed in *A. oryzae* (Takashima et al. 1998a). Both genes were cloned under the transcriptional control of the TAKA α-amylase promoter. Both recombinant enzymes showed narrow substrate specificities with avicel as the preferred substrate reaching levels of 0.01 and 0.02 U/mg for CBHI and CBHII, respectively.

The cDNA copy of *cbh1-4* of *P. chrysosporium* have been expressed in *A. niger* (this study) under the transcriptional control of the *gpd* promoter (Rose and Van Zyl 2003). Levels of 500 nkat/ml (30 U) were obtained on lichenan as substrate.

- ## β-glucosidases

The genomic copy of the β-glucosidase gene (*bgl4*) of *Humicola grisea* was cloned and expressed in *A. oryzae* under the control of the TAKA α-amylase promoter and terminator sequences (Takashima et al. 1999b). Optimal conditions were found to be 55°C and pH 6. BGL4 showed high activity (26 U/mg) towards PNPG (*p*-nitrophenyl B-D-glucopyranoside) and cellobiose, but weak activity towards PNPC. Native as well as recombinant BGL4 showed significant activity towards PNPGal (*p*-nitrophenyl B-D-galactoside).

The genomic copy of the β-glucosidase gene (*bgl2*) of *T. reesei* was cloned and expressed in *A. oryzae* under the transcriptional control of the TAKA α-amylase promoter and terminator sequences (Takashima et al. 1999b, Takashima et al. 1998a). BGL2 was produced with a specific activity of 24 U/mg (on PNPG) and has a pH and temperature optima of pH 6 and 40°C. BGL2 exhibited high activity towards PNPG, weak activity towards PNPGal and practically no activity towards PNPC.

- **Endo-β-xylanases**

Dalb∅ge and Heldt-Hansen (1994) reported the cloning and expression of three xylanase genes from *Humicola insolens*. Transcription was regulated by the TAKA α-amylase promoter and terminator sequences. The levels of activity was not determined. The cDNA copy of the *xyn2* of *T. reesei* was successfully expressed in *A. niger* (this study) under the transcriptional control of the *gpd* promoter (Rose and Van Zyl 2002). Levels of 8000 and 5000 nkat/ml was obtained in glucose and molasses medium, respectively.

2.6 Applications of *Aspergillus*

2.6.1 Environmental applications

- Waste and waste water treatment

Molasses is a cheap carbon source and is presently used in many industrial fermentations such as alcohol and amino acid production, baker's yeast cultivation, etc. (Peña Miranda et al. 1996). The use of this product also has an important environmental impact due to it's high organic matter content and it's dark colour, which still remains after completion of fermentation. Waste water from alcoholic fermentation plants contains a large amount of brown pigment and has a high oxygen demand. The use of *A. niger* 180 (derived from ATCC11414) to decolourise the waste water is currently evaluated. This organism achieved maximal colour elimination after 3 or 4 days of growth in the culture. A maximum of 69% colour elimination was achieved when $MgSO_4$, KH_2PO_4, NH_4NO_3 and a carbon source were added to the waste water.

Banana waste is available in many developing countries, with places such as Martinigue, where it constitutes about 15000 tons each year (Baldensperger et al. 1985).

Using *A. niger* in solid state fermentation, the protein content of the banana waste was increased by 12% (from 6% to 18%). The fermented banana waste can be used as cattle feed due to the increased protein content and decreased sugar content (from 84% to 64% dry weight).

Paper mill sludge, a waste discharge from the paper and pulp industry, represents a potential source of cellulose (Maheshwari et al. 1994). *T. reesei*, producing endo- and exoglucanases, was supplemented with *A. niger* producing β-glucosidases in mixed cultivation as an alternative for hypercellulase production. The best results were obtained with simultaneous inoculation of both organisms yielding 0.95 mg/ml protein secreted extracellularly.

Aspergillus awamori and *T. reesei* were used for the conversion of apple distillery waste with the mixed culture proving advantages (Friedrich et al. 1987). *Aspergillus* spp. proved effective in improving filtration, removing dissolved organic substances and producing β-glucosidases. *Trichoderma* strains, on the other hand, are effective in fibre degradation and the synthesis of mycelial protein and cellulose, xylan and pectin degrading enzymes. The two genera thus seems to complement each other in mixed cultivation.

Large quantities of toxic olive mill waste water are produced during the production of olive oil (Vassilev et al. 1997). The toxicity has been attributed to the phenolic part of the waste water, which is both phytotoxic and antimicrobial. The waste water, however, contains up to 11 kg of K_2O, 2 kg of P_2O_5 and 0.5 kg of MgO per m^3, making it ideal as a fertiliser if the toxic compounds could be removed. For this reason *A. niger* NB2 have been employed to ferment the waste in the presence of rock phosphate and ammonium sulphate. The fungus solubilised the rock phosphate to a concentration of 0.58 g/l, while the phenolic compounds were decreased from 6 to 2 g/l.

Apple pomace waste represents a considerable disposal problem (Berovic and Ostroversnik 1997). Apple pomace consists of the presscakes derived from pressing apples for juice/cider and includes the presscakes obtained from peeling core and peel wastes. It has a low fat content and is rich in carbohydrates, pectins, proteins and nitrogen free extract. Best results for fermentation by *A. niger* A 163 were obtained with 38% moisture content and a process temperature of 35°C. Maximum amounts of 25 g/kg

and 200 mg/kg of solid medium were obtained for polygalacturonases and pectinesterases, respectively.

Copper and zinc are abundant in swine feed due to their ability to promote immune system function and growth (Price et al. 2001). The unabsorbed metals are excreted and with time accumulate to phytotoxic levels in soil receiving swine manure and waste. Of all the fungi tested, *A. niger* was found to be best suited for the detoxification and was able to remove 91% of the copper and 70% of the zinc by means of internal absorption. The actual mechanism used for detoxification is still unknown.

- Cassava detoxification for human consumption

Cassava contains high concentrations of cyanogenic glycosides, which release nitriles upon hydrolysis by β-glucosidases (Birk et al. 1996). (Nitriles can further be hydrolysed either enzymatically or spontaneously at a pH > 5.) The cyanogenic glycosides are converted to cyanide upon digestion, making the consumption of non-treated or improperly treated cassava hazardous, since it can result in acute cyanide intoxication or even death. The traditional methods of cassava processing include boiling, drying and natural fermentation with the latter proving to be more commonly used. The detoxification of cassava by means of β-glucosidases are unfortunately inhibited due to acidification of the cassava by the micro-organisms populating the substrate. Fermentation of cassava by *A. niger* B-1, led to a decrease of 95% in the cyanide content, compared to a decrease in 50% after boiling and 25% after drying. An increase of 50% in the protein content and 140% in the fibre content of the cassava improved it's nutritional value as well.

- Rock phosphate solubilisation

Phosphorus is essential to plants, but the concentration in soil is extremely low (Vassilev et al. 1995). This problem can be overcome by using rock phosphate as fertiliser. Rock phosphate, however, is not available to plants in soil with a pH greater than 5.5. Even under optimal conditions the yields are lower than those obtained with soluble phosphate. It has been shown that low molecular mass organic acids can strongly increase solubilisation making filamentous fungi the ideal producer. *A. niger* was used to ferment

rice hulls, alperujo and sugar beet waste (all agroindustrial waste products) with the latter proving to be the best substrate. Sugar beet waste was supplemented with 3 g/l rock phosphate and resulted in 69% mineralisation, reaching a maximum of 292 mg/l phosphate produced.

2.6.2 Economical applications

- Native *Aspergillus* products

Fungi have been of great importance to mankind for several centuries. They have been used as food in the form of directly edible molds and in the production of fermented foods and drinks. More recently, fungi have proved to be useful in the production of primary and secondary metabolites (such as antibiotics and vitamins) and enzymes. The most significant fermentation use of fungi is most probably the production of β-lactam antibiotics, while their most economical use is still the production of fermentable foods. The metabolic versatility of the members of the genus *Aspergillus* is largely responsible for the success of this genus which have provided a rich source of organic acids, enzymes and food additives. The genus *Aspergillus* is the main contributor to the enzyme market (Table 3) with world sales of β-lactam antibiotics alone being estimated at $US 9 billion for 1997 (Gibbs et al. 2000). *Aspergilli* such as *A. nidulans*, *A. oryzae* and *A. niger* have been used with great success in the expression of a multitude of foreign genes. (See previous section on heterologous expression for more details.)

Table 3: The financial contribution of enzymes, expressed by *Aspergillus*, to the global economy (Harvey and McNeil 1994)

Enzymes	Market in millions of $US	Enzymes	Market in millions of $US
Alkaline proteases	150	Isomerases	45
Neutral proteases	70	Amylases	100
Rennins	60	Pectinases	40
Other proteases	50	Carbohydrates	10
Lipases	55	Other	600 (in 1994)

- Citric acid production

Citric acid is widely used in several industries such as the food, pharmaceutical and chemical industries with the demand exceeding 500 000 tons per year (Kirimura et al. 1999). Its' production by *A. niger* is tightly regulated by the composition of the culture medium (Alvarez-Vasquez et al. 2000, Gutiérrez-Rojas et al. 1995). Citric acid accumulation was significantly improved when manganese, zinc, iron and nitrogen were deficient in the medium and with the carbon source present at high concentrations. Glycerol is produced as an osmotic regulator and is responsible for the inhibition of mitochondrial $NADP^+$ dependent isocitrate dehydrogenase which leads to citric acid accumulation.

- Animal feeds

The addition of enzymes (especially cellulases, mannanases and xylanases) to animal diets seems to improve the nutritional value of the feed (Jackson et al. 1998, Fontes et al. 1995). Other enzymes used as dietary additives are amylases, pectinases glucanases, arabinoxylanases, proteases (acid and alkaline), phytases, esterases and lipases (Acamovic 2001). The commercial enzyme products are traditionally products of fermentation extract of bacterial (*Bacillus* spp.) or fungal (e.g. *Aspergillus* and *Trichoderma* spp.) origin (Howes et al. 1998). Enzymes produced by *A. niger* appear to be the most stable in the rumen with the endoglucanase and xylanase activities being stable for at least 6 hours (Morgavi et al. 2000b).

The efficiency of the enzymes is mainly due to the hydrolysis of β-glucan and arabinoxylan present in barley and rye based diets. The β-glucan, especially, influences the nutritional value of barley based feeds used in poultry rearing (Nahas and Lefrançois 2001). The glucan concentration is difficult to control and varies with cultivar, cultivation conditions, geographic origin, stage of ripeness during harvesting and storage condition. Both β-glucan and arabinoxylan form viscous gel-like structures in the small intestine of the animal, trapping nutrients (starch, proteins, fats, etc.), which would normally be accessible to the digestive enzymes (Fontes et al. 1995). The breakdown of these gels would release the trapped nutrients, which can then be absorbed. Naturally, the cellulases and xylanases should be active in the small intestine where the gel-like

structures form, they should be resistant to the proteinases of the small intestine and exhibit maximum catalytic activity in the pH range 6-8.

The presence of galactomannan in diets, has a strong antinutritive effect especially on monogastric animals (Jackson et all. 1999). An inclusion rate of 4% in feed, is enough to severely retard growth and decrease feed efficiency in broilers. It interferes with the glucose metabolism and insulin secretion rate in pigs. The latter impairs the uptake and utilisation of glucose and amino acids resulting in a reduced growth rate.

In ruminants, the digestion of plant material is accomplished though the microflora present in the rumen (Wang et al. 2001). This process is far from efficient; leaving considerable room for enhancement of fibrous feed utilisation. Studies on the use of exogenous enzymes have increased in recent years, but the results have been highly variable and were viewed with considerable scepticism (Wang et al. 2001, Nsereko et al. 2000). Little is known about the method in which exogenous fibrolytic enzymes improve feed utilisation in ruminants. The proposed modes of action include: (1) enhancing microbial attachment to, (2) colonisation of plant cell walls present in feeds and (3) enhancing the hydrolytic capacity of the rumen due to the additional enzyme activity and / or synergy with rumen microbial enzymes (Morgavi et al. 2000a, Nsereko et al. 2000). These factors lead to an increase in bacterial numbers and hence an increase in hydrolytic capacity (Wang et al. 2002, Nsereko et al. 2000).

Studies have shown that enzyme supplements can enhance the solubilisation of the fibrous component of ruminal diets (Dawson and Tricarico 1999). The indigestible degradation products (oligosaccharides or monosaccharides) are transferred to the gastrointestinal tract, altering the microfloral population (Acamovic 2001). This change in microbial activities and ruminal fermentation patterns, may be responsible for a large proportion of the beneficial attributes associated with exogenous enzymes in animal feeds. Furthermore, enzyme addition can cause unexpected and often unrelated effects, which may not always be beneficial (see Acamovic (2001) for examples). Therefore, it is difficult to predict the actual effect of the addition of commercial enzyme preparations. Until recently, it was assumed that the ruminal proteolytic activity would rapidly inactivate unprotected enzyme feed additives (Morgavi et al. 2001). More recent studies

have reported that enzyme stability depends largely on the origin of the enzyme (enzymes expressed by *Trichoderma* were found to be relatively stable in ruminal fluid and resistant to microbial degradation for a time period sufficiently long to act in the rumen), and type of activity. Proteolytic resistance is also enhanced by binding of the enzyme to the feed particles prior to exposure to the rumen environment (Wang et al. 2001). Enzymes are likely to be more affected by the host gastrointestinal proteases and pH than by the ruminal proteases (Morgavi et al. 2001).

- Single Cell Protein (SCP)

The search for non-conventional protein sources was spurred on by the increasing demand for food and feed protein. *A. niger* AS-101 has been used to produce single cell protein from alkali treated corncobs (Singh et al. 1991). The biomass was analysed after 6 days of fermentation and found to contain 30.4% crude protein, 46.7% carbohydrate and 12.9% crude fat (% of dried biomass). Zyla et al. (2000a, 2000b, 1996) also found the *A. niger* mycelium useful in poultry diets, due to the presence of phosphate releasing enzymes (such as phytases and acid phosphatases) trapped within the intracellular and membrane bound fractions of the mycelium. Microbial protein sources are of great value to the diet of ruminants and monogastric animals and can thus be used as a protein and enzyme supplement.

2.6.3 Economics of biocommodity products

The genus *Aspergillus* has no special growth requirements making it the ideal host for protein / enzyme production since the cost of the raw materials are often the dominant factor in determining the price of the commodity products (Lynd et al. 1999). The production of high volume / low value biocommodity products has an absolute requirement for inexpensive, readily available, high volume feedstock that is easy to handle and store (if not available all year round). Therefore, in order to keep the production cost to a minimum, a means of producing products from inexpensive abundant material has to be established. The inexpensive substrates can include various agricultural residues (corn stalks, wheat straws, potato or beet waste), wood residues (leftovers from harvested wood, and unharvested dead and diseased trees), specifically

grown crops (hybrid poplar, black locust, willow, silver maple, sugar cane, sugar beet, corn and sweet sorghum), and waste streams (municipal solid waste, recycled paper, bagasse from sugar manufacture, corn fibre, and sulfite waste) (Aristidou and Penttilä 2000). Naturally, the chemical composition of the biomass varies, but the biomass consists of approximately 75% carbohydrate polymers (cellulose and hemicellulose) and 25% lignin. From this point of view, the possible use of by-products produced by the sugar industry was further investigated.

2.6.3.1 Availability and uses of molasses and bagasse

South Africa produces millions of tons of sugar every year ranking it the eighth largest producer of sugar from sugar cane in the world (Van Niekerk 1981). As can be expected, large quantities of by-products are produced which include molasses, bagasse, sugar cane tops and filter press. Molasses (36 kg per ton of cane crushed) is the condensed residue, which remains after the removal of the crystallised sucrose from the concentrated sugar juice. It is used extensively in the manufacturing of yeast, in the fermentation industry for the production of potable and fuel alcohol, as a chemical feedstock, for the briquetting of chrome ore dust and fines, as well as a binder for refractory bricks and carbon black tyre.

Molasses is used in the animal feed industry mainly as an energy source, but also serves other purposes such as increasing feed intake (of unpalatable roughages) due to it being highly palatable for farm animals and ruminants. Due to the sticky nature of molasses, it helps in reducing the dustiness of feeds and feed factories and acts as a binding agent during the pelleting process. The main disadvantage of molasses as a feed ingredient is its high moisture content, which restricts its use in the balanced feed industry, and its high, imbalanced mineral content (Van Niekerk 1981).

Bagasse is the fibrous residue remaining after the sugar cane has been crushed and the sugar juice removed (Van Niekerk 1981). About 300 kg of bagasse is produced with every ton of sugar cane harvested (1.3 tons of bagasse produced with every 1 ton of sugar manufactured). Bagasse has numerous applications including fuel for sugar mill furnaces, animal feeds, the manufacture of paper, particle board, furfural, light weight concrete and is a possible substrate for single cell proteins. Bagasse can be used fresh

from the sugar mill or can be dried and processed into various feeds. It is often stored in baled form or is ensiled so as to extend its applications throughout the year and beyond the confines of sugar milling season.

Bagasse is difficult and expensive to transport due to its bulky nature (Van Niekerk 1981). This problem is overcome by drying and pelleting using limited amounts of molasses as a binding agent and for the increase in nutritional value. The bagasse is exposed to steam, high temperatures and pressures, during the pelleting process, which are believed to increase the digestibility of the material. The porous nature of bagasse makes it the ideal carrier for molasses and molasses-based liquid feed mixtures. Bagasse pith has the ability to absorb up to 80% by weight, and still remain free flowing.

2.6.3.2 Composition

Molasses is a heterogeneous product which varies in composition depending on factors such as the soil on which the sugar cane is cultivated, environmental factors such as temperature, rainfall / irrigation, season of production, crop variety and factory processes under which molasses is produced (Van Niekerk 1981). The approximate composition of molasses is summarised in Table 4.

Sucrose and water content can vary considerable depending on the production technology employed by the different sugar mills. Therefore, standardised molasses has been marketed with a water content of 25% in an attempt to reduce the variation in viscosity caused by differences in water content. The sugar content consists of mainly sucrose and small amounts of glucose, fructose, raffinose and unfermentable sugars. The non-sugar organic matter consists of pentosans, starch, organic acids, waxes, gums, sterols, pigments, crude proteins (non-protein nitrogen compounds such as amides, amino acids and other simple nitrogenous compounds) and vitamins. Since molasses contains little protein, it's nutritional value rests largely on its value as an energy source attributed to the high sugar content.

Dried whole bagasse contains about 45 - 55% cellulose, 20 - 30% hemicellulose, 25% pentosans and 15-26% lignin (Dixon 1988, Van Niekerk 1981). Bagasse contains little protein and is poorly digested. The high fibre content makes it ideal as an

Table 4: The approximate composition of standard molasses (Van Niekerk 1981)

Ingredient	75% DM* (g/kg)	Ingredient	75% DM (mg/kg)
Moisture	250	Copper	2.2
Crude protein	50	Zinc	2.7
Ether extract	1	Iron	101
Total ash	115	Cobalt	3.8
Nitrogen-free extract	584	Pantothenic acid (Vit. B5)	54-64
Sucrose	332	Biotin (Vit. H)	1.2-3.2
Total sugars	467	Folic acid (Vit. M)	ca 0.04
Gum	25.5	Inositol	ca 6000
Wax	4.7	Manganese	91
Starch	1.7	Pyridoxine (Vit. B6)	2.6-5.0
Calcium	8.8	Riboflavin (Vit. B2)	ca 2.5
Sodium	1.6	Thiamine (Vit. B1)	ca 1.8
Chlorine	21.1	Nicotinic acid	30-800
Magnesium	5.4	Choline	600-800
Potassium	33.3	Phosphorus	700
Sulphur	6.8	Crude fibre	0

DM* dry mass

inexpensive source of roughage in complete animal feeds. Two different methods are used for extraction of sucrose, the mill method and the diffuser method (Atsushi et al. 1984). With the mill method the extraction of sucrose is performed before the addition of lime milk, whereas with the diffuser method the lime milk is added before extraction. Bagasse produced by the mill method contains more pentosan, pectin and alkali soluble materials but less cellulose than that produced by the diffuser method. Bagasse is available in various forms (Table 5) which differ in composition (Van Niekerk 1981).

Pith is obtained by separating the whole bagasse by airflotation into two fractions, the harder rind / sugar cane barrel and the pith / parenchyma plus smaller pieces of the rind. Pelleted pith is prepared with steam and 6 - 10% molasses. Five percent NaOH can

be added prior to pelleting. Pelleted whole silage is prepared in the same way also containing 6 - 10% molasses.

Separation of pith and fibre is important for paper and board production since only the fibre is required for manufacturing (Van Niekerk 1981). In the animal feed industry, however, the pith is preferred as it acts as the ideal carrier for molasses or molasses-based liquid feed mixtures and also due to the attractive appearance in blended feeds. Due to the difficulty of separations of the different phases, the resultant products are non-uniform in composition.

Table 5: Proximate analysis of various forms of bagasse in g/kg (Van Niekerk 1981)

Ingredients	Dried whole bagasse	Bagasse pith	Pelleted pith	Pelleted whole silage	Pelleted NaOH pith
Moisture	66	62	65	67	90
Crude protein	20	22	26	24	30
Ether extract	9	10	10	9	9
Crude fibre	430	400	346	363	315
Ash	39	45	58	55	97
N F E	436	460	495	482	459
Calcium	2.3	2.3	2.4	2.4	2.2
Phosphorus	0.3	0.3	0.4	0.4	0.3
Sodium	0.6	0.8	1.0	1.0	3.0
Potassium	0.5	0.5	2.7	2.8	2.5

The advantages of working with bagasse includes (1) the long, tough and strong fibre, (2) natural resistance to decay, (3) high purity, (4) annual availability, (5) readily accessible infrastructure for bulk transport and (6) its a by-product which must be disposed of (Chapman 1955). The disadvantages include (1) the high moisture content of the fibres as it comes from the sugar mills, (2) the handling of huge volumes in a relatively short working period and (3) the problem of storing and preserving it under uniform conditions.

2.7 This study

This study was undertaken in light of the (1) large existing market for cellulases and hemicellulases, (2) the increasing pressure on countries to implement green technology for waste disposal and (3) the increasing demand for a more cost-effective means of rearing commercial farm animals.

The search for a host capable of expressing large quantities of functional enzymes (in this laboratory), started with the overproduction of enzymes in *Saccharomyces cerevisiae*, but inevitably led in the direction of fungi which are known to produce large quantities of native proteins. *A. niger*, *A. oryzae* and *T. reesei* are currently predominantly used as hosts for heterologous gene expression in fungi. *T. reesei* is presently being investigated by other research groups and was therefore eliminated for the purpose of this study. Expression in *T. reesei* and *A. oryzae* have received significant attention with regard to expression of heterologous polysaccharides (especially cellulases) in the last decade. Therefore *A. niger* was chosen as the work horse for this study. Although several foreign genes have been expressed in *A. niger,* polysaccharase encoding genes have largely been neglected. Most of the polysaccharase encoding genes expressed in *A. niger* were derived from other *Aspergillus* species and is for the purpose of this study, not considered to be truly heterologous (Verdoes et al. 1995).

The use of a constitutive fungal promoter for the production of hydrolases has been neglected in the past. Therefore the glyceraldehyde-3-phosphate-dehydrogenase promoter was chosen on the basis of being (1) a strong constitutive promoter and (2) being involved in the glycolysis pathway (considering glucose would mainly be used as carbon source).

Due to environmental concern and the cost involved in waste disposal, the emphasis is also shifting towards decreasing the quantity and toxicity of the waste produced. With South Africa being one of the largest producers of sugarcane in the world (and therefore also one of the largest producers of molasses waste), the possible use of molasses as cultivation substrate was further investigated. *A. niger*'s ability to grow on diluted molasses without any addition requirements, proved to be an unexpected bonus.

Producers of animal products aim to provide cost effective, high quality, homogeneous products in the shortest time possible with animal diets contributing to up to 80% of the total cost or rearing animals (Acamovic 2001). Thus, factors influencing the utilization of the diets will have a substantial effect on profits. This concept led to trial runs where the effect of some of the heterologous polysaccharases (the EgI, Xyn2 and ManI) produced in this study, were tested on poultry and ruminal diets.

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CHAPTER 3

Constitutive expression of the *Trichoderma reesei* β-1,4-xylanase gene (*xyn2*) and the β-1,4-endoglucanase gene (*egI*) in *Aspergillus niger* in molasses and defined glucose media.

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Constitutive expression of the *Trichoderma reesei* β-1,4-xylanase gene (*xyn2*) and the β-1,4-endoglucanase gene (*egI*) in *Aspergillus niger* in molasses and defined glucose medium

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3.1 Abstract

The xylanase II (*xyn2*) and endoglucanase I (*egI*) encoding regions of *Trichoderma reesei* QM6a were successfully expressed in *Aspergillus niger* D15 under the transcriptional control of the glyceraldehyde-6-phosphate dehydrogenase (*gpd*) promoter from *A. niger* and the *glaA* terminator of *A. awamori*. A stable *xyn2* transformant produced β-xylanase activity of 8000 nkat/ml and 5000 nkat/ml in shake flask cultures containing defined or 20% (v/v) molasses medium, respectively. The recombinant Xyn2 enzyme expressed highest activity at pH 5 - 6 and 50 - 60°C and retained more than 75% of its activity after 3 hours of incubation at 50°C. A stable *egI* transformant produced endo-β-1,4-glucanase activity of 2300 nkat/ml in shake flask cultures containing defined media and about half the activity in 20% molasses medium. Maximum endoglucanase activity was obtained at pH 5 and 60°C. Both Xyn2 and EgI retained >80% activity after incubation at 50°C for 3 hours. The heterologous Xyn2 and EgI represent a significant portion of the total extracellular proteins produced.

Aspergillus niger   xylanase   cellulase   heterologous expression gpd promoter

3.2 Introduction

Plant material consists mainly of cellulose, hemicellulose and lignin. Cellulose represents the largest form of fixed carbon in nature. The cellulose strand consists of β-1,4-linked glucopyranose units (Béguin and Aubert, 1994). Each glucose molecule is rotated at 180° relative to it's neighbor, making cellobiose the basic repeating unit. These molecules form inter- and intramolecular hydrogen bonding patterns which account for the rigid, insoluble microfibrils. Endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) are required for the degradation of cellulose to glucose (Aristidou and Penttilä, 2000).

Hemicelluloses are low molecular weight heteropolysaccharides with a wide variation in both structure and composition. Commonly occurring hemicellulases include the 1,3- and 1,4-β-galactans, mannans and xylans (Aristidou and Penttilä, 2000). Xylan is the main form of hemicelluloses present in plant cell walls and can constitute up to 35% of the dry weight of plants (Puls and Schuseil, 1993). The xylan structures vary from linear β-1,4-polyxylose main chains to highly branched polysaccharides containing acetyl, arabinosyl and glucuronosyl side chains. Endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) act synergistically to degrade xylan to xylose. α-Arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139) and acetyl xylan esterases (EC 3.2.1.72) are responsible for the debranching of the xylan backbone.

Cellulases and hemicellulases have a broad spectrum of industrial applications including the production of fuel ethanol (using agricultural waste products), the pulp and paper industry (reducing the amount of chlorine required for bleaching), the baking industry (increasing the volume of dough), the animal feed industry (improving digestion and production efficiency), the wine and fruit juice industry (clarification of juice), etc. (Aristidou and Pentilä, 2000; Oksanen et al. 2000; Colagrande et al. 1994). Therefore, a large potential market exists for cellulases and hemicellulases.

The advantages of using *Aspergillus* spp. as host for heterologous expression include its high secretion capacity, GRAS (Generally Regarded As Safe) status, rapid

growth on inexpensive media and a relatively well-studied genetic background. *Aspergillus* spp. have the ability to produce heterologous proteins in concentrations of grams per liter (Verdoes et al. 1995) and were used by various industries for the production of a diversity of commodities such as citric and gluconic acid, enzymes such as proteases, catalases, isomerases, α-galactosidases, rennin, lipase, phytase, glucoamylase, pectinase, glucose oxidase, α-amylase, as well as pharmaceuticals such as interleukin-6 and Fab (Archer, 2000; Gibbs et al. 2000). World sales of β-lactam antibiotics produced by *Aspergillus* spp. alone have been estimated at US$ 9 billion for 1997 alone (Gibbs et al. 2000).

Aspergillus spp. produce high levels of β-glucosidase activity, but have weak levels of endoglucanase activity (Duff et al. 1986). The cellulolytic fungus *Trichoderma reesei*, on the other hand, exhibits potent endoglucanase activity, but limited β-glucosidase activity (Takashima et al. 1999; Duff et al. 1986; Ghose et al. 1985). The endoglucanase EgI of *T. reesei* is of particular interest due to its ability to hydrolyze a wide range of substrates such as avicel, carboxymethyl cellulose (CMC), hydroxyethyl cellulose (HEC), barley β-glucan, acid swollen amorphous cellulose, lichenan, xylan and even galactomannan (Bailey et al. 1993b). However, the β-glucosidases of *T. reesei* account for only 1% of the total secreted protein. The insufficient activity is known to limit the saccharification of cellulose (Takashima et al. 1999). Therefore, the *Aspergillus* and *Trichoderma* genera have been used in mixed culture with great success since their cellulolytic enzyme systems compliment each other (Maheshwari et al. 1994; Chadha and Garcha, 1992; Friedrich et al. 1987; Duff et al. 1986; Ghose et al. 1985). Enzyme production by mixed cultivation, however, has inherent difficulties to optimize since the organisms require different growth conditions, media, etc. The production of cellulase enzyme cocktails from separate fungal hosts is also costly. This motivated us to evaluate recombinant *A. niger* strains capable of constitutively producing enzymes of *T. reesei* while using molasses, an industrial waste product of the sugar industry, as carbon source.

In this paper we describe the construction of a constitutive fungal expression cassette consisting of the glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_p) of *A. niger* and the glucoamylase terminator ($glaA_T$) of *Aspergillus awamori*. The *xyn2* and

egI encoding regions of *T. reesei* were subsequently constitutively expressed in *A. niger* under the transcriptional control of the *gpd* promoter sequences and the production of the recombinant enzymes in a simple industrial medium (molasses) compared to the production in a defined medium. The enzymatic properties of the recombinant Xyn2 and EgI were also characterized.

3.3 Material and methods

Media and Cultivation Conditions

Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5α and cultivated at 37°C in Terrific Broth and on Luria Bertani agar containing 100 μg/ml ampicillin for selective pressure (Sambrook et al. 1989). Fungal strains were cultivated at 30°C in 20% (v/v) molasses (Tongaat-Huletts, South Africa) or minimal media containing 0.5% (w/v) yeast extract, 0.2% (w/v) casamino acids, 1% (w/v) glucose, 6% NaNO$_3$ (w/v), trace elements and 0.01 M uridine (Punt and van den Hondel, 1992). Transformants were selected on minimal medium containing 10 mM acetamide and 15 mM CsCl, but lacking NaNO$_3$. Media were inoculated to a concentration of 1x10^5 spores per ml unless stated otherwise. *A. niger* D15 transformants were cultivated in 20% molasses solution and in double strength minimal media (2xMM) containing 10% glucose for enzyme activity determination.

Strains and Plasmids

The genotypes of the bacterial and fungal strains as well as the plasmids used in this study are summarised in Table 1.

Table 1. The genotype and sources of the strains and plasmids used in this study

Strains / plasmids	Genotype	Source
Strains:		
E. coli DH5α	*supE44 ΔlacU169 (Ø80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook et al. 1989
A. niger van Tieghem	Wild type	ATCC 10864
A. niger D15	*pyrG prtT phmA* (nonacidifying)	Wiebe et al. 2001
A. niger D15[pGT]	*A. niger* D15 with *gpd$_P$-glaA$_T$* integrated into the chromosome	This study
A. niger D15[*xyn2*]	*A. niger* D15 with *gpd$_P$-xyn2-glaA$_T$* integrated into the chromosome	This study
A. niger D15[*egI*]	*A. niger* D15 with *gpd$_P$-egI-glaA$_T$* integrated into the chromosome	This study
T. reesei QM6a	Wild type	ATCC 13631
Plasmids:		
pSPORT1	*bla*	Gibco BRL Life Technologies
pKSExtrendedglAPo	*bla glaA$_T$*	Stewart et al. 1996
pDLG5	*bla URA3 ADH2$_P$-xyn2-ADH2$_T$*	La Grange et al. 1996
p3SR2	*bla amdS*	Wernars et al. 1985
pSPORT-glaA$_T$	*bla glaA$_T$*	This study
pGT	*bla gpd$_P$-glaA$_T$*	This study
pGT-xyn2	*bla gpd$_P$-xyn2-glaA$_T$*	This study
pSPORT-egI	*bla egI*	This study
pGT-egI	*bla gpd$_P$-egI-glaA$_T$*	This study
pSPORT-gpd$_P$	*bla gpd$_P$*	This study
pSPORT-xyn2	*bla xyn2*	This study

DNA Manipulations and Amplification by PCR

Standard protocols were followed for all DNA manipulations and *E. coli* transformations (Sambrook et al. 1989). *A. niger* was transformed by means of spheroplasts using Lyzing enzymes (Sigma) in accordance to Punt and van den Hondel (1992).

Fungal strains were grown in minimal media for 72 hours. Mycelia were harvested and frozen under liquid nitrogen and DNA isolated according to La Grange et al. (1996). The *gpd* promoter (*gpd$_P$*) was amplified from the genome of *A. niger* ATCC10864 as a 1.2 kb fragment, using PCR and oligonucleotide primers GPDR and GPDL (Table 2). PWO polymerase (Roche) was used for amplification with the reaction set up in accordance to the supplier's specifications. The genomic copy of *egI* was amplified from the genome of *T. reesei* QM6a using primers TregR and TregL. The 1.6 kb genomic copy of *egI* was used as template to produce the cDNA via overlapping PCR, using primers TregL, EgverR (with the first 20 nucleotides being homologous to the entire 3rd exon and the last 20 nucleotides being homologous to the 3'-end of the 2nd exon) and EgintrR (with the first 20 nucleotides being homologous to the 5'-end of exon 2 and the last 17 nucleotides homologous to the 3'-end of exon 1). The standard PCR reaction yielded a 1.4 kb fragment containing the coding region of *egI* without introns, but with the secretion signal still intact.

Table 2. The DNA sequences and sizes of the oligodeoxyribonucleotide primers used in this study

Primer name	Sequence (restriction sites are underlined)	Primer size	Restriction Enzyme-Sites
GPDR	5' GTAC<u>GCGGCCGC</u>TGTTTAGATGTGTCTATGTGGC 3'	34	*Not*I
GPDL	5' GATC<u>GGATCC</u>GAGCATCACCAACATGGTAC 3'	30	*Bam*HI
TregR	5' <u>GCGGCCGC</u><u>AGATCT</u>AGTCAACGCTCTAAAGGCA 3'	33	*Not*I-*Bgl*II
TregL	5' <u>GCGGCCGC</u><u>GAATTC</u>AATGGCGCCCTCAGTTA 3'	30	*Not*I-*Eco*RI
EgverR	5' CTAAAGGCATTGCGAGTAGTAGTCGTTGCTATACTGGCAC 3'	40	
EgintrR	5' TATCTCCGGGGCCGTAGTAGCTTTTGTAGCCGCTGCC 3'	37	

Plasmid Construction

The *glaA* terminator from *A. awamori* was amplified from plasmid pKSExtendedglAPo (Stewart et al. 1996) as an 1.0-kb *Eco*RI-*Sal*I fragment and cloned into the corresponding

sites on pSPORT1, generating pSPORT-glaA$_T$. The amplified 1.2 kb *gpd$_P$* PCR fragment was cloned into pSPORT1 digested with *Sma*I, resulting in pSPORT-gpd$_P$. This plasmid was used to confirm the DNA sequence of the *gpd$_P$* DNA fragment. The *gpd$_P$* was subsequently retrieved by digesting pSPORT-gpd$_P$ with *Bam*HI and *Not*I and cloned into the corresponding sites in pSPORT-glaA$_T$, generating plasmid pGT. Plasmid pGT formed the expression vector used for the constitutive expression of recombinant genes in *A. niger* D15.

The *xyn2* gene (cDNA including the native secretion signal) was derived from pDLG5 (La Grange et al. 1996) by digestion with *Eco*RI and *Bgl*II. The 5' overhanging end was filled in by the addition of Klenow polymerase (Roche) and cloned into the *Sma*I site of pSPORT1. The *xyn2* was retrieved by digestion with *Eco*RI (subsequently filled in by using Klenow) and *Not*I, and cloned into pGT digested with *Sal*I (site filled in by using Klenow) and *Not*I, creating pGT-xyn2 (Figure 1A).

The PCR product containing the *egI* open reading frame (ORF) was cloned into pSPORT1 digested with *Sma*I, resulting in plasmid pSPORT-egI. This plasmid was used to confirm the DNA sequence of the *egI* gene. The *egI* was retrieved by digestion with *Not*I and the 1.4 kb DNA fragment cloned into the corresponding site in pGT, resulting in pGT-egI (Figure 1B).

DNA Hybridizations

Southern hybridizations were carried out according to Sambrook et al. (1989). The internal 540 bp *Eco*RI – *Xho*I fragment of *xyn2* and the internal 685 bp *Eco*RI – *Xho*I fragment of *egI* were labeled with [α-^{32}P]ATP using the Random Primed Kit (Roche), according to the supplier's specification. The number of integrations was determined by digesting the genome of both the *xyn2* and *egI* transformants overnight with *Hin*dIII. DNA were separated on a 0.8% agarose gel and used for traditional Southern blot analysis.
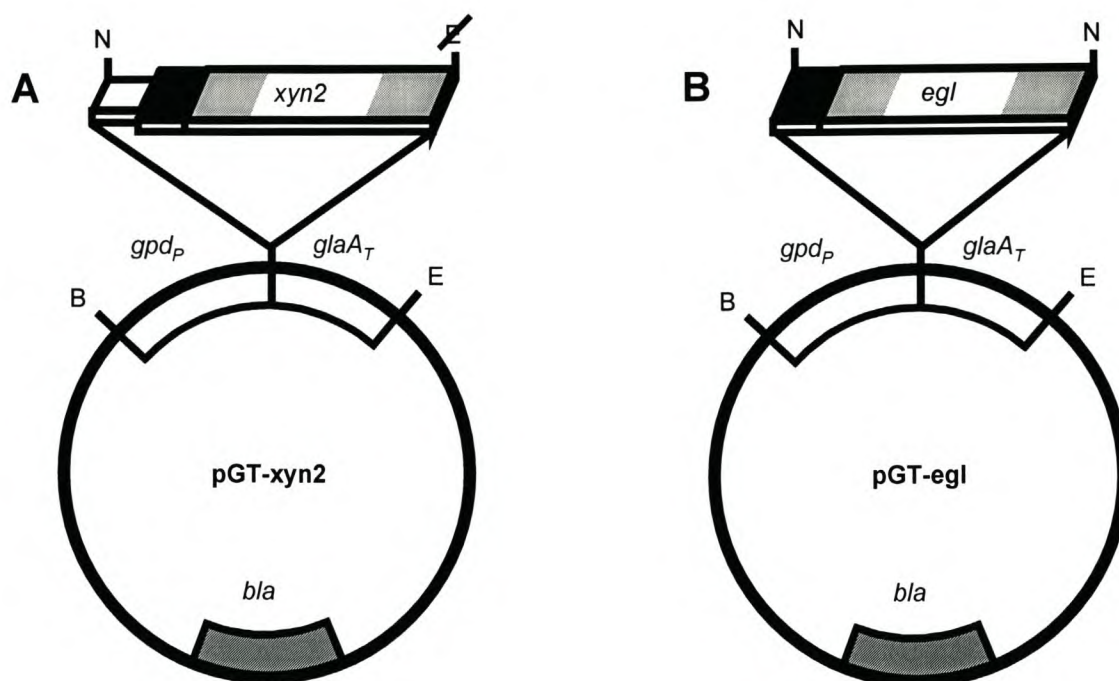
Figure 1. A schematic representation of expression vectors (**A**) pGT-xyn2 and (**B**) pGT-egI. The *xyn2* and *egl* genes are indicated with cross-hatched boxes, their secretion moieties with dotted boxes, the selectable marker (*bla*) by a hatched boxes, the *gpd* promoter and terminator sequences are indicated by the open boxes and the pSPORT1 sequences are indicated by thick lines, respectively. The restriction sites indicated are *Bam*HI (B), *Eco*RI (E) and *Not*I (N).

Enzymatic Assays

Transformants were screened on RBB-xylan and OBR-HECellulose plates containing 10% glucose for inhibition of the native xylanases and cellulases. Xylanase and endoglucanase activity, as well as pH and temperature optima determination of the Xyn2 and EgI was done as described by Bailey et al. (1992), using sodium phosphate buffer (0.05 M). The substrates used for liquid assays were 1% Birchwood xylan (Roth), 1% carboxymethyl cellulose (CMC, Sigma) and 0.1% lichenan (Sigma) resuspended in 0.05 M sodium citrate buffer (pH5 for Xyn2 and pH 5.7 for EgI). All enzymatic assays were done in triplicate. One unit of enzyme was defined as the activity producing 1 μmol

per minute of reducing sugars in glucose or xylose equivalents under these assay conditions.

Protein Isolation and Gel Electrophoresis

The supernatant produced by the different transformants were collected after 3 days of growth and freeze dried before determining the specific activity. Two mg of freeze dried supernatant (containing about 50 µg of total extracellular protein) was separated by 10% (in the case of the EgI) and 15% SDS-PAGE (in the case of Xyn2) using the Gibco Protein marker (Gibco) to estimate the size of the protein. The protein gel was stained with Coomassie brilliant blue and destained in accordance to Ausubel et al. (1998). Protein concentrations were determined with the aid of the Biorad Protein Assay (Biorad).

3.4 Results

Construction of recombinant *A. niger* strains containing *xyn2* and *egl* gene copies

Aspergillus niger D15, a pH mutant strain, was used as host for the heterologous production of the *T. reesei* xylanase II and endoglucanase I enzymes in molasses and defined glucose media. Plasmids pGT, pGT-egI and pGT-xyn2 were co-transformed with plasmid p3SR2 into *A. niger* D15, respectively, and the expression cassettes integrated into the *A. niger* genome in multiple sites (Wernars et al. 1985). Plasmid p3SR2 contained the *amd*S gene that allowed for selection of *A. niger* transformants now capable of acetamide and acrylamide utilization in the absence of other nitrogen sources. The transformant containing pGT (designated *A. niger* D15[pGT]) acted as negative control.

Chromosomal DNA was isolated from *A. niger* D15[pGT] as well as the *xyn2* and *egl* transformants (designated *A. niger* D15[*xyn2*] and *A. niger* D15[*egl*], respectively)

that expressed the highest level of recombinant enzyme activity. The DNA was digested overnight with *Hin*dIII, a restriction enzyme that does not cut within the *xyn2* or *egI* coding regions, and used for Southern blot analysis. Southern blot analysis of the digested chromosomal DNA showed that multiple copies of the genes have integrated into the genomes of the transformants. The number of the *xyn2* gene copies present in the genome of *A. niger* D15[*xyn2*] was determined to be at least five copies. The native *A. niger* xylanase genes were not detected (Figure 2A). At least two copies of the *egI* gene were present in the *A. niger* D15[*egI*] (Figure 2B).

Plate Assays for Enzymatic Activity

Spores of *A. niger* D15[*xyn2*] as well as the control strain, *A. niger* D15[pGT], were spotted onto RBB-xylan activity plates containing 10% (w/v) glucose. *A. niger* D15[*xyn2*] produced a zone after about 6 hours of growth, whereas, *A. niger* D15[pGT] only produced a zone after 48 hours, upon depletion of the glucose surrounding the colony (Figure 2C). *A. niger* D15[*egI*] started producing a clearing zone on OBR-HECellulose after 10 hours, whereas *A. niger* D15[pGT] only started to produce a zone after about 72 hours of growth (Figure 2D).

Effect of pH and Temperature on Activity Levels

The optimum levels of activity for the recombinant Xyn2 xylanase was achieved between pH 5 and 6 and at a temperature of 50 - 60°C (Figure 3A). This is similar to the pH and temperature optimum when the recombinant Xyn2 was produced by *Saccharomyces cerevisiae* (La Grange et al. 1996) as well as the native Xyn2 produced by *T. reesei* (Törrönen and Rouvinen, 1995) (see Table 3).
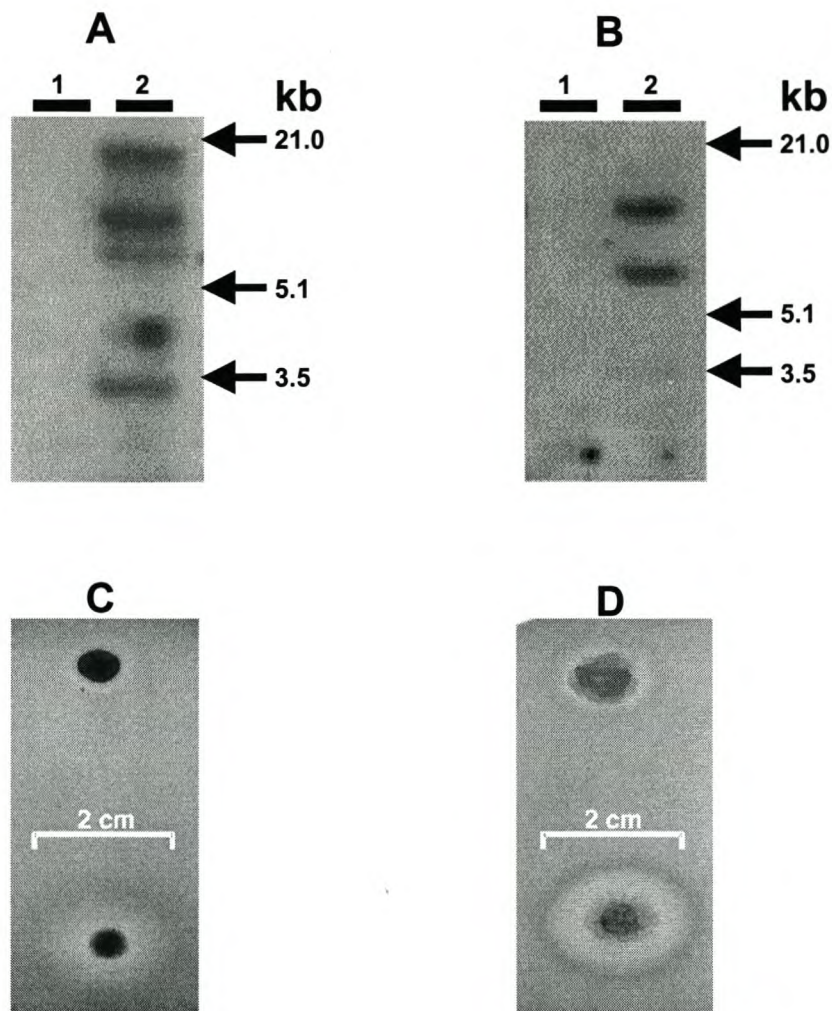
Figure 2. Southern blot analysis to determine the copy number of the *xyn2* and *egI* genes present on the genome of (**A**) *A. niger* D15[*xyn2*] and (**B**) *A. niger* D15[*egI*]. The total DNA was isolated and digested overnight with *Hin*dIII. Lane 1 contains the DNA isolated from *A. niger* D15[pGT] and lane 2 in **A** and **B** the DNA isolated from *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*], respectively. The internal 540 bp *Eco*RI – *Xho*I fragment of *xyn2* and the internal 685 bp *Eco*RI – *Xho*I fragment of *egI* were labeled with [α-^{32}P]ATP and used as DNA-probes. The marker sizes are indicated on the right. Each ^{32}P-highlighted DNA species represents a copy of the *xyn2* or *egI* gene, respectively. Recombinant xylanase produced by *A. niger* D15[*xyn2*] on RBB-xylan plates (**C**) and recombinant endoglucanase activity produced by the *A. niger* D15[*egI*] on OBR-HECellulose plates (**D**). The negative control, *A. niger* D15[pGT], is shown at the top with the transformant at the bottom. Colonies degrading RBB- or OBR-HECellulose are surrounded by pale clearing zones. The plates were photographed after 48 hours incubation at 30°C.

Table 3 summarises the characteristics of the native and heterologously produced Xyn2. The recombinant EgI endoglucanase produced by *A. niger*, exhibited its highest levels of activity at pH 5 and 60°C (Figure 3B).

Table 3. Comparison of the characteristics of Xyn2 and EgI when produced by different hosts

	T. reesei	*A. niger D15*	*Y. lipolytica*	*S. cerevisiae*
Xyn2:				
Enzyme activity (nkat/ml)	5400 [Bailey et al. 1993[a]]	8100		1200 [La Grange et al. 1996]
pH optimum	5-5.5 [Törrönen and Rouvinen 1995]	5 - 6		6 [La Grange et al. 1996]
Temp. optimum °C	56-60[a] [Dekker 1983]	50 - 60		60 [La Grange et al. 1996]
Protein Size in kDa	20 [Lappalainen et al 2000]	21		27 [La Grange et al. 1996]
EgI:				
Enzyme activity (nkat/ml) on CMC	1469.6 [Montenecourt 1983[b]]	2300	367.4 [22 U/ml Park et al. 2000]	119[c]
pH optimum	4-5 [Takashima et al. 1998, Zurbriggen et al. 1991]	5 [4[d] in Takashima et al. 1998]	4.8 [Park et al. 2000]	6 [Zurbriggen et al. 1991]
Temp. optimum °C	60°C [Zurbriggen et al. 1991]	60	50 [Park et al. 2000]	60 [Zurbriggen et al. 1991]
Protein size kDa	50 [Takashima et al. 1998, Kleywegt et al. 1997]	62-100 [67[d] in Takashima et al. 1998]	60-80 [Park et al. 2000]	60-100 [Takashima et al. 1998]

[a] The combined action of all the xylanases produced by *T. reesei*.

[b] The cellulolytic mutant RUT C-30 can produce levels of up to 225 IU/ml (3757.5 nkat/ml [Montenecourt 1983]

[c] Values obtained in this study using a multicopy episomal plasmid and the *ADH2* promoter for expression in SC^{-URA} medium (La Grange et al. 1996)

[d] Expression in *Aspergillus oryzae* as host
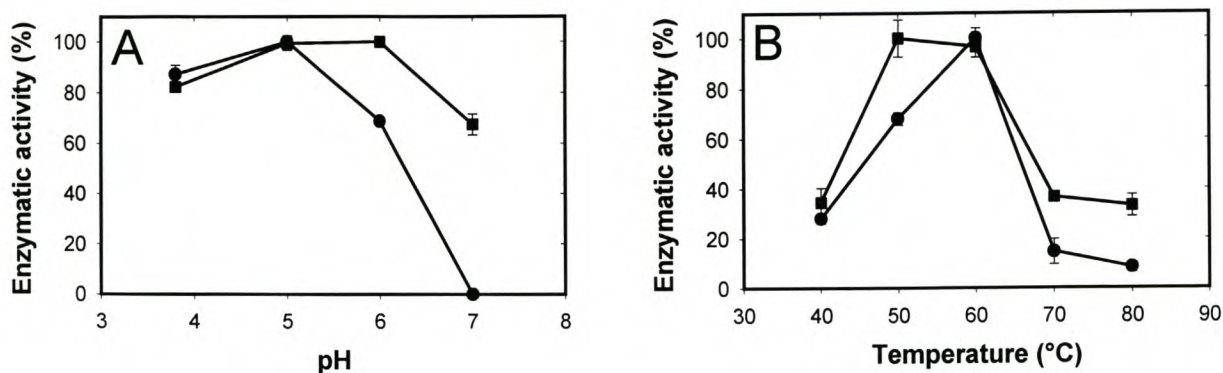
nd - not determined

Figure 3. The effect of (**A**) pH and (**B**) temperature on the enzymatic activity of the Xyn2 (■) and EgI (●) when produced by *A. niger* D15. The highest activity was measured at pH 5.5 and 55°C for the Xyn2 and pH 5 and 55 - 60°C for the EgI.

The thermostability of both the recombinant Xyn2 and EgI produced by *A. niger* was determined over a period of 3 hours (Figure 4). It was found that both the Xyn2 and EgI enzymes still retained more than 80% of their activity after 3 hours of incubation at 50°C but lost almost all activity within 2 hours at 70°C. However, EgI retained about 50% activity when incubated at 60°C for 3 hours.

Heterologous Enzyme Production

The production of heterologous Xyn2 and EgI by *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*], respectively, were followed over a time period of 15 days in both molasses as well as double strength liquid minimal media (2xMM). The highest level of activity for Xyn2 was achieved on day 9 in molasses and day 10 in 2xMM medium (Figure 5).
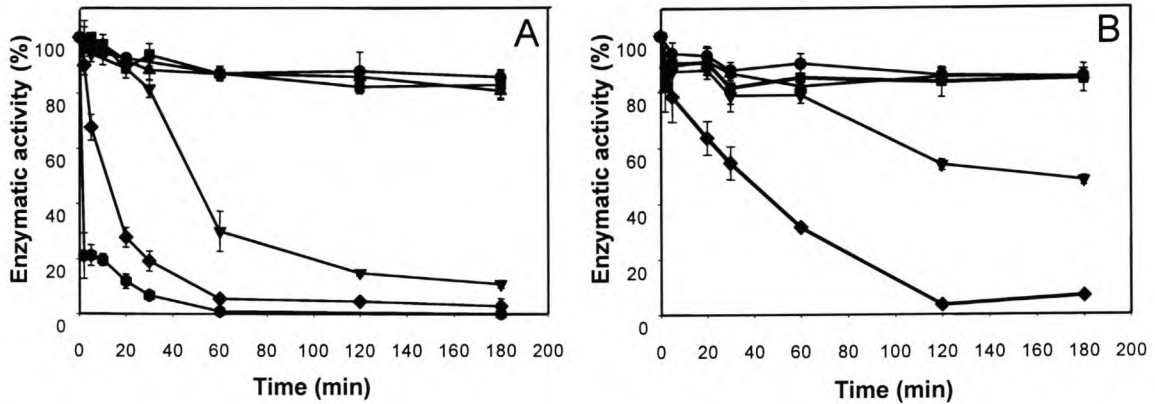
Figure 4. The thermostability of recombinant Xyn2 (**A**) and EgI (**B**) produced by *A. niger* D15 at 4°C (●), 40°C (■), 50°C (▲), 60°C (▼), 70°C (◆) and 80°C (filled hexagon). Freeze-dried enzyme (0.2 μg) was pre-incubated at the various temperatures in the absence of the substrate for up to 180 minutes before the remaining activity was determined. The activity determined prior to the incubations was taken as 100%.
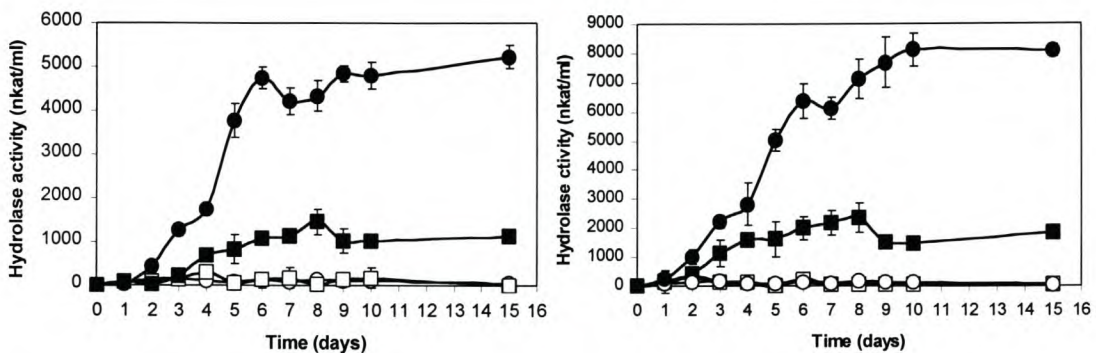

Figure 5. The heterologous production of xylanase (circles) and endoglucanase (squares) by *A. niger* D15[*xyn2*] (●) and *A. niger* D15[*egI*] (■) and *A. niger* D15[pGT] (O,□) was monitored over a period of 15 days in (**A**) molasses and (**B**) 2xMM medium. The pH of the media did not change significantly. The pH dropped from pH 5.5 to 4.5 after 3 days of cultivation and remained at pH 4.5 for the duration of the experiment. Enzyme activity assays were determined in triplicate using 6 parallel cultures. Enzyme activity was determined as described in La Grange et al. (1996).

The highest level of activity obtained with *A. niger* D15[*xyn2*] was 8000 nkat/ml, which is about 6 times more than that obtained with expression in *S. cerevisiae* and 1.5 times

more than that obtained in *T. reesei* (La Grange et al. 1996; Bailey et al. 1993a). The highest level of xylanase activity reached with growth in molasses was 5000 nkat/ml. *A. niger* D15[*egI*] produced at day 8, endoglucanase activity levels of 1400 and 2300 nkat/ml in molasses and 2xMM, respectively.

Heterologous Protein Isolation and SDS-PAGE Analysis

The supernatant of *A. niger* D15[pGT], *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*] was harvested on day 4 and freeze dried in order to concentrate the enzymes produced in 2xMM. In all cases the total extracellular protein content was determined as about 4% and the mixture was used without further purification. The activity of the recombinant Xyn2 in the unpurified supernatant was determined as 54 nkat/mg. The activity of the recombinant EgI was determined as 11 and 48 nkat/mg in the unpurified supernatant on CMC and lichenan as substrates, whereas *A. niger* D15[pGT] yielded only 1.8 and 1.3 nkat/mg of unpurified supernatant on CMC and lichenan, respectively.

Samples (2 mg) of the *A. niger* D15[*xyn2*] freeze-dried supernatant, corresponding to 50 µg of extracellular protein, was loaded onto a 15% SDS protein gel (Figure 6A). The Xyn2 protein was present as a single prominent band, while the rest of the native secreted proteins were barely visible. From the gel it was evident that the recombinant Xyn2 protein constituted a significant portion of the total amount of protein produced extracellularly by *A niger* D15[*xyn2*]. The recombinant Xyn2 enzyme has a molecular weight of about 21 kDa, which corresponds to the size of the native Xyn2 produced by *T. reesei* (Lappalainen et al. 2000).

Samples of the *A. niger* D15[*egI*] freeze-dried supernatant (50 µg of unpurified extracellular protein) was loaded onto a 10% SDS protein gel (Figure 6B). The recombinant EgI was present as a heterogenous protein species, constituting a large portion of the extracellular protein fraction. The recombinant EgI exhibited a molecular size varying from 62 - 100 kDa.
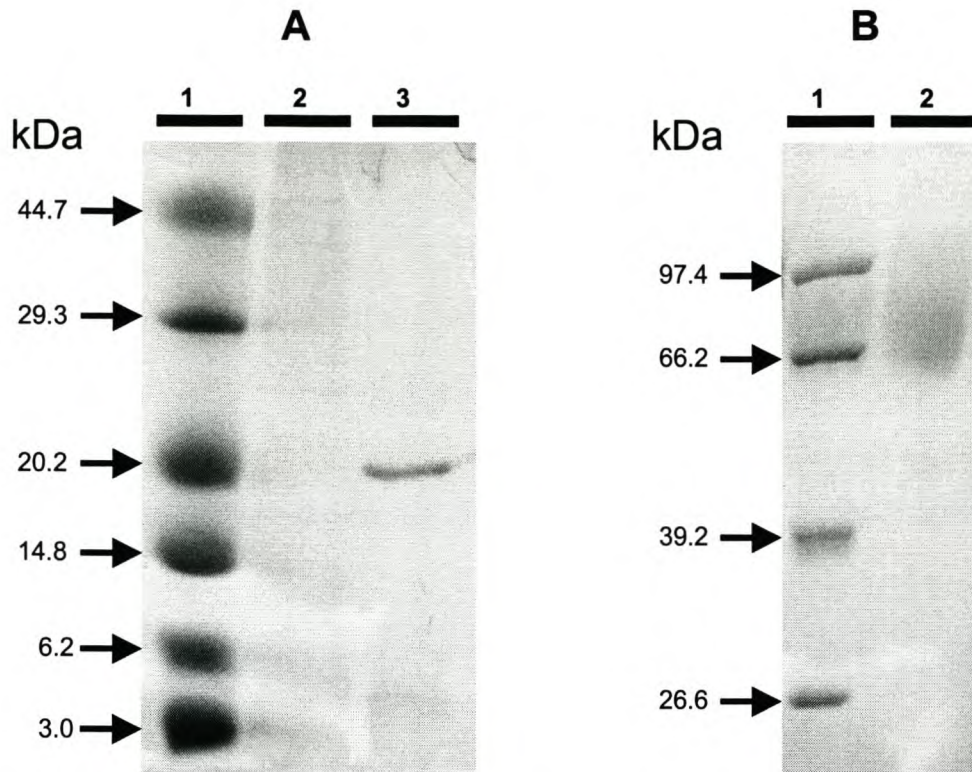
Figure 6. Separation of the total extracellular protein fractions (50 μg) of (**A**) *A. niger* D15[pGT] (lane 2) and *A. niger* D15[*xyn2*] (lane 3), and (**B**) *A. niger* D15[*egI*] (lane 2) on 15% and 10% SDS-PAGE, respectively. Lane 1 contains the molecular weight marker with the sizes depicted on the left and hand side. The recombinant Xyn2 is present as a single protein species of about 21 kDa (panel A, lane 3) while the recombinant EgI is a more heterogeneous protein species of > 60kDa (panel B, lane 2).

3.5 Discussion

cDNA copies of the *xyn2* and *egI* genes of *T. reesei* QM6a have been cloned and successfully expressed in the *A. niger* D15 strain. *A. niger* D15 is a nonacidifying pH mutant (*phmA*) derived from a protease-deficient (*prtT*) strain of AB1.13 (Gordon et al. 2000; Wiebe et al. 2001). Stable transformants were selected that constitutively expressed the *T. reesei* genes by means of the *gpd* promoter (Figure 1). The recombinant strains *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*] contained at least five and two copies of the *xyn2* and *egI* genes, respectively (Figure 2A, B). However, not all the gene copies may be intact or are functionally expressed. The integration site may also influence the

level of gene expression from the individual *xyn2* and *egI* gene copies (Verdoes et al. 1993, 1995).

The highest level of xylanase activity obtained from *A. niger* D15[*xyn2*] was 8000 nkat/ml. This activity level compared extremely well with the 5400 nkat/ml obtained from *T. reesei* Rut C-30 (Table 3) (Bailey et al. 1993a), when taking into account that the *T. reesei* activity represented the combined and synergistic action of the complete battery of xylanase enzymes, whereas the activity expressed in *A. niger* largely constituted that of the recombinant Xyn2 xylanase. The EgI endoglucanase activity obtained from *A. niger* D15[*egI*] was several fold higher than the levels obtained from other heterologous hosts (Table 3). *A. niger* is therefore the preferred host for heterologous expression of hydrolase genes, such as *xyn2* and *egI*.

The Xyn2 and EgI enzymes produced in different hosts had very similar biochemical characteristics (Tables 3). Our study indicated that recombinant EgI has a preference for CMC rather than lichenan, which was in contrast to the results obtained with expression of the *egI* in *S. cerevisiae* by Penttilä et al. (1987). Both the recombinant Xyn2 and EgI constituted the bulk of the extracellularly protein fraction produced by the recombinant *A. niger* D15[*xyn2*] and *A. niger* D15[*egI*] strains on molasses or glucose-based minimal (2xMM) media, respectively. The pH of the growth media was monitored throughout the study, and never dropped below pH 4.5, thus preventing activation of the native *A. niger* extracellular acid proteases. SDS-PAGE did not reveal any visible degradation of the enzymes. The production of native extracellular proteins by *A. niger* D15[pGT] on molasses or 2xMM media was very limited, confirming that the recombinant Xyn2 and EgI proteins were produced at high homogeneity (Figure 6).

The recombinant Xyn2 is similar in size to that of the native enzyme (Lappalainen et al. 2000). The EgI endoglucanase activity obtained was lower than what would be expected when considering the amount of protein present on the SDS-PAGE gel (Figure 6). This could imply that a significant amount of protein was inactive, as was the case reported by Aho et al. (1996), where only 2% of the heterologous produced EgI was found to be active. The endoglucanase protein was also more heterogenous in size when

expressed in *A. niger*, than with expression in *S. cerevisiae* (Penttilä et al. 1987). This phenomena, however, is not uncommon with expression of *T. reesei* enzymes in *Aspergillus* and can be ascribed to variable glycosylation patterns (Takashima et al. 1998). The glycosylation, responsible for the heterogenous nature of the protein, could be responsible for the lack of activity with the sugars covering the active site.

The use of the *gpd* promoter enabled the strain to produce the recombinant enzymes upon germination without the need for an inducer, simplifying the production of recombinant enzymes in bulk. This particular strain used, was a pH mutant and therefore did not significantly acidify the growth media as is often observed for *A. niger* strains. Therefore, this strain does not produce high amounts of the acidic proteases, which could extracellularly degrade the recombinant proteins produced. The use of a pH mutant of *A. niger* as host thus led to the production of a significant amount of extracellular protein. The control strain, *A. niger* D15[pGT], exhibited low levels of endoglucanase and xylanase activity when cultured on molasses or 2xMM media, despite the fact that *Aspergillus* spp. are known for their potent cellulase and xylanase activities. The levels of native *Aspergillus* glycoside hydrolases remained insignificantly low even after depletion of the glucose in the media.

In general, the levels of recombinant protein production could further be increased by increasing the inoculum concentration or using salt adaptation. Redkar et al. (1998) found that the secretion capacity of the fungus is directly related to the number of hyphal tips. Increased hyphal branching or salt adaptation (as a result of osmotic stress) could increase the production capacity of recombinant *Aspergillus* heterologously expressing glycoside hydrolases.

The ability of *A. niger* to grow on inexpensive industrial waste such as molasses, makes it ideal for waste control as molasses is a waste product of the sugar industry. It has the ability to support the growth of *A. niger* D15 without the requirement of additional nutrients, despite the fact that this *pyrG* strain requires uridine in the growth media. The high levels of enzyme production obtained, as observed for recombinant Xyn2 and EgI, in shake flask cultures and the ease of cultivation make it worthwhile to

consider the use of *A. niger* for industrial enzyme production on molasses as a commercially available carbon source.

3.6 Acknowledgements

3.7 References

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CHAPTER 4

The impact of cultivation conditions and strain properties on fungal growth and recombinant β-xylanase II production by *Aspergillus niger*.

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The impact of cultivation conditions and strains properties on fungal growth and recombinant β-xylanase II production by *Aspergillus niger*

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4.1 Abstract

The cultivation conditions of *Aspergillus* in submerged cultures largely determine its mycelial morphology and consequently affect the production of extracellular proteins. The impact of different cultivation conditions and strain properties on the heterologous *Trichoderma reesei* β-xylanase II production by a *pyrG⁻ Aspergillus niger* D15 was evaluated by monitoring β-xylanase activity. Optimal β-xylanase production and productivity was obtained in 20% molasses at 30°C medium. High spore inoculation (1×10^6 spores/ml) improved β-xylanase productivity only marginally, whereas an initial pH between 5.5 and 8.5 had no significant effect. The construction of a prototrophic strain containing extra copies of the *pyrG* gene, combined with salt adaptation in 2 M KCL yielded a recombinant strain that gave the highest recombinant β-xylanase production (5015 nkat/ml) and β-xylanase productivity (143.7 µkat/g).

Aspergillus niger, xylanase, heterologous, cultivation, molasses

4.2 Introduction

Filamentous fungi of the species *Aspergillus* are major producers of industrial enzymes (MacCabe et al. 2002). β-Xylanases are currently being used in different industrial processes, such as the reducing the amount of chlorine required for bleaching in the pulp and paper industry, increasing dough volume in the baking industry, improving digestion of animal feed, increasing clarification of wine and fruit juice (Aristidou and Pentilä, 2000; Oksanen et al. 2000; Colagrande et al. 1994). The enzyme production yields, production cost, and the cost of the feed stock in particular, are critical important determinants of the economical viability of applying these enzymes in industrial processes (Lynd et al. 1999). It is, therefore, important to establish a means of producing enzymes from inexpensive readily available material, such as byproducts from existing industrial processes, in order to keep the production cost to a minimum.

South Africa is a prominent producer of cane sugar in the world and annually produces large quantities of molasses as byproduct (Van Niekerk, 1981). Molasses is the condensed residue that remains after the removal of crystallized sucrose from the concentrated sugar juice. Molasses is a heterogeneous product, containing approximately 46.7 % total sugars of which 33.2% is sucrose, 2.11% chlorine, 3.3 % potassium, as well as a variety of amino acids and spore elements. It is readily available at low cost and used extensively in the manufacturing of bakers' yeast, in the fermentation industry for the production of potable alcohol and fuel alcohol, as well as a chemical feedstock.

Whereas growth and production of primary metabolites on molasses based industrial medium has been reported in literature (Vitolo et al. 1995; Jafelice et al. 1990), few studies have considered production of recombinant proteins on such medium. The xylanase II (*xyn2*) gene of *Trichoderma reesei* previously has been expressed in *A. niger* D15 (Rose and Van Zyl, 2002). In this paper we explored the impact of cultivation conditions and strain properties on growth and production of recombinant Xyn2 produced by *A. niger* D15 in a medium consisting solely of diluted molasses. Particular attention is paid to documenting instances where the response of growth (biomass production) and recombinant protein production differ, as such instances represent potentially important dilemmas that could be addressed in future work.

4.3 Material and methods

Strains, Plasmids, Media and Cultivation Conditions

The genotypes of the bacterial and fungal strains as well as the plasmids used in this study are summarized in Table 1. The construction of the plasmids and the origin of the recombinant fungal strains, *A. niger* D15[pGT] and *A. niger* D15[*xyn2*], were described in detail in Rose and Van Zyl (2002).

Table 1. The genotype and sources of the strains and plasmids used in this study

Strains / plasmids	Genotype*	Source
Strains:		
A. niger D15	*pyrG⁻ prtT phmA* (non-acidifying)	Wiebe et al. 2001
A. niger D15[pGT]	*A. niger* D15 with gpd_P-$glaA_T$ expression cassette and *amdS* gene	Rose and Van Zyl, 2002
A. niger D15[*xyn2*]	*A. niger* D15 with gpd_P-*xyn2*-$glaA_T$ expression cassette and *amdS*	Rose and Van Zyl, 2002
A. niger D15[pGT]PyrG⁺	*A. niger* D15[pGT] with *amdS* and *pyrG⁺* genes	This study
A. niger D15[*xyn2*]PyrG⁺	*A. niger* D15[*xyn2*] with *amdS* and *pyrG⁺* genes	This study
A. niger D15[pGT]SA	*A. niger* D15[pGT]PyrG⁺, salt adapted strain	This study
A. niger D15[*xyn2*]SA	*A. niger* D15[*xyn2*]PyrG⁺, salt adapted strain	This study
Plasmids*:		
pGT	*bla* gpd_P-$glaA_T$	Rose and Van Zyl, 2002
pGT-xyn2	*bla* gpd_P-*xyn2*-$glaA_T$	Rose and Van Zyl, 2002
pBS-pyrG-amdS	*bla amdS pyrG*	This laboratory (Dr. A. Plüddemann)

*All expression cassettes and listed genes are integrated into the chromosome of recombinant *A. niger* D15 strains

The molasses used in this study was supplied by Tongaat-Hulett (South Africa). Routinely, 20 ml of 20% molasses (native pH of 5.5) was inoculated to a final concentration of $1x10^5$ spores per ml, cultivated at 30°C at 100 rpm in 125 ml flasks unless stated otherwise. Although the *A. niger* D15[*xyn2*] and *A. niger* D15[pGT] strains require uridine for growth in synthetic media, they grow well in molasses without the addition of uridine and uridine was subsequently omitted from the media. The plasmid, pBS-pyrGamdS, was introduced and integrated into the genomes of *A. niger* D15[pGT] and *A. niger* D15[*xyn2*], resulting in the prototrophic *A. niger* D15[pGT]PyrG$^+$ and *A. niger* D15[*xyn2*]PyrG$^+$ strains (Table 1). DNA transformations were performed as described by Punt and van den Hondel (1992). Plates containing acetamide, but lacking uridine, were used for the selection of PyrG$^+$transformants (Punt and van den Hondel, 1992).

The procedure for salt adaptation was followed as described in Redkar et al. (1998). The fungus was plated out twice onto spore plates containing 0.5 M KCl and allowed to grow and sporulate for 4 - 6 days. The spores were subsequently transferred to plates containing 1 M, 1.5 M and 2 M KCl. Finally, spores were transferred three times to plates containing 2 M KCl. Spores of the salt adapted strains of *A. niger* D15[pGT]PyrG$^+$ and *A. niger* D15[*xyn2*]PyrG$^+$, subsequently called *A. niger* D15[pGT]SA and *A. niger* D15[*xyn2*]SA, respectively, were stored in a 2 M KCl solution.

Enzyme Assay

The β-xylanase activity was determined by means of liquid assay and performed at 50°C as described by La Grange et al. (1996). The substrate used for liquid assays was 1% birchwood xylan (Roth), resuspended in 0.05 M sodium citrate buffer (pH6). One unit of enzyme was defined as the activity producing 1 µmol per minute of reducing sugars in xylose equivalents under these assay conditions.

Molasses Optimization

Molasses contains a variety of substances (Van Niekerk, 1981). Since some of the substances may be inhibitory to the growth of the fungus, the optimal concentration of the molasses required for optimal Xyn2 production, was determined by using flasks containing 20 ml of 10, 20, 30, 40

and 50% molasses (v/v) inoculated with 1×10^5 spores/ml of the *A. niger* D15[pGT] and *A. niger* D15[*xyn2*] strains, respectively. The ideal inoculum concentration of *A. niger* D15[pGT] and *A. niger* D15[*xyn2*] spores was determined by inoculating 20 ml of 20% molasses, to a final concentration of 1×10^4, 1×10^5 and 1×10^6 spores per ml, respectively. The optimal temperature of cultivation was determined by inoculating 1×10^5 spores/ml of *A. niger* D15[pGT] and *A. niger* D15[*xyn2*] in 20 ml of 20% molasses and cultivating at 20, 25 and 30°C, respectively.

The optimum pH required for the germination of the spores was determined by inoculating 1×10^5 spores/ml of the *A. niger* D15[pGT] and *A. niger* D15[*xyn2*] strains in 20 ml of 20% molasses. The initial pH of the molasses was set at pH 5.5, 6.5, 7.5 and 8.5 by the addition of 10 M NaOH aseptically after autoclaving.

The *A. niger* D15[pGT], *A. niger* D15[*xyn2*] and *A. niger* D15[*xyn2*]PyrG$^+$ strains were inoculated to a spore concentration of 1×10^6 spores/ml in 20 ml of 20% molasses at pH 6.5 and cultivated under optimal conditions in order to determine if the uridine dependency of *A. niger* D15[*xyn2*] plays a significant role in the β-xylanase activity or biomass production. The salt adapted strain *A. niger* D15[*xyn2*]SA was compared to *A. niger* D15[*xyn2*]PyrG$^+$ (which was not salt adapted) by inoculating and cultivating them under optimal conditions to determine whether the salt adaptation had any effect on the amount of β-xylanase activity produced.

Biomass Determination

The recombinant *Aspergillus* strains were cultivated in molasses for 10 days. The biomass was harvested after 10 days using a pre-weighed myracloth filter and vacuum pump. The mycelia were washed with 20 ml of distilled water to remove the remaining molasses. Myracloth filters containing the biomass were dried at 70°C for 24 hours to remove all moisture. The final mass was determined and the mass of the filter subtracted.

4.4 Results and Discussion

The cultivation conditions of *Aspergillus* in submerged cultures largely determine the mycelial morphology and affects the production of extracellular proteins (Galbraith and Smith, 1969; Wönsten et al. 1991). The β-xylanase activity of recombinant *A. niger* D15[*xyn2*] expressing the

T. reesei xyn2 gene was monitored over a period of 10 days under different cultivation conditions to determine the optimum conditions required for extracellular Xyn2 production in molasses media (Figure 1A-D). The total biomass production (dry weight) after 10-day incubation periods was measured to determine the productivity (enzyme yield per biomass).
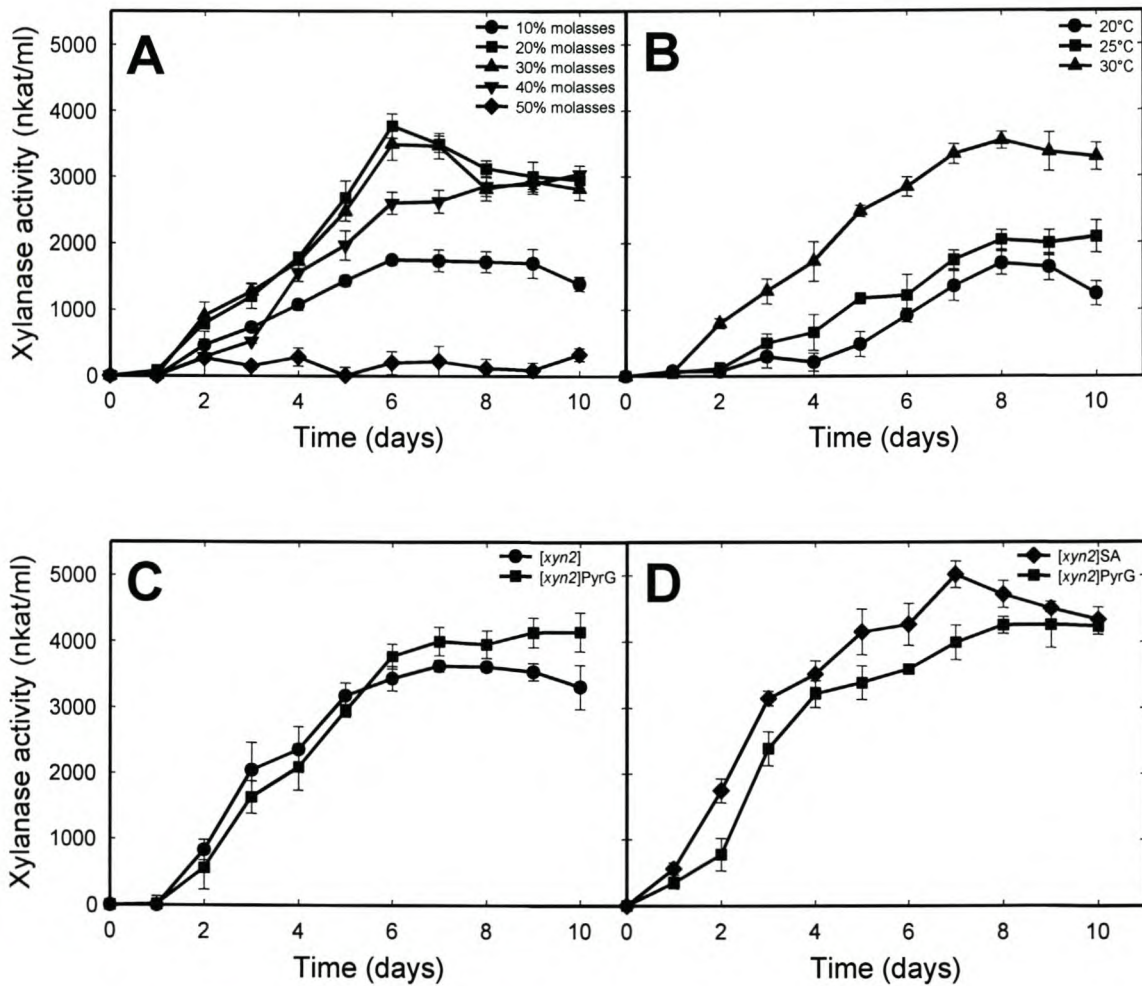

Figure 1. The heterologous production of Xyn2 by recombinant *A. niger* D15 strains was monitored over a period of ten days in different molasses media formulation and under different growth conditions: (**A**) media formulations containing 10-50% molasses concentration, (**B**) cultivation temperatures ranging from 20°C-30°C, (**C**) introduction of additional *pyrG* gene copies and (**D**) salt adaptation to 2M KCL.

The highest β-xylanase activities of 3779 and 3496 nkat/ml were produced by strain *A. niger* D15[*xyn2*] in 20 and 30% molasses, respectively (Figure 1A). Higher molasses concentrations

sustained more biomass production, but less enzyme production. The maximum biomass (58.4 g/l) was obtained at 40% molasses. However, at 50% molasses significantly less biomass was obtained after the same incubation period (Table 2). The viscosity at 50% molasses limited agitation in shake flasks and most probably did not allow for efficient oxygen transfer. Molasses also contains a variety of components, some of which could be inhibitory to the growth of the fungus at higher concentrations (Van Niekerk, 1981). The highest enzyme productivity was obtained at 20% molasses, suggesting it being the preferred concentration for optimal enzyme production. The β-xylanase production by *A. niger* D15[pGT] in all the experiments did not exceed 500 nkat / ml and was not included in Figure 1 A-D.

An increase in spore inoculum concentration from $1x10^4$ spores/ml to $1x10^5$ spores/ml and $1x10^6$ spores/ml resulted in a 18% and 26% increase in heterologous β-xylanase activity produced (Table 2). Protein secretion in mycelial fungi predominantly takes place at the hyphal tips and the secretion capacity of a fungus is thus directly proportional to the number of hyphal tips present in the culture (Wönsten et al. 1991; MacKenzie et al. 1994). Higher spore concentrations yield more germinating spores, thus more individual mycelial masses with more hyphal tips that form. However, despite the higher enzyme activity observed with increased spore concentration, no significant increase in biomass yield was noticed after 10 days. The latter phenomenon may be ascribed to the fact that the utilizable sugars in the media was not increased. Nevertheless, we conclude that higher concentrations ($1x10^6$ spores/ml in this study) are preferred for high heterologous β-xylanase production per biomass produced.

The efficiency of *Aspergillus* spore germination, the proteolytic system and morphology (pellet formation) were found to be affected by the pH of the growth media (Punt et al. 2002) with the optimum pH for spore germination being between pH 7 – 8, whereas the natural pH of molasses is 5.5. In this study the effect of pH at the time of inoculation was found to be not critical when considering both enzyme production and biomass production (Table 2). MacKenzie et al. (1994) found that lowering the cultivation temperature decreased biomass production, while increasing enzyme production. The effect of cultivating at different temperatures on the heterologous production of β-xylanase was determined with 30°C yielding the highest β-xylanase production and productivity (Figure 1B, Table 2). Cultivation at lower temperatures yielded less biomass, accompanied by a significant drop in the β-xylanase productivity.

Table 2. The maximum β-xylanase activity (nkat/ml), total biomass (g) and β-xylanase productivity (μkat/g) recombinant *A. niger* cultures were determined after cultivation of 10 days in different media formulations and under different growth condition.

Cultivation condition	β-Xylanase activity (nkat/ml)	Biomass (dry weight) (g/l)	β-Xylanase productivity (μkat/g)
Molasses concentration			
10%	1751 ± 66	19.7 ± 1.2	89.0 ± 5.8
20%	3774 ± 185	31.3 ± 2.5	121.0 ± 9.1
30%	3496 ± 240	47.6 ± 4.4	73.9 ± 6.8
40%	3034 ± 133	58.4 ± 9.5	53.2 ± 9.9
50%	324 ± 97	3.7 ± 0.4	87.8 ± 1.1
Spore concentration			
1×10^4 spores/ml	3089 ± 157	28.9 ± 0.7	114.8 ± 3.2
1×10^5 spores/ml	3779 ± 362	30.2 ± 2.3	125.9 ± 10.1
1×10^6 spores/ml	4174 ± 171	30.8 ± 2.2	135.9 ± 9.9
Initial pH			
5.5	2971 ± 185	32.7 ± 1.9	91.3 ± 5.4
6.5	3717 ± 365	35.4 ± 2.2	105.4 ± 6.6
7.5	3082 ± 217	36.6 ± 2.7	84.5 ± 6.4
8.5	3503 ± 193	34.9 ± 3.5	101.2 ± 10.2
Cultivation temperature			
20°C	1693 ± 174	20.6 ± 0.7	81.4 ± 2.4
25°C	2083 ± 238	25.4 ± 2.9	82.5 ± 7.9
30°C	3544 ± 131	28.1 ± 2.8	127.0 ± 12.2
Prototrophic strains			
D15[*xyn2*]PyrG⁺	4144 ± 293	37.9 ± 2.1	109.4 ± 6.1
Control(D15[*xyn2*])	3632 ± 85	34.4 ± 2.3	106.0 ± 7.6
Salt adaptation			
D15[*xyn2*]SA	5015 ± 198	34.9 ± 1.0	143.7 ± 4.4
Control(D15[*xyn2*]PyrG⁺)	4261 ± 345	35.5 ± 1.9	120.3 ± 6.7

The values in this table were obtained using six parallel cultures.

The recombinant strain *A. niger* D15[*xyn2*] is an uridine deficient strain that normally requires the addition of uridine to the growth media. Although the *A. niger* D15[*xyn2*] strain grew well without the addition of uridine (Figure 1A, B), the *pyrG* mutation could have a negative effect on the biomass produced when the uridine or uridine precursors / substitute in the

molasses or transport into the cell became limited. Additional *pyrG* gene copies were integrated into the genome of strain *A. niger* D15[*xyn2*] (generating strain *A. niger* D15[*xyn2*]PyrG$^+$) to complement the *pyrG*$^-$ mutation. Strain *A. niger* D15[*xyn2*]PyrG$^+$ was compared to *A. niger* D15[*xyn2*] with regard to β-xylanase production and productivity (Figure 1C). The β-xylanase activity and productivity increased with 14% and 3%, respectively. These results underlines the importance of using prototrophic strains independent of nutrient supplements.

Molasses (75% dry mass) contains significant amounts of potassium and chlorine (Van Niekerk, 1981). Redkar et al. (1998) found that salt adaptation affects gene expression levels. The effect of adapting strain *A. niger* D15[*xyn2*]pyrG$^+$ to 2 M KCl on β-xylanase and biomass production was evaluated. The salt-adapted strain *A. niger* D15[*xyn2*]SA gave the highest maximum β-xylanase activity (5015 nkat/ml) and β-xylanase productivity (143.7 μkat/g) obtained for any of the recombinant strains (Figure 1D, Table 2). This represent an improvement of more than 30% on strain *A. niger* D15[*xyn2*] before addition of the addisional *pyrG* gene copies and salt adaptation.

This study illustrated the effect of cultivation conditions on heterologous protein production by recombinant *Aspergillus* strains using molasses as growth medium. Optimal conditions for the production of *T. reesei* Xyn2 β-xylanase can be summarized as follows: inoculate 20% molasses at pH 6.5 with a spore inoculum of at least 1x10^6 spores/ml and cultivate at a temperature of 30 C. The importance of using prototrophic recombinant strains that are salt adapted was also demonstrated. It is important to take into account that the set of experiments described in this paper was performed with a single batch of molasses from Tongaat Hulett, South Africa. The composition of a different batch of molasses may differ depending on the region and season in which the sugar cane was harvested. Consequently, compared to the results obtained in this study, the fungal strains analysed here may react differently regarding growth, morphology and enzyme production when another batch of molasses is used as growth medium.

4.5 Acknowledgements

4.6 References

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CHAPTER 5

Constitutive expression of the *Phanerochaete chrysosporium* β-1,4-cellobiohydrolase gene (*cbh1-4*), the *Aspergillus aculeatus* β-1,4-endomannanase gene (*man1*) and the *Aspergillus kawachii* β-1,4-endoxylanase gene (xynC) in *Aspergillus niger*.

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5.1 Abstract

The major cellobiohydrolase (*cbh1-4*) of *Phanerochaete chrysosporium,* the endomannanase (*manI*) of *Aspergillus aculeatus* and the endoxylanase (*xynC*) of *Aspergillus kawachii* were successfully expressed in *Aspergillus niger* D15 under the transcriptional control of the glyceraldehyde-6-phosphate dehydrogenase (*gpd*) promoter derived from *A. niger* and the *glaA* terminator of *A. awamori.* Stable *A. niger* D15[*cbh1-4*], *A. niger* D15[*man1*] and *A. niger* D15[*xynC*] transformants, produced cellobiohydrolase activity of 500 nkat/ml, β-mannanase activity of > 6000 nkat/ml and β-xylanase activity of 900 nkat/ml (birchwood xylan) respectively. The recombinant Cbh1-4 enzyme yielded highest activity between pH 3.5 – 4.5 and 60 - 70°C depending on the substrate used. The ManI expressed highest levels of activity at pH 3.8 and between 75 - 80°C, while the Xyn3 reached optimal levels of activity at a pH < pH 3 and 55 – 60°C. The heterologous Cbh1-4, ManI and Xyn3 represents a significant portion of the total extracellular proteins produced by *Aspergillus* on minimal medium containing 10% glucose.

5.2 Introduction

Plant biomass contains a variety of compounds of which cellulose and hemicellulose are the most prominent (Schwarz, 2001). About half of the carbonaceous compounds in terrestrial biomass are cellulose, which makes cellulose the most abundant compound on earth. The cellulose strand consists of β-1,4-linked glucopyranose units with cellobiose as the basic repeating unit (Béguin and Aubert, 1994). The uniformity of the molecules promote spontaneous crystallization due to molecules forming inter- and intramolecular hydrogen bonding patterns resulting in tightly packed microfibrils (Schwarz, 2001). The combined action of endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) are required for the complete degradation of crystalline cellulose to glucose (Aristidou and Penttilä, 2000), with the action of the cellobiohydrolase being the slowest step in the process (Schwarz 2001).

Hemicelluloses are low molecular weight heterogenous chemical compounds with a wide variation in both structure and composition. Commonly occurring hemicellulases include the 1,3- and 1,4-β-galactans, mannans and xylans (Aristidou and Penttilä, 2000). Xylan and mannan are the main forms of hemicelluloses present in plant cell walls and can constitute up to 35% and 25% of the dry weight of plants, respectively (Puls and Schuseil, 1993). The xylan structures vary from linear β-1,4-polyxylose main chains to highly branched polysaccharides containing acetyl, arabinosyl and glucuronosyl side chains. Endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) act synergistically to degrade xylan to xylose. α-L-Arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139) and acetyl xylan esterases (EC 3.2.1.72) are responsible for the debranching of the xylan backbone.

Mannan can occur as either a homo- or a heteropolysaccharide in the form of glucomannan, galactomannan and galactoglucomannan (collectively known as β-mannans) (Jeffries, 1994). Glucomannan consists of β-1,4-linked D-mannose and D-glucose molecules, whereas galactomannan consists of a mannose backbone with galactose substituents on C-6. Galactoglucomannan has the same backbone chain as

glucomannan, but also contain α-1,6-linked galactose residues and may be acetylated at C-2 or C-3. In nature galactoglucomannans predominantly occur in softwoods, whereas galactomannans are mostly found in seeds of leguminous plants and carob beans (Setati et al. 2001). The hydrolysis of these substrates is accomplished through the action of endo-β-1,4-mannanase (EC 3.2.1.80), which randomly cleaves the β-mannosidic linkages within the main chain together with the exo-enzymes β-mannosidase (EC 3.2.1.25) and α-galactosidase (EC 3.2.1.22). Cellulases and hemicellulases have a broad spectrum of industrial applications including the production of fuel ethanol through fermentations, reducing the amount of chlorine required for bleaching in the pulp and paper industry, increasing dough volume in the baking industry, improving digestion of animal feed, increasing clarification of wine and fruit juice, etc. (Aristidou and Pentilä, 2000; Oksanen et al. 2000; Colagrande et al. 1994). Therefore, a large potential market exists for cellulases and hemicellulases.

The advantages of using *Aspergillus* spp. as host for heterologous expression include its high secretion capacity, GRAS (Generally Regarded As Safe) status, rapid growth on inexpensive media and a relatively well-studied genetic background. *Aspergillus* spp. has the ability to produce heterologous proteins in concentrations of grams per liter (Verdoes et al. 1995) and has been used by various industries for the production of a diversity of commodities such as citric and gluconic acid, enzymes such as proteases, catalases, isomerases, α-galactosidases, rennin, lipase, phytase, glucoamylase, pectinase, glucose oxidase, α-amylase, as well as pharmaceuticals such as interleukin-6 and Fab (Archer 2000; Gibbs et al. 2000). World sales of β-lactam antibiotics produced by *Aspergillus* spp. alone have been estimated at US$ 9 billion for 1997 alone (Gibbs et al. 2000).

The construction of a constitutive fungal expression cassette consisting of the glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd*$_p$) of *A. niger* and the glucoamylase terminator (*glaA*$_T$) of *Aspergillus awamori* have previously been described (Rose and Van Zyl, 2002). That study paved the way for this work which entailed the expression of the genes of enzymes which traditionally gave low yields and low enzyme activity when expressed in *Saccharomyces cerevisiae*. The *cbh1-4* of *Phanerochaete*

chrysosporium, *manI* of *Aspergillus aculeatus* and *xyn*3 of *Aspergillus kawachii* were constitutively expressed in *A. niger* under the transcriptional control of the *gpd* promoter. The enzymatic properties of the recombinant Cbh1-4, ManI and Xyn3 were also characterized.

5.3 Materials and methods

Media and Cultivation Conditions

Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5α and cultivated at 37°C in Terrific Broth and on Luria Bertani agar containing 100 µg/ml ampicillin for selective pressure (Sambrook et al. 1989). The fungal strains were cultivated at 30°C in minimal media containing 0.5% (w/v) yeast extract, 0.2% (w/v) casamino acids, 1% (w/v) glucose, 6% $NaNO_3$ (w/v), trace elements and 0.01 M uridine prior to spheroplast harvesting (Rose and van Zyl, 2002; Punt and van den Hondel, 1992). Transformants were selected on minimal medium containing 10 mM acetamide and 15 mM CsCl, but lacking casamino acids, uridine and $NaNO_3$. Media were inoculated to a concentration of $1x10^6$ spores per ml unless stated otherwise. *A. niger* D15 transformants were cultivated in double strength minimal media (2xMM) containing 10% glucose for enzyme activity determination.

Strains and Plasmids

The genotypes of the bacterial and fungal strains as well as the plasmids used in this study are summarized in Table 1.

DNA Manipulation

Standard protocols were followed for all DNA manipulations and *E. coli* transformations (Sambrook et al. 1989). *A. niger* D15 transformations were performed by means of spheroplasting using Lysing enzymes (Sigma) in accordance to Punt and van den Hondel (1992).

Table 1. The genotype and sources of the strains and plasmids used in this study

Strains	Genotype	Source
E. coli DH5α	*supE44 ΔlacU169 (∅80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook et al. 1989
A. niger D15	*pyrG prtT phmA* (non-acidifying)	Wiebe et al. 2001
A. niger D15[pGT]	*A. niger* D15 with gpd_P-$glaA_T$ integrated into the chromosome	Rose and Van Zyl, 2002
A. niger D15[*cbh1-4*]	*A. niger* D15 with gpd_P-$cbh1$-4-$glaA_T$ integrated into the chromosome	This study
A. niger D15[*man1*]	*A. niger* D15 with gpd_P-$man1$-$glaA_T$ integrated into the chromosome	This study
A. niger D15[*xynC*]	*A. niger* D15 with gpd_P-$xynC$-$glaA_T$ integrated into the chromosome	This study

Plasmids:

pBLUESCRIPT SK	*bla*	
pCBH	*bla URA3 $PGK1_P$-CBH1-4-$PGK1_T$*	Van Rensburg et al. 1998
pGT	bla GPD_P-GLA_T	Rose and Van Zyl, 2002
pJC3	*bla URA3 $PGK1_P$-xynC-$PGK1_T$*	Crous et al. 1995
pBS-pyrGamdS	*bla $pyrG_P$-pyrG-$pyrG_T$ $amdS_P$-amdS-$amdS_T$*	Plüddeman 2002
pBS-man1	*bla man1*	Setati et al. 2001
pBS-cbh1-4	*bla cbh1-4*	This study
pGT-cbh1-4	bla gpd_P-$cbh1$-4-$glaA_T$	This study
pGT-man1	bla gpd_P-$man1$-$glaA_T$	This study
pGT-xynC	bla gpd_P-$xynC$-$glaA_T$	This study

Plasmid Construction

Plasmid pGT (Rose and Van Zyl, 2002), was used as expression vector for the constitutive expression of the recombinant *cbh1-4*, *man1* and x*ynC* genes. The *cbh1-4* was retrieved from pCBH (Van Rensburg et al. 1998) as an *Eco*RI - *Xho*I fragment and subcloned into pBLUESCRIPT SK, generating pBS-cbh1-4. The *cbh1-4* gene was retrieved as a *Not*I – *Xho*I fragment and cloned into the corresponding sites of pGT, generating pGT-cbh1-4 (Figure 1). The manI gene was received as a *Eco*RI-*Xho*I

fragment cloned into the corresponding sites of pBLUESCRIPT. *Man1* was retrieved from pBLUESCRIPT-man1 (Setati et al. 2001) as a *Not*I-*Xho*I fragment and cloned into the *Not*I and *Sal*I sites of pGT, generating pGT-man1. XynC was retrieved from pJC1-xynC (Crous et al. 1996) as a *Eco*RI-*Xho*I fragment with the *Eco*RI overhang filled in with Klenow polymerase (Roche). The xynC was cloned into the *Not*I site (overhang filled in with Klenow polymerase) and *Sal*I sites of pGT, creating pGT-xynC. The gene products of gpd_P-*cbh1-4*-gla_T, gpd_P-*man1*-gla_T and gpd_P-*xynC*-gla_T have been designated Cbh1-4, ManI and Xyn3, respectively.
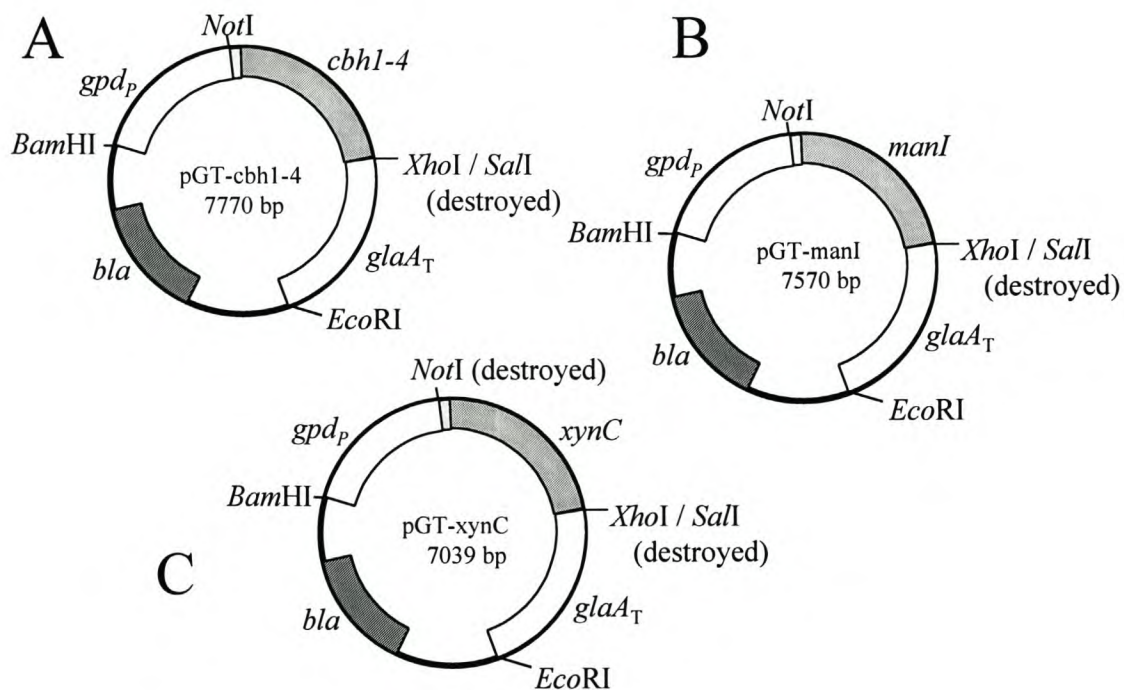

Figure 1. A schematic representation of expression vectors (**A**) pGT-cbh1-4, (**B**) pGT-manI and (**C**) pGT-xynC. The *cbh1-4*, *manI* and *xynC* genes are indicated with cross-hatched boxes, their secretion moieties with dotted boxes, the selectable marker (*bla*) by a hatched boxes, the *gpd* promoter and *glaA* terminator sequences are indicated by the open boxes and the pSPORT1 sequences are indicated by thick lines, respectively.

DNA Hybridizations

Southern hybridizations were carried out according to Sambrook et al. (1989). Chromosomal DNA was isolated after 3 days of cultivation using liquid nitrogen (La Grange et al. 1996). The number of integrations were determined by digesting the genome of the *A. niger* D15 transformants overnight using *Eco*RV. DNA was separated on a 0.8% agarose gel and used for traditional Southern blot analysis. The internal *Eco*RI-*Pst*I (726 bp) fragment of *cbh1-4*, the entire 1.2 and 0.67 kb coding region of *man1* and *xynC* were labeled with[A-^{32}P]ATP using the Random Primed Kit (Roche), according to the suppliers specifications.

Enzymatic Assays

The activity of Cbh1-4 using p-nitrophenyl β-D-cellobioside (PNPC) as substrate, was determined by incubation of 50 μl of the culture supernatant in 50 μl of PNPC (4 mM desolved in 0.05 M sodium citrate buffer pH 4.2) for 5 minutes at 70°C. The reaction was terminated by the addition of 1 ml of 1 M sodium carbonate. Color release was measured at OD 400 using p-nitrophenol as standard. Appropriate dilutions of the enzyme were made in 0.05 M sodium citrate buffer (pH 4.2). One unit of enzyme activity is defined as the activity producing 1 μmol of chromophore per minute under these assay conditions.

The temperature and pH optima of the enzymes in question were determined (using 0.05 M citrate phosphate buffer) as described by Bailey et al. (1992). The cellobiohydrolase activity of Cbh1-4 was also determined using 0.2% lichenan (Sigma) and 1% CMC (Sigma) as substrates. The activity of Man1 and Xyn3 was determined using 0.25% galactomannan (Sigma) and 1% birchwood xylan (Roth), respectively. The amount of reducing sugars released during the degradation of lichenan / CMC, mannan and xylan, was determined by the dinitrosalicylic acid method using glucose, mannose and xylose respectively as standard (Miller et al. 1960). One unit of enzyme was defined as the activity producing 1 μmol reducing sugar per minute in glucose, mannose or xylose equivalents, under the optimal assay conditions.

Synergism between Cbh1-4 and EGI of *Trichoderma reesei* (Rose and Van Zyl, 2001) was investigated by following the standard DNS method using 0.2% lichenan (pH 4.2) and a solution of 1 mg/ml of freeze dried Cbh1-4 and 0.5 mg/ml of freeze dried EGI. The conditions of the assay was altered to accommodate the EGI to an incubation time of 5 minutes and using 60°C.

Protein Isolation and Gel Electrophoresis

The supernatant of the different strains were collected after 7 days of cultivation in double strength medium and freeze dried before determining the specific activity. Two mg of freeze dried material (approximately 50 µg of total extracellular protein) were dialized and separated by 8% (in the case of Cbh1-4 and ManI) and 15% (in the case of Xyn3) SDS-PAGE using the low range (14.4 to 97.4 kDa) premixed protein molecular weight marker (Roche) to estimate the size of the proteins. Coomassie brilliant blue was used to visualize the protein bands (Ausubel et al. 1998).

5.4 Results

Construction of recombinant *A. niger* D15 strains containing the *cbh1-4*, *man1* and *xynC* gene copies

Plasmids pGT, pGT-cbh1-4, pGT-man1 and pGT-xynC were individually co-transformed with plasmid pBS-pyrGamdS (Plüddeman 2002) to *A. niger* D15, resulting in transformants *A. niger* D15[pGT], *A. niger* D15[*cbh1-4*], *A. niger* D15[*man1*] and *A. niger* D15[*xynC*]. Plasmid pBS-pyrGamdS contains the *amdS* and the *pyrG* marker genes that were used for the double selection of the transformants. *PyrG* enabled the transformants to grow in the absence of uridine, whereas *amdS* allowed the utilization of acetamide or acrylamide as the sole carbon and nitrogen source. Transformant *A. niger* D15[pGT] was used as reference strain in all experiments.

Transformants that produced the highest levels of activity were selected for further study. Chromosomal DNA was isolated from *A. niger* D15[pGT] and *A. niger* D15[*cbh1-4*], *A. niger* D15[*man1*] and *A. niger* D15[*xynC*]. The DNA was digested overnight with *Eco*RV, an enzyme which does not cut within the coding regions of the

cbh1-4, man1 or *xynC* genes. The Southern blot analyses revealed at least one copy of the *cbh1-4, man1* and *xyn3* expression cassettes integrated into the genome of *A. niger* D15.
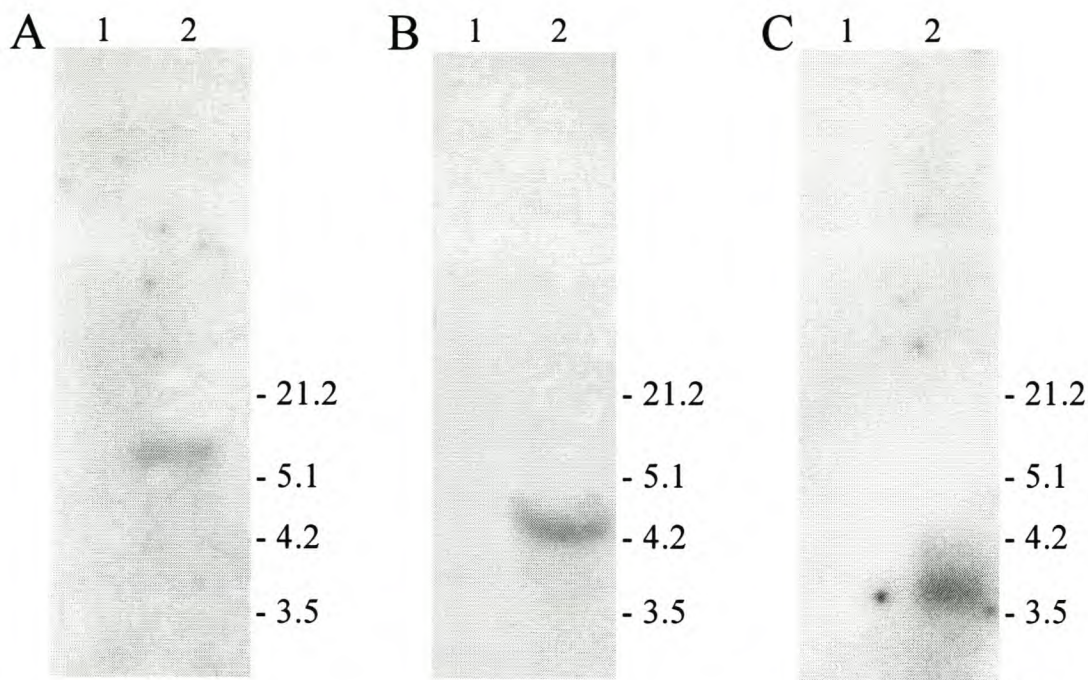

Figure 2. Southern blot analysis to determine the copy number of the *cbh1-4, man1* and *xynC* genes present on the genome of (**A**) *A. niger* D15[*cbh1-4*], (**B**) *A. niger* D15[*man1*] and (**C**) *A. niger* D15[*xynC*]. The total DNA was isolated and digested overnight with *Eco*RV. Lane 1 (A, B and C) contains the DNA isolated from *A. niger* D15[pGT] and lane 2 the DNA isolated from (**A**) *A. niger* D15[*cbh1-4*], (**B**) *A. niger* D15[*man1*] and (**C**) *A. niger* D15[*xynC*], respectively. The internal 726 bp *Eco*RI – *Pst*I fragment of *cbh1-4* and the entire 1.2 and 0.67 kb coding regions of *man1* and *xynC* were labeled with [α-^{32}P]ATP and used as DNA-probes. The marker sizes are indicated on the right. Each ^{32}P-highlighted DNA species represents a copy of the *cbh1-4, man1* or *xynC* genes, respectively.

Effect of pH and Temperature on Enzyme Activity

The pH and temperature optima of Cbh1-4 was determined using lichenan, CMC and PNPC (Figure 3A and B). The optimum temperature was determined between 60 and 70°C using CMC, lichenan and PNPC. The pH optimum was determined between 3.5 and 4.5 depending on the substrate used. The standard deviations on the graph using CMC as substrate, was omitted due to them ranging more than 30%. The optimum

conditions for the cellobiohydrolase activity of Cbh1-4 was taken as pH 3.5 at the temperature optimum of 70°C.
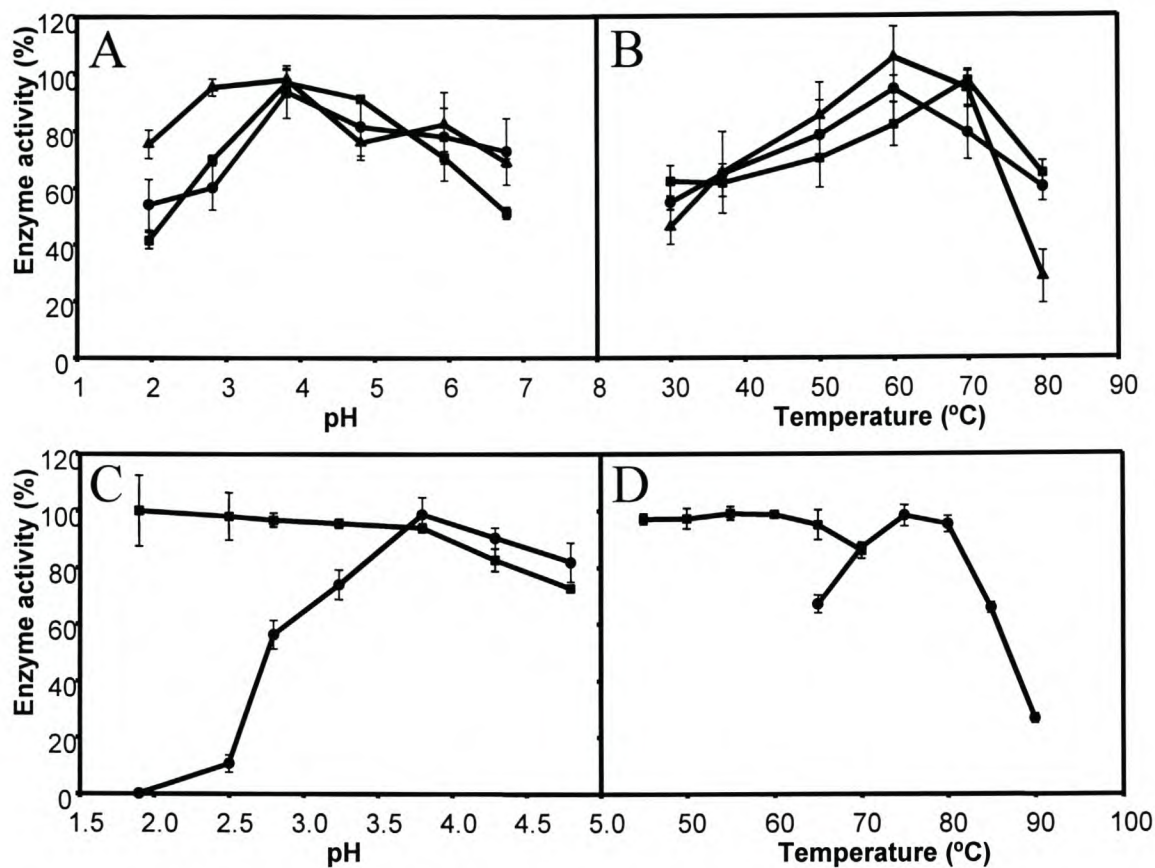

Figure 3. The effect of (**A**) pH and (**B**) temperature on Cbh1-4 (using ■ PNPC, ● CMC and ▲ lichenan as substrates), and (**C**) pH and (**D**) temperature on the enzymatic activity of ManI (●) and XynC (■) when produced by *A. niger* D15. The highest activity was measured at pH 3.5 – 4.5 and 60 - 70°C for the Cbh1-4 (depending on the substrate used), pH 3.8 and 75 - 80°C for the ManI and pH 3 and 55 - 60°C for Xyn3.

The activity of Man1 was determined using 0.25% galactomannan (Sigma) (pH 3.8) at a temperature of 75°C. The optimum conditions for the mannanase activity of Man1 was taken at pH 3.8 and 70°C (for practical reasons it was decided to perform the assay at 70 and not at 75°C although the latter appeared to be the optimum temperature). The pH optimum of Man1 was determined around pH 3.8 (Figure 3C) which is similar to that observed with expression of the native enzyme (pH 3), as well as with expression of the *man1* gene in *S. cerevisiae* (Table 2). The temperature optimum of Man1 was

determined between 75 and 80°C (Figure 3D), which differs significantly from the 60°C optimum achieved with the native enzyme as well as with expression in *S. cerevisiae*.

Table 2. Comparison of the characteristics of Cbh1-4, ManI and Xyn3 when produced by different hosts

	Native enzyme	*S. cerevisiae* Y294	*A. niger* D15
Cbh1-4			
Activity achieved		-	400 nkat/ml
pH optimum		4.8* [Van Rensburg et al. 1996]	3.5 – 4.5
Temp. optimum °C		50* [Van Rensburg et al. 1996]	65 - 70
Protein size (kDa)		-	85
ManI			
Activity achieved		521 nkat/ml [Setati et al. 2001]	6000 nkat/ml
pH optimum	3 [Setati et al. 2001]	3 [Setati et al. 2001]	3.8
Temp. optimum °C	60 [Setati et al. 2001]	60 [Setati et al. 2001]	75-80
Protein size (kDa)	45 [Setati et al. 2001]	50 [Setati et al. 2001]	45 - 50
Xyn3			
Activity achieved		300 nkat/ml [Crous et al. 1995]	600 nkat/ml
pH optimum		<3 [Crous et al. 1995, Ito et al. 1992]	< 3
Temp. optimum °C		60 [Crous et al. 1995]	55 - 60
Protein size (kDa)	19.8 (putative) 29 (glycosylated) [Ito et al. 1992]	24.5 (glycosylated) [Crous et al. 1995]	22

*assays were performed at pH 4.8 and 50°C, but was not determined to be the optimal conditions.

The optimum pH of Xyn3 was determined to be below pH 3 (Figure 3C). pH 3 was taken as the optimum pH since the substrate becomes unstable when the pH is lowered further. The latter leads to large standard deviations. The temperature optimum was determined to be between 55-60°C (Figure 3D). The temperature and pH optima is similar to that reported by Crous et al. (1995) for expression of the *xynC* gene in *S. cerevisiae* (Table 2).

Heterologous Enzyme Production

The production of the heterologous Cbh1-4 by *A. niger* D15[*cbh1-4*], ManI by *A. niger* D15[*man1*] and Xyn3 by *A. niger* D15[*xynC*] were followed over a time period of 10 days in 2xMM (Figure 4) The highest levels of cellobiohydrolase activity produced by *A. niger* D15[cbh1-4] was 500 nkat/ml, achieved on day 5, while the reference strain produced baseline levels of up to 100 nkat/ml. The highest level of β-mannanase activity by *A. niger* D15[*man1*] was produced on day 8 (6800 nkat/ml), although the levels started to stabilize from day 6 at above 6000 nkat/ml (Figure 4). These levels of activity exceded the levels of activity obtained with expression of *man1* in *S. cerevisiae* (521 nkat/ml) approximately 13 fold. Xyn3 produced it's highest level of activity on day 5 (> 900 nkat/ml), which was followed by a rapid decrease in activity. This corresponds to the approximate time when the fungus starts to lyse / break down (visual observation). These levels of expression is about three times that obtained with expression in the *xynC* in *S. cerevisiae* (300 nkat/ml; Crous et al. 1995).

Synergism was established between the Cbh1-4 and the EgI (Rose and Van Zyl, 2002) of *Trichoderma reesei*. A 1 mg/ml solution of Cbh1-4 and 0.5 mg/ml solution of EGI was prepared. The solutions were mixed in appropriate volumes and the DNS assays performed. From the data in Table 3 it is evident that the two enzymes compliment each other with the best scenario being 0.5 mg/ml of Cbh1-4 and 0.25 mg/ml of EGI leading to 14.4% synergism. The effect of incubation of the Cbh1-4 with the substrate (0.2% lichenan) prior to the actual DNS assay was investigated (Table 4). Treatment nr 4 is the traditional way in which the assay is conducted; heating the substrate to the appropriate temperature, adding the Cbh1-4, incubating for 5', followed by the addition of DNS and boiling the mixture for 15'. The combined activities of

treatments 1 and 2 should in theory present the same value as that of treatment 3 if the actual catalytic activity was the only factor to be taken into consideration, but from the data presented in Table 4 it is clear that the combined treatments (1+2) yielded at least 30% less activity then was expected.
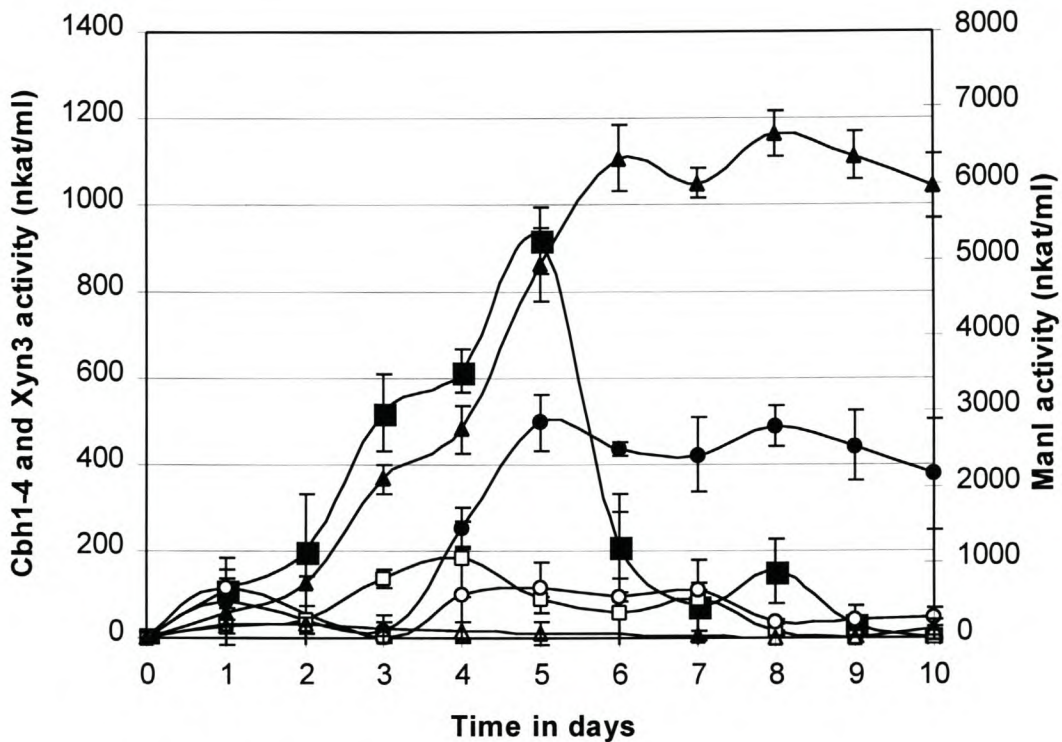

Figure 4. The heterologous production of Cbh1-4 (●), ManI (▲) and Xyn3 (■) by *A. niger* D15[*cbh1-4*], *A. niger* D15[*manI*] and *A. niger* D15[*xyn3*] was monitored over a period of 10 days in 2xMM medium and compared to the activity produced by *A. niger* D15[pGT] (○, △ and □ representing the levels of cellulase, mannanase and xylanase expressed by the reference strain). The pH of the media did not change significantly. The pH dropped from pH 5.5 to 4.5 after 3 days of cultivation and remained at pH 4.5 for the duration of the experiment. Enzyme activity assays were determined in triplicate using 6 parallel cultures. Enzyme activity was determined as described in La Grange et al. (1996).

Table 3. Data from the combined action of the Cbh1-4 and EGI on lichenan to confirm the presence of synergism between the two enzymes.

Enzyme combination	Activity nkat/ml*	Theoretical act. nkat/ml	Synergism
100% Cbh1-4	4.26 (0.04)		
75% Cbh1-4 : 25% EGI	3.56 (0.18)	3.29	0.27 (7.6%)
50% Cbh1-4 : 50% EGI	2.71 (0.42)	2.32	0.39 (14.4%)
25% Cbh1-4 : 75% EGI	1.48 (0.06)	1.35	0.13 (8.78%)
100% EGI	0.38 (0.31)		

*standard deviations are in brackets

Table 4. Investigation of the effect of incubation of the Cbh1-4 (1.5 mg/ml) with the substrate (0.2% lichenan) on ice prior to the DNS assay.

Treatment		Activity (nkat/ml)	Relative activity as %
1	5' on ice (- Cbh1-4)	0.10 (0.02)	1.2
2	5' on ice without Cbh1-4, then 5'at 70°C (+ Cbh1-4)	5.27 (0.28)	67.3
3	5' on ice (+Cbh1-4), then 5' at 70°C	7.83 (0.6)	100
4	5'at 70°C (+ Cbh1-4)	6.78 (0.49)	86.59

standard deviations are in brackets

Heterologous Protein Isolation and SDS-PAGE Analysis

Two mg samples of the *A. niger* D15[pGT], *A. niger* D15[*cbh1-4*] and *A. niger* D15[*man1*] freeze-dried supernatant (50 µg of unpurified extracellular protein) was separated by 8% SDS PAGE (Figure 5A). The recombinant Cbh1-4 was present as two protein species, while the ManI protein was present as a more diffused band. The recombinant Cbh1-4 exhibited a molecular size of about 85 and 90 kDa whereas ManI exhibited a molecular size varying from 45 - 50 kDa.
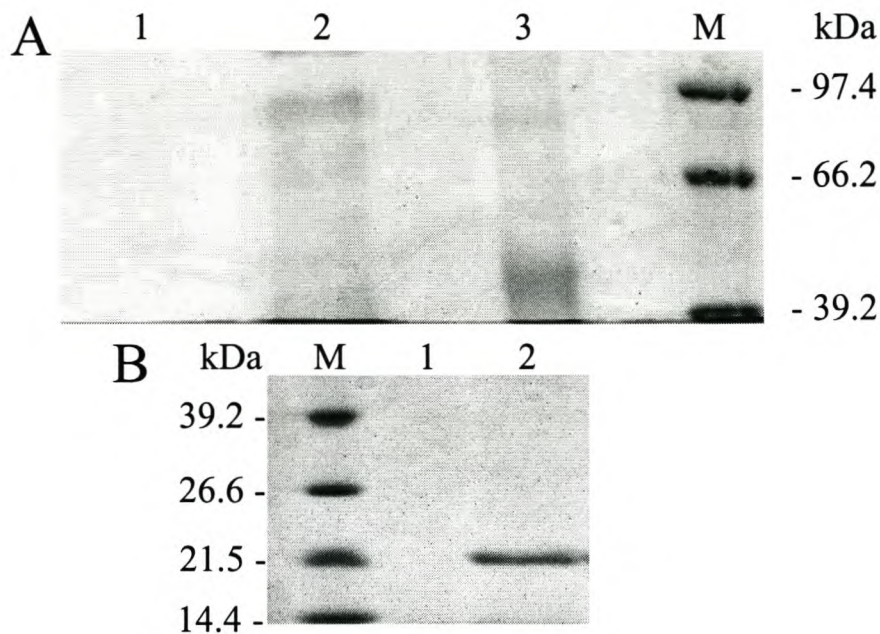
Figure 5. Separation of the total extracellular protein fractions (2 mg) of (**A**) *A. niger* D15[pGT] (lane 1), *A. niger* D15[*cbh1-4*] (lane 2), and *A. niger* D15[*manI*] (lane 3) on 8% SDS-PAGE, and (**B**) *A. niger* D15[pGT] (lane 1), *A. niger* D15[*xynC*] (lane 2) on 15% SDS-PAGE. Lane M contains the molecular weight marker with the sizes depicted on the right and left hand side of (**A**) and (**B**) respectively. The recombinant Cbh1 4 is present as 2 heterogeneous protein species of about 85 and 90 kDa, whereas ManI is present as a heterogenous species varying in size from 45 - 50 kDa. The recombinant Xyn3 is present as a single protein species of about 22 kDa.

A 2 mg freeze dried sample of the *A. niger* D15[pGT] and *A. niger* D15[*xynC*] supernatant, was separated by a 15% SDS PAGE (Figure 5C). The Xyn3 protein was present as a single prominent band of about 22 kDa. The size of the protein corresponds well with the results obtained with the expression of *xynC* in *S. cerevisiae* as well as the native enzyme (Table 2). From Figure 5A and B it was evident that the recombinant proteins constituted a significant portion of the total amount of protein produced extracellularly, since the rest of the native secreted proteins were barely visible.

5.5 Discussion

The strain *A. niger* D15 have previously proven to be an excellent host for the production of heterologous proteins (Rose and van Zyl, 2002). This *A. niger* D15 strain is non-acidifying and derived from a protease-deficient (*prtT*) strain of AB1.13 (Gordon et al. 2000; Wiebe et al. 2001). These characteristics enables the fungus to produce large quantities of protein without the fear of degradation by natively produced acid proteases. Furthermore, an excellent expression system have been established using the constitutive *gpd* promoter of *A. niger* and the *gla* terminator of *A. awamori* (Rose and Van Zyl, 2002).

cDNA copies of the *cbh1-4* of *P. chrysosporium*, *man1* of *A. aculeatus* and the *xynC* of *A. kawachii* have been cloned and successfully expressed in *A. niger* D15 (Figure 1). Stable transformants were selected on the bases of constitutively expressing the Cbh1-4, ManI and Xyn3 enzymes. The recombinant strains *A. niger* D15[*cbh1-4*], *A. niger* D15[*man1*] and *A. niger* D15[*xynC*] contain at least one copy of the *cbh1-4*, *man1* and *xynC* genes, respectively (Figure 2). Little data is available on the biochemical characteristics of the Cbh1-4 of *P. chrysosporium*, therefore we were unable to draw a comparison with previous studies. The highest level of activity obtained on 0.2% lichenan was about 500 nkat/ml. The freeze dried sample yielded activities of 0.79 and 2.5 nkat/mg on lichenan and PNPC respectively.

The *A. niger* D15[*man1*] expressed endomannanase activity levels of up to 6800 nkat/ml. These levels are about 13 times higher than with expression of the *man1* gene in *S. cerevisiae*. The pH optimum and size of the ManI protein was similar regardless of the host used for expression. Interestingly, though, the optimum temperature preferred by the ManI expressed by *A. niger* D15[man1] (75 - 80°C) was at least 25°C higher than with expression in *A. aculeatus* or *S. cerevisiae*.

The highest levels of xylanase activity obtained for *A. niger* [*xynC*] was 900 nkat/ml, which was about 3 times more than that obtained using *S. cerevisiae* as host (Crous et al. 1995). This however does not compare well with a previous study where levels of 8000 nkat/ml were obtained with the expression of the *xyn2* of *Trichoderma reesei* in *A. niger* D15, using the same media composition and expression system (Rose

and Van Zyl, 2002). The biochemical characteristics of the Xyn3, however, was similar to that of the native enzyme as well as the enzyme expressed in *S. cerevisiae* (Table 2). No visual form of degradation could be detected on the SDS-PAGE that could account for the low levels of activity. The pH stability of the enzyme was determined by incubating the Xyn3 for 90 min at different pH levels before determining the remaining activity. The enzyme proved to be stable over the pH range of 3 – 8. One possible explanation for the low levels of β-xylanase activity could be linked to the site of integration. Integration of the expression cassettes takes place at random, which can greatly influence the level of gene expression (Verdoes et al. 1995). This could be one of the reasons for the low levels of Xyn3 activity compared to the activity obtained with the ManI, which is also present as a single copy cassette.

From the data presented in Table 4 we also conclude that a low temperature incubation of the Cbh1-4 in the presence of the substrate prior to performing the DNS assay is required for optimal levels of activity. This might be explained by the cellulose binding domain (CBD) requiring low temperatures in order to bind properly to the substrate which is a prerequisite for the catalytic domain to perform it's function. Although some hydrolysis does take place during the incubation period on ice, the main portion of the catalytic activity takes place at the optimum temperature required for the enzyme in question, which in this case is 70°C. It is however important to note that this ice incubation only had a positive effect when using lichenan as substrate, but not with avicel. The ice incubation was repeated using the EgI of *T. reesei*, which also contains a CBD, but with no improvement in the levels of activity. Therefor, ice incubation should not be taken as the norm for all substrates available or for all enzymes containing a CBD.

5.6 Acknowledgements

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CHAPTER 6

Investigation of the application of enzyme technology for poultry rearing.

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Investigation of the application of enzyme technology for poultry rearing

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6.1 Abstract

The *egI* and *xyn2* genes of *Trichoderma reesei* and *man1* gene of *Aspergillus aculeatus* have been successfully expressed in *Aspergillus niger* D15. The heterologously produced EgI, Xyn2, ManI hydrolases and a native hydrolase cocktail of *Aspergillus niger* (named Asp) was evaluated at different concentrations as feed supplements in broiler diets. The recombinant ManI at a concentration of 320 nkat/kg feed, seemed to produce the best results in terms of AWG (Average Weight Gain). Recombinant EgI (553 nkat/kg) produced similar results to that of the recombinant ManI in terms of BW (Body Weight) at the end of the study. Although the total cellulase activity of Asp and the recombinant EgI were similar, the EgI seemed to performe better than the Asp, indicating that the EgI is more suited for this specific feed used, than the Asp. The Xyn2 showed a steady increase in AWG and BW with an increase in concentration, indicating that the optimum Xyn2 concentration is > or equal to 1813 nkat/kg and was not established in this study. The feed conversion ratio (FCR) supported the AWG and BW results. This also indicated a decrease in the amount of feed required for all four enzyme treatments.

key words: broiler performance, enzyme, supplements

6.2 Introduction

Producers of animal products aim to provide cost effective, high quality, homogeneous products in the shortest time possible (Acamovic, 2001). Good quality animal products are produced when a high health and welfare status is maintained throughout the growth period. Due to environmental concern and the cost involved in waste disposal, the emphasis is also shifting towards decreasing the quantity and toxicity of the annual waste produced.

Feedstuffs for diets can contribute to up to 80% of the total cost or rearing poultry. Thus, factors influencing the effective utilization of the diets will have a substantial effect on the cost of poultry production. Enzyme supplements are widely used in poultry diets to improve nutrient utilization, product quality and increase the choice of ingredients acceptable for inclusion in diets (Acamovic, 2001; Malathi and Devegowda, 2001; Zyla et al. 2000a, b). The effectiveness of the enzymes depends on a number of feed related factors such as the nature of the dietary components, whether or not the diets have been processed and the quality of the feed. The enzyme related factors include the substrate specificity / substrate preference, levels of activity at the different conditions (such as pH and temperature of the feed during storage and gizzard conditions) and enzyme stability (shelf life and protease resistance).

Despite the abundance of agricultural products (corn, wheat etc.) available, their inclusion in feed are limited due to the presence of non-starch polysaccharides (NSP) which consists mainly of glucan and arabinoxylan. NSP increase the gut viscosity, leading to nutrient entrapment that affects the growth and performance of the animal (Chesson, 2001). Enzyme supplements, such as cellulases / glucanases and xylanases, are able to degrade these NSP, therefore reducing the viscosity and improving the nutritive value of the feed stuffs (Malathi and Devegowda, 2001). Although assays have been established for the *in vitro* testing of enzyme effectiveness on different feeds, these tests are incomplete and not always reliable since the effect on the microbial population cannot be monitored. A change in the microbial harmony can play a significant role in the health and overall wellbeing of the animal.

The present study was conducted in order to determine the effect different concentrations of endoglucanase, endoxylanase and endomannanase enzymes has on the performance of broiler chickens when included as diet supplements. For this study the heterologously produced EgI (*Trichoderma reesei*), Xyn2 (*T. reesei*), ManI (*Aspergillus aculeatus*) enzymes and a native cellulase enzyme cocktail of *Aspergillus niger* (named Asp) were evaluated.

6.3 Materials and methods

Enzymes

The microbial enzymes used in this study was prepared by cultivating *A. niger* D15[*egl*] expressing the endoglucanase *egl* gene of *T. reesei*, *A. niger* D15[*xyn2*] expressing the xylanase *xyn2* gene of *T. reesei*, *A. niger* D15[*man1*] expressing the mannanase *man1* gene of *A. aculeatus* and *A. niger* D15[pGT] (producing the native cellulase cocktail, Asp) in 2xMM, double strength medium (Rose and Van Zyl, 2003a, 2002). Cultivation conditions were such that only baseline levels of native cellulose degrading enzymes could be detected in Asp, whereas the heterologous enzymes, EgI, Xyn2 and ManI dominated in the other samples (Rose and Van Zyl, 2003a, 2002).

The supernatant of the four strains were harvested (through filtering using myracloth) after 8 days of cultivation, freeze dried, ground and stored in airtight containers at -20°C. Before use, the enzymes were individually premixed with 50 g maize starch (maizena) prior to mixing with the chicken feed. Starch, (maltodextrin in particular) proved to have a positive effect on the stability of dried enzymes stored at 4 and 30°C for up to 8 months (Belghith et al. 2001). For economical reasons maizena was used instead of chemically defined maltodextrin. For a control experiment, only maizena was added to the chicken feed.

Enzyme activity measurements

The enzyme activity of the individual enzymes were determined at 40°C and pH 4.5, corresponding to the conditions in the gizzard (Zyla et al. 2000a, b). The substrates used for determining the enzyme activities were 1% carboxymethyl cellulose (CMC supplied

by Sigma) for the EgI and Asp, 1% birchwood xylan (Roth) for the Xyn2 and Asp and 0.25% galactomannan (locust bean gum supplied by Sigma) for the ManI and Asp, dissolved in 0.05M citrate buffer (pH 4.5). The reducing sugars were determined by means of 3,5-dinitro-salicylic acid (DNS) according to Miller *et al*. (1960). All enzyme activities were determined in triplicate. One unit of enzyme activity was defined as the activity producing 1 μmol per minute of reducing sugars (in glucose, xylose or mannose equivalents depending on the substrate used) under these assay conditions. Enzyme activities are summarised in Table 1. No enzyme activity could be detected in the maizena prior to the enzyme additions.

Table 1. The activities of the different enzyme supplements present in the feed

Activity of the enzymes[1] measured, given as nkat					
Treatment (Assay substrate)	**Neg. Con.**	**Asp** (CMC)	**EgI** (CMC)	**Xyn2** (Xylan)	**ManI** (Mannan)
Treatment stage					
mixed in maizena[2]	0	1000	1036	1700	391
mixed with broiler feed[3]	0	1066	1105	1813	426

[1] activities were determined at 40°C and pH 4.5 regardless of the actual temperature and pH optima of the enzymes.

[2] activity measured (as nkat/g) after dilution of the enzymes in 50 g of maizena, but prior to mixing with the feed

[3] activity measured (as nkat/kg) after mixing enzymes with the feed

Chicken feed

The details regarding the composition of the chicken feed is given in Table 2 and the nutritional value of the feed in Table 3. Enzyme treated chicken feed was stored at room temperature (16°C during the day). The four enzymes and the maizena negative control (named Neg. Con.) were mixed with the feed. The four enzyme treated feed batches were mixed with the Neg. Con. feed batch in a ratio of 100:0, 75:25, 50:50, 25:75 and 0:100 respectively, yielding 17 treatments in total. The feed was used without enzyme

addition for day 1-7 to allow uninhibited microfloral establishment in the gut of the broiler chickens, after which the feed containing the different concentrations of enzymes were used for day 8-21.

Table 2. The composition of the broiler diet

Composition	%	Composition	%
Wheat	53.00	Limestone	0.95
Soybean 48*	30.57	Monocalcium phosphate	0.65
Fish meal 65	11.08	Salt	0.02
Oil - sunflower	3.30	Sodium bicarbonate	0.12
DL-methionine	0.05	vit+min premix	0.25

* 29.02% nonstarch polysaccharide (Malathi and Devegowda, 2001)

Table 3. Nutritional value of the broiler diet

Nutrient	Formulated value	Nutrient	Formulated value
dry matter (%)	89.10	EE[3]	10.67
total fat (%)	5.90	total crude protein (%)	27.44
crude fibre (%)	2.59	lysine (%)	1.41
ash (%)	4.35	methionine (%)	0.49
threonine[1] (%)	0.88	isoleucine (%)	1.11
tryptophan (%)	0.28	leucine (%)	1.80
arginine (%)	1.61	phenylalanine (%)	1.15
histidine (%)	0.61	valine (%)	1.18
TEAA[2] (%)	13.79	sodium (%)	0.18
calcium (%)	1.00	chloride (%)	0.23
phosphorous (%)	0.50	AMEn[4] (MJ/kg)	12.40
methionine+cystine (%)	0.82	phenylalanine+tyrosine (%)	1.95

[1] amino acids are expressed as percentage available, [2] TEAA: Total essential amino acids, [3] EE: Effective energy, [4] AMEn: apparent metabolizable energy

Animals, experimental design and measurements

Two hundred and fifty five broiler chickens (Ross 308, seven day old) of mixed sex were obtained from a commercial hatchery. Birds were housed in stainless steel battery brooders with wire-mesh floors. The initial temperature was set at 28°C for day one to seven after which the temperature was reduced by 1°C per day. Lighting was supplied for 23 hours followed by 1 hour of darkness. Feed and water were supplied continuously. The experimental design consists of 17 dietary treatments, given to 5 pen replicates of 3 chickens allotted at random. Broilers were weighed and the average weight gain (AWG as %), body weight (BW) and feed conversion ratio (FCR) were calculated.

6.4 Results and Discussion

Four different enzymes mixtures Asp, EgI, Xyn2 and ManI were evaluated, at different concentrations as feed supplements in broiler diets. The different levels of activities taken at the different stages of the study are given in Table 1. The values determined before addition of the enzymes to the feed were 1000, 1036, 1700, 391 nkat/g for the Asp, Eg1, Xyn2 and ManI. The cellulase activity (on CMC as substrate) of Asp and EgI were adjusted to the same concentration to determine the effect of the different cellulases present. The Neg. Con. (maizena) showed no activity on either of the substrates used. After dilution with the feed the theoretical additional activity should be 1066, 1105, 1813, 426 and 0.0 nkat/kg for Asp, EgI, Xyn2, ManI, and Neg. Con. respectively, for feeds with the 100:0 ratio of enzyme treated feed: maizena treated feeds.

The BW (Figure 1) of the broilers were determined for the period 1-21 days. The optimal enzyme concentration required for the maximum BW was 75% for the Asp, 50% for the EgI, 100% for Xyn2 and 75% for ManI. The AWG (Figure 2) of the broilers were determined for the period 8-21 days, confirming the results of Figure 1. The optimal enzyme concentration required for the maximum AWG is 75% for Asp, 50% for EgI, 100% for Xyn2 and 75% for ManI. However, ManI at a concentration of 75%, yielded the most promising results in terms of AWG (12.82% increase), although the BW obtained was similar to that obtained with 50% EgI (14% increase). A further increase in

enzyme concentration of the Asp, EgI and ManI (higher than the optimal concentration) failed to improve the BW and AWG of the broilers.
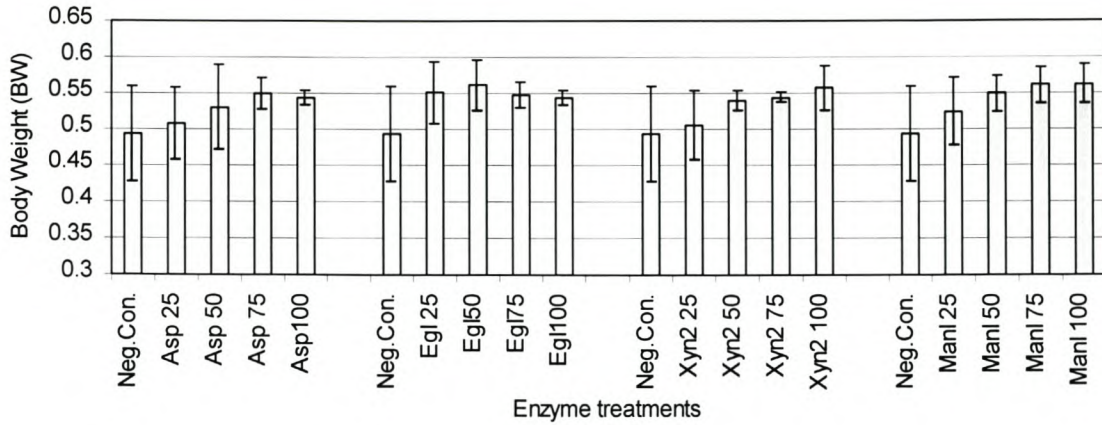

Figure 1. The body weight (BW) of the broilers after 21 days exposure to different concentrations of the enzyme supplements Asp, EgI, Xyn2 and ManI.
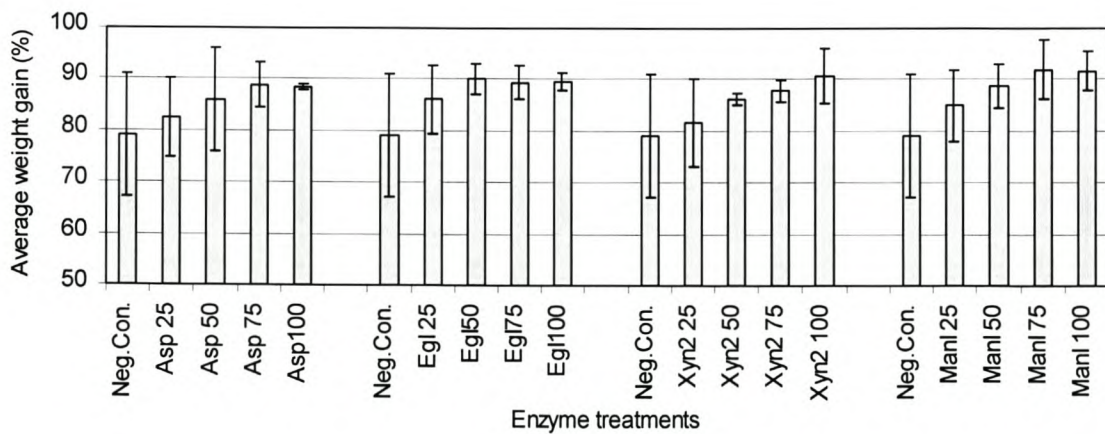

Figure 2. The average weight gain (AWG) of the broilers after 14 days exposure to different concentrations of the enzyme supplements Asp, EgI, Xyn2 and ManI.

Asp contains mainly CMC activity (which is similar to that of EgI). The EgI, though, at a concentration of 50%, produced the best result (compared to Asp) which gave a better yield than Asp with an optimum at 75%, indicating EgI as the preferred cellulase in this study. The Xyn2 showed a steady increase in BW as well as AWG with an increase in enzyme concentration. This phenomenon indicates that the optimal Xyn2 concentration was not established, but is possibly equal to or more than 1813 nkat/kg feed.

The FCR (for the period 8-21 days) is given in Figure 3. The smaller the FCR, the more energy can be obtained from that amount of feed. The Asp, EgI and Xyn2, performed best at the maximum enzyme concentration, whereas ManI yielded the best results at a concentration of 50%. These results indicate that enzyme addition tends to contribute to a decrease in the amount of feed required by the broilers. It also supports the results of Figures 1 and 2, that the EgI of *T. reesei* is more suited as a feed supplement for the feed used in this study, than the Asp. In terms of FCR the EgI also seems to performs better (a decrease of 19.6%) than the Xyn2 and ManI additions.
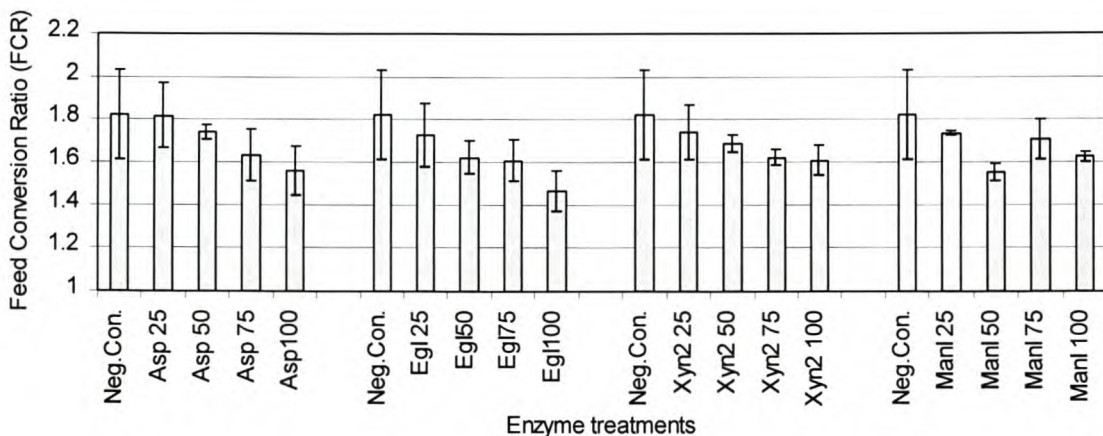

Figure 3. The average feed conversion ratio (FCR) of the broilers after 14 days exposure to different concentrations of the enzyme supplements Asp, EgI, Xyn2 and ManI.

The expected efficiency of the added exogenous enzymes relies greatly on the initial grain quality used in the feeds, the type of grain / feed used and the condition of the

animals prior to the commence of the study, the origin of the enzymes used and the enzyme concentration. Therefore, it is difficult to deduct any general conclusions from a study such as this one. In this case the EgI seemed to produce better results than the Asp, although they contain approximately the same amount of cellulase activity. The scenario might be different when another type of grain is used in the feed or when another cellulase of different origin is used. The Asp and EgI gave the best results in terms of FCR, which might suggest that cellulase enzymes provide a more significant contribution to feed efficiency, than other polysaccharide degrading enzymes. This might be explained by the cellulose representing a larger portion of the feed (than the other polysaccharides), thus contributing to the larger portion of the gel-like structure that normally form in the gut of poultry. The addition of cellulases, therefore not only contribute to the release of trapped nutrients, but also provide oligo and monosaccharides for the microflora population and makes the starch (in the grains) more accessible for amylase degradation.

Although ManI seemed to yield promising results in terms of AWG and BW, the results were less optimistic in terms of FCR. This might imply that the ManI not only increased the amount of energy released from the feed, but also increase the flow of the feed through the gut of the animal, thus allowing the bird to consume more than was the case with the other enzyme preparations.

This study was conducted in such a way as to make it economically feasible for use in third world countries, (such as South Africa), and applicable in practise. Therefore the use of low levels of enzyme activities, the optimisation of the enzyme concentrations required for a specific feedstock, the use of maizena (supplied by all local general dealers) to increase the enzyme stability and thus the shelf life, the simple method of harvesting (by filtering) and concentration of enzymes (through freeze drying).

The cultivation of *A. niger* transformants for enzyme production, is the most expensive part of this study, since chemically defined medium was used in this case (Rose and Van Zyl, 2002). *Aspergillus* spp. however, have the ability to grow on a variety of inexpensive substrates and industrial by-products, (such as sugar cane molasses), agricultural waste and specifically grown crops. With the economic implications in mind, we have already started optimising the conditions for using

molasses as substrate for heterologous enzyme production (Rose and Van Zyl, 2003b). Molasses waste water, however, has an important environmental impact due to the high organic content and the dark colour (Peña Miranda et al. 1996). *A. niger*, though, has the additional ability to eliminate 69% of the colour component in the waste water after 4 days of cultivation. Therefore, from an environmental and economical point of view, the possible use of molasses waste water (derived from already existing fermentation plants) could be further investigated as cultivation medium for the expression of heterologous enzymes.

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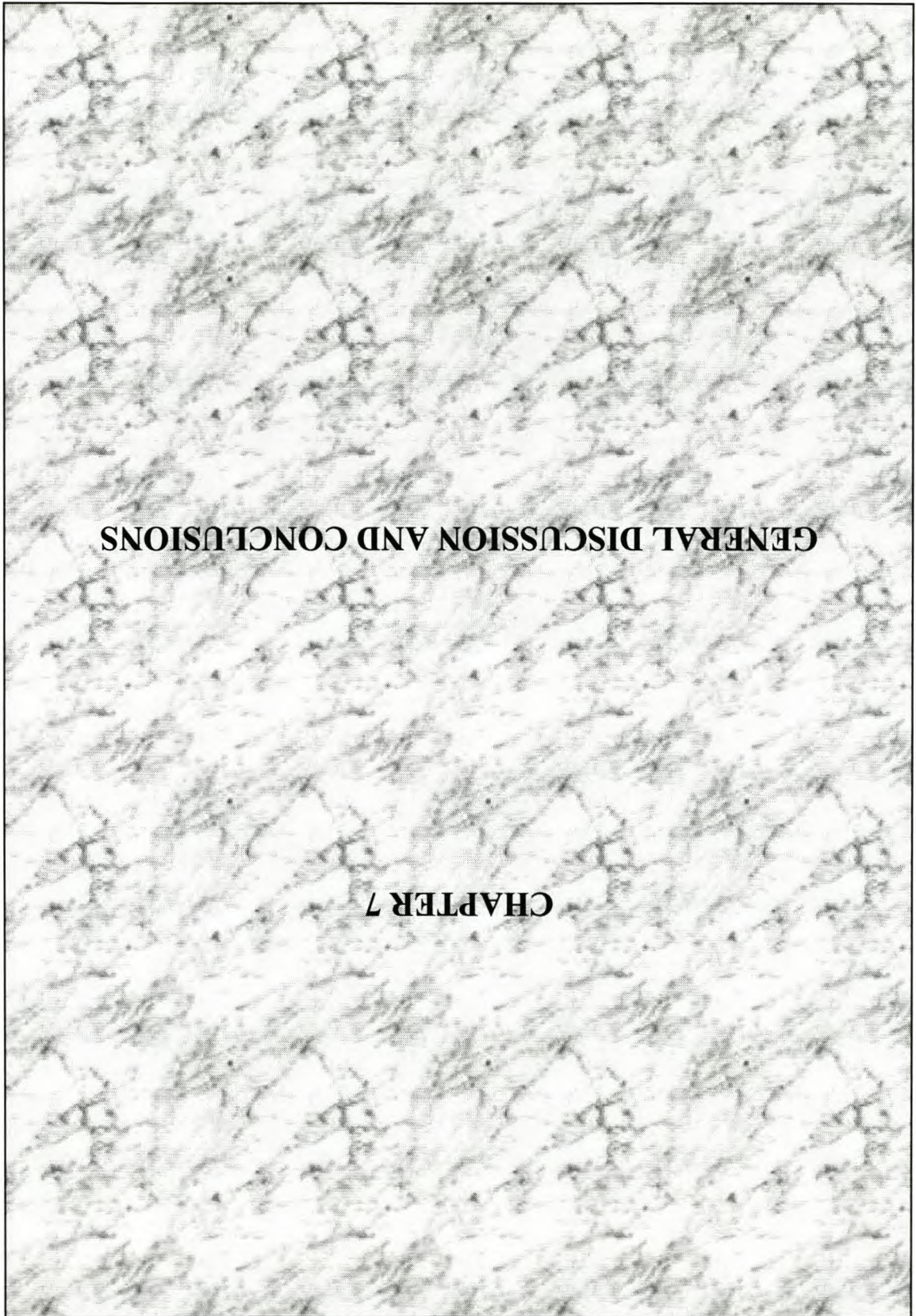
CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7. GENERAL DISCUSSION AND CONCLUSIONS

7.1 Introduction

Saccharomyces cerevisiae was the first fungal host used for foreign gene expression on industrial scale and is still pre-dominantly used due to the variety of vector systems and promoters available, the long association with the food and beverage industry, the established fermentation technologies and the ease of product purification (when the desired product is secreted). Although *S. cerevisiae* offers an attractive tool for expression of recombinant proteins, some limitations associated with poor expression capacity, inadequate post translational modification of proteins and low product yields could not be overcome. This led to the investigation and ultimately successful exploitation of alternative yeast, such as *Pichia pastoris* and *Pichia stipitis*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe*. These strains all have unique qualities which provide a variety of tools that can be used to relieve some of the limitations encountered with *S. cerevisiae* as host, but the production level (in general) of the heterologous proteins still remained low.

Filamentous fungi, in general, have a greater secretion capacity than yeast and most can easily be cultivated on inexpensive media. It is not surprising that *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei* have dominated the research done in the field of heterologous expression of genes in fungi. For the purpose of this study we decided to use *A. niger* as host for foreign gene expression due its GRAS (Generally Regarded As Safe) status, high secretion capacity and use in industrial processes (such as citric acid, enzyme and therapeutical protein production). Interestingly enough, (1) the expression of polysaccharases has almost exclusively been dominated by *A. oryzae* (see Table 2) and (2) the use of constitutive fungal promoters was neglected and might not have been investigated before. We therefore decided to investigate and evaluate the use of the constitutive glyceraldehyde-3-phosphate-dehydrogenase (*gpd*) promoter as part of the study.

7.2 Conclusions

Specific milestones achieved and conclusions derived from this study included:

- An expression cassette for constitutive expression of genes in *A. niger,* using the *gpd* promoter of *A. niger* and *glaA* terminator of *A. awamori*, was constructed.

- Co-transformation of the plasmid containing the *amdS* marker and the plasmids containing the expression cassettes was performed with a success rate (transformants containing both plasmids) of about 70%.

- The *egI* and *xyn2* genes of *T. reesei,* the *cbh1-4* gene of *P. chrysosporium*, the *manI* gene of *A. aculeatus* and the *xyn3* gene of *A. kawachii* was effectively expressed in recombinant *A. niger* D15 strains.

- The EgI, Xyn2, Cbh1-4, ManI and Xyn3 enzymes were successfully produced and secreted as functional enzymes with high levels of activity. The enzymes were characterised and compared to the natively produced equivalents.

- The production of the EgI and Xyn2 enzymes using molasses as cultivation medium was demonstrated as alternative to expensive chemically defined synthetic medium. The use of molasses broadens the profit margin, paving the way for possible large scale enzyme production.

- The levels of enzyme activity produced was mainly affected by the sugar (or molasses) concentration, the temperature of cultivation, the use of a prototrophic strain and a process called salt adaptation. This underlines the importance of process optimisation to facilitate high levels of protein production. Although the size of the spore inoculum and the initial pH of the medium (which has an affect on the spore germination) had little effect in molasses medium, they should not be disregarded when using alternative medium formulations for cultivation.

- Salt adaptation has additional features such as (1) less biomass is produced, thus less waste, (2) it requires 2 M KCl / NaCl which makes the cultivation medium more expensive and (3) is time consuming since the initial salt adaptation process can take up to 2 months to complete.

- The EgI, Xyn2 and ManI enzymes were used as feed additive for broiler chickens. The results indicated that enzyme additions may lead to an increase in body

weight (BW), an increase in average weight gain (AWG) and a decrease in feed conversion ratio (FCR).

- We were able to produce high levels of expression using the constitutive *gpd* promoter. Therefore, we concluded that the *gpd* promoter functioned as well as (if not better) than most of the inducible promoters used in previous studies where high level of expression was the main objective.

7.3 Unsuccessful gene expression

A few setbacks have been encountered in this study and will be discussed briefly:

The laccase, *lcc1* of *Trametes versicolor* was cloned onto pGT, under the transcriptional control of the *gpd* promoter and *glaA* terminator. The plasmid was transformed to *A. niger* D15 with the transformants (*A. niger* D15[*lcc1*]) producing strong initial activity. The *A. niger* D15[*lcc1*] transformants repeatedly lost their laccase activity after two generations making them too unstable to work with.

With the *A. niger* D15[*cbh1-4*] transformants, the integrations were stable long enough to complete the purification process which forms part of the transformation protocol (three generations), but most of the transformants lost their cellobiohydrolase activity after approximately four to five generations. The cellobiohydrolase, *cbhI* of *T. reesei* (provided by Mike Himmel, NREL) and three green fluorescent protein (GFP) genes (provided by CLONETECH) were successfully cloned under the transcriptional control of the *gpd* promoter and *gla*A terminator. The presence of the genes were confirmed by means of PCR, but no activity could be detected. The same problem arose with the attempt to express the laccase (*lac1*) derived from *Pleurorus ostreatus*.

Not only is the transformation and purification process time consuming (about 4 weeks), but foreign gene expression is not always successful. It is possible that there might be a common factor involved which leads to unstable transformants. Unfortunately little information is available addressing the problem of unstable transformants and unsuccessful gene expression, due to the difficulty of getting negative results published and therefore the tendency to omit such information in literature.

The construction of episomal plasmids or targeted integration (into a specific part of the genome) would shed some light on the difficulties encountered in studies such as this one. Although linearisation of the vectors before DNA transformation increases the targeting of integration, the copy number of the gene cassette cannot be controlled by this means, since random integration could still take place. Due to the lack of episomal plasmids and the influence of the integration site on expression levels, several transformants need to be screened by means of liquid cultivation and assays, where possible. In this study we found that the copy number of the gene should not be taken as a measure for level of expression, that plate assays were inconclusive (mostly due to the difference in germination and growth rate of the different transformants) and that liquid assays are preferred for the screening of transformants.

Since plasmid integrations in *A. niger* are not always stable, adequate supplies of stock cultures has to be preserved and sub-culturing avoided as much as possible. This implies than when a transformant is lost, it cannot simply be replaced by means of another transformation, since no two transformants are alike. This problem (as well as the tedious screening of transformants) can be eliminated with the use of targeted integrations as well as stable episomal plasmids.

Therefore, the construction of episomal plasmids should be of high priority since it can open a whole new field of study (including promoter studies, comparing gene expression levels, determining the effect of gene copy number, genomic libraries with easy plasmid recovery, etc.), provided the plasmids maintain stable copy numbers through generations.

7.4 Future research

The genus *Aspergillus* is capable of expressing and secreting high levels of authentic enzymes. This study proved that by using *A. niger* as host the levels of production of successfully expressed genes can be significantly higher than with expression of the same genes in other frequently used hosts.

The boundaries of this study could be extended to include the following short term objectives:

(1) the development of a wild type or industrial *A. niger* strain, successfully expressing foreign genes, which can be used to produce commercially important enzymes (required by industries such as the animal feed industry, the wine industry, paper and pulp industry, etc.),

(2) the evaluation of other waste products for possible use as cultivation substrate,

(3) the construction of stable episomal plasmids maintaining a high copy number (thus leading to higher levels of expression),

(4) the isolation and evaluation of other promoters for transcriptional regulation of foreign genes,

(5) the use of fermenters for the large scale cultivation of *A. niger* (currently under way),

(6) the evaluation of the *A. niger* mycelium as a feed additive for the poultry industry.

The long term objectives for the production of enzymes as animal feed supplements could involve:

(1) the cloning of other cellulase and hemicellulase genes from organisms native to South African soil, which in theory should be more suited for the degradation of locally grown animal feeds,

(2) expressing these genes in a wild type or industrial strain of *A. niger*,

(3) evaluating the enzymes for use in locally produced animal feeds and

(4) producing the preferred enzymes commercially using locally produced waste as cultivation substrate

(5) The ultimate aim of studies like these is to design diets with a lower nutrient density than the conventional, but which is enhanced to conventional values by the addition of enzymes

.

An additional advantage to a project such as this one, is that the fungus itself need not go to waste. It can in fact be used as a single cell protein supplement in animal feed due to the high protein (30.4%) and carbohydrate content (46.7%) (Singh et al. 1991). Zyla et al. (2000) also found the *A. niger* mycelium useful in poultry diets, due to the presence of

phosphate releasing enzymes (such as phytases and acid phosphatases) trapped within the intracellular and membrane bound fractions of the mycelium. Therefore, with the proper planning and infrastructure, waste could be used and purified (during the fermentation process) before disgarding, no additional waste is produced (since the mycelium can also be used for animal feed), enzymes could be harvested, etc., leaving a large profit margin. This could create an industry that is environmentally friendly in every sense of the word.

7.5 References

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APPENDIX I

A method of providing a recombinant strain producing lignocellulosic hydrolases.

DrG Ref.: 599514

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TITLE OF INVENTION

A method of providing a recombinant fungus strain producing lignocellulosic hydrolases.

FIELD OF INVENTION

5   The present invention relates to a method of providing a recombinant fungus strain.

More particularly the invention relates to a method of providing a fungus strain with the capability of producing lignocellulosic hydrolases, a method of producing lignocellulosic hydrolases on cheap sugars syrups, for example 10   molasses, and to a recombinant DNA for use in transforming a fungus strain strain to produce lignocellulosic hydrolases.

BACKGROUND TO INVENTION

Cellulose and hemicellulose are the most abundant polymers present in nature. Lignocellulosic hydrolases (called cellulases and hemicellulases) are 15   enzymes needed for the enzymatic degradation of cellulose and hemicellulose to monomeric sugars, such as hexoses (glucose, mannose, and galactose) and pentoses (xylose, and arabinose). Cellulases comprise of β-endoglucanases, β-cellobiohydrolases and β-glucosidases, whereas hemicellulases comprise of β-xylanase, β-xylosidase, β-mannanases 20   β-mannosidases, and a whole variety of accessory enzymes, that include α-arabinofuranosidases, α-glucuronidases, acetyl xylan esterase, and phenolic esterases. The production of lignocellulosic hydrolases have a broad spectrum of industrial applications.

It is an object of the invention to suggest a method of providing a fungus 25   strain with the capability of producing lignocellulosic hydrolases, a method of

producng lignocellulosic hydrolases on cheap sugars syrups, for example molasses, and to a recombinant DNA for use in transforming a fungus strain strain to produce lignocellulosic hydrolases.

SUMMARY OF INVENTION

5 According to the invention, there is provided a method of providing a Aspergillus fungus strain with the capability of of producing lignocellulosic hydrolases for example the β-xylanase II (Xyn2) and β-endoglucanase I (EgI) of Trichoderma reesei, the method including transforming a fungus strain with a DNA nucleotide sequence including a gene encoding a β-xylanase enzyme, 10 a gene encoding a β-endoglucanase enzyme, and suitable promoter for promoting transcription of these genes in the transformed Aspergillus fungus and expression and secretion of the enzymes β-xylanase and β-endoglucanase.

Further according to the invention there is provided a method of producing 15 lignocellulosic hydrolases by an fungus strain on cheap sugars syrups for example molasses, DNA comprising a gene which encodes a β-xylanase and a gene which encodes a β-1,4-endoglucanase, and suitable promoter for promoting transcription of these genes in the fungus, and expression and secretion of the β-xylanase and β-endoglucanase enzymes.

20 Yet further according to the invention, there is provided recombinant DNA for use in transforming yeast so as to provide it with a capability of degrading xylan and cellulose, the DNA including:

a gene encoding a β-xylanase enzyme;

a gene encoding a β-endoglucanase enzyme; and

a suitable promoter for promoting transcription of these genes in the transformed yeast and expression of the β-xylanase and β-endoglucanase enzymes.

The nucleotide sequence or DNA of the transformed Aspergillus may include a suitable terminator DNA sequence for promoting efficient expression of the β-xylanase and β-1,4-endoglucanase genes.

The strain may be Aspergillus strain.

The gene encoding β-xylanase may be the xyn2 gene from a fungus such as Trichoderma reesei QM 6a which produces the endo-1,4-β-xylanase enzyme.

The gene encoding β-1,4-endoglucanase may be the eg1 gene from a fungus such as Trichoderma reesei QM 6a which produces the endo-1,4-β-glucanase enzyme.

The fungus promoter and terminator sequences may be the gpd promoter and glaA terminator DNA sequence from Aspergillus niger and Aspergillus awamori, respectively.

The DNA vector containing the genes for β-xylanase and endo-1,4-β-endoglucanase, as well as promoter and terminator sequences, may be the Escherichia coli vector pSPORT1.

The cloning may be effected as follows:

(a)   The construction of the fungus expression vector containing the gpd promoter and glaA terminator DNA regions, called plasmid pGT. The glaA terminator from Aspergillus awamori was amplified from plasmid pKSExtendedglAPo (donated to us by Dan Cullen, Forest Products Laboratory, USDA, Forest Service, One Gifford Pinchot Drive, Madison, WI USA) with the PCR technique with the aid of oligodeoxyribonucleotide DNA

primers designed by conventional techniques and cloned as a 1000-bp DNA fragment into plasmid pDLG1 (la Grange et al., 1996), generating plasmid pDLG1-glaA. The glaA terminator was retrieved from this plasmid and cloned into the corresponding sites on pSPORT1 generating pGlaA. The gpd promoter was amplified from Aspergillus niger ATCC10864 with the PCR technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques and cloned as a 1270-bp DNA fragment into plasmid pGlaA, generating plasmid pGT.

(b) The xyn2 gene from Trichoderma reesei QM 6a was cloned as a 780-bp DNA fragment into plasmid pGT to generate plasmid pGT-xyn2.

(c) Genomic DNA was isolated from Trichoderma reesei QM 6a and used as template for the amplification of the eg1 gene. with the PCR technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques. This DNA fragment still contained non-coding intron sequences. The introns were removed from the genomic copy of the eg1 gene by subsequent amplification with the PCR technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques and cloned as a 1400-bp DNA fragment into plasmid pGT to generate plasmid pGT-eg1.

Hereinafter the term "a fungal expression cassette" is used to denote a recombinant DNA molecule according to the invention which includes the fungal gpd promoter and glaA terminator DNA sequences, preferably the gpd promoter DNA sequence of Aspergillus niger ATCC10864 and the glaA terminator DNA sequence of Aspergillus awamori NRRL 3112, ADH2 promoter and terminator DNA sequences resident on the Aspergillus / Escherichia coli integrative vector pGT.

Furthermore hereinafter the term "a xylanase fungal expression cassette" is used to denote a recombinant DNA molecule according to the invention which includes the xyn2 gene, preferably the xyn2 gene from Trichoderma reesei QM 6a (La Grange et al., 1996), and the fungal gpd promoter and glaA

5   terminator DNA sequences, preferably the gpd promoter DNA sequence of Aspergillus niger ATCC10864 and the glaA terminator DNA sequence of Aspergillus awamori resident on the Aspergillus / Escherichia coli integrative vector pGT-xyn2.

Also hereinafter the term "a endoglucanase fungal expression cassette" is

10   used to denote a recombinant DNA molecule according to the invention which includes the egl gene, preferably the egl gene from Trichoderma reesei QM 6a, and the fungal gpd promoter and glaA terminator DNA sequences, preferably the gpd promoter DNA sequence of Aspergillus niger ATCC10864 and the glaA terminator DNA sequence of Aspergillus awamori resident on

15   the Aspergillus / Escherichia coli integrative vector pGT1-eg1.

The method of the invention has been found to be particularly effective for transforming an Aspergillus fungal strain into a microorganism capable of producing β-xylanase and endo-1,4-β-glucanase.

As mentioned above, cellulose and xylans (major hemicellulose component)

20   form the major constituents of all plant material and are therefore one of the must abundant renewable carbon sources available on earth. Endo-1,4-β-endoglucanase are capable of degrading amorphous cellulose by splitting the poly-D-glucose chains into smaller oligo-D-glucose subunits. β-Xylanase are capable of degrading xylan by splitting the poly-D-xylose

25   chains into smaller oligo-D-xylose subunits. Endo-1,4-β-endoglucanase, also in combination with β-xylanase, have a broad spectrum of industrial applications.

Fuel Ethanol: There is a growing consumer demand for environmentally safe, renewable fuel, such as fuel ethanol. Enzymatic treatment of steam-pretreated wood and other agricultural waste products is still the most effective and safe way of converting the cellulose and hemicellulose to sugars, that could be fermented to ethanol (Von Sivers and Zacchi, 1995).

Paper and Pulp Industry: The treatment of unbleached cellulose pulp with β-xylanase enzymes removes parts of the residual lignin, thus reducing the amount of active chlorine needed for chemical bleaching of the pulp (Viikari et al., 1994).

Baking Industry: In the baking industry, the treatment of bread dough with β-xylanase enzymes can result in an increase in the specific volume (raising) of bread, without negative side effects on dough handling (stickiness of the dough) (Maat et al., 1992; Wong and Saddler, 1993).

Citric acid production: Traditionally, Aspergillus niger strains have been used for the production of citric acid on an industrial scale, using cheap sugars syrups, such as molasses (Kubicek and Röhr, 1986; Röhr, 1998). The production of hydrolases by recombinant Aspergillus grown on medium containing both sugarcane molasses and lignocellulosic material, for example sugarcane bagasse, could result in the release of additional reducing sugars from bagasse that can be converted to citric acid, thus enhancing the citric acid yields.

Animal Feed Industry: The addition of β-xylanase and endo-1,4-β-glucanase enzymes (called fibrolytic enzymes by the animal feed industries) to ruminants improve the digestion and production efficiency in ruminants interest. Several studies showed that the enzymes of Aspergillus and particularly Trichoderma species enhance ruminal fibre digestion, resulting in more than 10% incease in feed conversion ratio (FCR) and average daily gain

(ADG), particularly in lactating and growing cattle (Annison, 1997; Dawson, 1993; Newbold, 1995).

Poultry Feed Industry: Poor nutrient uptake and sticky droppings in chicks can be resolved by adding β-xylanase enzymes to poultry diets, improving both the weight gain of chicks and their feed conversion efficiency (Wong and Saddler, 1993).

BRIEF DESCRIPTION OF DRAWINGS, DIAGRAMS AND REPRESENTATIONS

The invention will now be described by way of example with reference to the accompanying schematic drawings, diagrams and representations.

In the drawings, diagrams and representations there is shown the procedure of rendering an Aspergillus strain capable of producing β-xylanase and endo-1,4-β-glucanase on sugar syrups by way of the method according to the invention:

Figure 1   the construction of the Aspergillus expression vector, plasmid pGT;

Figure 2   the construction of a xyn2 β-xylanase expression cassette in plasmid pGT, yielding plasmid pGT-xyn2;

Figure 3A   the presence of multiple copies of the xyn2 β-xylanase expression cassette integrated into the Aspergillus genome DNA;

Figure 3B   the separation of extracellular protein produced by Aspergillus producing the XynII β-xylanase on 20% SDS-PAGE;

Figure 4A   An example for the production of β-xylanase by the recombinant _Aspergillus_ in molasses growth medium;

Figure 4B   An example for the production of β-xylanase by the recombinant _Aspergillus_ in synthetic sucrose growth medium;

Figure 5   the construction of a _eg1_ endo-1,4-β-glucanase expression cassette in plasmid pGT, yielding plasmid pGT-eg1;

Figure 6A   the presence of multiple copies of the _egl_ endo-1,4-β-glucanase expression cassette integrated into the _Aspergillus_ genome DNA;

Figure 6B   the separation of extracellular protein produced by _Aspergillus_ producing the _Egl_ endo-1,4-β-glucanase on 20% SDS-PAGE;

Figure 7A   An example for the production of endo-1,4-β-glucanase by the recombinant _Aspergillus_ in molasses growth medium;

Figure 7B   An example for the production of endo-1,4-β-glucanase by the recombinant _Aspergillus_ in synthetic sucrose growth medium;

DETAILED DESCRIPTION OF DRAWINGS, DIAGRAMS AND REPRESENTATIONS

STEP 1

The construction of the fungus expression vector containing the _gpd_ promoter and _glaA_ terminator DNA regions, called plasmid pGT, is shown in Fig. 1. The _glaA_ terminator from _Aspergillus awamori_ was amplified from plasmid pKSExtendedglAPo (donated to us by Dan Cullen) with the PCR technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques and cloned as a 1000-bp _SalI_-_BglII_ DNA fragment into the corresponding restriction enzyme sites of plasmid pDLG1 (la Grange

et al., 1996), generating plasmid pDLG1-glaA. The glaA terminator was retrieved from this plasmid as a 1000-bp SalI / EcoRI DNA fragment and cloned into the corresponding sites on pSPORT1, generating pGlaA. The gpd promoter was amplified from Aspergillus niger ATCC10864 with the PCR

5   technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques and cloned as a 1270-bp DNA fragment blunt ended into the SmaI site of plasmid pSPORT, generating plasmid pSPORT-GPD. The two following synthetic oligodeoxyribonucleotides were used for the amplification of the gpd promoter region:

10   GPDR 5'-GTACGCGGCCGCTGTTTAGATGTGTCTATGTGGC-3'

GPDL 5'-GATCGGATCCGAGCATCACCAACATGGTAC-3'

The 1270-bp gpd promoter DNA fragment was retrieved from plasmid pSPORT-GPD as a BamHI and NotI fragment and cloned into the corresponding sites of plasmid pGlaA, generating plasmid pGT (Fig. 1).

15   **STEP 2**

For the purpose of expressing the β-xylanase xyn2 gene of Trichoderma reesei QM6a in Aspergillus, the xyn2 gene was introduced into the Aspergillus expression vector, plasmid pGT. The xyn2 gene was retrieved from plasmid pDLG5 (La Grange et al. 1996) as a 780-bp EcoRI / BglII DNA

20   fragment. The fragment was rendered blunt ended by treating with the Klenow polymerase (Roche) enzyme and cloned into the SmaI site of pSPORT1. The xyn2 was subsequently retrieved as an EcoRI / NotI fragment (after the EcoRI was first made blunt ended with Klenow polymerase), and cloned in pGT digested with SalI (made blunt ended with

25   Klenow polymerase) and NotI, creating pGT-xyn2 (Fig. 2).

Plasmid pGT-xyn2 was introduced into Aspergillus D15 by the spheroplasting method of Ballance et al. (1983), using Novozyme (supplied by Sigma Chemical Co. St. Louis, USA). Plasmid pGT-xyn2 was transformed, together with plasmid p32R2 (kindly provided by Prof. Cees van den Hondel,

Department of Molecular Genetics and Gene Technology, TNO Nutrition and Food Research Institute, Utrechtseweg 48, Zeist, The Netherlands) and selection for successful transformants was performed on medium containing acetamide as nitrogen source. The plasmid p32R2 contains the amdS gene,

5   which allow acetamide utilization in the absence of a nitrogen source (containing 0.5% yeast extract, 0.2% casamino acids, 1-5% glucose, 10 mM acetamide and 15 mM CsCl, trace elements and 0.01 M uridine).

The presence of the xyn2 gene in the Aspergillus genome was determined by isolating chromosomal DNA from a stable xyn2 transformant that expressed

10   the highest level of β-xylanase activity. The genomic DNA was isolated according to La Grange et al. (1996) and digested overnight with the restriction enzymes BamHI and HindIII (which do not cut within the xyn2 cassette) and used for conventional Southern blot analysis (Sambrook et al., 1989). The copy number of the xyn2 gene in the recombinant Aspergillus

15   strain was determined to be at least 5 copies (Fig. 3A).

The production of β-xylanase activity by the recombinant Aspergillus strain was followed over a time period of 10 days in both molasses (20% molasses) as well as double strength liquid minimal media (containing 1.0% yeast extract, 0.4% casamino acids, 12% $NaNO_3$, trace elements and 0.02 M

20   uridine) containing 20 % sucrose. The β-xylanase activity was determined as described by Bailey et al. (1992), but modified by La Grange et al. (1996). The substrate used for liquid assays was 1% Birchwood (Sigma), resuspended in 0.05 M sodium citrate buffer (buffered at pH 5.5). The highest level of β-xylanase activity was achieved on day 9 in both molasses

25   and in minimal media (Fig. 4A&B). The highest level of β-xylanase activity was was 7000 nkat/ml, which is about 5.8 times more than that obtained with expression in Saccharomyces cerevisiae and 15 times more than that obtained in Trichoderma reesei (La Grange et al. 1996). Surprisingly, the pH in both media stays reasonably stable, despite the tendency of Aspergillus to

30   acidify the media they grow in.

The purity and size of the recombinant XynII β-xylanase produced by the Aspergillus was verified by 20% SDS polyacrylamide gelelectrophoresis (PAGE). The supernatant of the recombinant Aspergillus was harvested on day 4 and freeze dried before use. The β-xylanase activity was determined

5 as 5429 nkat/mg unpurified supernatant and a single protein species with a molecular weight of 21 kilo Daltons (kDa) was observed, indicating the production of homogenous β-xylanase of high purity (Fig. 3B).

The Aspergillus niger D15 strain used in this study is a pyrG mutant and the addition of 0.01M uridine to the growth medium is required to sustain growth.

10 The Aspergillus niger D15 transformants containing the xyn2 and egl genes thus still require the addition of 0.01M uridine to the growth media, making cultivation of the strains in large volumes unnecessary expensive. Therefore, both the xyn2 and egl transformants were re-transformed with plasmid pBluescript-pyrGamdS (Plüddemann and van Zyl, 2001) that contains both

15 the amdS and the pyrG markers. The integration of the pyrG gene into the Aspergillus niger D15 transformants genome now allow the transformants to grow on media deprived of uridine while expressing either the Xyn2 endoxylanase or EgI endoglucanase activity.

20 **STEP 3**

For the purpose of expressing the endo-1,4-β-endoglucanase egl gene of Trichoderma reesei QM6a in Aspergillus, the egl gene was introduced into the Aspergillus expression vector, plasmid pGT. The genomic copy of egl was amplified from the genome of Trichoderma reesei using PWO polimerase

25 (Roche) and the following primers:

TregR   5'-GCGGCCGCAGATCTAGTCAACGCTCTAAAGGCA-3'
TregL   5'-GCGGCCGCGAATTCAATGGCGCCCTCAGTTA-3'.

The genomic copy was used as template to produce the cDNA via PCR using primers TregL (above sequence) as well as:

EgverR 5'-CTAAAGGCATTGCGAGTAGTAGTCGTTGCTATACTGGCAC-3' (with the first 20 nucleotides being homologous to the 5'-end of the exon 3ʳ and the last 20 nucleotides being homologous to the 3'-end of the exon 2)

EgintrR 5'-TATCTCCGGGGCCGTAGTAGCTTTTGTAGCCGCTGCC-3' (with
₅ the first 20 nucleotides being homologous to the 5'-end of exon 2 and the last 20 nucleotides homologous to the 3'-end of exon 1) in a standard PCR reaction. The PCR reaction mix was set up in accordance to the suppliers specifications.

The cDNA copy of EGI was obtained through PCR (see Fig. 5). The 1400-bp
₁₀ PCR product was cloned blunt end into plasmid pSPORT1 digested with *Sma*I, generating pSPORT-eg which was used for sequencing purposes. The egI gene was retrieved by digestion with NotI and the 1400-bp DNA fragment cloned into the corresponding site in plasmid pGT, resulting in pGT-egI (Fig. 5).

₁₅ Plasmid pGT-egI was introduced into Aspergillus D15 by the spheroplasting method of Ballance et al. (1983). Plasmid pGT- egI was transformed, together with plasmid p32R2 and selection for successful transformants was performed on medium containing acetamide as nitrogen source.

The presence of the egI gene in the Aspergillus genome was determined by
₂₀ isolating chromosomal DNA from a stable egI transformant that expressed the highest level of β-xylanase activity. The DNA was digested overnight with the restriction enzyme HindIII (which do not cut within the egI cassette) and used for conventional Southern blot analysis (Sambrook et al., 1989). The copy number of the egI gene in the recombinant Aspergillus strain was determined
₂₅ to be at least 2 copies (Fig. 6A).

The production of endo-1,4-β-endoglucanase activity by the recombinant Aspergillus strain was followed over a time period of 10 days in both molasses (20% molasses) as well as double strength liquid minimal media. The endo-1,4-β-endoglucanase was determined, as described by La Grange

et al. (1996). The substrate used for liquid assays was 1% carboxymethyl cellulose (CMC, Sigma) resuspended in 0.05 M sodium citrate buffer (buffered at pH 5.0). The highest level of endo-1,4-β-endoglucanase activity for was achieved on day 8 in both molasses and in minimal media (Fig. 7A&B). The highest level of endo-1,4-β-endoglucanase activity was 1309 and 2041 nkat/ml in molasses and double strength minimal medium, respectively. The same enzyme shows β-xylanase activity of 436 and 811 nkat/ml in molasses and double strength minimal medium, respectively.

The purity and size of the recombinant Egl endo-1,4-β-endoglucanase produced by the Aspergillus was verified by 10% SDS PAGE. The endo-1,4-β-endoglucanase was produced as a predominent protein species representing >90% of the total protein produced. However, the protein appears to be more heterogenous of nature, with a molecular weight varying between 62 – 100 kDa (Fig. 6B).

The Aspergillus niger D15 strain used in this study is a pyrG mutant and the addition of 0.01M uridine to the growth medium is required to sustain growth. The Aspergillus niger D15 transformants containing the xyn2 and egl genes thus still require the addition of 0.01M uridine to the growth media, making cultivation of the strains in large volumes unnecessary expensive. Therefore, both the xyn2 and egl transformants were re-transformed with plasmid pBluescript-pyrGamdS (Plüddemann and van Zyl, 2001) that contains both the amdS and the pyrG markers. The integration of the pyrG gene into the Aspergillus niger D15 transformants genome now allow the transformants to grow on media deprived of uridine while expressing either the Xyn2 endoxylanase or Egl endoglucanase activity.

STEP 4

Alternatively, for the purpose of expressing the cbhI gene of Trichoderma reesei QM6a in Aspergillus, the cDNA copy of the cbhI gene may be retrieved

from plasmid pB210-5a (kindly provided by W.S. Adney of NREL, USA). The cbhI gene may be cloned as a 1.7 kb Sal1 / Xho1 DNA fragment, blunt ended by the Klenow polymerase, into plasmid pGT within the Not1 site between the gpd promoter and glaA terminator sequences. The resulting plasmid may be

5   pGT-cbhI and may be transferred to Aspergillus, as discussed in Step 1 and 2.

Furthermore, for the purpose of expressing the cbhII gene of Trichoderma reesei QM6a in Aspergillus the cbhII gene of Trichoderma reesei QM6a may be isolated by cultivating the fungus in synthetic complete (SC) medium

10   (Rose et al. 1990; containing all the necessary growth factors), with 0.2% finely ground pine and 0.2% xylose as carbon source to induce for the cellulolytic enzymes. Total cellular RNA from Trichoderma reesei may be prepared as described previously (La Grange et al., 1996). The Trichoderma reesei cbh2 gene may be amplified from first-strand cDNA prepared from

15   mRNA with the PCR technique with the aid of two oligonucleotides:

TRECBH2-left (5'-GTACGAATTCTATTGCACCATGATTGTCG-3')

TRECBH2-right (5'- CCCATCGTTCCTGTAAGGAGATCTGATC-3')

The cbhII gene of Trichoderma reesei may be cloned as a blunt ended 2800-bp EcoRI / BglII DNA fragment into the Not1 site between the gpd promoter

20   and glaA terminator sequences. The resulting plasmid may be pGT-cbhII and may be transferred to Aspergillus, as discussed in Step 1 and 2.

Also furthermore, for the purpose of expressing the laccase lcc2 gene of Trametes versicolor SBUG1050 in Aspergillus, the lcc2 gene may be isolated as a 1500-bp EcoRI / XhoI DNA fragment from plasmid pBluescript/lcc2

25   (Cassland and Jönsson, 1999), blunt ended with Klenow polymerase and inserted into the NotI site of expression vector pGT1. The resulting plasmid

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may be pGT-lcc2 and may be transferred to <u>Aspergillus</u>, as discussed in Step 1 and 2.

For the purpose of expressing the <u>cbh1-4</u> gene of <u>Phanerochaete chrysosporium</u> (strain BKM-1767 = ATCC 24725) in <u>Aspergillus</u>, the <u>cbh1-4</u> gene may be retrieved from plasmid pCBH (Van Rensburg et al. 1996, 1998) as a <u>Bgl</u>II / <u>Eco</u>RI DNA fragment and cloned into the <u>Bam</u>HI and <u>Eco</u>RI sites on pBluescript. The <u>cbh1-4</u> gene may then be retrieved as a <u>Not</u>I / <u>Xho</u>I DNA fragment of 1440 bp and cloned onto pGT within the <u>Not</u>I and <u>Sal</u>I sites between the <u>gpd</u> promoter and <u>gla</u> terminator sequences.

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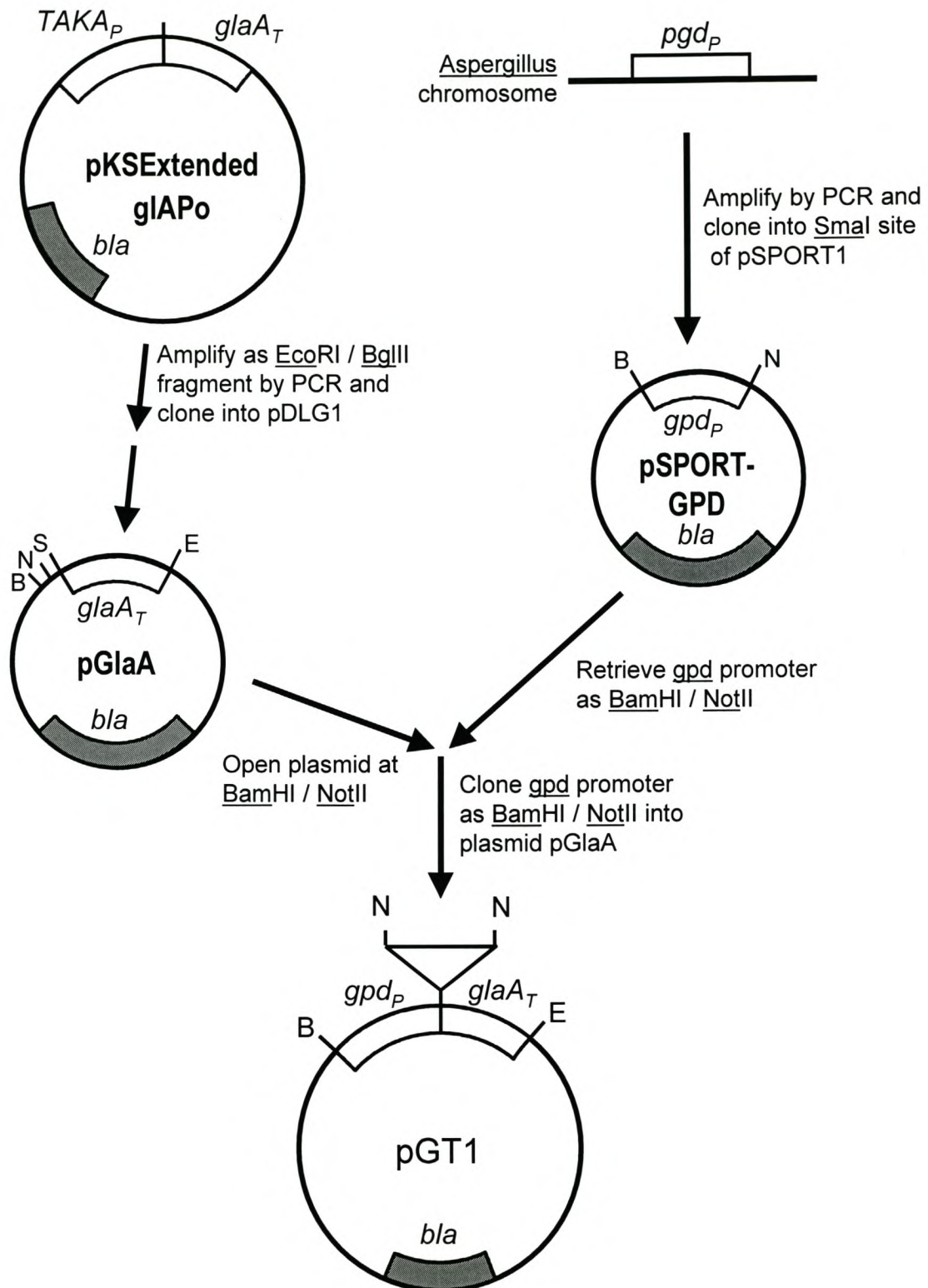
Figure 1: Construction of the Aspergillus expression vector pGT1. The restriction sites indicated are BamHI (B), EcoRI (E), HindIII (H), NotI (N) and SalI (S)
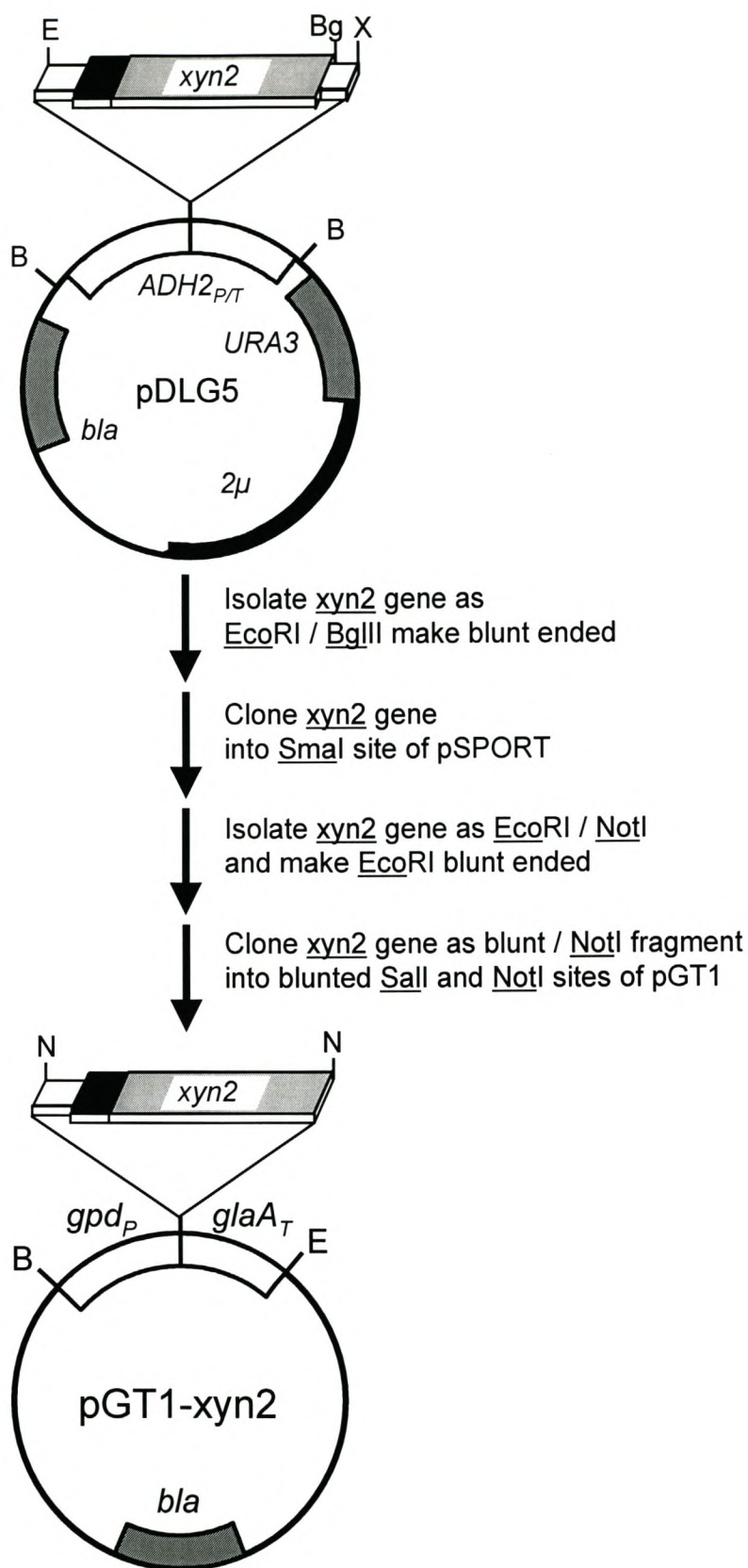
Figure 2: Construction of the β-xylanase expression vector pGT1-xyn2. The restriction sites indicated are BamHI (B), EcoRI (E), HindIII (H), NotI (N) and SaII (S)
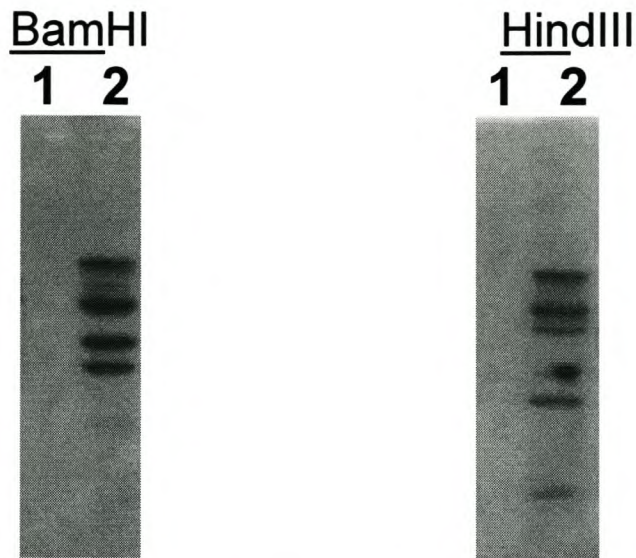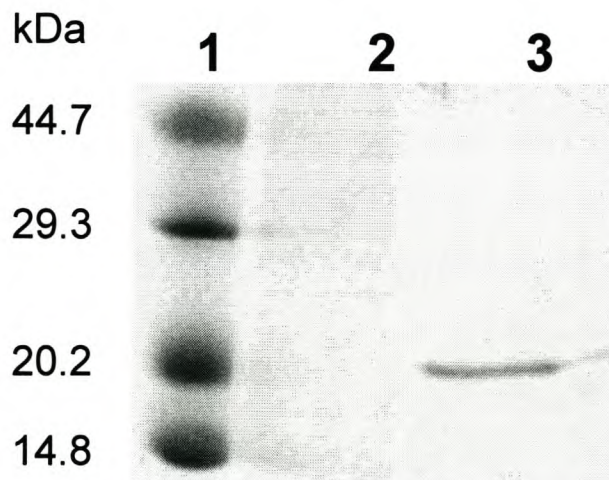
Figure 3A: Southern blot analysis to determine the number of xyn2 copies integrated into the Aspergillus genome. Lane 1 contains the DNA isolated from the negative control and lane 2 the DNA isolated form the transformant. The xyn2 gene was detected with ^{32}P-labelled xyn2 DNA probe.

Figure 3B: SDS-PAGE (15%) of the XynII protein produced by the Aspergillus

transformant. Lane 1 contains the molecular weight marker with the sizes depicted on the left hand side. Lane 2 contains 50 µg of extracellular protein produced by Aspergillus strain. Lane 3 contains 50 µg of extracellular protein produced by the Aspergillus transformant strain.
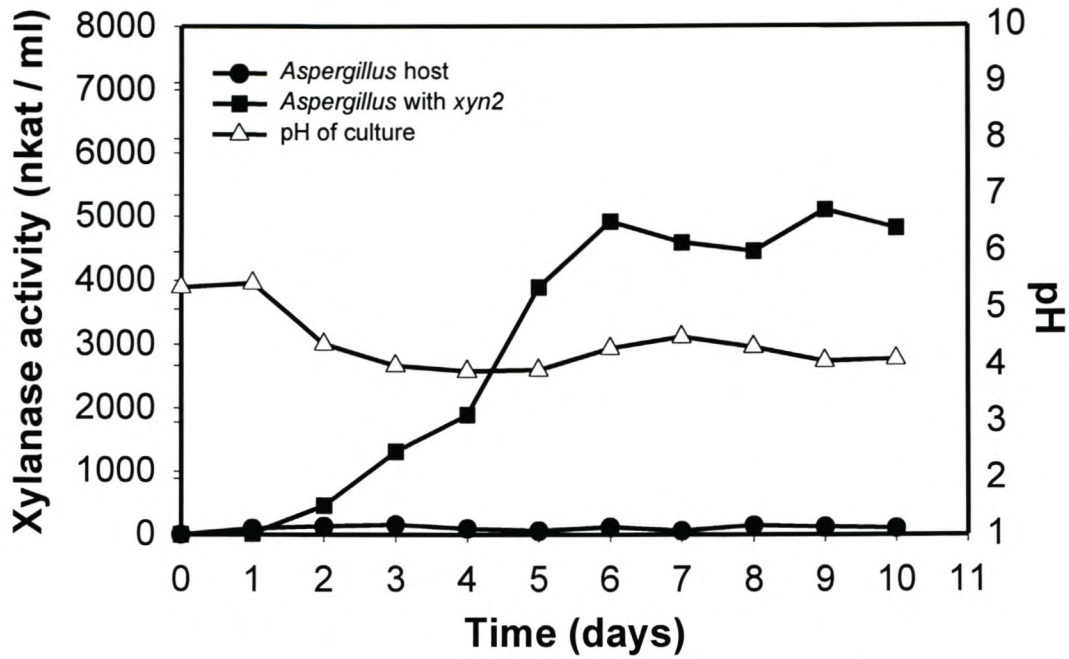
Figure 4A: The production of β-xylanase by the xyn2 Aspergillus transformant over a period of 10 days in 20% molasses medium.
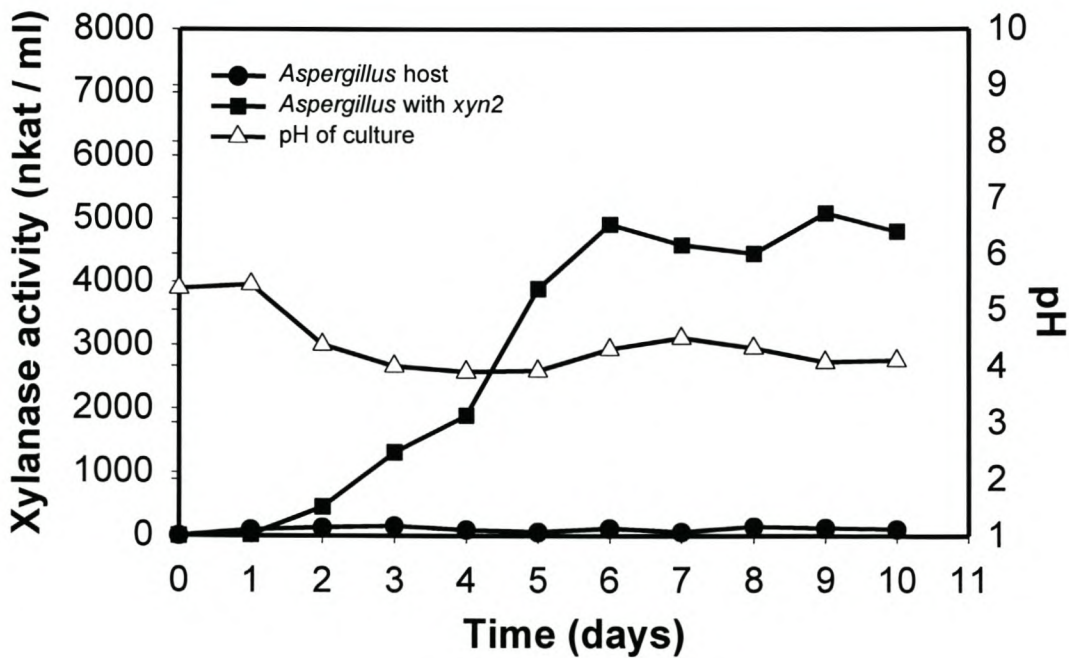
5

Figure 4B: The production of β-xylanase by the xyn2 Aspergillus transformant over a period of 10 days in double strength minimal medium.
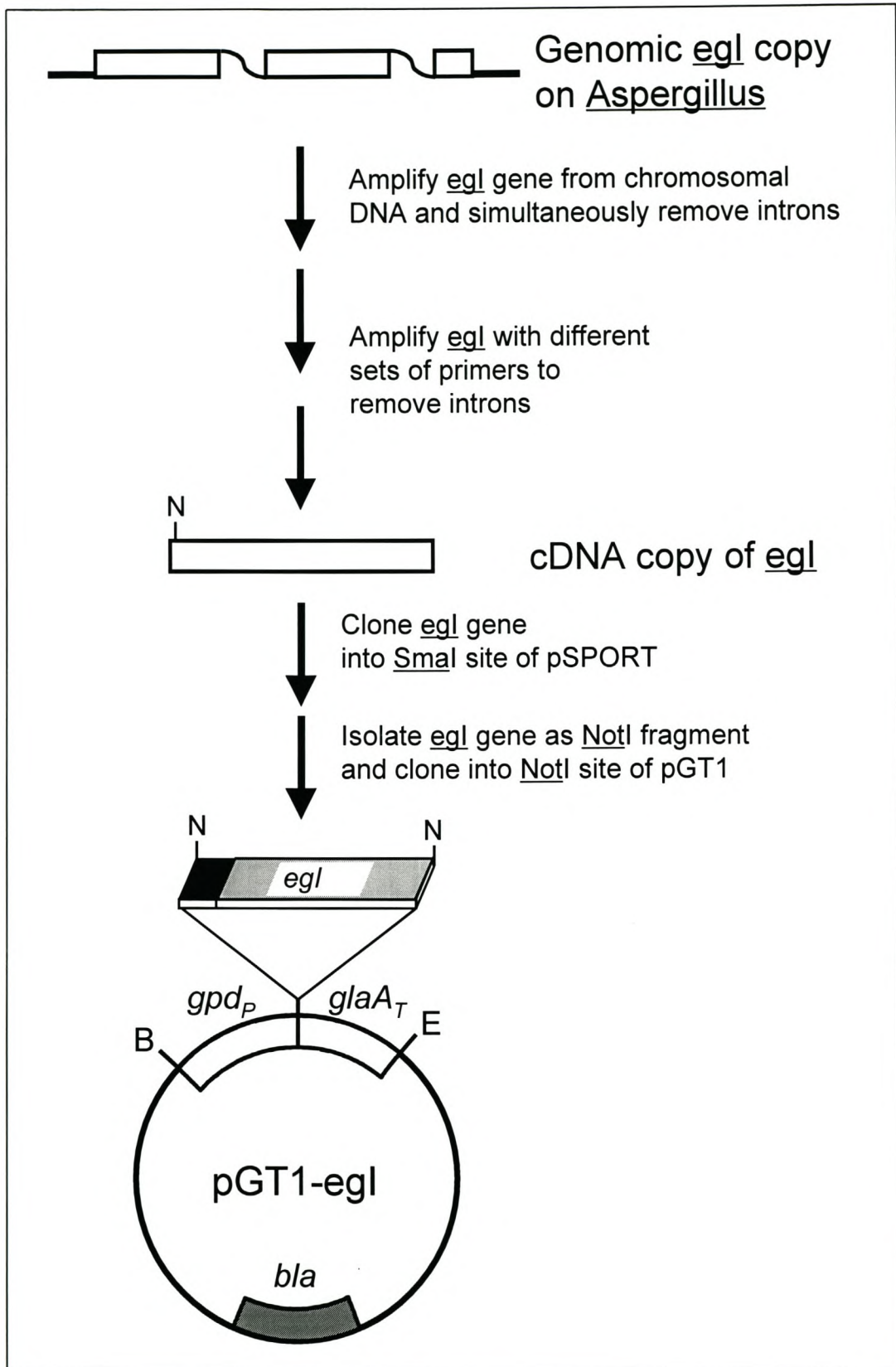
Figure 5: Construction of the β-xylanase expression vector pGT1-egl. The restriction sites indicated are BamHI (B), *Eco*RI (E), HindIII (H), NotI (N) and SalI (S)
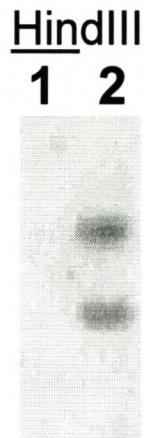
Figure 6A: Southern blot analysis to determine the number of egl copies integrated into the Aspergillus genome. Lane 1 contains the DNA isolated from the negative control and lane 2 the DNA isolated form the transformant. The egl gene was detected with ^{32}P-labelled egl DNA probe.
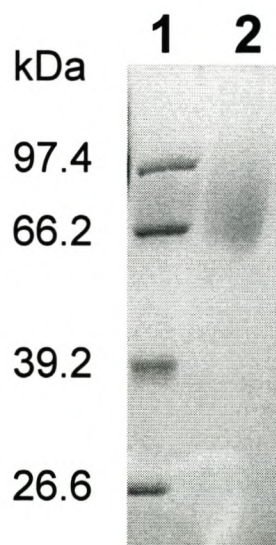

Figure 6B: SDS-PAGE (10%) of the Egl protein produced by the Aspergillus transformant. Lane 1 contains the molecular weight marker with the sizes depicted on the left hand side. Lane 2 contains 50 μg of extracellular protein produced by the Aspergillus transformant strain.

Figure 7A: The production of β-xylanase and endo-1,4-β-endoglucanase (CMCase) by the egl Aspergillus transformant over a period of 10 days in 20% molasses medium.

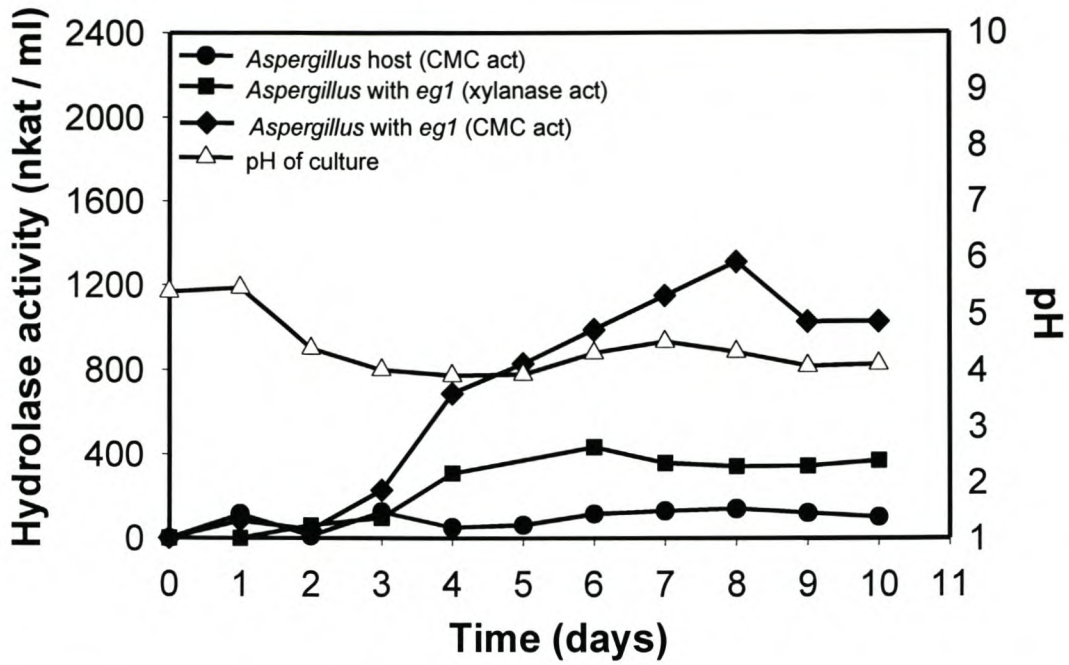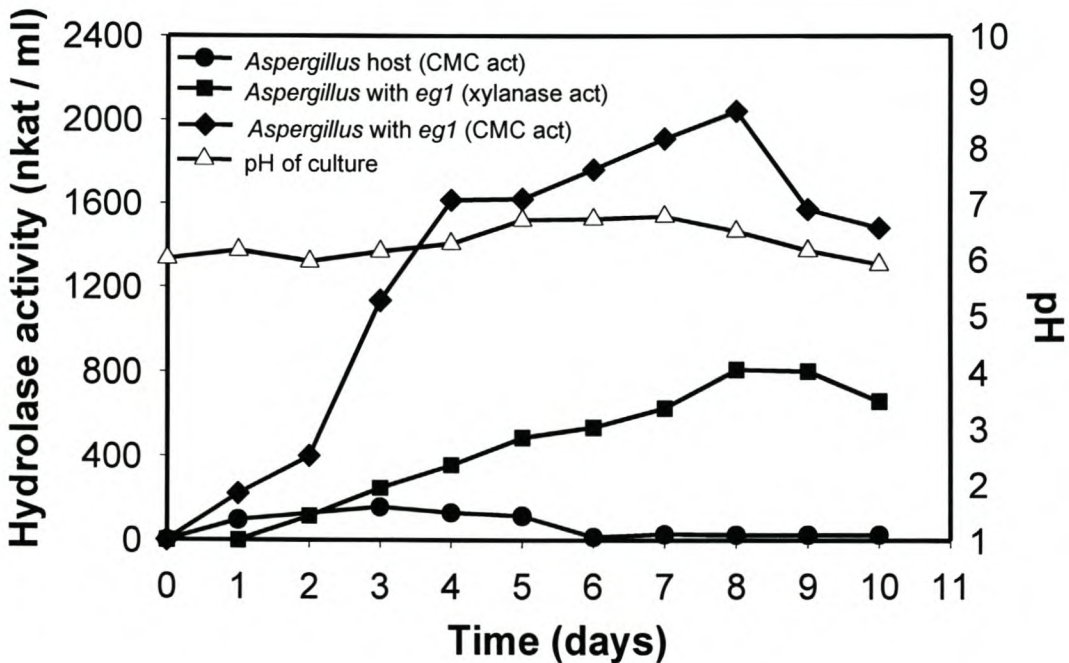**Figure 7B:** The production of β-xylanase and endo-1,4-β-endoglucanase (CMCase) by the egl Aspergillus transformant over a period of 10 days in double strength minimal medium.

APPENDIX II

A method for providing a yeast and fungal strain to produce β-mannanase and for producing coffee extracts.

DrG Ref.: 607114spec-amended

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TITLE OF INVENTION

A method for providing a yeast and fungal strain to produce β-mannanase and for producing coffee extracts.

FIELD OF INVENTION

5 The present invention relates to a method for producing coffee extracts.

More particularly, the invention relates to a method for producing coffee extracts from *Arabica* and *Robusta* beans.

The present invention also relates to a method of providing a yeast strain and an *Aspergillus* fungus strain with the capability of producing an endo-1,4-β-

10 mannanase (β-mannanases).

More particularly the invention relates to a method of providing a yeast strain with the capability of producing a β-mannanase and to a recombinant DNA for use in transforming a yeast strain to produce a β-mannanase.

Also, in particularly the invention relates to a method of providing an *Aspergillus*

15 fungus strain with the capability of producing a β-mannanase and to a recombinant DNA for use in transforming an *Aspergillus* fungus strain to produce a β-mannanase.

BACKGROUND TO INVENTION

Polysaccharides such as arabinogalactan, mannan, and cellulose constitute

20 nearly 50 % of the green coffee bean weight (Nunes *et al.*, 2001; Sachslehner *et al.*, 2000). The major polysaccharide of this fraction is a water-insoluble, crystalline mannan that forms approximately 20-30% of the dry weight of *Arabica* and *Robusta* beans (Sachslehner *et al.*, 2000). These carbohydrates play an

important role in the retention of volatile substances due to their capacity to bind aromatic compounds at the adsorptive sites (Nunes *et al.*, 2001; Trugo, 1985).

Modern instant coffee production entails, cleaning, roasting and grinding of coffee beans, followed by a split extraction and concentration to achieve a high
5   solids concentrate for a low-energy spray-drying operation (Stoltze and Masters, 1979). During the roasting procedure of the coffee beans the physical and chemical properties of carbohydrates change drastically (Sachslehner *et al.*, 2000). For instance, green coffees contain 62 % arabinogalactan, 24 % galactomannan, and glucans in the high-molecular weight material extracted with
10   water, whereas roasted coffees contain 69 % galactomannan and 28 % arabinogalactan (Nunes *et al.*, 2001). After roasting and grinding, extraction is the key operation in the large-scale manufacture of instant coffee in which both soluble solids and volatile aroma/flavor compounds are extracted (Clarke, 1987). Technically produced extracts from roasted *Arabica* and *Robusta* coffee contain
15   20-36 % carbohydrates depending on the degree of extraction. They are predominantly composed of mannan and galactan in about the same proportions, with glucan and araban making up only 1-3 % of the extracts (Thaler, 1979).

Coffee extraction techniques led to extracts of around 25 % w/w soluble solids
20   concentration (Clarke, 1987). Spray-drying on these concentrations can provide coffee of the required physical form, however, there will be substantial loss of volatile compounds. Consequently, pre-concentration methods for coffee extracts prior to drying have been introduced to circumvent extensive losses (Clarke, 1987). The extracts can be concentrated to 45 % w/w soluble solids
25   concentration in vacuum evaporators, resulting in lower costs of removing water during drying (Stoltze and Masters, 1979). However, concentrations above 42 % w/w solids are difficult to reach, due to the high viscosity of coffee extracts, also

highly viscous extracts will require longer pre-concentration times (Clarke, 1987). The viscosity of coffee extracts can be reduced by hydrolysing the mannan portion to short oligosaccharides (Sachslehner *et al.*, 2000; Wong and Saddler, 1993). This would make it possible to concentrate the extracts to concentrations higher than 42 % w/w soluble solids.

β-Mannanases hydrolyse linear mannan polysaccharides and complex substituted mannan polysaccharides such as glucomannan, galactomannan and galactoglucomannan into oligosaccharides of various chain lengths (Sabini *et al.*, 2000; Setati *et al.*, 2001). Hydrolysis of substituted mannans is greatly affected by the degree and pattern of substitution so that as the galactose content increases, the rate of hydrolysis (V_{max}) by β-mannanase decreases and the K_m increases (McCleary, 1983). β-Mannanases can therefore be used to perform hydrolysis experiments in which the intention is only to modify the properties of polysaccharides without complete degradation. The viscosity of polysaccharides is proportional to chain length, branching, and entanglement, and reduction of viscosity can be effected through partial hydrolysis.

Considering the high galactomannan content (69%) in roasted coffee beans, β-mannanases can also be considered for enzymatic pre-treatment of ground coffee beans before extraction (percolation) to (i) improve coffee solid yields and (ii) improve extraction of volatile substances.

It is an object of the invention to suggest a novel method for producing coffee extracts.

It is further an object of the invention to suggest a method of providing a yeast strain with the capability of producing β-mannanase and to a recombinant DNA for use in transforming a yeast strain to produce β-mannanase enzyme.

Yet further, another object of the invention to suggest a method of providing a fungus strain with the capability of producing β-mannanase and to a recombinant DNA for use in transforming a fungus strain to overproduce β-mannanase enzyme.

5 **SUMMARY OF INVENTION**

According to the invention, a method of producing coffee extracts includes the step of performing extraction on coffee beans by an enzyme-aided extraction procedure to produce a coffee extract.

10 According to the invention, a method of producing coffee extracts includes the step of performing extraction on coffee beans by an enzyme-aided extraction procedure to produce a coffee extract.

The method may include the step of hydrolysing the coffee extract with an enzyme.

Also, according to the invention, a method of producing coffee extracts includes 15 the steps of performing extraction on coffee beans and of hydrolysing galactomannan present in the coffee beans.

The galactomannan may be hydrolysed by an enzyme.

The enzyme may be recombinant β-mannanase.

The recombinant β-mannanase may be produced from *Saccharomyces* 20 *cerevisiae* Y294(pMES1).

The recombinant β-mannanase may be produced from *Aspergillus niger* D15(pGT-man1).

The coffee extract may have between 8 % and 40 % w/v concentration.

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The coffee beans may be roasted.

The coffee beans may be *Arabica* and/or *Robusta* beans.

The coffee beans may contain 20-36% carbohydrates, predominantly in the form of β-1,4-mannan and β-1,3-galactan.

5   β-1,4-mannan and β-1,3-galactan may be hydrolysed by the enzyme.

The coffee extract may have a concentration higher than 42%.

The extraction may occur at a temperature below 80 degrees Celsius.

The enzyme-aided extraction procedure may occur at 30 - 60°C

The method may include the step of drying the coffee extract.

10   The method may be used to influence the viscosity, the retention of volatile compounds and the rate of drying of coffee extracts

According to the invention, there is provided a method of providing a yeast strain with the capability of producing a β-mannanase, the method including transforming a yeast strain with a DNA nucleotide sequence including a gene

15   encoding a β-mannanase enzyme and suitable promoter for promoting transcription of these genes in the transformed yeast and expression and secretion of the enzyme β-mannanase.

Furthermore, according to the invention, there is provided recombinant DNA for use in transforming yeast so as to provide it with a capability of producing

20   β-mannanase, the DNA including:

a gene encoding a β-mannanase enzyme;

suitable promoter for promoting transcription of these genes in the transformed yeast and producing of the β-mannanase enzymes.

The nucleotide sequence or DNA of the transformed yeast may include a suitable terminator DNA sequence for promoting efficient expression of the β-mannanase gene.

The yeast strain may be *Saccharomyces cerevisiae*.

The gene encoding β-mannanase may be the *man1* gene from a fungus such as *Aspergillus aculeatus* MRC11624 which produces the β-mannanase enzyme.

The yeast promoter and terminator sequences may be the *PGK1* promoter and terminator DNA sequence.

The DNA vector containing the gene for β-mannanase, as well as promoter and terminator sequences, may be the yeast / *Escherichia coli* shuttle vector YEp352.

The cloning may be effected as follows:

(a) The construction of the yeast expression vector containing the *ADH2* promoter and terminator DNA regions, called plasmid pDLG1, e.g. as generated in the Applicants' laboratory (La Grange *et al.*, 1996).

(b) The *man1* gene from a *Aspergillus aculeatus* MRC11624 culture is amplified with the PCR technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques and cloned as a 1180-bp *Eco*RI/*Xho*1 DNA fragment into plasmid pDLG1 to generate plasmid pMES1 (Figure 5A) (Setati *et al.*, 2001).

(c)   Total RNA is isolated from a *Aspergillus aculeatus* MRC11624 culture prepared on locust bean gum as carbon source.

(d)   Poly-A mRNA is purified from the total RNA and first strand complementary DNA (cDNA) is prepared for the poly-A mRNA.

5   (e)   oligodeoxyribonucleotide DNA primers designed by conventional techniques.

Hereinafter the term "a mannanase yeast expression cassette" is used to denote a recombinant DNA molecule according to the invention which includes the *man1* gene, preferably the *man1* gene from *Aspergillus aculeatus* MRC11624

10   (Setati *et al.*, 2001), and the yeast *ADH2* promoter and terminator DNA sequences, preferably the *ADH2* promoter and terminator DNA sequences resident on the yeast / *Escherichia coli* shuttle vector pMES1 (Setati *et al.*, 2001).

The method of the invention has been found to be particularly effective for transforming a *Saccharomyces cerevisiae* yeast strain into a microorganism

15   capable of producing β-mannanase enzyme.

Yet further according to the invention, there is provided a method of providing a fungus strain with the capability of producing a β-mannanase, the method including transforming a fungus strain with a DNA nucleotide sequence including a gene encoding a β-mannanase enzyme and suitable promoter for promoting

20   transcription of these genes in the transformed yeast and expression and secretion of the enzyme β-mannanase.

Furthermore, according to the invention, there is provided recombinant DNA for use in transforming the fungus strain so as to provide it with a capability of producing β-mannanase, the DNA including:

a gene encoding an β-mannanase enzyme;

suitable promoter for promoting transcription of these genes in the transformed fungus strain and producing of the β-mannanase enzymes.

The nucleotide sequence or DNA of the transformed fungus strain may include a suitable terminator DNA sequence for promoting efficient expression of the β-mannanase gene.

The fungus strain may be *Aspergillus niger* D15.

The gene encoding β-mannanase may be the *man1* gene from a fungus such as *Aspergillus aculeatus* MRC11624 which produces the β-mannanase enzyme.

The fungus promoter and terminator sequences may be the *gdp* promoter and *glaA* terminator DNA sequence.

The DNA vector containing the gene for β-mannanase, as well as promoter and terminator sequences, may be the *Aspergillus / Escherichia coli* shuttle vector pGT.

The cloning may be effected as follows:

(f)   The construction of the yeast expression vector containing the *gpd* promoter and *glaA* terminator DNA sequences, called plasmid pGT, e.g. as generated in the Applicants' laboratory (Rose and Van Zyl, 2002).

(g)   The *man1* gene from a *Aspergillus aculeatus* MRC11624 culture is amplified with the PCR technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques and cloned as a 1180-bp *Eco*RI/*Xho*1 DNA fragment into plasmid pGT to generate plasmid pGT-man1 (Figure 5B).

Hereinafter the term "a mannanase fungus expression cassette" is used to denote a recombinant DNA molecule according to the invention which includes the *man1* gene, preferably the *man1* gene from *Aspergillus aculeatus* MRC11624 (Setati *et al.*, 2001), and the fungus *gpd* promoter and *glaA*
5   terminator DNA sequences, preferably the *gdp* promoter and terminator DNA sequences resident on the fungus / *Escherichia coli* shuttle vector pGT-man1 (Figure 5B).

The method of the invention has been found to be particularly effective for transforming a *Aspergillus niger* yeast strain into a microorganism capable of
10   producing β-mannanase enzyme.

BRIEF DESCRIPTION OF DRAWINGS, DIAGRAMS AND REPRESENTATIONS

The invention will now be described by way of example with reference to the accompanying schematic Figures.

15   In the drawings there is shown in:

Figure 1   Hydrolysis of locust bean gum galactomannan with the recombinant β-mannanse from *Saccharomyces cerevisiae* Y294(pMES1). Viscosity was measured with a Brookfield viscometer at 40°C and the reducing sugars were measured using
20   the DNS assay;

Figure 2   The flow properties of coffee extracts determined with 20 %, 40 %, and 60 % extracts. Closed symbols represent the flow curves, and open symbols represent the viscosity curves;

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Figure 3   The effect of β-mannanase on the viscosity of coffee extracts and their fluid dynamics. Change in viscosity of 20 % and 40 % extracts was monitored before treatment (closed symbols) and after treatment (open symbols); and

5   Figure 4   GC profiles of coffee samples. C-3 is the control sample, A-2 is the autoclaved sample, and E-1 is the enzyme treated sample.

Furthermore, in the drawings, diagrams and representations there is shown the amino acid sequence of Man1 mannanase of *A. aculeatus* and the plasmid pMES used to render *Saccharomyces cerevisiae* yeast strain capable to produce

10   β-mannanase: Yet further, in the drawings, diagrams and representations there is shown the plasmid pMES used to render *Aspergillus niger* fungus strain capable to produce β-mannanase

Figure 5A   diagram of a *man1* β-mannanase yeast expression cassette in plasmid pMES1;

15   Figure 5B   diagram of a *man1* β-mannanase yeast expression cassette in plasmid pGT-man1.

Figure 6   Amino acid sequence of β-mannanase of *Aspergillus aculeatus* MRC11624.   The The neutral Ser→Thr substitution in the β-mannanase of *A. aculeatus* MRC11624 versus the published

20   sequence of the β-mannanase of *A. aculeatus* KSM510 is encircled.

DETAILED DESCRIPTION OF FIGURES

The invention will now be described by way of example with reference to the accompanying schematic Figures and experiment.

A.   EXPERIMENT

(a)   Enzyme Production

The recombinant β-mannanase producing yeast strain *Saccharomyces cerevisiae* Y294(pMES1) (Setati *et al.*, 2001) was cultivated in minimal sythetic complete medium supplemented with 4 % glucose, 0,17 % yeast nitrogen base without ammonium sulphate, and an amino acid drop-out mix without methionine. The cultures were incubated at 30°C on a rotary shaker for 4 days, after which the cells were removed by centrifugation. The supernant (enzyme source) was concentrated through the minitan cross-flow ultrafiltration devise (Millipore Corporation, Bedford, Massachusetts, USA). The filtrate was freeze-dried and used for hydrolysis experiments.

(b)   Hydrolysis of locust bean gum (galactomannan)

A 1 % (w/v) locust bean gum solution was prepared in 50 mM citrate buffer pH 5 and used as a substrate for viscosity analysis. The viscosity analyses were performed using a Brookfield viscometer model (Brookfield Engineering Laboratories, Inc., Stoughton, Mass. USA). The initial viscosity was determined at 40°C, after which the β-mannanase enzyme was added to the final concentration of 2 nkat/mg substrate. The viscosity of the reaction mixture was measured at different time points and samples were collected concurrently and analysed for reducing sugars. The reaction was terminated by boiling for 5 min. The reducing sugars were determined using the modified DNS method as previously described (Stålbrand *et al.*, 1993).

(c)   The flow dynamics of coffee extracts

Ground *Arabica* coffee (Boveldt pure South African) was supplied by SAPEKOE (Pty) Ltd. (Tzaneen, SA). Coffee extracts were prepared by pre-wetting 400 g of

coffee in 5 mM citrate buffer at pH 5 for 5 hrs. Extraction was carried out overnight at 80°C with constant stirring. The soluble extract was collected by filtration through a miracloth (CALBIOCHEM), and freeze-dried. The dried extract was used to prepare different extract concentrations (20 %, 40 % and 60 % w/v) for viscosity analysis. The flow dynamics of the extracts were studied at 30°C on a HAAKE RV12 Viscometer (HAAKE Mess-Technik GmbH & Co., Germany). The effect of β-mannanase on the viscosity, and flow dynamics of the coffee extract was evaluated. The enzyme was added to a final concentration of 2 nkat/mg extract and 100 μg/ml BSA was added to stabilize the enzyme. The reaction was carried out at pH 5, 50°C for 3 h followed enzyme inactivation at 100°C for 5 min.

(d)   Extraction processes

Thirty grams of ground *Arabica* coffee were pre-wet to 50°C in 100 ml of citrate buffer pH for 5 h, followed by hydrolysis with β-mannanase for 5 h with constant stirring. The enzyme was inactivated, and the extraction was continued for another 2 h. In a parallel experiment, 30 g of coffee was pre-wet under the same conditions, followed by two autoclave cycles, at 121°C for 20 min per cycle. As control, the same amount of coffee was pre-wet with buffer for 5 h, then freeze-dried. In all the treatments, 0.02 % sodium azide was added to prevent microbial growth. The soluble extracts were freeze-dried and volatile compounds were analysed by gas chromatography.

(e)   Analytical methods

Gas chromatography analysis was performed on 3.57 g of coffee samples. SPME fiber (100 μm Polydimethylsiloxane) was used for sampling. Extraction was carried out for 120 min at 65°C (head space), and desorption at 230°C for 5 min. Compounds were separated on PS089 (0.25 μM film), 40 m x 0.25 mm GC

column using 38 cm/s He as a carrier gas. The GC program was 40°C (0 min) to 230°C at 4°C/min.

(f)   The effect of β-mannanase on the viscosity of locust bean gum

Locust bean gum was hydrolysed with β-mannanase for a period of 120 min. The change in viscosity of the substrate and the release of reducing sugars were monitored continuously. A rapid loss of viscosity was observed in the first 20 minutes of the reaction (Figure 1). This was accompanied by a gradual increase in the concentration of reducing sugars. These results indicated that there is a rapid decrease in the average molecular weight of galactomannan, which is followed by a steady state. The viscosity remained constant whereas the concentration of reducing sugars increased continuously.

(g)   Flow dynamics of coffee extracts

Ground *Arabica* coffee was extracted with water at 80°C and the extract was freeze-dried. The fluid properties of 20 %, 40 % and 60 % w/v coffee extracts were studied. Figure 2 shows the flow curves and viscosity curves of the different extract concentrations. The extracts showed similar rheological properties, and exhibited non-Newtonian flow characteristics. The viscosity of the extracts decreased with increase in shear rate resulting in pseudoplastic behaviour. These results compare with those reported by Sachslehner *et al.* (2000), and indicate that galactomannan found in coffee extracts impart on-Newtonian flow properties to the extracts.

Treatment of the 20 % and 40 % extracts with β-mannanase resulted in significant drop of viscosity (Figure 3) indicating that the enzyme is capable of hydrolysing coffee galactomannan. The extracts still retained their pseudoplastic properties after hydrolysis, this implies that other polysaccharides such as

arabinogalactan could have similar flow properties as galactomannan and that they are not hydrolysed by the β-mannanase. Sachslehner *et al.* (2000) reported that enzymatic treatment of coffee extract with the β-mannanase from *Sclerotium rolfsii* resulted not only in the cleavage of mannan but also with possible degradation of galactan. An enzyme-aided treatment of coffee extracts could play a pivotal role in instant coffee production since the hydrolysis of mannan will reduce the viscosity of the extracts, thus improving the preconcentration process. In addition, the remaining polysaccharides will impart enough viscosity to give coffeee its characteristic body.

(h)   The effect of enzyme-aided extraction on volatile compounds

It has previously been shown that hydrolysis of coffee galactomannan with β-mannanase results in reduction of viscosity, and that the viscosity remained constant after a few hours of hydrolysis (Sachslehner *et al.*, 2000). Therefore, it was expected in the experiment that hydrolysis of coffee extracts at 50°C for 5 h would generate high concentrations of oligosaccharides which together with high molecular weight arabinogalactan have the capacity to bind volatile compounds (Nunes *et al.*, 2001; Trugo, 1985). Due to the mildness of the treatment, retention of higher concentrations of volatile compounds would be achieved. Figure 4, shows the chromatograms obtained from coffee extract samples that were extracted by enzyme-aided treatment (E-1), and through autoclaving at 121°C (A-2). The profiles of volatile compounds were compared to those detected in ground coffee. The three samples displayed similar profiles which, indicated that they contained the same type of volatile compounds. However, the differences in peak sizes indicate that the autoclaved sample had a significant loss of volatile compounds, relative to the control. Enzyme treatment does also seem to affect the amount of compounds retained, resulting in improved retention of some compounds and slight losses of others.

The preliminary results indicate that β-mannanase is capable of hydrolysing coffee galactomannan, leading to substantial reduction in viscosity. In addition the results seem to indicate that enzyme-aided extraction might be a valuable techniques for instant coffee production since this could allow extractions at lower temperatures, and therefore, better retention of volatile compounds. In addition, lower extract viscosity would allow recovery of concentrates higher than 42 % after pre-concentration, and also improve the drying process due to the lower water content of the extracts.

B. CONSTRUCTRION OF RECOMBINANT YEAST AND FUNGAL STRAINS

(a) Isolation of β-mannanase (man1) gene

For the purpose of isolating the *man1* gene, the fungus *Aspergillus aculeatus* MRC11624 was cultivated in minimal medium containing: 0,3% locust been gum [Sigma], 0.1% bacto tryptone; 0.5% yeast extract; 0.1% $NaNO_3$; 0.001% $FeSO_4.7H_2O$; pH 5.5 in shaking flasks for 48 hours at 30°C. Total RNA was isolated essentially according to Crous *et al.* (1995): The poly $(A)^+$mRNA was purified from total RNA using the Oligotex™ mRNA isolation kit (Qiagen, Hilden, Germany). First strand cDNA was synthesised from 116 ng of mRNA using a first strand cDNA synthesis kit (Roche Molecular Biochemicals, Ottweiler, Germany), and used as template for amplification of the β-mannanase encoding gene *man1* by PCR on a Biometra Trio Thermoblock TB1 (Biometra Biomedizinische Analytik, Göttingen, Germany). The primers used were designed based on the sequence of the *man1* gene of *A. aculeatus* strain KSM510 (Accession. No. L35487) (Christgau *et al.*, 1994)

1. MANR (28-mer, the *Eco*RI restriction site is underlined)

    (5'-GATCGAATTCCACCACCACACAACCAAG-3')

2.   MANL (28-mer; the *Xho*I restriction site is underlined)

(5′-CTAGCTCGAGCGCCAACAGTCTACTTCG-3').

The cDNA amplified *man1* gene was ligated to pBLUESCRIPT and sequenced, and the nucleotide sequence showed 99.7% identity with the sequence of the

5   *A. aculeatus* KSM510 as reported by Christgau *et al.* (1994) (GenBank accession number L35487). Three base-pair descrepancies were detected on the DNA level and one resulted in an amino acid sequence difference (Ser → Thr) at position 225 (Figure 6). This region is variable according to sequence alignment by Hilge *et al.* (1998) and is not crucial for its structure and function.

10   The neutral Ser→Thr substitution is thus not likely to affect the enzyme activity.

(b)   construction of recombinant yeast and fungal strains

For the purpose of constructing a yeast strain capable of producing β-mannanase, plasmid pMES1 was engineered. The PCR product was pre-digested with the restriction enzymes *Eco*RI and *Xho*I, the DNA purified through

15   agarose gel electrophoresis and ligated as a 1180-bp *Eco*RI/*Xho*I DNA fragment into the *Eco*RI and *Xho*I sites between the *ADH2* promoter and terminator in plasmid pDLG1, thereby generating plasmid pMES1, which constitutes a β-mannanase yeast expression cassette according to the invention (Figure 5A). The DNA nucleotide sequence of the *man1* gene encoding β-mannanase

20   enzyme is presented in Figure 3B.. Plasmid pMES1 was transformed into *Saccharomyces cerevisiae* strain Y294 following the DMSO-lithium acetate method described by Hill *et al.* (1991). Auto-selective *Saccharomyces cerevisiae* strains contain this plasmid was constructed by replacing the *FUR1* gene on the chromosome with an *fur1::LEU2* disruptive allele, as described by La Grange *et*

25   *al.* (1996).

For the purpose of constructing a fungus strain capable of producing endo-1,4-β-mannanase, plasmid pGT-man1 was engineered. The *manI* gene was retreived as a *Eco*RI-*Xho*I DNA fragment from plasmid pMES1 (Setati *et al.*, 2001) and cloned into the corresponding sites of *Escherichia coli* plasmid pBLUESCRIPT.

5 The *man1* gene was retrieved from pBLUESCRIPT-man1 as a *Not*I-*Xho*I DNA fragment and cloned into the *Not*I and *Sal*I sites of plasmid pGT (Rose and Van Zyl, 2002), generating plasmid pGT-man1 (Figure 5B). Plasmid pGT-man1 was transformed into *Aspergillus niger* strain D15 by the spheroplasting method of Ballance *et al.* (1983). Plasmid pGT-man1 was transformed, together with

10 plasmid p32R2 and selection for successful transformants was performed on medium containing acetamide as nitrogen source (Rose and Van Zyl, 2002).

(b) assay of β-mannanase enzyme activity

β-Mannanase activity was assayed using the method described by Stålbrand *et al.* (1993) with 0.5% locust bean gum (Sigma) as substrate at 50°C. Appropriate

15 dilutions of the cell-free culture solution in 50 mM Na-citrate buffer (pH 6.0) were used as enzyme source. The amount of released sugar was determined by the dinitrosalicylic acid method described by Miller *et al.* (1960). β-Mannanase activity was expressed in nkat/ml. Extracellular activity was determined using the culture supernatant as source of enzyme.

20 ### (c) production levels of recombinant β-mannanase

The maximum β-mannanase activity obtained from the recombinant *fur1::LEU2 Saccharomyces cerevisiae* Y294(pMES1) strain (521 nkat/ml) and recombinant *Aspergillus niger* D15(pGT-man1) strain (6 000 nkat/ml) compares very well with that of *Aspergillus aculeatus* MRC11624 (about 500 nkat/ml) and other

25 recombinant strains reported in literature (Table 1), if one takes into account that

the *Aspergillus aculeatus* MRC11624 culture supernatant contains the auxiliary enzymes involved in mannan degradation.

Table 1. β-Mannanase activity levels measured from expression in different hosts.

Expression Host	Enzyme Activity (nkat/ml)	Reference
S. lividans IAF10-164	1450	Arcand *et al.*, 1993
S. lividans 1326	1917	Marga *et al.*, 1996
E. coli JM109	81.7	Mendoza *et al.* 1995
E. coli RR28	1.33	Gibbs *et al.*, 1992
S. cerevisiae DBY746	0.22	Stålbrand *et al.*, 1995
S. cerevisiae Y294(pMES1)	521	This patent
A. niger D15	6000	This patent

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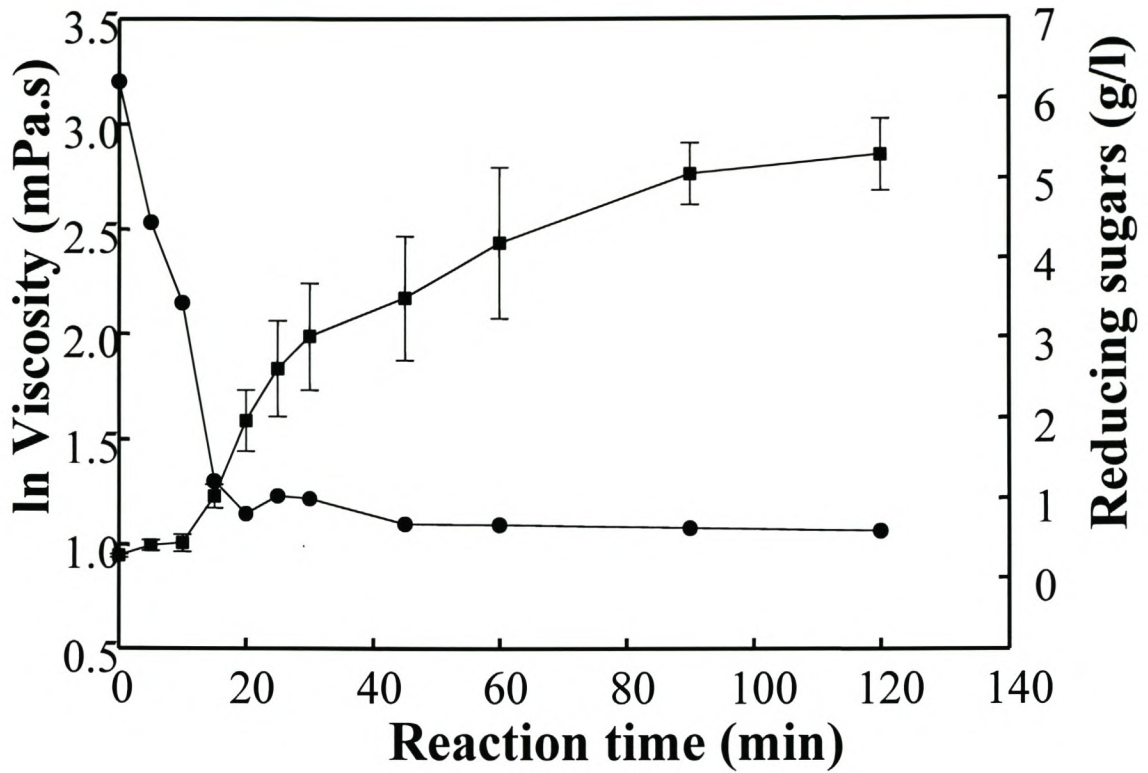**Date:** 14 October 2002

DrG Ref: 607114

Figure 1: Hydrolysis of locust bean gum galactomannan with the recombinant β-mannanse from *Saccharomyces cerevisiae* Y294(pMES1). Viscosity (●) was measured with a Brookfield viscometer at 40°C and the reducing sugars (■) were measured using the DNS assay.
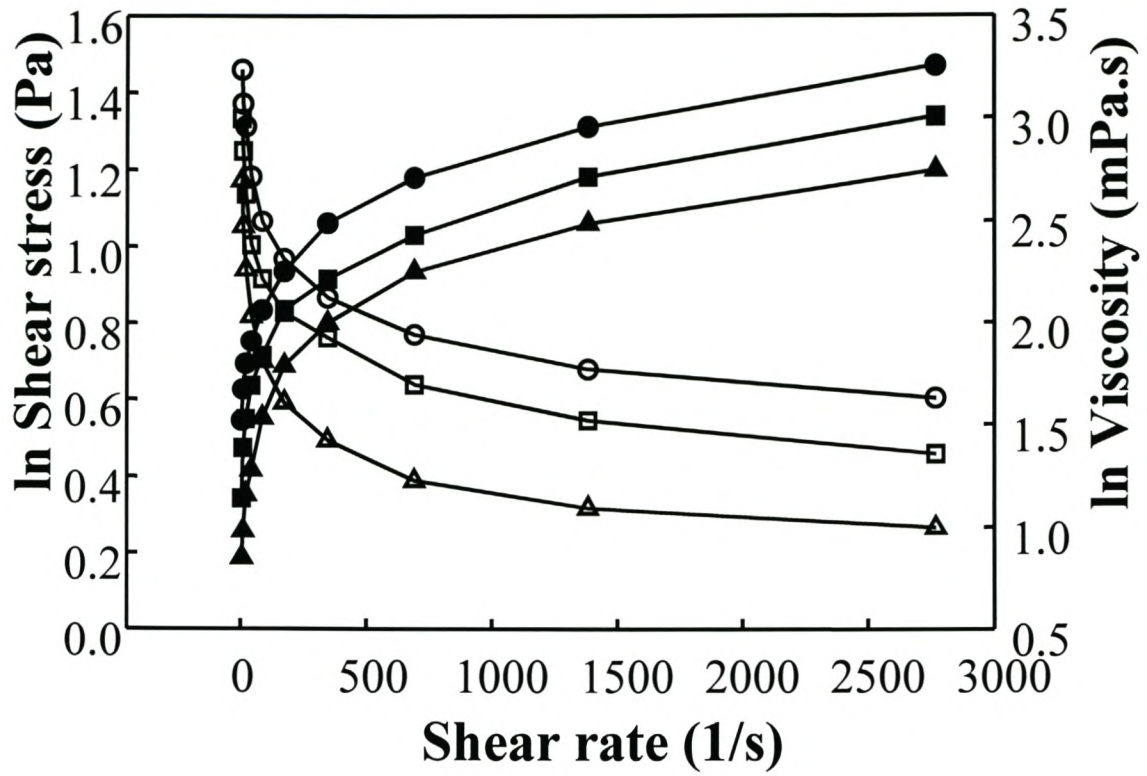
Figure 2: The flow properties of coffee extracts determined with 20 % (▲), 40 % (■), and 60 % (●) extracts. Closed symbols represent the flow curves, and open symbols represent the viscosity curves.
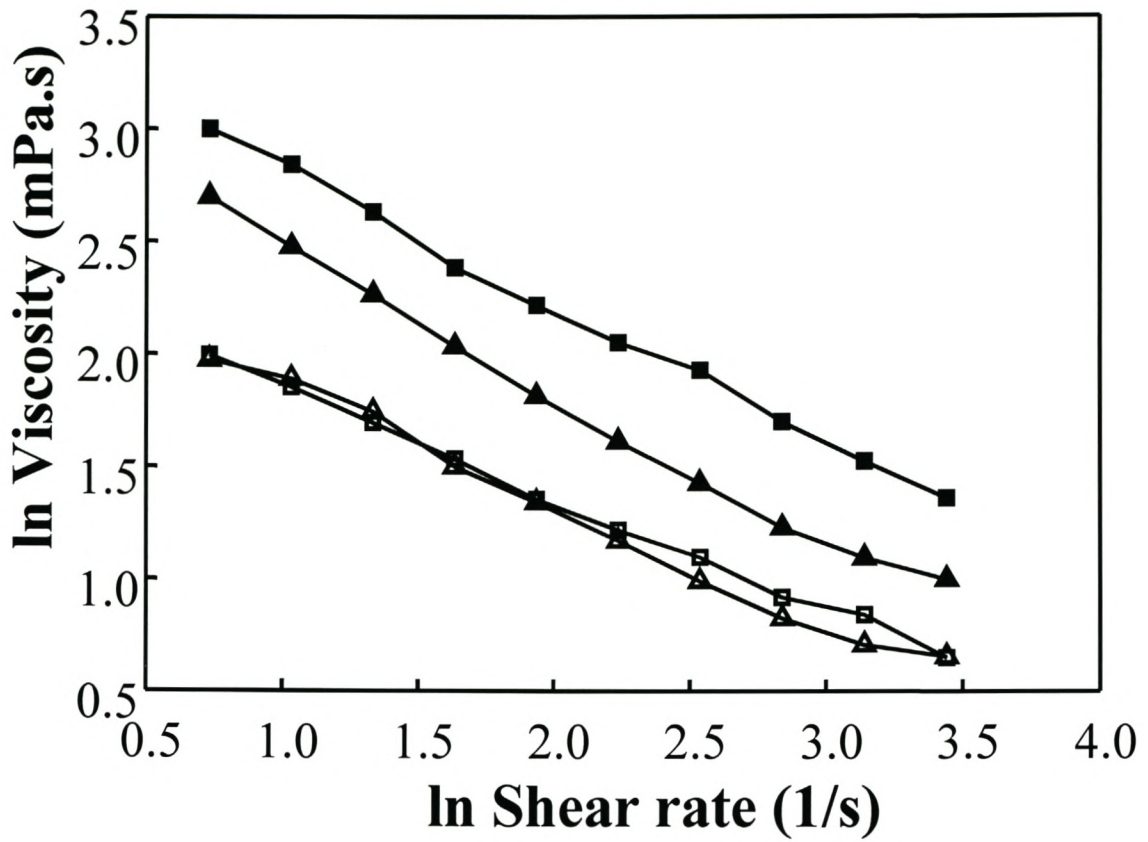
5

10

Figure 3: The effect of β-mannanase on the viscosity of coffee extracts and their fluid dynamics. Change in viscosity of 20 % (▲) and 40 % (■) extracts was monitored before treatment (closed symbols) and after treatment (open symbols).
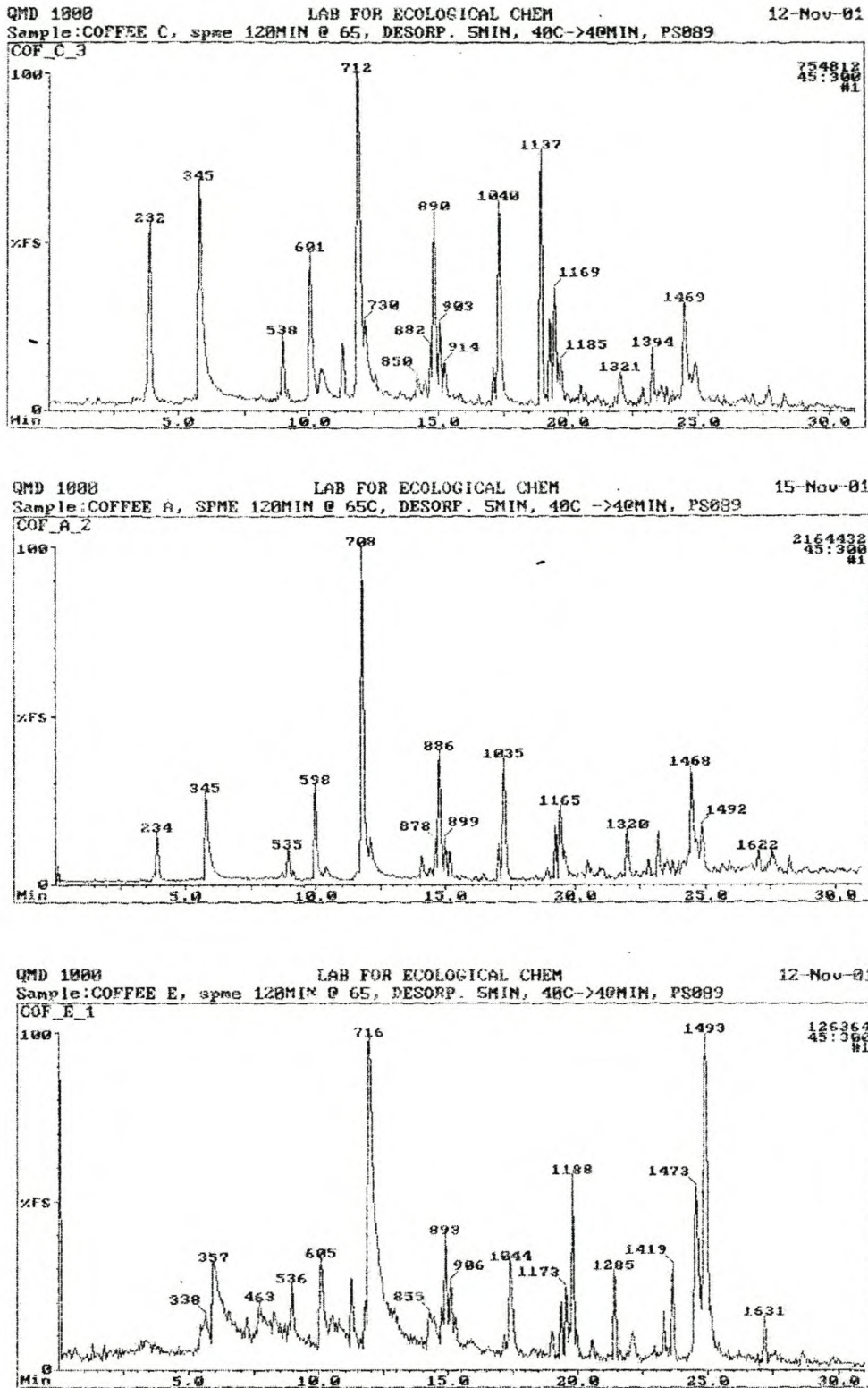
178

Figure 4: GC profiles of coffee samples. C-3 is the control sample, A-2 is the autoclaved sample, and E-1 is the enzyme treated sample.
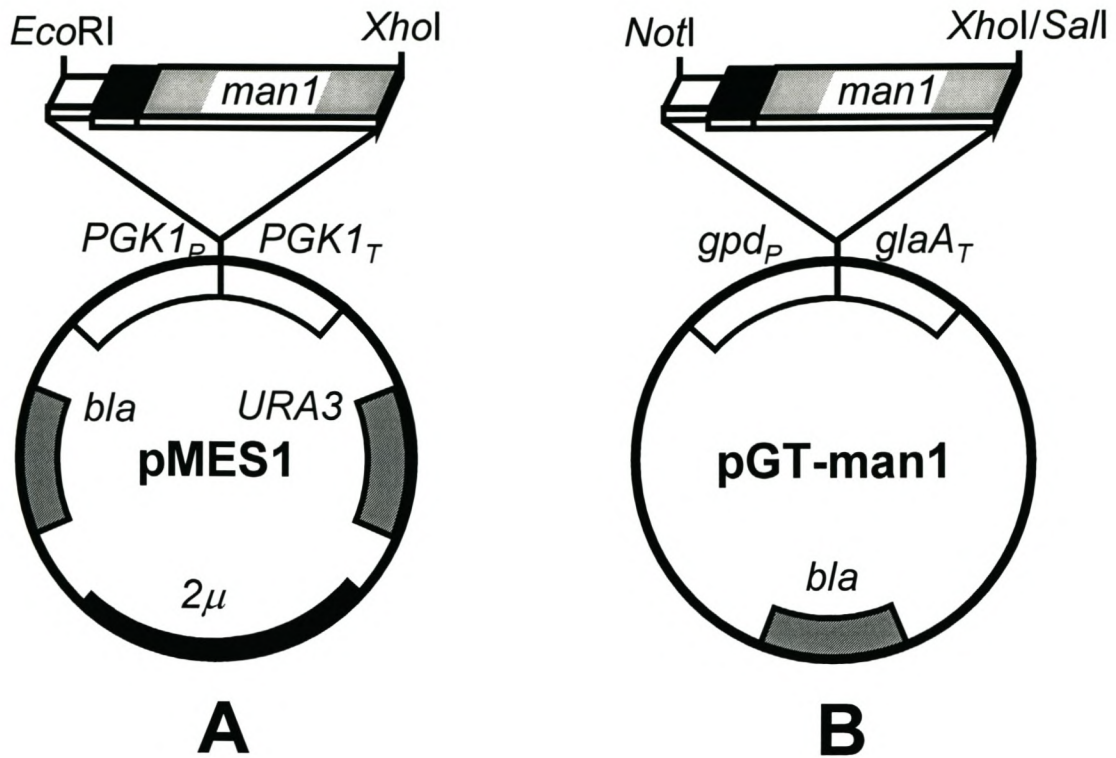
Figure 5: A diagram of (A) a *man1* β-mannanase yeast expression cassette in plasmid pMES1 and (B) a *man1* β-mannanase *Aspergillus niger* expression cassette in plasmid pGT-man1.

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  1                    11                   21                   30
  M K L S H M L L S L A S L G V A T A L P R T P N H N A A T T

  31                   41                   51                   60
  A F P S T S G L H F T I D G K T G Y F A G T N S Y W I G F L

  61                   71                   81                   90
  T N N D D V D L V M S Q L A A S D L K I L R V W G F N D V N

  91                   101                  111                  120
  T K P T D G T V W Y Q L H A N G T S T I N T G A D G L Q R L

  121                  131                  141                  150
  D Y V V T S A E K Y G V K L I I N F V N E W T D Y G G M Q A

  151                  161                  171                  180
  Y V T A Y G A A A Q T D F Y T N T A I Q A A Y K N Y I K A V

  181                  191                  201                  210
  V S R Y S S S A A I F A W E L A N E P R C Q G C D T S V L Y

  211                  221                  231                  240
  N W I S D T S K Y I K S L D (T) K H L V T I G D E G F G L D V

  241                  251                  261                  270
  D S D G S Y P Y T Y G E G L N F T K N L G I S T I D F G T L

  271                  281                  291                  300
  H L Y P D S W G T S Y D W G N G W I T A H A A A C K A V G K

  301                  311                  321                  330
  P C L L E E Y G V T S N H C A V E S P W Q Q T A G N A T G I

  331                  341                  351                  360
  S G D L Y W Q Y G T T F S W G Q S P N D G N T F Y Y N T S D

  361                  371          377
  F T C L V T D H V A A I N A Q S K
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Figure 6: Amino acid sequence of β-mannanase of *Aspergillus aculeatus* MRC11624. The The neutral Ser→Thr substitution in the β-mannanase of *A. aculeatus* MRC11624 versus the submitted sequence (GenBank accession number L35487) of the β-mannanase of *A. aculeatus* KSM510 is encircled.