EXPRESSION AND EVALUATION OF ENZYMES REQUIRED FOR THE DEGRADATION OF GALACTOMANNAN

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Thesis presented in partial fulfilment of the
requirements for the degree of
Masters of Science
in the faculty of Science
at Stellenbosch University

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MARCH 2013

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SUMMARY

The need for a cost-effective and environmentally friendly substitute for fossil fuels has resulted in significant attention to the production of bioethanol. Lignocellulose being the most abundant renewable resource on the planet consists of cellulose, hemicelluloses and lignin. It can be exploited as a source of fermentable sugars for the conversion to ethanol which may serve as the ultimate fossil fuel replacement. Hemicelluloses, contributing one third of lignocellulose, consists of xylan and mannan. Mannan consists of glucomannan, galactomannan and galactoglucomannan. A cocktail of enzymes are required for its complete hydrolysis, including β-mannanase, β-mannosidase, α-galactosidase, β-glucosidase and acetyl-mannan esterases. A need has arisen for the development of a recombinant microorganism capable of converting lignocelluloses to bioethanol through an economically feasible process.

The yeast *Saccharomyces cerevisiae* naturally ferments hexose sugars into ethanol and has been used in various industrial applications due to its robustness in industrial processes, its well-developed expression systems, its frequent use as a model organism for heterologous gene expression and its current GRAS (**G**enerally **R**egarded **A**s **S**afe) status. This yeast is unable to naturally utilise complex lignocelluloses. Recombinant biotechnology can be implemented to overcome this limiting factor. Due to certain restraints by the yeast *S. cerevisiae* such as hyperglycosylation and poor secretion capacity, alternative hosts such as *Aspergillus niger* has also been considered for heterologous protein production.

The *Aspergillus aculeatus* β-mannanase (*man1*) and *Talaromyces emersonii* α-galactosidase (*Agal*) genes were expressed in *S. cerevisiae* Y294. The cDNA of *A. niger* β-mannosidase (*cAnmndA*) and synthetic *Cellvibrio mixtus* β-mannosidase (*CmMan5A*) were expressed in *A. niger*. The sequence coding for the native secretion signal from *CmMan5A* was removed and replaced with the *XYNSEC* sequence (yielding *XYNSEC-CmMan5A*) and expressed in *E. coli* DH5α. The recombinant Man1, Agal, cAnmndA, CmMan5A and XYNSEC-CmMan5A displayed optimal pH of 5.47, 2.37, 3.4, 3.4 and 5.47, respectively, and optimal temperatures of 70°C for Man1, Agal, cAnmndA and CmMan5A and 50°C for XYNSEC-CmMan5A. Activity levels of Man1, Agal, cAnmndA, CmMan5A and XYNSEC-CmMan5A peaked at 36.08, 256.83, 11.61, 7.58 and 2.14 nkat/ml, respectively. Co-expression of *Agal* and *man1* led to a decrease in enzyme secretion and therefore individual expression of these genes should be considered rather than co-expression. The

enzymatic activity of Man1, Agal and CmMan5A resulted in a significant decrease in the viscosity of galactomannan when used synergistically. This study confirmed successful production of galactomannan hydrolysing enzymes by the yeast *S. cerevisiae* and the fungus *A. niger*, as well as providing insight into the synergistic effect of these enzymes on the viscosity of galactomannan.

OPSOMMING

Die behoefte vir 'n koste-effektiewe en omgewingsvriendelike plaasvervanger vir fossielbrandstowwe het tot 'n beduidende belangstelling in die produksie van bio-etanol gelei. Lignosellulose synde die volopste hernubare hulpbron op die planeet bestaan uit sellulose, hemiselluloses en lignien. Dit kan as 'n bron van fermenteerbare suikers vir die omskakeling na etanol benut word, wat kan dien vir uiteindelike fossielbrandstofvervanging. Hemiselluloses, wat bydra tot 'n derde van lignosellulose, bestaan uit xilaan en mannaan. Mannaan bestaan uit glukomannaan, galaktomannaan en galaktoglukomannaan. 'n Mengsel van ensieme word vir die volledige hidroliese van mannaan benodig, insluitende β-mannanase, β-mannosidase, α-galaktosidase, β-glukosidase en asetiel-mannaan esterases. 'n Behoefte bestaan vir die ontwikkeling van 'n rekombinante mikroörganisme wat in staat is tot die omskakeling van lignoselluloses na bio-etanol deur middel van 'n ekonomies lewensvatbare proses.

Die gis *Saccharomyces cerevisiae* kan heksoe suikers na etanol omskakel en word gebruik in verskeie industriële toepassings as gevolg van sy robuustheid in industriële prosesse, goed ontwikkelde uitdrukking sisteme, gereelde gebruik as 'n model-organisme vir heteroloë uitdrukking van gene en huidige GRAS (**G**enerally **R**egarded **A**s **S**afe) status. Die gis is nie daartoe in staat om komplekse lignosellulose te benut nie. Rekombinante biotegnologie kan egter geïmplementeer word om hierdie beperkende faktor te oorkom. As gevolg van sekere beperkinge van die gis *S. cerevisiae* soos hiperglikosilering en lae sekresie kapasiteit, is alternatiewe gashere soos *Aspergillus niger* ook oorweeg vir heteroloë proteïenproduksie.

Die *Aspergillus aculeatus* β-mannanase (*man1*) en *Talaromyces emersonii* α-galaktosidase (*Agal*) gene is in *S. cerevisiae* Y294 uitgedruk. Die cDNA van *A. niger* β-mannosidase (*cAnmndA*) en sintetiese *Cellvibrio mixtus* β-mannosidase (*CmMan5A*) is in *A. niger* uitgedruk. Die DNA volgorde wat kodeer vir die natuurlike sekresiesein van *CmMan5A* is verwyder en vervang met die *XYNSEC* volgorde (gegewe *XYNSEC-CmMan5A*) en uitgedruk in *E. coli* DH5α. Die rekombinante Man1, Agal, cAnmndA, CmMan5A en XYNSEC-CmMan5A vertoon optimale pH kondisies van 5.47, 2.37, 3.4, 3.4 en 5.47, onderskeidelik, en die optimale temperatuur van 70°C vir Man1, Agal, cAnmndA en CmMan5A en 50°C vir XYNSEC-CmMan5A. Aktiwiteitsvlakke van Man1, Agal, cAnmndA, CmMan5A en XYNSEC-CmMan5A het 'n maksimum bereik op 36.08, 256.83, 11.61, 7.58 en 2.14 nkat/ml, onderskeidelik. Gesamentlike uitdrukking van *Agal* en *man1* het

tot 'n afname in ensiemsekresie gelei en dus moet individuele uitdrukking van hierdie gene eerder as gesamentlike-uitdrukking oorweeg word. Die ensiematiese aktiwiteite van Man1, Agal en CmMan5A het tot 'n beduidende afname in die viskositeit van galaktomannaan gelei wanneer dit sinergisties gebruik word. Hierdie studie bevestig suksesvolle produksie van galaktomannaan hidrolitiese ensieme in die gis *S. cerevisiae* en die fungus *A. niger*, en verskaf insig in die sinergistiese effek van hierdie ensieme op die viskositeit van galaktomannaan.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutes for their invaluable contributions to the successful completion of this study:

Prof. WH van Zyl, Department of Microbiology, University of Stellenbosch, who acted as my supervisor and for accepting me into his research team;

Prof. M Bloom, Department of Microbiology, University of Stellenbosch, for accepting me into the Honours class, and for assisting me with gaining financial support;

Co-workers in lab 353 and 335 for their support and guidance;

The National Research Foundation for financial support;

My friends and family, especially my parents for supporting me throughout my studies;

Natalie Smyth for her patience and belief in me;

Dr. Shaunita Rose, Department of Microbiology, University of Stellenbosch, who acted as my co-supervisor, for giving me the chance and for believing in me, for supporting and guiding me, and for pushing me to the best of my abilities.

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CHAPTER 3:

Expression and valuation of enzymes required for the degradation of galactomannan

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION

Plant cell walls consist of complex polymers such as cellulose, hemicellulose and lignin (McNeil et al. 1984, Moreira and Filho 2008, Scheller and Ulvskov 2010). Together they maintain structural integrity in the plant cell walls (Klemm et al. 2005). This association as well as the crystalline nature of cellulose, renders it inaccessible and recalcitrant to enzymatic hydrolysis (van Rensburg et al. 1998). Hemicelluloses are the second most abundant renewable carbon source on earth and consist of mainly mannan and xylan (Lynd et al. 2002, De O. Petkowicz et al. 2001). The different forms of mannan include glucomannan, galactomannan and galactoglucomannan (Moreira and Filho 2008). The search for alternative fuels to replace the depleting petroleum-based fuels has been an ongoing quest, were the most promising solution is the production of ethanol from lignocellulose biomass (van Dyk and Pletschke 2012). Due to its renewable nature and abundance, lignocellulosic biomass is an appropriate candidate for replacing petroleum-based fuels (Beukes and Pletschke 2011, Gao et al. 2011).

Hydrolytic enzymes are naturally produced by most organisms and are involved mainly in breaking down complex substrates (such as carbohydrates, proteins, lipids and polyphenols) to simple units that can be assimilated easily. Microbial hydrolases are the most extensively studied and were introduced into commercial industries in the 1960s (Dalbøge 1997). The majority of commercialised microbial enzymes are produced from a small number of fungi (*Aspergillus*, *Fusarium*, *Trichoderma*, *Humicola*, *Mucor* and *Rhizomucor*) and bacterial (*Bacillus*, *Pseudomonas*) (Dalbøge and Lange 1998, van Zyl et al. 2010). These microorganisms secrete cocktails of hydrolytic enzymes that degrade the polymeric substrates through synergistic action. The hydrolysis of mannan requires enzymes β-mannanases (1,4-β-D-mannan mannohyrolases), β-mannosidases (1,4-β-D-mannopyranoside hydrolases), α-galactosidases (1,6-α-D-galactoside galactohydrolases), β-glucosidases (1,4-β-D-glucoside glucohydrolases) and galactomannan acetylesterases (Moreira and Filho 2008).

Saccharomyces cerevisiae has a long fermentation history with the wine and brewing industries. It is also the most popular host for heterologous protein expression, due to its GRAS status and the ease with which it can be genetically manipulated (Gellissen and Hollenberg 1997, Müller et al. 1998). Unfortunately, *S. cerevisiae* is unable to utilise lignocellulose, limiting the range of substrates that can be used in industrial fermentations.

Construction of a polysaccharide-degrading *S. cerevisiae* strain with the ability to utilise renewable, natural substrates may provide an economically feasible way to produce commercially important commodities such as biofuels (Den Haan et al. 2007).

Filamentous fungi are versatile organisms with the ability to grow on inexpensive readily available material such as agricultural residues (for example corn stalks and wheat straw), wood residues (such as un-harvested dead and diseased trees), specifically grown crops (such as sugar cane and sorghum) and waste streams (such as municipal solid waste, recycled paper and bagasse) (Aristidou and Penttilä 2000). The use of inexpensive media for cultivation together with the GRAS status, its long history in the food industry and the significant contribution to the production of antibiotics makes *Aspergillus niger* an ideal host for the production of viable enzymes. Yet, *A. niger* is unable to produce high levels of ethanol. Combining the good attributes of *A. niger* and *S. cerevisiae* will result in the construction of a polysaccharide degrading *S. cerevisiae* strain with the ability to utilise renewable, natural substrates. It may provide an economically feasible way to produce commercially important commodities such as biofuels (Den Haan et al. 2007).

2. AIMS OF THIS STUDY

The objective for this study was the expression and evaluation of β-mannanase, β-mannosidase and α-galactosidase enzymes that are required for the degradation of galactomannan, such as Locust bean gum (LBG). The use of multiple expression hosts is used due to the unsuccessful expression of β-mannosidases in *S. cerevisiae* (see appendix A).

The specific aims of this study were as follows:

- Subcloning and functional expression of the *Aspergillus aculeatus man1* and synthetic *Talaromyces emersonii* α-galactosidase (*Agal*) in the yeast *S. cerevisiae* Y294;
- Amplifying the cDNA copy of *Aspergillus niger* β-mannosidase (*cAnmndA*)
- Functional expression of *cAnmndA* and a synthetic *Cellvibrio mixtus* β-mannosidase (*CmMan5A*) in *A. niger*;
- Partial characterisation of the Man1, Agal and CmMan5A enzymes

- Determine the synergistic effect of Man1, Agal and CmMan5A on the viscosity of LBG.

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1. INTRODUCTION

The quest for alternative fuels began in 1975, with the sudden increase in oil prices and the realisation that the world's oil supply is finite. All modern economies are powered by fossil fuels. This dependence is mostly attributed to their use in transportation, industrial processes, households and the generation of electricity (Lin and Tanaka 2006). The rate of fuel consumption is exceeding the rate of production causing fuel price increases imposed by the Middle Eastern countries controlling the oil market (Figure 1). The focus has shifted to finding economical ways to produce ethanol, preferably from abundantly available, biodegradable and renewable raw materials. Ethanol is an excellent transportation fuel and in some respects superior to gasoline (Lynd et al. 1991a, Lynd et al. 1991b). Unblended ethanol burns more cleanly, has a higher octane rating, can be burned with greater efficiency, is thought to produce smaller amounts of ozone precursors (thus decreasing urban air pollution) and is particularly beneficial with respect to low net carbon dioxide release into the atmosphere. Ethanol is considerably less toxic to humans than gasoline (or methanol). Due to its low volatility, low combustion products and its photochemical reactivity, combustion of ethanol results in low levels of smog-producing compounds (Wyman and Hinman 1990). Furthermore, bio-ethanol (via fermentation) offers a more favourable trade balance, enhanced energy security and represents a new commodity for the agricultural economy.

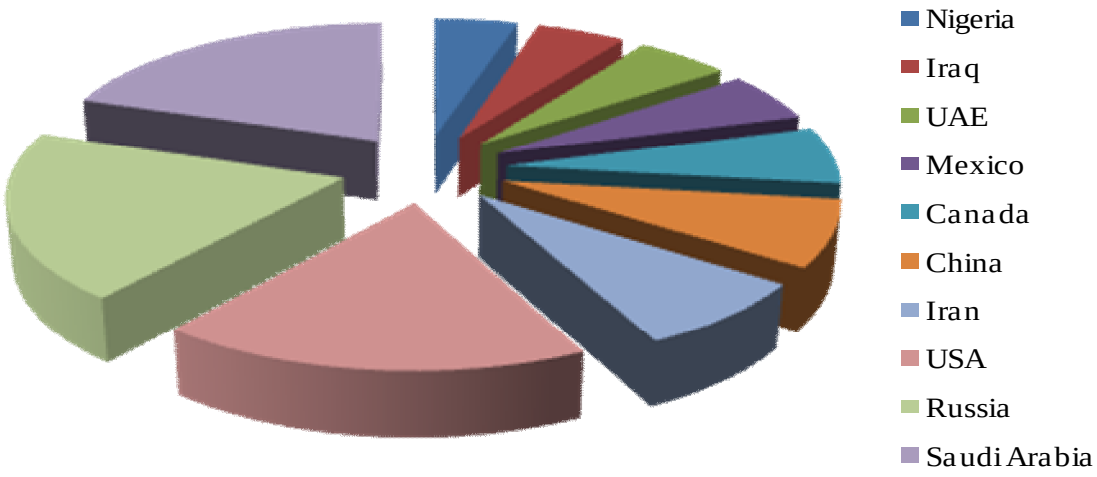

Figure 1: The top ten global oil producers for 2007 (https://www.cia.gov/library/publications/the-world-factbook/).

The amount of solar energy received at the earth's surface is 2.5 x 10^{21} Btu/year, which far exceeds the present human usage of 2.0 x 10^{17} Btu/year (Demain et al. 2005). The amount of energy from the sun, which is used for photosynthesis, is 10 times that of world's total human usage. Globally, terrestrial plants produce 1.3 x 10^{10} metric tons (dry weight basis) of plant material per year, which is equivalent to 7 x 10^{9} metric tons of coal or about two-thirds of the world's energy requirement. Cellulosic feedstocks from agriculture and other sources amount to about 180 million tons per year (Demain et al. 2005). Furthermore, vast amounts of cellulose are available as agricultural wastes (Table 1) making lignocellulose by far the most abundant renewable natural resource. The inexpensive and plentiful nature of cellulosic biomass created interest in its possible use as a renewable source of energy.

Table 1: The composition of common agricultural residues and wastes (Kaur et al. 1998, McKendry 2002, Prasad et al. 2007)

Agricultural residue	**Cellulose**	**Hemicellulose**	**Lignin**
Hardwood	40-50	25-40	18-35
Softwood	45-50	25-35	25-35
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Wheat straw	33-40	20-25	15-20

Hemicelluloses are structural polysaccharides found in plant cell walls in close association with cellulose and lignin (Figure 2), forming the lignocellulosic biomass (Saha 2003). Cellulose and hemicelluloses are macromolecules constructed from simple sugars, whereas lignin is an aromatic polymer synthesised from phenylpropanoid precursors. These polymers are intertwined through non-covalent forces and covalent cross-linkages, producing the intricately weaved cell wall of plants.

The hemicelluloses are estimated to account for one third of all components available in plants and are the second most abundant heteropolymer present in nature (Table 2) (Chaikumpollert et al. 2004). The hemicellulose distribution varies in woods, but it can contribute to 25-30% of the dry weight (Pérez et al. 2002). The majority of the hemicelluloses are relatively small molecules containing 70 to 200 monosaccharide residues. The hardwood hemicelluloses are generally larger molecules with 150 to 200 residues (Moreira and Filho 2008).

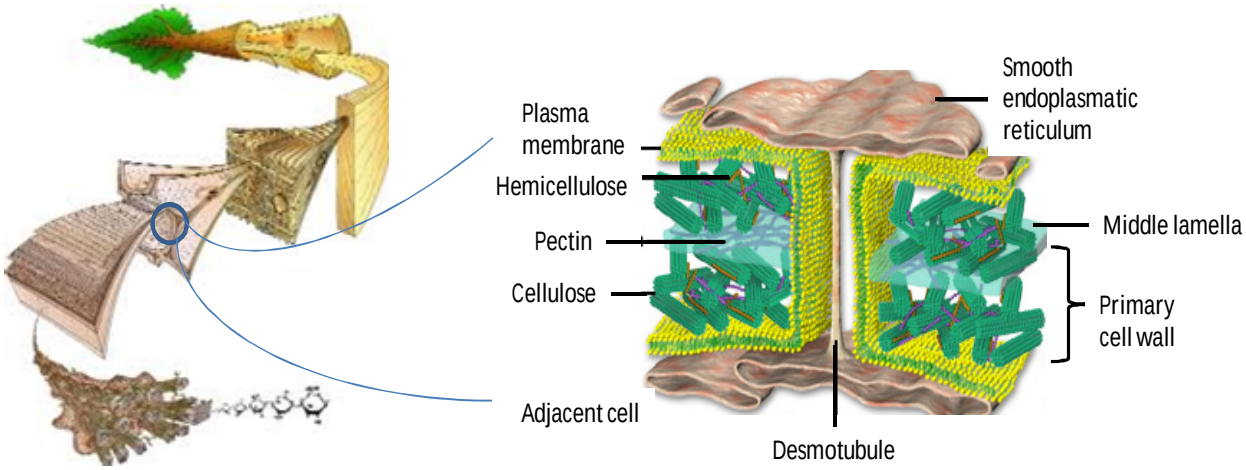

Figure 2: Schematic representation of the intricately woven lignocellulose components of the cell wall (Boudet et al. 2003).

Table 2: The major hemicellulose components in softwood and hardwood (Moreira and Filho 2008, Scheller and Ulvskov 2010, Timell 1965, Timell 1964)

Wood	**Hemicellulose type**	**Amount (%)**	**Composition**			**DP**
			Units	**Molar ratios**	**Linkage**	>
Soft wood	Galactoglucomannan	5-8	β-D-mannopyranose β-D-glucopyranose β-D-galactopyranose Acetyl	3 1 1 1	1-4 1-4 1-6	100
	Glucomannan	10-15	β-D-mannopyranose β-D-glucopyranose β-D-galactopyranose Acetyl	4 1 0.1 1	1-4 1-4 1-6	100
	Arabinoglucuronoxylan	7-10	β-D-xylopyranose 4-*O*-Me-α-D-glucopyranosyluronic acid α-L-arabinofuranose	10 2 1.3	1-4 1-2 1-3	100
Hard wood	Glucuronoxylan	15-30	β-D-mannopyranose 4-*O*-Me-α-D-glucopyranosyluronic acid Acetyl	10 1 7	1-4 1-2	200
	Glucomannan	2-5	β-D-mannopyranose β-D-glucopyranose	1-2 1	1-4 1-4	200

2. MANNAN STRUCTURE

Hemicelluloses include a variety of polysaccharides with linear or branched polymers derived from sugars such as D-xylose, D-galactose, D-mannose, D-glucose and L-arabinose (Moreira and Filho 2008). Hemicelluloses are classified according to the main sugar unit. The main-chain sugars in hemicellulose structure are predominantly linked together by β-glycosidic bonds (Polizeli et al. 2005). Mannans are the major constituents of the hemicellulose fraction in softwoods and show wide spread distribution in plant tissues (De O. Petkowicz et al. 2001). In plants, they present a structural role (Brennan et al. 1996, Liepman et al. 2007) as well as displaying a storage function as non-starch carbohydrate reserves in endosperm walls and vacuoles of seeds and vegetative tissues (Moreira and Filho 2008).

The different types of mannan can be divided into four subfamilies: linear mannan, glucomannan, galactomannan and galactoglucomannan (De O. Petkowicz et al. 2001). Each of these polysaccharides presents a β-1,4-linked backbone containing mannose or a combination of glucose and mannose residues (Liepman et al. 2007). In addition, the mannan backbone can be substituted with side chains of α-1,6-linked galactose residues.

Linear mannans are homopolysaccharides composed of linear main chains of 1,4-linked β-D-mannopyranosyl residues and contain less than 5% of galactose. They are the major structural units in woods and in seeds of many plants (such as ivory nuts and green coffee beans) (Aspinall 1959), and typically present in the endosperms of Palmae (such as *Phytelephas macrocarpa*) (De O. Petkowicz et al. 2001). The mannans from ivory nuts can be separated into two components: A and B (Petkowicz et al. 2007). Mannan A is a dense polysaccharide extracted with alkali that possesses granular form and crystalline structure. Mannan B cannot be extracted directly and is built up of microfibrils similar to cellulose microfibrils and shows a less crystalline structure (Aspinall 1959). Mannan B is insoluble in aqueous NaOH and contains some water molecules in its lattice (De O. Petkowicz et al. 2001). Both polymers are insoluble in water, but differ in molecular size. Mannan A has a lower molecular weight, while mannan B presents a higher molecular weight polysaccharide.

Plant galactomannans consist of water-soluble 1,4-linked β-D-mannopyranosyl residues with side chains of single 1,6-linked α-D-galactopyranosyl groups attached along the chain (Parvathy et al. 2005, Shobha et al. 2005). Differences in the distribution of D-galactosyl

units along the mannan structure are found in galactomannans from different origins (Bresolin et al. 1997). True galactomannans are those mannans containing more than 5% (w/w) D-galactose residues (Aspinall 1959). Figure 3 show typical structures of Locust bean gum and tara gum with linear main chain of β-1,4-linked mannose units and an α-1,6-galactose side chain (Duffaud et al. 1997, Sittikijyothin et al. 2005).

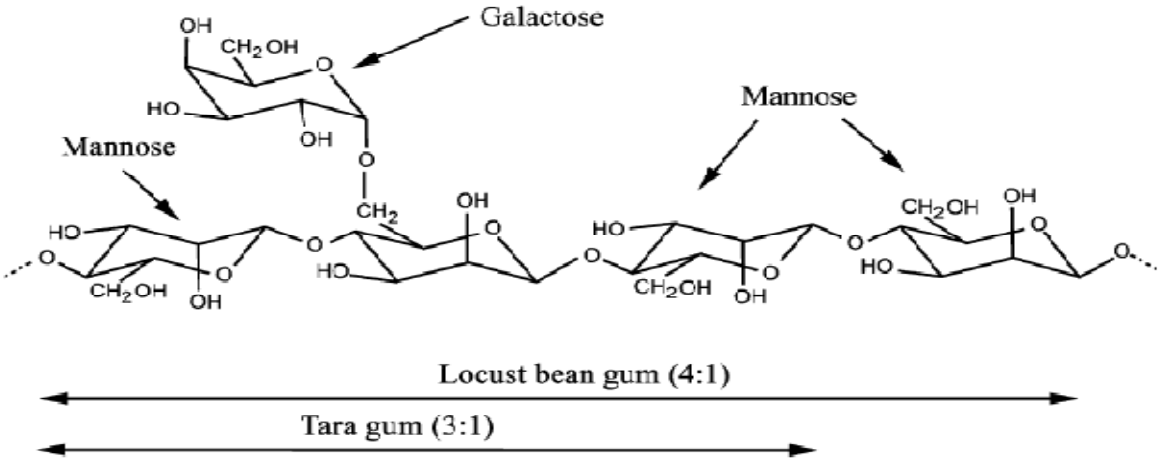

Figure 3: Structure of Locust bean gum and tara gum, displaying a linear backbone of 1,4-linked β-D-mannose units attached by a single α-D-galactose residue at the C-6 of the mannose with β-1,6-glycosidic bonds. In guar gum the linear backbone is substituted every two residues by an α-D-galactose residue at C-6 of a mannose with 1,6-glycosidic bonds (Moreira and Filho 2008).

Glucomannans contain chains of randomly arranged β-1,4-linked D-mannose and β-1,4-linked D-glucose residues in a 3:1 ratio (Moreira and Filho 2008, Northcote 1962). Hardwoods contain glucomannan with a mannose:glucose ratio of 1.5–2:1 (Hongshu et al. 2002, Timell 1967). The mannose residues of glucomannnan provide the branching points in the polysaccharide by 1,6- and/or 1,3-linkages (Aspinall et al. 1962). They account for half of the hemicellulose fractions of coniferous woods (Aspinall 1959) and occur together with galactoglucomannans. Mannans are present in small amounts in the hemicellulose components of hardwood and represent 3–5% of the total cell wall material (Northcote 1962). Some D-galactose residues may be attached to the main mannose chain through α-1,6-linked terminal units with a mannose:glucose:galactose ratio of 3:1:0.1 (Moreira and Filho 2008). In this case, it consists of residues of mannose:glucose:galactose in the ratio of 3:1:0.1. These residues act as flexible groups that can provide non-covalent

connecting bridges with water and other matrix polysaccharides (Northcote 1962). Glucomannans of seed plants, coniferous woods and to a lesser extent from some hardwoods are found to be in close association with cellulose and xylans as cell wall components (Aspinall 1959). The conformation of glucomannan chains is similar to those of cellulose.

Galactoglucomannans are polysaccharides containing D-galactose residues attached to both D-glucosyl and D-mannosyl units as α-1,6-linked terminal branches (Aspinall et al. 1962). They are the predominant hemicelluloses present in softwoods (Timell 1965). The mannose:glucose:galactose residues are reported to be in the molar ratio of 3:1:1 (Moreira and Filho 2008, Timell 1967). The galactoglucomannan solubility in water is due to its D-galactose side-chains that prevent the macromolecules from aligning themselves with strong hydrogen bonds (Timell 1965).

3. XYLAN

Xylan is the second most abundant polysaccharide in nature and the main hemicellulose found in plant cell walls, constituting 30 – 35% of the total dry weight (Joseleau et al. 1992). Xylan exists in the interface between lignin and cellulose adding to the stability of plant structure. Consistent with their structural chemistry and side-group substitutions, the xylans seem to be interspersed, intertwined and covalently linked at various points with the overlying sheath of lignin. Xylan produces a coat around underlying strands of cellulose (Biely 1985) via hydrogen bonding (Joseleau et al. 1992). The xylan layer with its covalent linkage to lignin and its non-covalent interaction with cellulose may be important in maintaining the integrity of the cellulose *in situ* and in helping to protect the fibres against degradation by cellulases (Uffen 1997).

Xylan is the major hemicellulose in hardwood, but is less abundant in softwood (Figure 4). The structure of xylans can differ depending on their origin, but will always contain a β-1,4-linked D-xylose backbone (Ebringerová and Heinze 2000). Although most xylans are branched structures, some linear forms have been identified (Eda et al. 1976). The xylan from hardwood is *0*-acetyl-4-*0*-methylglucuronoxylan consists of at least 70 β-xylopyranose residues containing acetyl, arabinosyl and glucuronosyl substituents (Beg et al. 2001). Every tenth xylose residue carries a 4-*0*-methylglucuronic acid attached to the C-2 position of xylose. Hardwood xylans are highly acetylated which contributes to the partial solubility of

xylan in water. Acetylation is more frequent at the C-3 than at the C-2 position (Beg et al. 2001). These acetyl groups are readily removed when xylan is subjected to alkali extraction (Antranikian 1997). Xylans from softwood are composed of arabino-4-*O*-methylglucuroxylans. They have a higher 4-*O*-methylglucuronic acid content than hardwood xylans. Softwood xylans are not acetylated but contain an α-L-arabinofuranose units linked by α-1,3-glycosidic bonds at the C-3 position of the xylose (Beg et al. 2001). The ratio of β-D-xylopyranose:4-*O*-methyl-α-D-glucuronic acid:L-arabinofuranose is 100:20:13 (Beg et al. 2001).

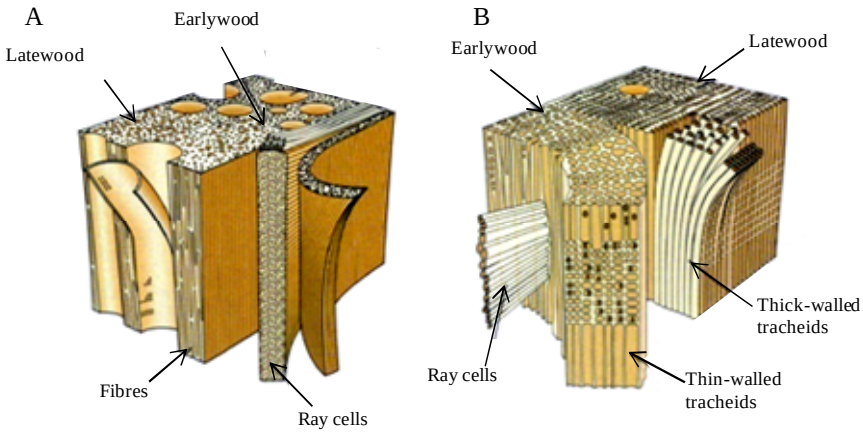

Figure 4: Schematic displaying the difference between (A) hardwood and (B) softwood. The cell structure of softwoods is much simpler than that of hardwoods (Arno 1993).

Homoxylans consist exclusively of xylosyl residues. This type of xylan is not widespread in nature and has been isolated from esparto grass (Chanda et al. 1950), tobacco stalks (Eda et al. 1976) and guar seed husk (Montgomery et al. 1956). Xylans with β-1,3-linked backbone have been reported in marine algae (Dekker and Richards 1976).

4. LIGNIN

Lignin is a complex polyphenolic compound present in softwood at a concentration of 20 - 30% and in hardwood at 18 – 25% (Scheller and Ulvskov 2010). It is responsible for cell wall rigidity and durability occurring mostly in the secondary cell wall of plants (Mosier et al. 2005). They also provide the vascular system with the hydrophobicity needed

for transport of water and solutes. Lignins represent a major obstacle in chemical pulping, forage digestibility and processing of plant biomass to biofuels. Lignins are generally problematic, therefore these industries would benefit from processing biomass containing either less lignin or a lignin that is easier to degrade (Vanholme et al. 2008).

Three major groups of lignin can be distinguished. Coniferyl alcohol is the main precursor in softwoods in which case dehydrogenation produces guaiacyl lignin. In hardwoods, dehydrogenation of *p*-sinapyl alcohol and *p*-coumaryl alcohol forms guaiacyl-syringyl lignin. Grasses contain guaiacyl-syringyl-*p*-hydroxyphenyl-lignin (Eriksson and Rzedowski 1969, Grabber 2005). Unlike cellulose or hemicelluloses, lignin is not readily biologically degraded due to the absence of hydrolysable bonds. It consists of random stable carbon-carbon and ether linkages between monomeric units (Mosier et al. 2005, Pérez et al. 2002). A reduction in the concentration, hydrophobicity and cross-linking of lignin enhances enzymatic hydrolysis of the structural polysaccharides in cell walls (Grabber 2005).

5. GLYCOSYL HYDROLASES

Microbial hydrolytic enzymes had been identified that can cleave almost all chemical bonds found in plant structures. These enzymes are often modular, and in addition to catalytic domains, they have modules for carbohydrate binding (CBM) and cellulose surface modification and disruption (Bayer et al. 1998, Saloheimo et al. 2002, Ximenes et al. 2005). Two types of enzyme are involved in the breaking down of hemicellulose. The exohydrolases act on the terminal glycosidic linkages and release terminal monosaccharide or disaccharide units from the non-reducing or reducing end, while endohydrolases cleave internal glycosidic bonds at random or at specific positions (Moreira and Filho 2008). The two major cleavage preferences correlate to active site architecture (Dominguez et al. 1995, Sabini et al. 2000a, Sabini et al. 2000b). Endo-acting enzymes such as endoglucanases and β-mannanases often have cleft shaped active sites whereas exo-acting enzymes (β-galactosidases and β-mannosidases) often have pocket-shaped active sites (Aleshin et al. 1994, Juers et al. 1999). Interestingly, enzymes with exo-activity may display endo-activity and enzymes with endo-activity can similarly display exo-activity, hence the architecture of an active site may not necessarily give an indication of the cleavage preferences (Stahlberg et al. 1993, Tomme et al. 1996).

Glycoside hydrolase enzymes are mainly involved in the degradation of plant polysaccharides (Davies and Henrissat 1995). They are grouped into enzyme families according to amino acid sequence similarities and hydrophobic cluster analysis (Henrissat and Bairoch 1993, Lemesle-Varloot et al. 1990). A continuously updated list of the GH families is available on the Carbohydrate-Active Enzyme database (CAZY) (http://www.cazy.org). Families compiled over time have shown a direct relation between classification and their tertiary structure (Henrissat 1991). Glycosyl hydrolases can further be grouped into clans based on the tertiary structure at the active site (Henrissat et al. 1995, Juers et al. 1999).

The glycosidic hydrolases employ either an inversion or retention of the anomeric configuration (Desmet and Soetaert 2011). The retention mechanism follows a double displacement mechanism involving 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 (Kulkarni et al. 1999). Water attacks the anomeric center of the intermediate in a general base-catalysed process to yield the product and release the enzyme in its original state (Figure 5A). Depending on the enzymatic conditions, the water attack can be replaced by either reactive donor molecules or high concentrations of oligosaccharide donor molecules (Faijes and Planas 2007), resulting in a transglycosylation reaction (Harjunpää et al. 1999, Kurakake and Komaki 2001, McCleary and Matheson 1983, Schröder et al. 2004). In certain circumstances molecules that are not natural substrates of β-mannanases can be produced via transglycosylation (Davies and Henrissat 1995, Gübitz et al. 1996a, Gübitza et al. 2000). Inverting glycosidases follow a single displacement mechanism, catalysing a direct nucleophilic attack of water on the anomeric carbon. One carboxylic residue (the catalytic base) assists the water molecule by accepting a proton, while the other residue (the catalytic acid) activates the leaving group by donating a proton (Figure 5B).

6. MANNAN DEGRADING ENZYMES

Hemicellulose degradation requires the concerted action of various hydrolytic enzymes due to its complex structure. The interwoven associations between hemicelluloses and cellulose fibrils also contribute to the complexity of the substrate. In plants, the mannan-degrading

enzymes play a key role in the growth, maturation and ripening of plants (Moreira and Filho 2008).

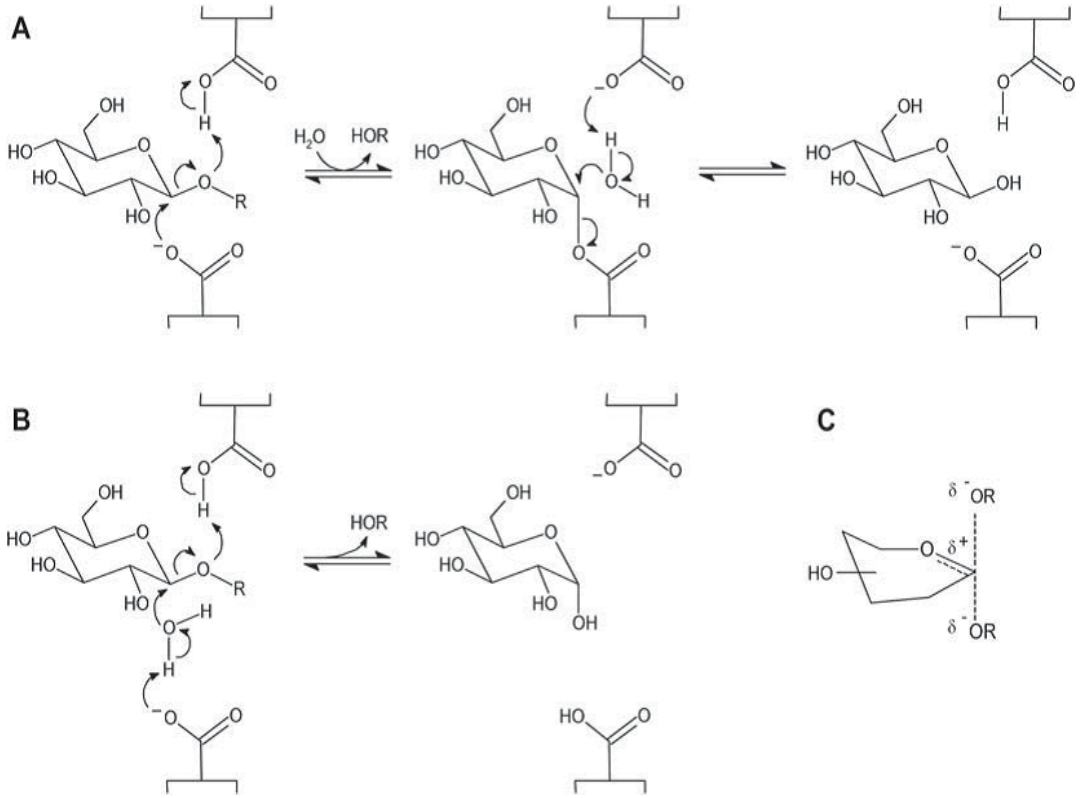

Figure 5: Reaction mechanism of glucosidases. (A) Retaining glucosidases follow a double displacement mechanism, while (B) inverting glucosidases follow a single displacement mechanism. (C) Represents the transition state (Desmet and Soetaert 2011, Withers 2001).

Microbial degradation begins with endo-β-1,4-mannanases (1,4-β-D-mannan mannohydrolases, EC 3.2.1.78) that cleave the β-1,4-mannopyranosyl linkages in the mannan backbone (Figure 6) resulting in oligosaccharides of different lengths (Stoll et al. 2000). The α-galactosidases (1,6-α-D-galactoside galactohydrolases, EC 3.2.1.22) remove the galactose units from the mannan backbone (McCutchen et al. 1996). The hydrolysis of the oligomannans is performed by the enzyme β-mannosidase (1,4-β-D-mannopyranoside hydrolases, EC 3.2.1.25), releasing single mannose units (Moreira and Filho 2008). Additional enzymes, namely β-glucosidases (1,4-β-D-glucoside glucohydrolases, EC 3.2.1.21) and acetyl mannan esterases (EC 3.1.1.6), catalyse the removal of glucose and acetic acid, respectively (Moreira and Filho 2008). The removal of side-chain substituents, attached at various points on the mannan structure, creates more sites for subsequent enzymatic hydrolysis (Moreira and Filho 2008).

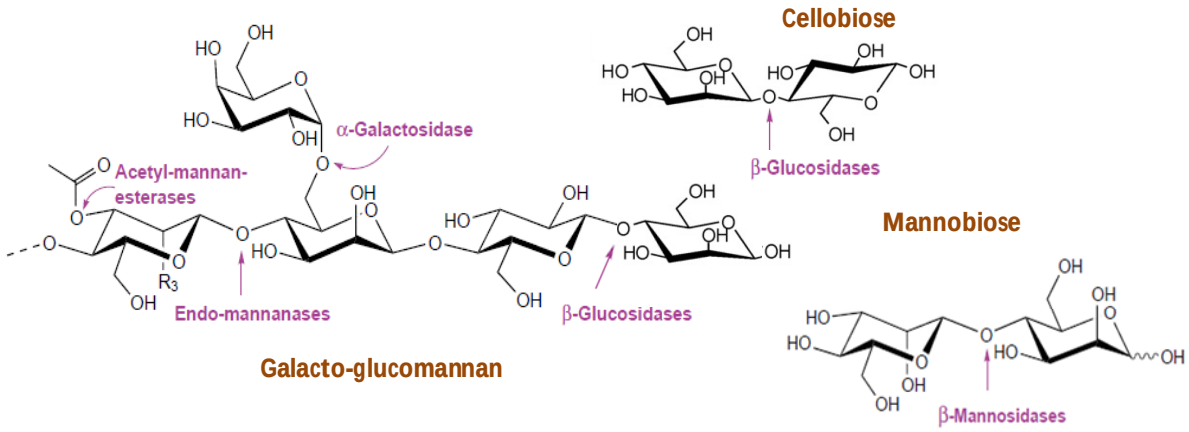

Figure 6: Galactoglucomannan displaying α-1,6-linked galactose sidechains, where the O-2 and O-3 of the mannose units can be substituted with acetate groups, as well as the various enzymes required for degradation (Shallom and Shoham 2003).

7. ENDO β-1,4-MANNANASE

Numerous β-mannanases have been isolated and characterised from bacteria (Akino et al. 1989, Braithwaite et al. 1995), fungi (Ademark et al. 2001, Ademark et al. 1999, Ademark et al. 1998, Christgau et al. 1994, Setati et al. 2001), plants (Derek Bewley et al. 1997, Marraccini et al. 2001) and animals (Xu et al. 2002a, Xu et al. 2002b, Yamaura et al. 1996). In plants, β-mannanases are involved in seed germination as well as fruit ripening (Nonogaki et al. 2000, Nonogaki and Morohashi 1999). Fungal β-mannanases (from *Aspergillus tamarii* (Civas et al. 1984), *Trichoderma reesei* (Stålbrand et al. 1993) and *Aspergillus niger* (Ademark et al. 1998)) are produced extracellularly, but can be cell wall bound (Dhawan and Kaur 2007). The β-mannanases cleaves the back bone chain of glucomannan, galactomannan and glucogalactomannan resulting in new chain ends (Stoll et al. 2000). The degradation is affected by the extent and pattern of substitution of the mannan backbone as well as the ratio of glucose to mannose (Moreira and Filho 2008, De Vries and Visser 2001). In glucomannan, the pattern of distribution of *O*-acetyl groups may also affect the susceptibility of hydrolysis. The presence of galactose residues on the mannan backbone significantly hinders the activity of β-mannanases (McCleary and Matheson 1983), but this effect is small if the galactose residues in the vicinity of the cleavage point are all present on the same side of the main chain (McCleary 1979). The predominant products of β-mannanases are mannobiose and

mannotriose, confirming their true endohydrolytic property (Ademark et al. 1998, Civas et al. 1984, De Vries and Visser 2001, Reese and Shibata 1965).

Mannanases from *A. tamarii* (Civas et al. 1984), *T. reesei* (Stålbrand et al. 1993) and *A. niger* (Ademark et al. 1998) all produce mainly mannobiose, mannotriose and higher oligosaccharides. A chain length of four sugar residues is requires for the binding of β-mannanases to ensure hydrolysis (McCleary and Matheson 1983, Sabini et al. 2000a, Sabini et al. 2000b). The substrate binding surface can be split into different subsites, where the subsites are numbered from –n to +n (Figure 7), where n is an integer and are bound from non-reducing to reducing ends of the mannan substrate respectively (Davies et al. 1997). Cleavage of the glycosidic bond occurs between the subsites +1 and -1 (McCleary and Matheson 1983). The majority of β-mannanases hydrolyse manno-oligosaccharides up to a DP of 4 (Biely and Kremnický 1998, McCleary 1988). Although the β-mannanase activity on mannotriose has been observed, the rate of hydrolysis is significantly lower, indicating a preference for at least 4 subsites (Akino et al. 1989, Harjunpää et al. 1995). Generally β-mannanases rarely cleave mannobiose (Benech et al. 2007), yet a β-mannanase from *A. aculeatus* released mannose in addition to mannotriose and mannobiose when hydrolysing ivory nut mannan (Setati et al. 2001).

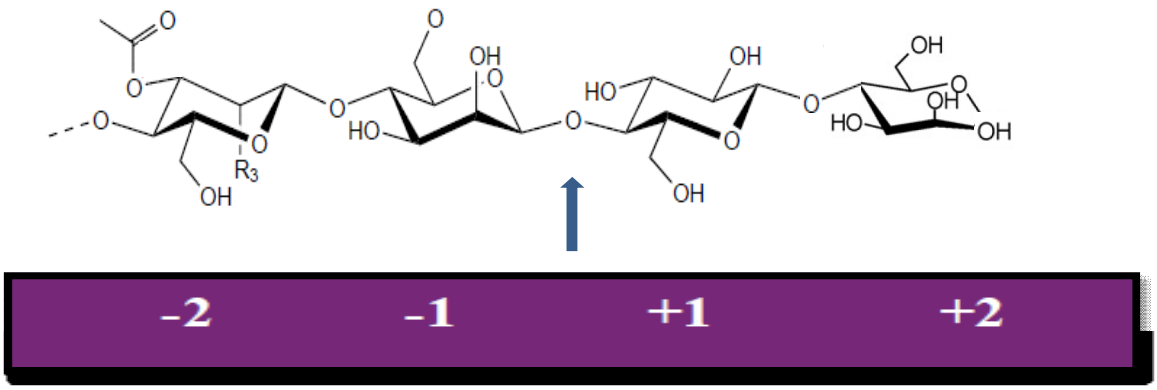

Figure 7: Schematic representation of the enzyme-substrate interaction and subsite binding of β-mannanase enzyme and substrate (β-1,4-mannan chain) (McCleary and Matheson 1983).

Genetic regulation of the β-mannanase gene expression is poorly understood compared to that of cellulases and xylanases. Nevertheless, β-mannanases are well represented in the fungal kingdom, where their regulation simulates that of other known hemicellulases (van Zyl et al. 2010). Some organisms are able to produce more than one enzyme of similar

function, often indicating different specificity. Various strains of the basidiomycete *Sclerotium rolfsii* have been shown to secrete several different β-mannanases at levels exceeding that of xylanases and endoglucanases (Grosswindhager et al. 1999, Haltrich et al. 1994). Cellulose acts as best inducer for β-mannanases in *S. rolfsii*, whereas mannans and manno-oligosaccharides are less efficient. This fungus produces a 42 kDa, 58 kDa and a 61 kDa β-mannanase (Gübitz et al. 1996a, Gübitz et al. 1996b, Sachslehner et al. 2000), where the first hydrolyses smaller fragments from mannan and was shown to be active against mannotetraose and mannotriose. The 58 kDa β-mannanase displayed activity on mannotetraose, mannotriose and mannobiose, whereas the 61 kDa β-mannanase displayed random breakdown of mannan, with a decrease in viscosity of mannan solutions (Gübitz et al. 1996b, Sachslehner et al. 2000). The induction of β-mannanases, β-xylanases and β-endoglucanases loosely correlate which may suggest a common induction system (Sachslehner et al. 1998). Indication of a second gene regulation mechanism appears when continued levels of β-mannanases are observed following glucose depletion as the sole carbon source (van Zyl et al. 2010).

The β-mannanases are also commonly found as part of the hemicellulase repertoire of hydrolases produced by ascomycetes fungi. The transcriptional activiater, XylR, is responsible for global hemicellulase induction (Arisan-Atac et al. 1993, Margolles-Clark et al. 1997). Yet, cellulose induces β-mannanase production in *Trichoderma* spp. (Arisan-Atac et al. 1993, Margolles-Clark et al. 1997). It shows weak proliferation when grown on mannan as a sole carbon source, indicating the presence of a different induction system. The β-mannanase production in *Aspergillus* spp. shows induction by growth on mannan-rich substrates (such as Palm kernel meal or defatted coconut kernel meal), but is presumably not regulated by XylR (Lin and Chen 2004, Stricker et al. 2008).

The optimal pH of β-mannanases varies between neutral and acidic with temperature optima ranging from 40 to 70°C (Table 3). The β-mannanases from thermophiles have shown functionality at much higher temperatures (Gibbs et al. 1999, Parker et al. 2001, Politz et al. 2000, Sunna et al. 2000). Molecular weights vary from 30 kDa to 130 kDa (Cann et al. 1999, Stoll et al. 1999, Sunna et al. 2000). Most β-mannanases have an isoelectric point between 4 and 8, but some enzymes have varying isoelectric points and molecular weights (Akino et al. 1989, Marraccini et al. 2001, Stålbrand et al. 1993). Such enzymes could be isoforms from the same gene as a result of differences in post-translational

modifications (Akino et al. 1989, Stålbrand et al. 1995) or can be produced from completely different genes (Millward-Sadler et al. 1996, Millward-Sadler et al. 1994).

Based on amino acid sequence similarity, β-mannanases are classified as glycoside hydrolase (GH) family 5 and 26 (Henrissat 1991, Henrissat and Bairoch 1993). GH family 5 represent the mannan-degrading enzymes from bacteria (*Caldocellum saccharolyticum, Cladibacillus, Vibrio* species), fungi (*A. aculaetus, T. reesei, Agaricus bisporus*) and eukaryotic (*Lycopersicon esculentum* and *Mytilus edulis*) origin (Dhawan and Kaur 2007, Larsson et al. 2006, Ximenes et al. 2005). GH family 26 mannanases are mostly from bacterial origin (*Bacillus* spp., *Cellvibrio japonicus, Pseudomonas fluorescens, Rhodothermus marinus*), but also contain mannanases of the anaerobic fungus (*Piromyces* spp.) (Dhawan and Kaur 2007). The β-mannanases from the same genus such as *Cladocellulosiruptor* and *Bacillus* have been placed in both families 5 and 26 (Akino et al. 1989, Gibbs et al. 1992, Gibbs et al. 1996, Mendoza et al. 1994, Mendoza et al. 1995) indicating that the enzymes from the same organism can have different evolutionary origins.

The three dimensional structures and X-ray crystallography for β-mannanases from *T. reesei* and *Thermobufida fusca* have been determined (Figure 8). The active site can be visualised as a cleft and has eight conserved amino acids for *T. fusca* (Gilbert 2010, Hilge et al. 1998). The crystal structure of these β-mannanases displays an open cleft shaped active site with strictly conserved catalytic glutamate residues present on β-strands 4 and 7 (Bourgault et al. 2005, Gilbert et al. 2008, Larsson et al. 2006, Le Nours et al. 2005).

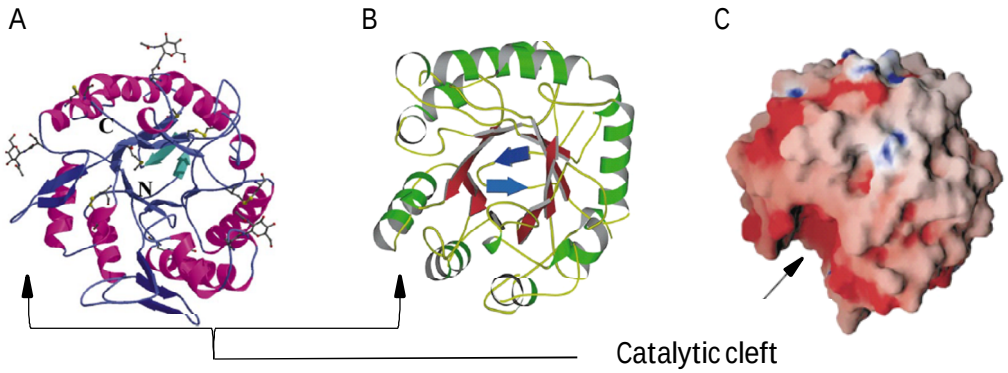

Figure 8: The secondary structure of the (A) *T. reesei* β-mannanase reveals a three-stranded and a two-stranded β-sheet (blue) that lie in close proximity to the C-terminus (Sabini et al. 2000a). The (B) *T. fusca* β-mannanase displaying β-strands (blue and red) and helices (green spirals). (C) Surface electrostatic potential distribution, with positive (blue) and negative (red) potentials. The catalytic site is visualised as a cleft containing 8 conserved amino acids (Hilge et al. 1998).

8. β-1,4-MANNOSIDASE

Complete hydrolysis of β-mannans requires β-mannosidases (β–D-mannoside mannohydrolase EC 3.2.1.25) that hydrolyse manno-oligosaccharides to mannose (Moreira and Filho 2008). The β-mannosidases have been isolated and characterised from fungi (Ademark et al. 2001, Ademark et al. 1999, Ademark et al. 1998, Bauer et al. 1996, Setati et al. 2001), bacteria (Bauer et al. 1996, Duffaud et al. 1997, Stoll et al. 2000), archaebacteria (Béki et al. 2003), plants (McCleary et al. 1982, Mo and Bewley 2002) and animals (Charrier and Rouland 2001). Molecular weights of β-mannosidases range between 50 – 130 kDa and can consist of several subunits (Parker et al. 2001, Bauer et al. 1996). The optimum temperature can range between 40°C-70°C and pH optima from 4 - 5.5 (Table 3). Bacterial β-mannosidases generally have neutral pI and acidic isoelectric points. Depending on the native host organism, β-mannosidases can display different functions. Bacteria and fungi normally produce β-mannosidases that degrade mannan from plants. Certain β-mannosidases from plants release the storage polysaccharides in seed endosperm during germination (McCleary and Matheson 1983, Mo and Bewley 2002). Higher eukaryotes, such as mammals, produce β-mannosidases that hydrolyse terminal non-reducing mannopyranoside linkages of glycoproteins (Chen et al. 1995).

The β-mannosidases can display activity on glucosides and mannosides (Bauer et al. 1996). They are capable of cleaving manno-oligosaccharides with a DP of up to 4 (Ademark et al. 1999, Han et al. 2010). Native β-mannosidase from *A. niger* can cleave oligosaccharides with a DP of up to 6. Like mannanases, the rate of hydrolysis shown is dependent on the degree and pattern of the side-chain substitutions (Ademark et al. 1999). Eukaryotic (human, bovine, caprine) β-mannosidases removes the *N*-linked oligosaccharides of glycoproteins (Chen et al. 1995, Alkhayat et al. 1998). The lack of a functional β-mannosidase in humans leads to deleterious storage of Man-β-1,4-GlcNAc, known as β-mannosidosis, a congenital disorder associated with a range of neurological involvement, including various degrees of mental retardation, hearing loss and speech impairment, hypotonia, epilepsy and peripheral neuropathy (Alkhayat et al. 1998).

The chromogenic substrate *p*-nitophenyl β-D-mannopyranoside (*p*NPM) is commonly used to determine β-mannosidase activity. Only a few β-mannosidases have been shown to release mannose from the non-reducing end of mannan-based polymers (Araujo and Ward 1990, Hirata et al. 1998, Kulminskaya et al. 1999). *A. niger*, *T. reesei* and *A. awamori* produce

extracellular β-mannosidases (Stoll et al. 2000), whereas *Aureobasidium pullulans* and *C. fimi* produces intracellular β-mannosidases that require a membrane-embedded mannobiose permease to transports the dissacharide into the cells (Kremnický and Biely 1997, Stoll et al. 1999,). Yet, *A. pullulans* and *C. fimi* also produce extracellular mannosidases when β-1,4-mannobiose is present in the medium (Dias et al. 2004).

Like mannanases, β-mannosidases also have the ability to transglycosylate certain mannose containing substrates (Béki et al. 2003, Gomes et al. 2007, Kurakake and Komaki 2001). A new β-mannosidase from *Streptomyces* spp. S27, expressed in *E. coli* BL21, displayed low transglycosylation activity. Small amounts of methylmannobiose were synthesised when incubated with *p*-nitrophenyl-β-D-mannopyranoside as glycosyl donor and methyl-β-D-mannopyranoside as acceptor (Shi et al. 2011). The *A. awamori* β-mannosidase was shown to transfer mannose residues to alcohols and fructose when using mannobiose prepared from Konjak as substrate. Fructose displayed high acceptor specificity implying the possible production of novel heteromanno-oligosaccharides (Kurakake and Komaki 2001). Transglycosylation by *T. reesei* β-mannosidase resulted in the synthesis of novel di- and tri-*p*NP-mannosides (Eneyskaya et al. 2009).

Most β-mannosidases are classified as GH family 2, with the exception of the enzyme produced by *Pyrococcus furiosus* which was placed in GH family 1 (Bauer et al. 1996, Henrissat 1991, Henrissat and Davies 1997). Families 1 and 2 (http://www.cazy.org) form part of the GH-A clan (Henrissat 1991, Henrissat and Davies 1997). GH family 2 also includes β-glucuronidase and β-galactosidase enzymes. Some enzymes have functional differences and do not correspond to the family consensus pattern, but they are none-the-less still confirmed as GH family 2 members. Glu-519 was shown as the conserved catalytic nucleophile in a β-mannosidase 2A from *C. fimi* (Stoll et al. 2000) and corresponds to the same residue that was identified within a β-galactosidase (*E. coli* β-galactosidase) and β-glucuronidase (Gebler et al. 1992, Wong et al. 1998) as catalytic nucleophiles. Even though mannosidases form a sub-family, they still adopt the three-dimensional structures of GH family 2.

Table 3: Characteristics of β-mannanases and β-mannosidases

Origin	Host	GH Family	Temp °C opt	pH opt	pI	MW kDa	K_m	V_{max}	Reference
Mannanases									
Agaricus bisporus CEL4	*S. cerevisiae*	5							(Tang et al. 2001)
Agaricus bisporus CEL4	*P. pastoris*	5							(Tang et al. 2001)
Armillariella tabescens	*P. pastoris*	5	60						(Wang et al. 2009)
Aspergilus aculeatus	*A. niger*	5	75						(van Zyl et al. 2009)
Aspergilus aculeatus	*S. cerevisiae*	5	50	3 - 6		50	0.3[a,b]	82[c]	(Setati et al. 2001)
Aspergilus aculeatus	*Y. lipolytica*	5							(Roth et al. 2009)
Aspergilus aculeatus	*A. oryzae*	5	60 - 70	5	4.5	45			(Christgau et al. 1994)
Aspergillus fumigates	*A. sojae*	5	60	5					(Duruksu et al. 2009)
Aspergillus fumigates	*P. pastoris*	5	45	5					(Duruksu et al. 2009)
Aspergillus niger	*P. pastoris*	5	80	4					(Do et al. 2009)
Aspergillus niger		5	50	4	3.7	40			(Ademark et al. 1998)
Aspergillus sulphurous	*P. pastoris*	5	40	6		48	0.93[a,b]	344.83[d]	(Chen et al. 2007)
Aspergillus terreus	*P. pastoris*	5	55						(Huang et al. 2007)
Trichoderma reesei	*P. pastoris*	5	70						(Wei et al. 2005)
Trichoderma reesei	*S. cerevisiae*	5	70	3 - 4	3.6 - 6.5	51 - 53			(Stålbrand et al. 1995, Stålbrand et al. 1993)
Sclerotium rolfsii		5	74	2.9	3.5	61	2.05[b,h]		(Gübitz et al. 1996b)
Bacertiodes ovatus		26	37	6.5	4.8 - 6.9	61/190			(Gherardini and Salyers 1987)
Bacillus circulans K-1	*E. coli*	5	65	6.9	5.4 - 6.2	62			(Yoshida et al. 1998, Yosida et al. 1997)
Bacillus sp. Strain AM-001	*E. coli*	26	60	9	5.9	58			(Akino et al. 1989)
Bacillus subtilis NM-39		26	55	5	4.8	38			(Mendoza et al. 1994, Mendoza et al. 1995)
Thermotoga neopolitana 5068			92	6.9	5.1	65	0.23[a,b]	3.8[c]	(Duffaud et al. 1997)
Streptomyces lividans		5	58	6.8	3.5	36	0.77[a,b]	207[c]	(Arcand et al. 1993)
Mannosidases									
Streptomyces sp. S27	*E. coli*	2	50	7		96	0.23[f]		(Shi et al. 2011)
Aspergillus awamori			60-70	5					(Kurakake and Komaki 2001)
Trichosporon cutaneum JCM 2947			40	7		114	0.25[f]	91.7[d]	(Oda and Tonomura 1996)
Thermotoga neapolitana		2	87	8	6	100	3.1[f]	36.9[d]	(Duffaud et al. 1997)
Aspergillus niger		2	70	2.5 - 5	5	135	0.3[f]	500[g]	(Ademark et al. 1999)
Aspergillus aculeatus	*A. oryzae*	2				130			(Kanamasa et al. 2001)
Sclerotium rolfsii			55	2.5	4.5	57.5			(Gübitz et al. 1996a)
Thermotoga neapolitana		2	87	7.7	5.6	95			(Parker et al. 2001)
Trichoderma reesei				3.5	4.8	105	0.12[f]		(Kulminskaya et al. 1999)
Cellulomonas fimi ATCC 484		2	55	7		103			(Stoll et al. 1999)
Pyrococcus furiosus		1	105	7.4	6.9	59			(Bauer et al. 1996)
Thermobifida fusca TM51	*S. lividans*	2	53	7.17	4.87	94	0.18[f]	5.96[i]	(Béki et al. 2003)
Thermoascus aurantiacus			76	3	4.8	99	1.1[e,f]	61[g]	(Gomes et al. 2007)

[a] *K*m value for Locust bean gum
[b] mg/mL
[c] IU/mL
[d] U/mg
[e] *K*m value for *p*-nitrophenyl-β-D-mannopyranosidase
[f] mM
[g] nkat/mg
[h] *K*m value for mannan
[i] µmol/min/mg

9. β-GLUCOSIDASE

The exo-acting glycosyl hydrolase enzyme, β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21), catalyses the release of terminal, non-reducing β-D-glucose residues in various β-D-glucosides including glucomannan and galactoglucomannan (Bauer et al. 1996, Lin et al. 1999). Most purified β-glucosidases are competitively inhibited by glucose and cellobiose (Bauer et al. 1996, Gomes et al. 2000, Lin et al. 1999) and are unable to degrade long β-1,4-linked glucose chains (Bauer et al. 1996, Lin et al. 1999). The β-glucosidases are grouped into GH families 1 and 3 (Henrissat and Bairoch 1993). The β-glucosidases have diverse properties and cellular locations. Most GH 3 β-glucosidases have similar retaining mechanisms and broad substrate specificity (Decker et al. 2001, Saloheimo et al. 2002).

10. α-GALACTOSIDASE

The α-galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) liberates the α-1,6-linked non-reducing galactose residues from the main mannan chain (Ademark et al. 2001, McCutchen et al. 1996). Two types of distinct substrate specificities have been identified. Some enzymes cleave α-1,6-linked galactose units linked to the inner mannose residues of galactoglucomannan whereas the other group shows preference for substrates where the galactose is linked to the non-reducing end of a substrate such as melibiose and raffinose (Halstead et al. 2000, Kaneko et al. 1991, Luonteri et al. 1998). The α-galactosidases have been placed in GH families 4, 27, 36 and 57 (Henrissat 1991). Bacterial α-galactosidases are mostly grouped in GH families 4 and 36, while eukaryotic enzymes are grouped into GH family 27. In general GH families 4 and 27 α-galactosidases can release galactose from polymeric substrates, whereas GH family 36 enzymes lack this ability (Ademark et al. 2001, Luonteri et al. 1998). Some fungal α-galactosidases are produced as a mixture of isoenzymes and can have different enzyme-substrate specificities. The α-linked D-galactose residues are released from hemicelluloses (such as xylan and galactomannan), by α-galactosidases belonging to GH family 27 and GH family 36. These α-galactosidases act via a double-displacement mechanism and are considered to have a common evolutionary origin (Rigden 2002). Various enzymes belonging to the GH family 27 also show α-*N*-acetylgalactosaminidase activity implying that not all GH family 27 α-galactosidases are involved in hemicellulose degradation (Kulik et al. 2010). The GH 36

α-galactosidases are often larger in size and are more active against mono-, di- and oligosaccharides, such as melibiose and raffinose (Ademark et al. 2001).

Similä et al. (2010) cloned and characterised the first gene encoding an extracellular α-D-galactosidase from the thermophilic fungus *T. emersonii*. The enzyme displayed a 24 amino acid secretion signal peptide. The translated protein had highest identity with other fungal α-galactosidases belonging to GH family 27. The enzyme displayed optimal activity at pH 4.5 and 70°C. The enzyme was however competitively inhibited by galactose.

11. ACETYL-MANNAN ESTERASES

Acetyl esterases liberate acetic acid from acetylated mannan substrates (Ratto et al. 1993). Esterases isolated from fungal sources displayed varying substrate specificities. The esterases from *A. niger* and *T. reesei* preferably liberate acetic acid from polymeric acetyl galactoglucomannan (Tenkanen et al. 1993, Tenkanen et al. 1995). An *A. oryzae* esterase has broad substrate specificity and can liberate phenolic side groups from xylan (Tenkanen et al. 1993, Tenkanen et al. 1995). Acetyl esterases in combination with β-mannanases can dramatically increase the hydrolysis of mannan polysaccharides (Tenkanen et al. 1995), however the gene encoding this specific enzyme has not yet been identified nor characterised in other fungi. Tenkanen et al. (1995) reported that the hydrolysis yield of the esterase of *A. oryzae* on *O*-acetyl-galactoglucomannan increased to 87% when the esterase was used in combination wth the β-mannanase from *T. reesei*.

12. ENZYME SYNERGY

Synergy is the cooperation between two hydrolytic enzymes in such a way that their actual combined hydrolysis exceeds the theoretical sum of their individual hydrolysis. Homosynergy is the interaction between two main-chain enzymes (for example, β-mannanase and β-mannosidase) or two side-chain enzymes (for example, α-galactosidase and acetyl mannan esterase). Heterosynergy is the synergistic interaction between side-chain and main-chain enzymes (for example, β-mannanase and α-galactosidase). Numerous examples of synergistic activity have been reported for combinations of β-mannosidase, β-mannanase, β-glucosidase or α-galactosidase. When using Locust bean gum as substrate more reducing

sugars were liberated by the synergistic action of β-mannosidase and β-mannanase from *Streptomyces* spp. S27 (Shi et al. 2011). Mannans, glucomannans and galactomannans were shown to completely hydrolyse when exposed to β-mannosidases isolated from *Sclerotium rolfsii*, liberating monosaccharides from the mannans. The activity of the enzyme was enhanced by the addition of β-mannanases. Synergistically, both enzymes randomly cleaved fragments larger than mannobiose from the mannans (Gübitz et al. 1996a). The addition of purified α-galactosidase to β-mannosidase isolated from *A. niger* showed that the action of these enzymes significantly enhanced degradation of galactomanno-oligosaccharides into galactose and mannose (Ademark et al. 2001).

13. INDUSTRIAL APPLICATIONS OF MANNAN AND MANNANASES

Mannan, also known as gum, has various applications and is used in numerous industries. Gum extraction is inexpensive, non-toxic and has GRAS (**G**enerally **R**egarded **A**s **S**afe) status (Moreira and Filho 2008). They are produced in large amounts and used in the manufacturing of food, paper, textile, pharmaceutical, cosmetics and mining (Moreira and Filho 2008). Gums are extracted from seeds and include Locust bean gum. Gums are mostly extracted from plants of the Luguminoseae family like *Caesalpinia spinosa* (carob seeds), *Ceratonia siliqua* (Tara seeds) as well as other plants like *Cyamopsis tetragonoloba* (Guar seeds) and *Cassia grandis* (Duffaud et al. 1997, Joshi and Kapoor 2003, Shobha et al. 2005). These gums have film-forming abilities and excellent heat shock protection that can be applied in frozen foods. They act as stabilisers in low-fat and non-fat dairy products and have many fat-replacement applications acting as a fat-imitator (Fernández et al. 2007, Hsu and Chung 1999, Ishurd et al. 2006). Other mannans, like linear mannan from *Aloe vera*, have immuno-pharmacological and therapeutic properties (Aspinall 1959).

Given the natural abundance of mannan, many microorganisms produce enzyme systems to hydrolyse mannan completely into simple sugars that can be used as energy and carbon sources for various animals (Jiang et al. 2006). The increasing availability of genome sequences, bioinformatic tools and expression cloning (Xu et al. 2002b) has facilitated the acquisition of coding sequences for novel and previously characterised enzymes, hence the increasing number of publications and patent applications describing heterologous enzyme-producing strains. Additionally, protein engineering approaches are creating

enzymes with improved functionality under extreme pH and temperature conditions increasing the industrial applications (van Zyl et al. 2010).

The majority of industrially used enzymes are hydrolytic, including proteases and glycoside hydrolases (Kirk et al. 2002). The global market for industrial enzymes was estimate at $2 billion in 2004 with an annual growth rate predicted at 4 to 5% (Turner et al. 2007). Interest in mannan-degrading enzyme systems has increased in the past decade, because of their biotechnological applications. Some applications will be discussed briefly, but other applications include the extraction of vegetable oils from leguminous seeds, improvement in the consistency of beer, biopulping of wood, etc. (Heck et al. 2005, Singh et al. 2003).

13.1. BIOFUELS

Production of second generation bioethanol (from lignocellulosic substrates) has received much attention in the past two decades. Residues from various industries and origins can serve as sources for bioethanol production. Interestingly, many commercial cellulase cocktails contain low levels of mannanases (Berlin et al. 2007). The application of mannanases for catalysing the hydrolysis of β-1,4-mannans could be as important as the application of xylanases. The hydrolysis of all polysaccharides is of interest and evidence of synergy between mannan-degrading enzymes and cellulases was demonstrated by a 5-fold increase in glucose yields (Jørgensen et al. 2010). Palm kernel press cake was recently reported to contain 50% hexose sugars in the form of glucan and galactomannan (van Zyl et al. 2010). It was possible, without thermochemical pre-treatment, to obtain 88% of the theoretical mannose yields. An optimised cocktail of cellulases, β-mannanases and β-mannosidases proved efficient in hydrolysing Palm kernel press cake polysaccharides, and when combined with a simultaneous saccharification and fermentation strategy, realised ethanol yields of 200 g ethanol/kg Palm kernel press cake. Enhanced oil cake residues obtained after fermentation contain less fibre and are protein enriched—17% to 28% in the case of Palm kernel press cake. Palm kernel press cake could be used for the production of mannose and MOS (manno-oligosaccharides) as the mannan component has been shown to be easily digested by enzymes. The remainder can serve as a feedstock for bioethanol production and lastly the protein enriched residues could be added to animal feeds making Palm kernel an ideal versatile substrate.

For plant biomass to become a viable feedstock for meeting the future demand for liquid fuels, efficient and cost effective processes must exist to breakdown cellulosic materials into their primary components. The development of a feasible biological delignification process should be possible if lignin-degrading microorganisms (*Phanerochaete chrysosporum* and *Phlebia radiata*), their ecophysiological requirements, and optimal bioreactor design are effectively coordinated. New fermentation technology for converting xylose to ethanol also needs to be developed to make the overall conversion process more cost-effective. The fermentation of glucose, the main constituent of the cellulose hydrolysate, to ethanol can be carried out efficiently. On the other hand, although bioconversion of xylose (the main pentose sugar obtained on hydrolysis of hemicellulose) to ethanol presents a biochemical challenge, especially if it is present along side glucose, it needs to be achieved to make the biomass-to-ethanol process economical. A consolidated bio-processing of biomass into ethanol would provide the most cost-effective route to renewable fuels. Although a diverse range of bacteria and fungi possess the enzymatic machinery capable of hydrolysing plant-derived polymers, none discovered so far meet the requirements for an industrial strength biocatalyst for the direct conversion of biomass to combustible fuels (Elkins et al. 2010). Synthetic biology combined with a better fundamental understanding of enzymatic cellulose hydrolysis at the molecular level is enabling the rational engineering of microorganisms for utilising cellulosic materials with simultaneous conversion to fuel (Elkins et al. 2010). As no naturally occurring organism can satisfy all necessary specifications (high yield, high productivity, wide-substrate range, ethanol tolerance, tolerance to inhibitors present in hydrolysates and biomass disposal cost). Therefore, the utilisation of modern genetic engineering techniques are aimed at developing/constructing organisms that are endowed with most of the desirable properties for such bioprocesses.

It is anticipated that the largest gains in cost competitiveness in terms of producing fuels from biomass could be realised through the consolidation of several production steps into a streamlined process where hydrolytic enzymes are simultaneously produced *in situ* by a solventogenic, fermentative microbe (Galbe and Zacchi 2002, Lynd et al. 2008). Consolidated bioprocess (CBP) requires a highly engineered microbial workhorse that has been developed for several different process-specific characteristics (Figure 9). These desirable traits include enzyme production and stability, balanced growth on hexoses and pentoses, tolerance to pretreatment inhibitors, maximal product yield and production rates, solvent tolerance, and the ability to persevere through process fluctuations.

(Elkins et al. 2010). This means that all types of sugars in cellulose and hemicellulose must be converted to ethanol, and that microorganisms must be obtained that efficiently perform this conversion under industrial condition (Chandrakant and Bisaria 1998, Gong et al. 1999, Lee 1997).

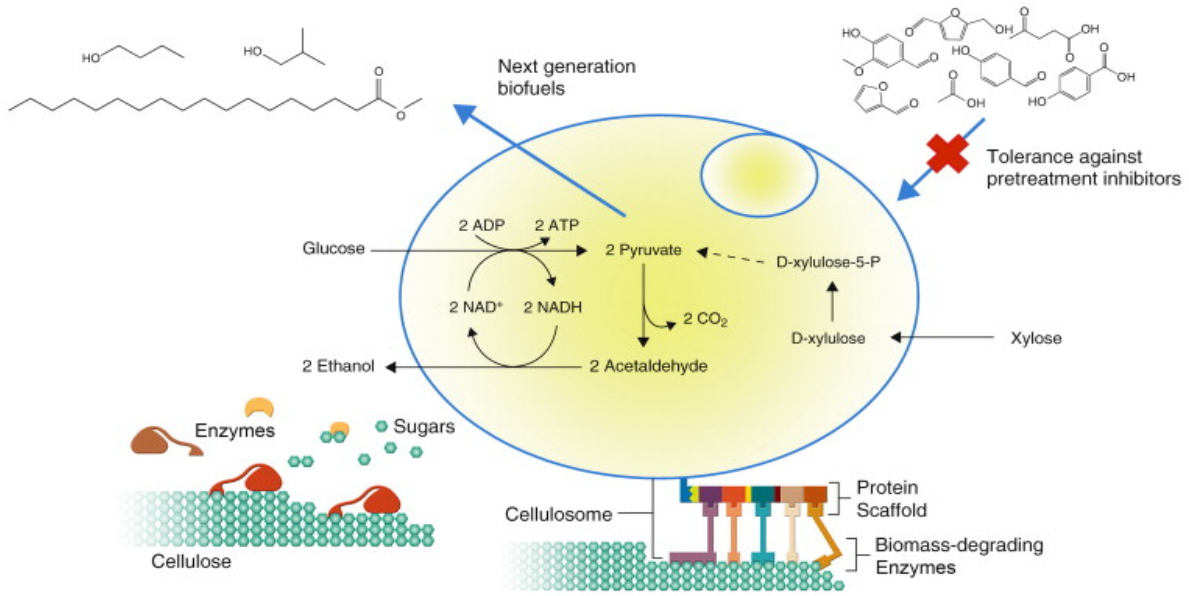

Figure 9: A conceptual whole cell biocatalyst for producing liquid transportation fuels. The optimal organism would express a synergistic mixture of highly active exo- and endoglucanases, glucosidases and xylanases (Elkins et al. 2010).

Process concepts for the conversion of lignocellulosic feedstocks to ethanol generally include a pre-treatment or fractionation step in which the chopped raw material is exposed to acidic or alkaline pH, at high temperature, so that the hemicellulose fraction is partially hydrolysed to monomeric and oligomeric sugars, rendering the cellulose fraction susceptible for hydrolysis (Figure 10), then follows either acidic or enzymatic hydrolysis of the cellulose fraction. The hemicellulose and cellulose hydrolysates are fermented to ethanol, and the ethanol is recovered by distillation (Figure 10). Furthermore, the lignin fraction can be used throughout the process to generate the necessary heat by burning, and the waste streams are evaporated and burned or fermented to methane to recover energy to be used in the process and commercialised as an added-value co-product (Hahn-Hägerdal et al. 2007, Wingren et al. 2003).

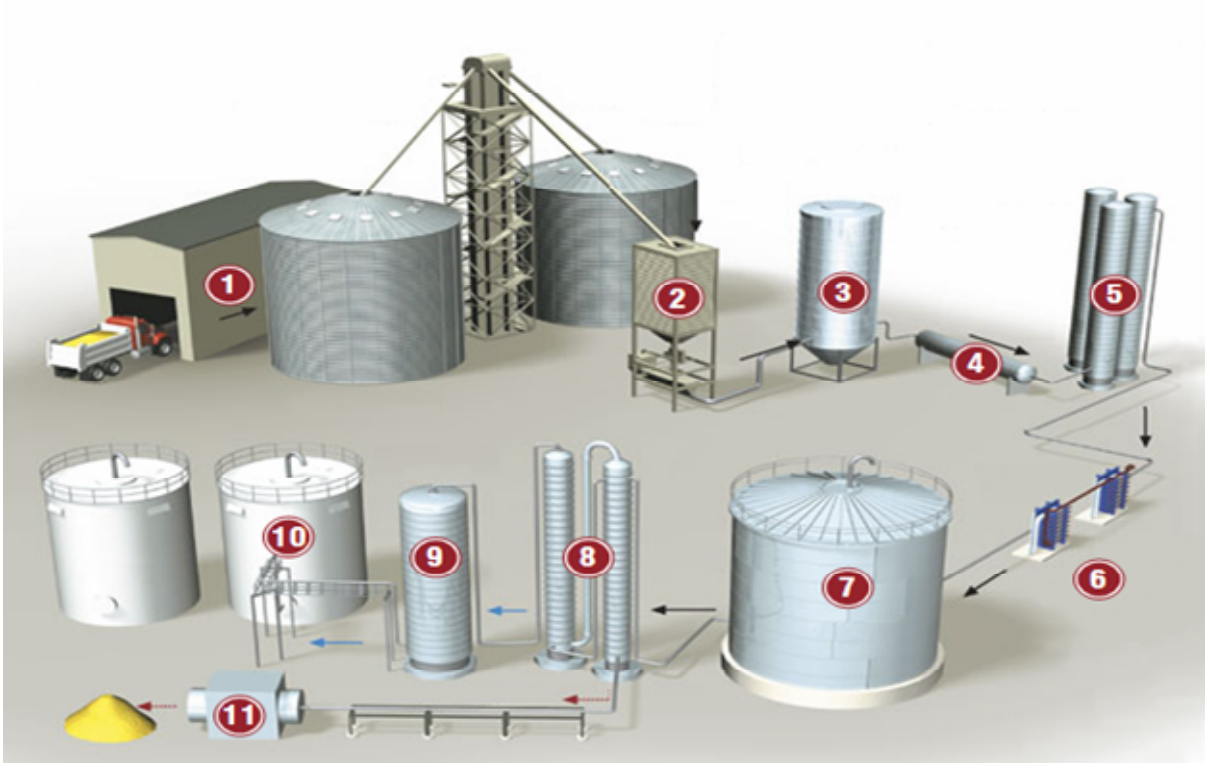

Figure 10: A schematic illustration of the process design for lignocellulosic bioethanol production. (1) unloading (2) milling (3) mashing (4) cooking (5) hydrolysis (6) cooling (7) fermentation (8) distillation (9) dehydration (10) storage (11) stillage treatment (Hahn Hägerdal et al. 2007, http://www.biofuels-platform.ch/en/infos/bioethanol.php)

13.2. COFFEE

Mannan is the main polysaccharide present in *Arabica* and *Robusta* coffee beans with galactomannan constituting 20 – 30% of its dry weight (Sachslehner et al. 2000). Mannan is the main polysaccharide component of the coffee bean extracts and is responsible for their high viscosity, which negatively affects the technological processing of instant coffee. Different mannanase preparations are used for the hydrolysis of coffee mannan, thus reducing significantly the viscosity of coffee extracts (Dhawan and Kaur 2007, Sachslehner et al. 2000). Hydrolysis of galactomannans present in a liquid coffee extract inhibits gel formation during freeze drying of the instant coffee. Polysaccharides, obtained by alcohol precipitation, form nearly half of the coffee extract dry weight. Coffee mannan is isolated from green defatted beans by delignification, acid wash and subsequent alkali extraction resulting in a yield of 12.8%. Additionally coffee extract polysaccharides are

separated by alcohol precipitation and are found to form nearly half of the coffee extract dry weight. These isolated mannans as well as the mannans in the coffee extract are efficiently hydrolysed by the mannanase, which resulted in significant viscosity reductions (Sachslehner et al. 2000). Concurrently, the reducing sugar content increased continuously due to the release of various manno-oligosaccharides including mannotetraose, mannotriose and mannobiose. Immobilised or soluble, crude mannanase preparations can be successfully employed for the degradation of coffee mannan (Nunes and Coimbra 1998, Nunes et al. 2006). However, fungal β-mannanases are best suited to this application as spent coffee ground has a slightly acidic pH of 5 (van Zyl et al. 2010).

13.3. ANIMAL FEED

The β-mannans are polysaccharides commonly found in feed ingredients such as soybean meal, Palm kernel meal, copra meal, guar gum meal and sesame meal (Dhawan and Kaur 2007). Soybean meal and full fat soy is used almost universally as protein sources in poultry, ruminant (Chandrasekharaiah et al. 2001, Moss and Givens 1994), pig (Pettey et al. 2002) and rabbit diets (Dhawan and Kaur 2007). Meals have some common properties which include high fibre content, low palatability, lack of several essential amino acids and high viscosity coupled with several non-nutritional properties such as mannan, galactomannan, xylan and arabinoxylan. Their utilisation in the intestine is limited. The β-mannans have been found to be highly deleterious to animal performance, severely compromising weight gain and feed conversion as well as glucose and water absorption. Incorporation of β-mannanases into these diets results in decreased intestinal viscosity, thus improving both the weight gain of chicks and their feed conversion efficiency.

More recently, the beneficial effect of β-mannanase addition to diets containing soybean meal has been documented in broilers (Daskiran et al. 2004, Jackson et al. 2004, Lee et al. 2003), layers (Wu et al. 2005), turkeys (Odetallah et al. 2002) and swine (Pettey et al. 2002). Using endo-mannanase alone may only produce a small proportion of mannose and thus only a small amount of mannan is likely to be absorbed in the intestine of broilers (Dhawan and Kaur 2007). Most commercial mannanases on the market almost exclusively contain endo β-mannanases, therefore manno-oligosaccharides, mannotriose and mannobiose as well as a small amount of mannose are generated when this type of enzyme is included in the diet. Since only mannose can be absorbed in the intestine, the mannobiose and

manno-oligosaccharides are unable to supply energy directly to the host. Yet the production of manno-oligosaccharides can improve a chicken's health (van Laere et al. 1999) by increasing the population of specific bacteria (such as *Bifidiobacteria*). These types of carbohydrates are a source of feed for bacteria in the caeca and thus suppressing the pathogenic organisms. Manno-oligosaccharides are used to flush out the pathogenic bacteria which attach to the manno-oligosaccharides (Dhawan and Kaur 2007).

13.4. NON-NUTRITIONAL FEED

Dietary fibres act as prebiotics, displaying beneficial effects on intestinal microflora and improve human health. Konjac flour glucomannan has been shown to increase faecal probiotics (Fan et al. 2009a, Fan et al. 2009b) with, more specifically, partially hydrolysed glucomannans showing greater potency than their unhydrolysed counterparts (Al-Ghazzewi et al. 2007, Chen et al. 2007). Konjac flour is produced from the corms of *Amorphophallus konjac*. It is commonly used as a gelling and thickening agent in foods and beverages, but has a relatively low commercial standing. With the aim of increasing the value of konjac flour, an industrially relevant means of producing manno-oligosaccharides (MOS) from konjac flour has been investigated (Al-Ghazzewi et al. 2007). With MOS providing comparable prebiotic effects (in a model system) to oligofructose (van Zyl et al. 2010), the application of MOS as functional food additives represents a potentially commercial important application for β-mannanases. Furthermore, clinical studies have highlighted the positive effects of MOS derived from different plant sources on human health. Consumption of 3 g MOS per day derived from extracts of coffee spent grounds (galactomannans), have been shown to decrease fat utilisation and increase fat excretion without negatively effecting fat metabolism (Yeh et al. 2010).

Guar gum is a water-soluble polysaccharide derived from the endosperm of guar seeds of *C. tetragonoloba* trees and consists primarily of galactomannans. Owing to its high viscosity, partially hydrolysed guar gum (PHGG) is used in beverages and has been shown to provide relief from irritable bowel syndrome (IBS) symptoms (Zhang et al. 2009). Although not significantly better than wheat bran in relieving IBS symptoms, PHGG is preferred by patients and a lower dosage is required to achieve relief. These factors may facilitate the more successful application of PHGG as a dietary fibre supplement. The addition of a commercial preparation of PHGG to World Health Organisation oral rehydration solution was shown to

reduce the duration and stool output in children suffering from acute non-cholera diarrhoea, thus facilitating their recovery (Kumao et al. 2006).

13.5. DETERGENTS

Alkaline mannanases have found application in laundry segments as stain removal boosters (Dhawan and Kaur 2007). Stains containing mannan are difficult to remove since mannans have high tendency to adsorb to cellulose fibres. Mannanases cleave the β-1,4-linkage between mannose units in guar and break down the gum polymer into smaller carbohydrate fragments. These smaller, more water soluble polysaccharide fragments remain free from the fabric and are siphoned out of the wash. The gluing effect of the mannan leads to trapping of particulate soils released during the wash cycle. In other words, not only does mannan stains reappear, but mannan can also be transferred to otherwise clean fabrics during washing and result in greying of the fabric (Chauhan et al. 2012). Treatment with cleaning or detergent compositions comprising mannanases can improve whiteness as well as prevent binding of certain soils to the cellulosic material. Accordingly, mannanases are used in cleaning compositions, including laundry, personal cleansing and oral/dental compositions. The cleaning composition would comprise a mannanase and an enzyme selected from cellulases, proteases, lipases, amylases, pectin degrading enzymes and xyloglucanases to provide superior cleaning performance (Moreira and Filho 2008).

13.6. PHARMACEUTICAL APPLICATIONS

D-mannose derived from beech and birch wood hydrolysates is sold commercially as an excipient (van Zyl et al. 2010). Mannose is used extensively by the pharmaceutical industry (Fu et al. 2006) due to its high solubility which contributes to the structure-forming and fast dissolving properties of tablets. The role of mannose as a remedy for urinary tract infection has also been suggested (van Zyl et al. 2010). Mannose is currently obtained from chromatographic separation of sulphite spent liquors (Alam et al. 2000). Substrates rich in mannan such as Palm kernel and copra meals represent low cost alternative sources of mannose. The addition of cellulase to β-mannanase/β-mannosidase preparations did not increase mannose yields implying that glucans or cellulose did not prevent access of the mannan-degrading enzymes to the substrate (van Zyl et al. 2010). The addition of an

α-galactosidase to the β-mannanase/β-mannosidase cocktail did not significantly improve mannose yields implying low levels of galactose side chains. Proteins present in the substrate did not appear to restrict access of the enzymes to the polysaccharides as addition of a protease preparation did not increase mannose yields (Jørgensen et al. 2010). Increasing β-mannosidase activity relative to endo-β-mannanase did increase mannose yields, especially when high solid loadings were used. These results indicate that β-mannosidases and endo-β-mannanases are sufficient to degrade the substrate to obtain high levels of mannose.

13.7. BIOBLEACHING

The extraction of lignin from wood fibres is an essential step in bleaching of pulps. Pulp pre-treatment under alkaline conditions hydrolyses hemicelluloses covalently bound to lignin and thus facilitates subsequent lignin removal. The major drawback of alkaline treatment is that it creates an environmental pollution problem due to release of chlorinated compounds. As an alternative, use of mannanases along with other enzymes like xylanases, can equally facilitate lignin removal in pulp bleaching and give results comparable to alkaline pre-treatment (Dhawan and Kaur 2007). Softwoods (from which the majority of pulps are derived) contain as much as 15–20% hemicellulose in the form of galactomannan. The β-mannanase and the accessory enzymes are able to cleave the mannan component in the pulp selectively without affecting the cellulose (Moreira and Filho 2008). Mannanases would therefore make excellent candidates for use in enzymatic bleaching of softwood pulps (Benech et al. 2007, Gübitz et al. 1997). Pulping is carried out at high temperature and pH conditions and requires mannanases which are active at these conditions (He et al. 2008, Pan et al. 2011). Mannanases are also used in chlorine-free bleaching (chemical pulps, semi-chemical pulps, mechanical pulps or kraft pulps) to increase the pulp brightness, thus decreasing or eliminating the need for hydrogen peroxide in the bleaching process (Tenkanen et al. 1997).

14. HETEROLOGOUS EXPRESSION IN YEAST

Yeasts offer the ease of microbial growth and gene manipulation found in bacteria along with the eukaryotic environment and ability to perform many eukaryote-specific post-translational modifications, such as proteolytic processing, folding, disulfide bridge formation and

glycosylation (Eckart and Bussineau 1996). Bacteria lack these capabilities and often produce eukaryotic proteins that are misfolded, insoluble or inactive (Cereghino and Cregg 1999). Recent advances include the use of yeasts to manufacture large quantities of foreign proteins (for research and therapeutics), the synthesis of life saving drugs for the pharmaceutical (Cereghino and Cregg 1999) industry as well as the use of yeasts to determine the functional and regulatory dynamics of recombinant proteins.

The yeast *S. cerevisiae* is one of the main hosts considered for bioethanol production and application in CBP, mainly due to its long and successful history with ethanol production for the beer and wine industries (Lynd et al. 2002). The yeast, however, does not natively produce enzymes such as cellulases and hemicellulases. Yet, the successful production of these enzymes has been obtained through DNA manipulation techniques, using an array of vector systems and expression cassettes (La Grange et al. 2001, van Rooyen et al. 2005). Foreign gene expression in yeasts consists of four steps: (1) cloning of a foreign protein-coding DNA sequence within an expression cassette containing a yeast promoter and transcriptional terminator sequence; (2) transformation and stable maintenance of this gene cassette in the host; (3) synthesis of the foreign protein under specified culture conditions; and (4) purification of the heterologous protein and comparison with its native counterpart (Cereghino and Cregg 1999).

Highly expressed genes tend to use a narrow set of codons corresponding to the most abundant species of tRNA (Bulmer 1987, Gouy and Gautier 1982, Ikemura 1985). Codons in a gene may be bottlenecks especially in the case of foreign gene expression in a host where the use of codons in highly expressed genes does not resemble the use of codons in the species from which the foreign gene originated (Lithwick and Margalit 2003). Different patterns of codon usage found in the genomes of different species are widely recognised as a possible cause of low protein yields during heterologous protein expression (Holm 1986, Kane 1995, Varenne and Lazdunski 1986). Minimising the risk of tRNA depletion during translation is of practical importance for expression experiments, especially when the expressed gene has many codons that are rarely used in the host. To overcome this problem, the non-optimal codons in the introduced gene can be substituted for codons that correspond to the more abundant tRNA species, and yields can thereby be increased considerably (Wu et al. 2006). This process is commonly referred to as codon optimisation and is a general approach to improving heterologous expression where genes are moved from their native genomes into alternatives that exhibit different patterns of codon usage.

Many studies have manipulated codon usage of a coding sequence in an attempt to increase translational efficiency (Gustafsson et al. 2004). Some have successfully improved protein expression (Deng 1997, Feng et al. 2000, Frelin et al. 2004, Kotula and Curtis 1991, Sinclair and Choy 2002) but others have failed (Alexeyev and Winkler 1999, Wu et al. 2004). The usage of rare codons may have negative consequences for the expression other than just the amount of protein obtained. The quality of the expressed protein is dependent on the codons, as rare codons are associated with an increased chance of misincorporation (Calderone et al. 1996, Forman et al. 1998, McNulty et al. 2003, Seetharam et al. 1988).

In *S. cerevisiae* the preferred codons in highly expressed genes, have a GC content comparable to the mean GC content for the whole genome (Sharp et al. 1993). This implies that a GC content does not necessarily play a large role in whether the gene is highly expressed or not. Also, the correlation between predicted and actual expression data is unclear when relying solely on characteristics of coding DNA sequences (Friberg et al. 2004).

15. ALTERNATIVE HOSTS

The yeast, *S. cerevisiae* has many limitations regarding the expression of recombinant proteins including poor expression capacity, hyperglycosylation, protease activity and low product yield (Cregg et al. 2000). A limited number of yeast species (inclusing *Hansenula*, *Pichia*, *Candida* and *Torulopsis*) are able to grow on methanol as a sole energy and carbon source (Lee and Komagata 1980). *Pichia pastoris* has been utilised to produce ~300 foreign proteins since 1984 (Cereghino and Cregg 2000, Cregg et al. 2000, Faber et al. 1995, Lin Cereghino et al. 2001, Macauley-Patrick et al. 2005, Weidner et al. 2010). Several factors contribute to this systems popularity. The use of a strong alcohol oxidase I (*AOX1*) promoter, the ability to stably integrate expression plasmids at specific sites in the *P. pastoris* genome in single or multicopy and the ability to culture strains at high density in fermenters (Cereghino and Cregg 1999). The *AOX1* promoter is strongly repressed by glucose and most other carbon sources, but is induced in cells shifted to methanol as a sole carbon source (Tschopp et al. 1987). Cultures are shifted from glucose to a methanol medium to induce rapid high-level expression once the desired optical density is reached (Clare et al. 1998). Unfortunately, methanol is a potential fire hazard and can cause blindness upon consumption.

It might therefore not be approved for use in the production of food additives. The glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter provides a constitutively high level of expression on glucose, glycerol and methanol media (Waterham et al. 1997). Using the *GAP* promoter does not allow for repression of the recombinant protein, limiting its use to foreign genes where the products are a burden to the cell. A good compromise is the promoter derived from the *P. pastoris FLD1* gene, can be induced either by methanol or methylamine (a nontoxic nitrogen source) in glucose containing media. Expression levels from the methylamine-induced *FLD1* promoter are comparable to those obtained with the *AOX1* promoter in methanol (Shen et al. 1998).

Improvements in other yeast systems (Table 4) such as the identification of new, strong promoters for foreign protein expression in *Y. lipolytica* and *K. lactis* have been explored (Müller et al. 1998, Saliola et al. 1999). Several new yeast expression systems have been reported, including *Pichia methanolica*, which shares attributes of *P. pastoris* and *H. polymorpha*, including the ability to be readily grown to high cell densities and the availability of expression vectors that contain the tightly regulated alcohol oxidase promoter to control expression of foreign genes (Raymond et al. 1998).

The attraction of filamentous fungi as production hosts is based on their natural ability to secrete large amounts of proteins (mainly hydrolytic enzymes) into the growth medium (Nevalainen et al. 2005, Verdoes et al. 1995). In traditional fermentation technology, filamentous fungi are dominant producers of a range of primary metabolites including organic acids such as citric, gluconic, fumaric, kojic, itaconic acid and fatty acids (Ward 2012). Eukaryotic posttranslational protein processing machinery is an added bonus for heterologous proteins requiring elaborate posttranslational modification (such as protein glycosylation, proteolytic cleavage or formation of multiple disulfide bonds).

Filamentous fungi have gone through intricate strain improvement programmes for industrial exploitation (Verdoes et al. 1995). Obtaining high protein secreting mutants has been done through traditional random mutagenesis, for which the characteristics have further been modified by genetic engineering (Nevalainen et al. 2005). Achieving high expression of heterologous genes requires a strong promoter to drive robust transcription. In both the model filamentous fungi and industrially important fungi, a variety of either constitutive or inducible promoters, involved in diverse physiological processes in the host cell and some originating from *E. coli*, have been evaluated for application (Meyer et al. 2011,

Su et al. 2012). In an overall comparison with other available expression systems for heterologous proteins, applied in liquid culture, filamentous fungi perform well and provide a potentially high yielding and relatively inexpensive option. Filamentous fungi that dominate the markets as production hosts are the asexually reproducing *A. niger*, *A. oryzae* and *T. reesei* with *A. niger* being able to produce up to 30 g/l of glucoamylase and *T. reesei* reportedly producing up to 100 g/l of extracellular protein (Demain and Vaishnav 2009).

Table 4: Comparison of the features of yeast expression systems (Cereghino and Cregg 1999)

Species name	Promoter	Regulation	Reference
Methanol utilising			
Candida boidinii	*AOD1*	Methanol induced	(Sakai et al. 1994)
Hansenula polymorpha	*MOX*	Methanol induced	(Gellissen and Hollenberg 1997)
Pichia methanolica	*AUG1*	Methanol induced	(Raymond et al. 1998)
Pichia pastoris	*AOX1*	Methanol induced	(Cregg et al. 2000)
	GAP	Strong constitutive	(Waterham et al. 1997)
	FLD1	Methanol/methylamine induced	(Shen et al. 1998)
	PEX8	Moderate methanol induced	(Johnson et al. 1999)
	YPT1	Moderate constitutive	(Sears et al. 1998)
Lactose utilising			
Kluyveromyces lactis	*LAC4*	Lactose induced	(van den Berg et al. 1990)
	PGK1	Strong constitutive	(Rocha et al. 1996)
	ADH4	Ethanol induced	(Saliola et al. 1999)
Starch utilising			
Schwanniomyces occidentalis	*AMY1*	Maltose/starch induced	(Piontek et al. 1998)
	GAM1	Maltose/starch induced	(Piontek et al. 1998)
Xylose utilising			
Pichia stipitis	*XYL1*	Xylose induced	(Piontek et al. 1998)
Alkane and fatty acid utilising			
Yarrowia lipolytica	*XPR2*	Peptone induced	(Ogrydziak and Scharf 1982)
	TEF	Strong constitutive	(Müller et al. 1998)
	RPS7	Strong constitutive	(Müller et al. 1998)

(*ADH4*) alcohol dehydrogenase, (*AMY1*) α-amylase, (*AOX, AUG1, AOD1, MOX*) alcohol oxidase in species shown, (*FLD1*) formaldehyde dehydrogenase, (*GAP*) glyceraldehyde-3-phosphate dehydrogenase, (*GAM1*) glucoamylase, (*LAC4*) β-galactosidase, (*PEX8*) peroxin 8, (*PGK1*) phosphoglycerate kinase from *S. cerevisiae*, (*RPS7*) ribosomal protein S7, (*TEF*) translation elongation factor-1a, (*XPR2*) extracellular protease, (*YPT1*) GTPase involved in secretion

16. METABOLISM OF HEMICELLULOSE AND DERIVED MONOMERS

Certain microorganisms are able to degrade cellulose and hemicellulose to oligosaccharides and monosaccharides. However, that is not a characteristic of the most promising ethanol producers (Gírio et al. 2010). In natural environments, hydrolysis of hemicellulose is performed by a variety of enzymes that work synergistically and, in some cases, organised in complexes. Microorganisms can be divided in three groups according to their strategies to hydrolyse hemicelluloses (Shallom and Shoham 2003). Complete hydrolysis to

monosaccharides and disaccharides, by the synergistic action of extracellular hemicellulases, is adopted by several fungi, like *Fusarium*, *Trichoderma* and *Aspergillus* species. Bacteria like *Bacilli*, perform partial extracellular hydrolysis to oligosaccharides combined with hydrolysis by cell-associated or intracellular hemicellulases. This mechanism has advantages regarding sugar competition against non-hemicellulolytic microorganisms. Hydrolysis can also be accomplished by cellulosomes, extracellular cell-associated multienzyme complexes, harbouring cellulases and hemicellulases. This structure is considered a versatile extracellular organelle whose functions can be tailored by incorporating different dockerin-containing subunits, as described for *Clostridium thermocellum* (Demain et al. 2005).

Monosaccharides, disaccharides and oligosaccharides need to be transported across the cell membrane following hemicellulose hydrolysis. Transport systems for a specified sugar depend on the microorganism and the surrounding conditions, including growth substrate, sugar concentration, oxygen availability, temperature and pH. It has often been found that more than one transporter for the same sugar may operate simultaneously. Two classes of mediated transport systems are common for microbial sugar uptake (Figure 11): (1) facilitated diffusion, which uses a concentration gradient and does not require metabolic energy; and (2) active transport, which requires energy for membrane potential as ATP or as phosphoenolpyruvate (PEP) (Gírio et al. 2010).

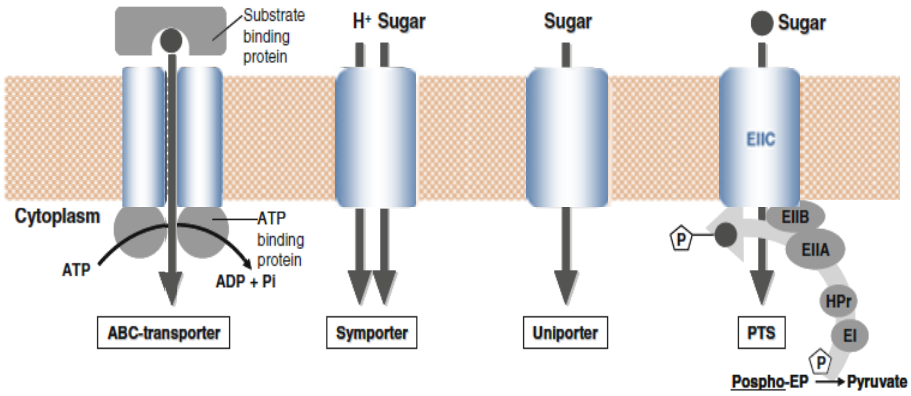

Figure 11: General view of the organisation and the mode of action of sugar transporters. In the carbohydrate phosphotransferase system (PTS), phosphoenolpyruvate donate a phosphoryl group to enzyme I of the PTS. Histidine-containing phosphocarrier protein delivers the phosphoryl group to the sugar-specific permease (EIIA) complex. Sugars accept the phosphoryl group from the EIIB. Phosphor-EP indicates phosphoenolpyruvate (Jojima et al. 2010).

The hemicellulose derived hexoses enter the upper part of glycolysis (*E. coli* and *S. cerevisiae*) through transport-dependant or independant phosphorylation followed by isomerisation (in the case of D-mannose) and the Leloir pathway for D-galactose. The catabolism of hemicellulose-derived pentoses (L-arabinose and D-xylose) is closely related, sharing common intermediates (Figure 12). Both D-xylose and L-arabinose are metabolised through the Pentose Phosphate Pathway (PPP). These pentoses can be converted into D-xylulose-5-phosphate, the PPP intermediate, through different pathways.

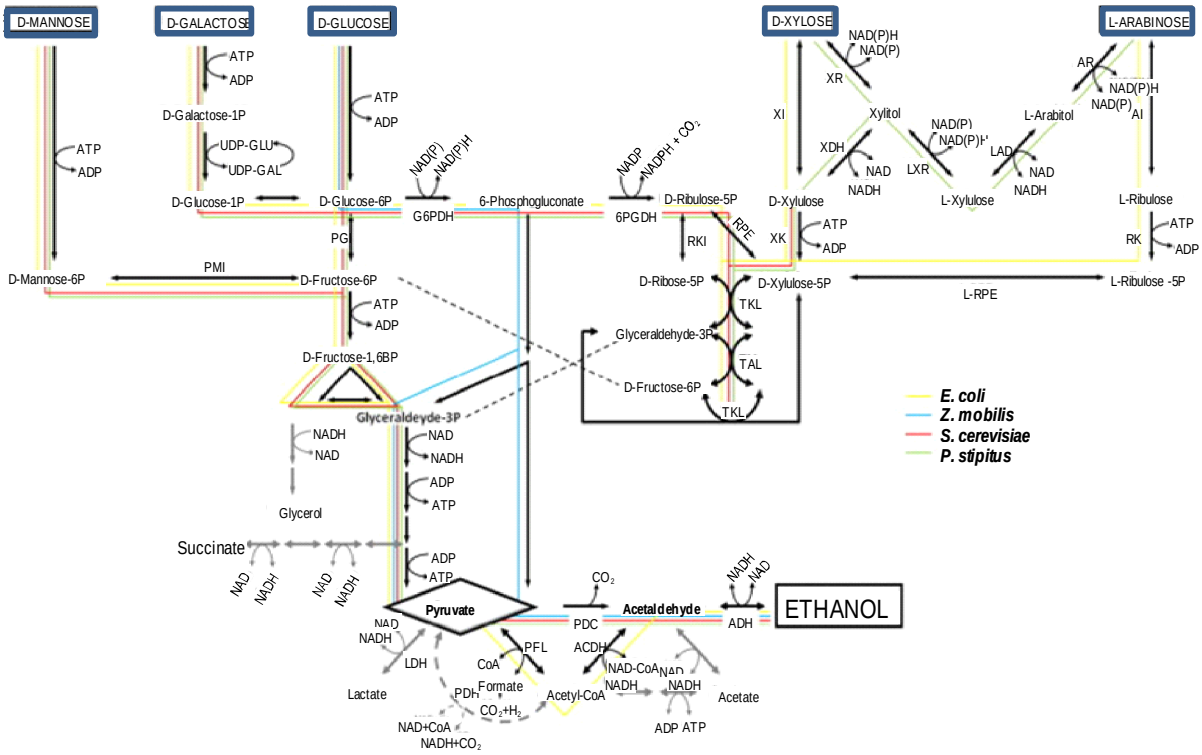

Figure 12: Fermentative pathways of hemicellulose-derived monosaccharides in *E. coli*, *Z. mobilis*, *S. cerevisiae* and *P. stipitis*. Natural traits are represented for each microorganism. *E. coli*, *S. cerevisiae* and *P. stipitis* use glycolysis and PPP to convert monosaccharides into pyruvate (D-fructose-6P and glyceraldeyde-3P are common intermediates between glycolysis and PPP). *Z. mobilis* utilise the Entner–Doudoroff (ED) pathway to convert glucose into pyruvate. In *Z. mobilis*, *S. cerevisiae* and *P. stipitis*, pyruvate is converted to ethanol via PDC/ADH, while in *E. coli*, acetyl-CoA is an intermediate. In *E. coli*, *S. cerevisiae* and *P. stipitis*, D-galactose is metabolised through the Leloir pathway and, as D-mannose, is converted into a glycolysis intermediate. *E. coli* and *P. stipitis* utilise pentoses (D-xylose and L-arabinose) through different pathways: through isomerases in the bacteria and reductases/dehydrogenases in the yeast. Abbrev: (PGI) glucose-6P isomerase (G6PDH) glucose-6P dehydrogenase (6-PGDH) 6-phosphogluconate dehydrogenase (RPE) D-ribulose-5P-3-epimerase (RKI) D-ribose-5P ketolisomerase (TKL) transketolase (TAL) transaldolase (PMI) mannose-6P isomerase (LDH) lactate dehydrogenase (PFL) pyruvate formate lyase (PDH) pyruvate dehydrogenase (acetyl-transferring) (ACDH) acetaldehyde dehydrogenase (acetylating) (PDC) pyruvate decarboxylase (ADH) alcohol dehydrogenase (XR) D-xylose reductase (XDH) xylitol dehydrogenase (XI) xylose isomerise (XK) xylulokinase (AI) L-arabinose isomerise (RK) L-ribulokinase (L-RPE) L-ribulose-5P-4-epimerase (AR) L-arabinose reductase (LAD) L-arabitol-4-dehydrogenase (LXR) L-xylulose reductase (Gírio et al. 2010).

In bacteria, the isomerisation step is followed by a phosphorylation reaction (and additional epimerisation, in the case of L-arabinose catabolism). Fungi employs redox reactions and use NAD(P)(H) as cofactors. Most bacteria utilise the xylose isomerase pathway in D-xylose catabolism. The presence of D-xylose isomerase has been reported in some yeasts (Gírio et al. 2010, Vongsuvanlert and Tani 1988) and filamentous fungi (Banerjee et al. 1994, Harhangi et al. 2003, Madhavan et al. 2009). Efficient ethanol producers like *S. cerevisiae* and *Z. mobilis* utilise different routes to convert sugars to pyruvate (EMP Pathway and ED Pathway, respectively). These microorganisms convert pyruvate into ethanol through pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). *E. coli* produces ethanol through mix-acid metabolism involving the production of acetyl-CoA as intermediate of ethanol production.

17. THIS STUDY

Fungi form an important role in the ecosystem (Adrio and Demain 2003, Polizeli et al. 2005). They decompose polysaccharides and recycle inorganic and organic material. For many decomposers the plant cell wall polysaccharides are their primary carbon energy source (Adrio and Demain 2003, Polizeli et al. 2005). Fungi have different enzymatic affinities for the three different cell wall components (cellulose, hemicellulose and lignin). White rot fungi primarily degrade the lignin component of wood (the cellulose and hemicellulose components to a lesser extent) giving the wood a bleached appearance. A brown rot fungus selectively removes hemicelluloses and cellulose, leaving mostly modified lignin (Irbe et al. 2001).

The genus *Aspergillus* is a group of filamentous fungi comprising pathogenic fungi (*A. fumigatus*, *A. flavus* and *A. parasiticus*) as well as industrially important black aspergilli (*A. oryzae*, *A. niger* and *A. tubingensis*). Several of these species (and their products) have obtained a GRAS (**G**enerally **R**egarded **A**s **S**afe) status and is receiving increased interest as hosts for heterologous protein production (van Zyl et al. 2010). The wide range of enzymes produced by *Aspergillus* for the degradation of plant cell wall polysaccharides is of particular importance to the food and feed industries. The one major limitation to fungi is that they are unable to produce high levels of ethanol.

The yeast *S. cerevisiae* has long been associated with the food and beverage industry (Ostergaard et al. 2000). This yeast is attractive as a tool for expression of recombinant proteins due to the variety of vector systems and promoters available, as well as the ease of product purification (Cereghino and Cregg 1999). It is currently under investigation for the possible role as host for bioethanol production through genetic engineering and expression of (primarily) fungal genes. The ideal yeast strain will have to be able to degrade cellulose, xylan and mannan effectively in order to obtain complete conversion of biomass to ethanol. Much progress has been made on the degradation of cellulose and xylan by *S. cerevisiae*, while mannan degradation has received less attention. This study focuses on the enzymes (Man1, Agal, CmMan5A) required for complete hydrolysis of galactomannan (Locust bean gum) and the influence of synergistic activity on the viscosity of Locust bean gum. The purpose of this study is to pave the way towards providing a cost effective means to decrease the quantity of agricultural and municipal lignocellulolytic waste while producing sustainable second generation biofuels.

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EXPRESSION AND EVALUATION OF ENZYMES REQUIRED FOR THE DEGRADATION OF GALACTOMANNAN

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3.1. ABSTRACT

The *Aspergillus aculeatus* endo-β-mannanse (*man1*) and *Talaromyces emersonii* α-galactosidase (*Agal*) genes were expressed in *S. cerevisiae* Y294. The *A. niger* β-mannosidase (*cAnmndA*) and synthetic *Cellvibrio mixtus* β-mannosidase (*CmMan5A*) were expressed in *A. niger*. The Man1, Agal, cAnmndA and CmMan5A enzymes displayed optimal pH of 5.47, 2.37, 3.4 and 3.4, respectively, and optimal temperatures of 70°C. Activity levels for Man1 and Agal peaked at 36.08 and 256.83 nkat/ml, respectively. Activity against *p*NPM for cAnmndA and CmMan5A were obtained at 11.61 and 7.58 nkat/ml, respectively. The protein species of deglycosylated Man1 and Agal revealed sizes of 40 and 60 kDa, respectively. The enzymatic behaviour of Man1, Agal and CmMan5A resulted in a significant decrease in the viscosity of galactomannan (Locust bean gum) when used synergistically, confirming the hydrolytic properties thereof. Co-expression of the *man1* and *Agal* genes in *S. cerevisiae* Y294[Agal-man1] displayed a significant decrease in enzyme secretion compared to individual expression of the respective genes.

Keywords: *A. niger, S. cerevisiae*, β-mannanase, β-mannosidase, α-galactosidase

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3.2. INTRODUCTION

The increase in oil prices and negative impact of fossil fuels has led to a search for alternative forms of fuel. Bioethanol is used as a gasoline replacement in numerous parts of the world (Sun and Cheng 2002). Consolidated bioprocessing, a single step process of converting lignocellulolytic material to ethanol by one microorganism would enable a cost-effective and commercially viable method for the production of bioethanol (Lynd et al. 2002). *Saccharomyces cerevisiae* has proven to be the most efficient microorganism for ethanol production from sugars such as glucose and mannose. This yeast has been used extensively in the production of heterologous proteins and has a long history with the fermentation of wine and brewing industries, due to its ease of genetic manipulations and GRAS status (**G**enerally **R**egarded **A**s **S**afe) (Gellissen and Hollenberg 1997, Müller et al. 1998).

Depending on the source, plant biomass consists of 40-45% cellulose, 25-50% hemicellulose and 10-40% lignin, (Moreira and Filho 2008). Mannan, together with xylan, constitutes the major hemicellulose components contributing to as much as one third of the lignocelluloses portion. Mannan consists of glucomannan, galactomannan and galactoglucomannan, complete hydrolysis of which involves the concerted effort of several enzymes, namely β-mannanases (1,4-β-D-mannan mannohydrolases, EC 3.2.1.78), β-mannosidases (1,4-β-D-mannopyranoside hydrolases, EC 3.2.1.25), α-galactosidases (1,6-α-galactoside galactohydrolases, EC 3.2.1.22), β-glucosidases (1,4-β-D-glucoside glucohydrolases, EC 3.2.1.21) and acetyl-mannan esterases (Moreira and Filho 2008). Degradation begins with the endo-β-1,4-mannanases cleaving the β-1,4-mannopyranosyl linkages in the mannan backbone, resulting in oligosaccharides of varying length (Stoll et al. 2000). Hydrolysis of the oligomannans is performed by the β-mannosidases, releasing single mannose units (Moreira and Filho 2008). The α-gaactosidases remove the galactose units from the mannan backbone (McCutchen et al. 1996), while β-glucosidases and acetyl-mannan esterases catalyse the removal of glucose and acetic acid, respectively (Moreira and Filho 2008).

Mannan degrading enzymes are naturally produced by numerous organisms and are involved in the breakdown of complex structures to simple units. The filamentous fungi, *Aspergillus niger* produce an array of mannan degrading enzymes and have been extensively studied (Dalbøge 1997). The Aspergilli are versatile organisms with the ability to grow on

inexpensive material, such as agricultural waste (Aristidou and Penttilä 2000). Together with its GRAS status and long standing history in the food industry, *A. niger* makes for an ideal host for the production of heterologous proteins.

With the inability for *S. cerevisiae* to grow on complex sugars present in lignocellulose and *A. niger*'s inability to produce high levels of ethanol, combining the positive attributes of these two microorganisms resulted in the construction of a polysaccharide degrading *S. cerevisiae* strain with the capabilities of utilising renewable, natural substrates. The β-mannanase (*man1*) from *A. aculeatus* and α-galactosidase (*Agal*) of *T. emersonii* were functionally expressed in *S. cerevisiae* Y294. The β-mannosidase (*cAnmndA* and *CmMan5A*) from *A. niger* and *C. mixtus*, respectively, were functionally expressed in *A. niger* D15. The enzymes were partially characterised and evaluated on Locust bean gum.

3.3. MATERIALS AND METHODS

3.3.1. MEDIA AND CULTIVATION

All chemicals used were of analytical grade. *Escherichia coli* DH5α was used as host strain for the recombinant DNA manipulations and plasmid propagation. All bacterial cultivations took place at 37°C in Terrific Broth (12 g/l tryptone, 24 g/l yeast extract, 4 ml/l glycerol, 0.1 M phosphate buffer) containing 100 µg/ml ampicillin (Sambrook et al. 2001). All *S. cerevisiae* strains were aerobically cultivated on a rotary shaker (200 rpm) at 30˚C in 125 ml Erlenmeyer flasks containing 25 ml synthetic complete medium (1.7 g/l yeast nitrogen base without amino acids (Difco laboratories), 20 g/l glucose and supplemented with appropriate amino acids). The *S. cerevisiae* Y294 transformants were selected and maintained on agar plates of the same composition, whereas the parental strain was maintained on YPD. *Aspergillus niger* parental strain was maintained on spore plates (1 g/l yeast extract, 2 g/l casamino acids, 10 g/l glucose, 0.4 g/l magnesium sulphate heptahydrate, 2 g/l peptone, 0.01 M uridine, 6 g/l $NaNO_3$ and trace elements) (Punt and Van den Hondel 1992). For heterologous protein expression, the *A. niger* fungal strains were cultivated in double strength minimal media (2x MM, 100 g/l glucose) lacking uridine (Rose and Van Zyl 2002).

3.3.2. STRAINS AND PLASMIDS

The relevant genotypes of yeast, fungal and bacterial strains as well as relevant plasmids used in this study are listed in Table 1.

Table 1: Strains and plasmids used in this study

Yeast strains:		
S. cS. cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-289*	ATCC 201160
S. cS. cerevisiae Y294[BBH1]	*URA3 ENO1$_P$-ENO1$_T$*	Njokweni et al. (2012)
S. cS. cerevisiae Y294[BBH4]	*URA3 ENO1$_P$-XYNSEC-ENO1$_T$*	This study
S. cS. cerevisiae Y294[Agal-man1]	*URA3 ENO1$_P$-Agal-ENO1$_T$*; *ENO1$_P$-man1-ENO1$_T$*	This study
S. cS. cerevisiae Y294[man1]	*URA3 ENO1$_P$-man1-ENO1$_T$*	This study
S. cS. cerevisiae Y294[Agal]	*URA3 ENO1$_P$-Agal-ENO1$_T$*	This study
Fungal strains:		
A. niger 10864	Wild type	ATCC 10864
A. niger D15	*pyrG prtT phmA* (non-acidifying)	Wiebe et al. (2001)
A. niger D15[GTP2]	*pyrG$^+$ gpd$_P$-glaA$_T$*	
A. niger D15[CmMan5A]	*pyrG$^+$ gpd$_P$-CmMan5A-glaA$_T$*	This study
A. niger D15[cAnmndA]	*pyrG$^+$ gpd$_P$-cAnmndA-glaA$_T$*	This study
Bacterial strains*:*		
E. coli DH5α	*fhuA2Δ(argF-lacZ)U169 phoAV44Φ80 Δ(lacZ) M15gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Sambrook and Russel (2001)
E. coli DH5α[Bluescript]	*bla*	This study
E. coli DH5α[XYNSEC-CmMan5A]	*bla XYNSEC-CmMan5A*	This study
Plasmids:		
pBBH1	*bla URA3 ENO1$_P$-ENO1$_T$*	Njokweni et al. (2012)
pBBH4	*bla URA3 ENO1$_P$-XYNSEC-ENO1$_T$*	Njokweni et al. (2012)
pBBH1-man1	*bla URA3 ENO1$_P$-man1-ENO1$_T$*	This laboratory
pBBH1-man1-Agal	*bla URA3 ENO1$_P$-Agal-ENO1$_T$*; *ENO1$_P$-man1-ENO1$_T$*	This study
pBBH1-Agal	*bla URA3 ENO1$_P$-Agal-ENO1$_T$*	This study
pBBH4-XYNSEC-CmMan5A	*bla URA3 ENO1$_P$-XYNSEC-CmMan5A-ENO1$_T$*	This study
pGTP2	*bla gpd$_P$-glaA$_T$; pyrG$_P$-pyrG-pyrG$_T$*	This laboratory
pGTP2-gAnmndA	*bla gpd$_P$-gAnmndA-glaA$_T$*; *pyrG$_P$-pyrG-pyrG$_T$*	This laboratory
pGTP2-cAnmndA	*bla gpd$_P$-cAnmndA-glaA$_T$*; *pyrG$_P$-pyrG-pyrG$_T$*	This study
pGTP2-CmMan5A	*bla gpd$_P$-CmMan5A-glaA$_T$*; *pyrG$_P$-pyrG-pyrG$_T$*	This study
pBluescript II SK (+)	*bla*	Stratagene
pBluescript-man1	*bla man1*	Setati et al. (2001)
pBluescript-XYNSEC-CmMan5A	*bla XYNSEC-CmMan5A*	This study
pMA-RQ-CmMan5A	*bla CmMan5A*	GeneArt
pRDH213-Agal	*URA3 ENO1$_P$-Agal-ENO1$_T$*	This laboratory

3.3.3. DNA MANIPULATIONS AND AMPLIFICATION BY PCR

Standard protocols were followed for all DNA manipulations (Sambrook et al. 2001). All enzymes used for restriction digestions and ligations were purchased from Roche (South Africa) and used as recommended by the supplier. The *A. niger* 10864 strains were grown in minimal media for 72 hours, mycelia harvested and frozen in liquid nitrogen prior to total DNA and RNA isolation according to La Grange et al. (1996). The sample was treated with DNase (Roche) to remove the DNA. Total cDNA was created using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit with the OligoT primer (Fermentas). The *cAnmndA* (2.8 kb) was amplified using a GeneAmp® PCR system 9700 (Applied Biosystems), TaKaRa^{TM} Ex Taq^{TM} Polymerase (TaKaRa Bio Inc.) and oligo-primers AnmndA-R and AnmndA-L (Table 2). Resulting PCR product was eluted from agarose gel (1%) using Zymoclean™ Gel DNA Recovery Kit (Zymo Research).

Table 2: Primer sequences used in this study

Primer name	Sequence (Restriction sites underlined)	Tm (°C)	RE site
AnMndA-R	5'-TAGGCGCGCCTGCGAATGCTATTGATAAT-3'	63	*Asc*I
AnMndA-L	5'-GCTTAATTAACCCTTCTAGCTGTACGC-3'	57	*Pac*I
MndAint-R	5'-GCTGCCAATACAAGGA-3'	49	
mndAint-L	5'-GTCTATGTCCTGAACACG-3'	49	
CmMan5A2-R	5'-TGGCGCGCCCTCGAG-3'	62	*Xho*I
CmMan5AXYNSEC2-L	5'-TTCGCGAGTTGCTGAATCTAATTCTGCTG-3'	60	*Nru*I

3.3.4. PLASMID CONSTRUCTION

A brief overview of the construction of some of the vectors is shown in Figs. 1 and 2. Construction of pBBH1-Agal-man1 involved digestion of pRDH213-Agal with *Pac*I and *Asc*I restriction enzymes. The *Aga*l (1.3 kb fragment) was subsequently ligated into pBBH1 (digested with *Pac*I and *Asc*I) resulting in pBBH1-Agal. This construct was digested with *Bam*HI and *Bgl*II and the 2.8 kb *ENO1$_P$-Agal-ENO1$_T$* cassette ligated into pBBH1-man1 (following linearisation with *Bam*HI) to yield the final pBBH1-Agal-man1 construct.

Following cDNA synthesis, the *cAnmndA* was ligated into pTZ57R (Fermentas) using the InsTAclone PCR cloning kit (Fermentas). Sequence verification was confirmed with the

dideoxy chain termination method, with an ABI PRISM^{TM} 3100 Genetic Analyser. The gene was subsequently retrieved by digestion with restriction enzymes, *Pac*I and *Asc*I, and ligated into the corresponding sites on pGTP2, yielding pGTP2-cAnmndA.

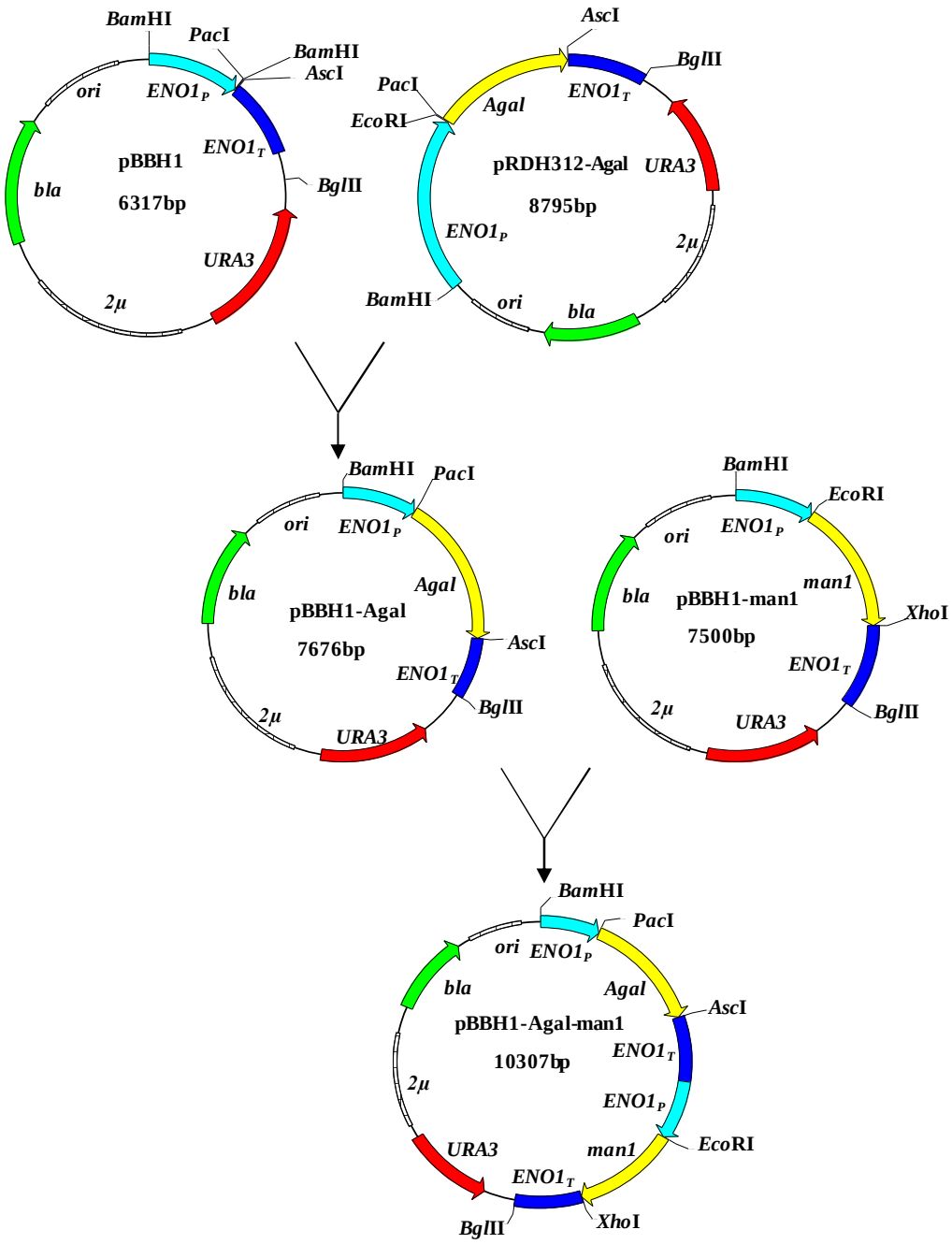

Fig. 1: Overview of pBBH1-AgaI-man1 plasmid construction (note vector inserts are not to scale, images represent graphical indication and are not necessarily comparably scalable).

The synthetically designed *Cellvibrio mixtus* β-mannosidase (*CmMan5A*) was obtained from GeneArt® containing only codons favoured by *S. cerevisiae*. Construction of pGTP2-CmMan5A involved excising the synthetic gene from pMA-RQ-CmMan5A (*Pac*I

and *Asc*I) and cloning into pGTP2 at the *Pac*I and *Asc*I sites. For construction of pBluescript-XYNSEC-CmMan5A, the *CmMan5A* gene was amplified from the pMA-RQ-CmMan5A and cloned into pBBH4 at the *Nru*I and *Xho*I sites (thereby incorporating the *XYNSEC* secretion signal) generating pBBH4-XYNSEC-CmMan5A. Subsequent digestion of this vector with *Eco*RI and *Xho*I yielded a 1.4 kb fragment, which was cloned into pBluescript at the corresponding sites resulting in the vector pBluescript-XYNSEC-CmMan5A. The *XYNSEC* DNA sequence codes for the secretion signal of *xyn2* of *Trichoderma reesei*.

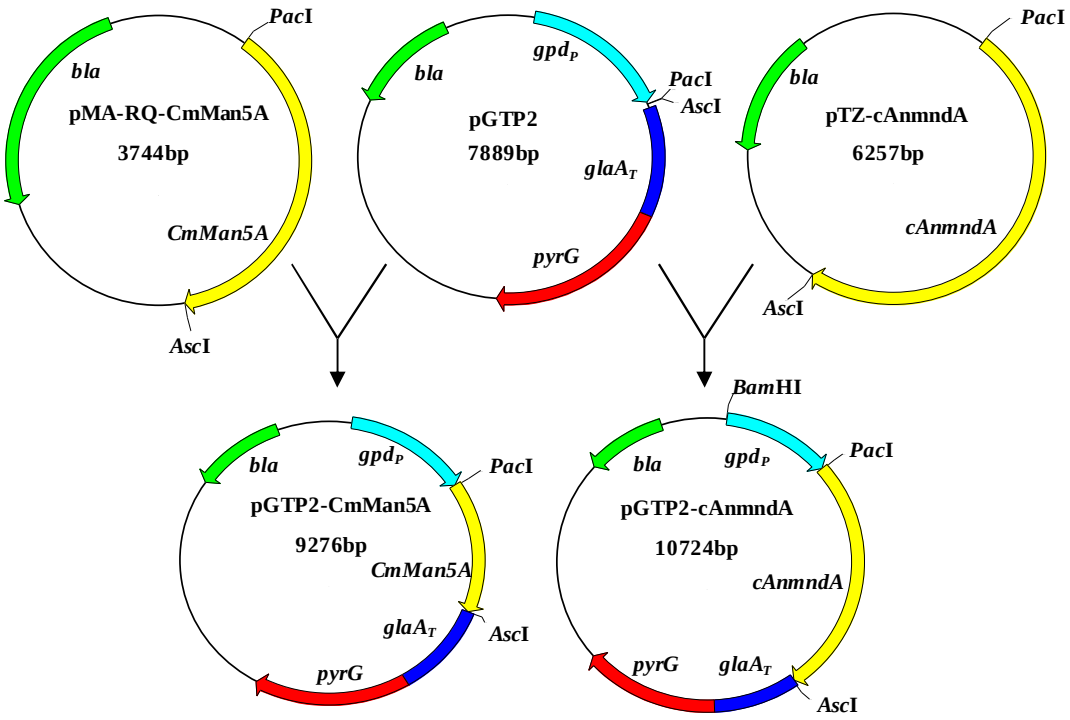

Fig. 2: Overview of mannosidase vector construction (note vector inserts are not to scale, images represent graphical indication and are not necessarily comparably scalable).

3.3.5. STRAIN DEVELOPMENT

Electrocompetent *S. cerevisiae* Y294 cells were prepared as described by Cho et al. (1999). The electrocompetent cells containing 1 µg of vector DNA were transferred to chilled 0.2 cm electroporation cuvette and pulsed at 1.4 kV, 200 Ω, 25 µF. The cells were resuspended in 1 ml YPDS and incubated at 30°C for 1 hour, after which the culture was plated onto SC^{-URA}

agar plates for selection. The *A. niger* D15 strain was transformed by means of spheroplasts using Lysing enzymes (Sigma) in accordance to Punt and van den Hondel (1992).

3.3.6. GROWTH DETERMINATION CONDITIONS

Yeast and bacterial pre-cultures were grown overnight to late stationary phase and used to inoculate fresh pre-warmed medium to an optical density (OD_{600}) of 0.1. Samples were taken at regular intervals and optical density measured using an X-MARK™ microtitre plate reader (Biorad, Hercules, CA, USA) to determine growth rate. All stains were cultured in triplicates. Bacterial cultures were supplemented with 100 µg/ml ampicillin every 24 hours in order to maintain plasmid stability.

3.3.7. PLATE ENZYME ASSAYS

The presence of extracellular β-mannanase activity was confirmed by using OBR-mannan prepared according to (Biely et al. 1985). OBR-mannan plates were prepared containing SC^{-URA} and 0.5% (w/v) OBR-mannan. The recombinant *S. cerevisiae* strains were transferred (spotted) to the OBR-mannan plates, where the secretion of β-mannanase was visualised by a clear halo or zone around the colony after incubation at 30°C for 24 hours.

3.3.8. LIQUID ACTIVITY ASSAYS

The β-mannanase and α-galactosidase activity was measured using the substrate Locust bean gum (LBG) and *p*-nitrophenyl-α-D-galactopyranoside (*p*NPGal), respectively. The cells were centrifuged at 13000 rpm for 2 minutes and the resulting supernatant used to determine the extracellular β-mannanase and α-galactosidase activities. Secreted β-mannanase activity was

determined using a modified dinitrosalicylic acid (DNS) reducing sugar assay (Bailey et al. 1992) with 0.5% (w/v) LBG as substrate. Recombinant strains were cultivated in their respective media. Samples were taken at regular intervals. Three 8 µl supernatant samples of each culture were pipetted into a 96-well PCR plate well containing 72 µl 0.5% LBG (w/v) substrate in 0.05 M citrate buffer (pH 5.0). The supernatant-substrate mixture was incubated at 50°C for 10 minutes in a 96-well thermocycler (GeneAmp® PCR system 9700 (Applied Biosystems)). Following the incubation step, 120 µl of DNS (10 g/l 2-hydroxy-3,5-dinitrobenzoate, 200 g/l potassium sodium-tartrate, 10 g/l sodium hydroxide, 2 g/l phenol, 0.5 g/l sodium sulfite) solution was added to each incubated sample. The samples were heated to 99.9°C for 5 minutes and then cooled to 4°C for 1 minute. The liquids were transferred with a multichannel micropipette into 96-well round-bottomed microtitre plates. The colorimetric changes were measured at OD_{540} with an X-MARK™ microtitre plate reader (Biorad, Hercules, CA, USA). Standard curves were prepared using mannose (Sigma, Stockholm, Sweden) at concentrations of 2.0-10 mg/ml. From the standard curves the enzyme activities of each culture supernatant could be calculated. Supernatants were diluted appropriately to ensure absorbance values fell within the range of the mannose standard curve. Background sugar levels in the supernatant samples were also detected and subtracted from the activity values.

Secreted α-galactosidase activity was determined by incubation of supernatant with 4 mM *p*NPGal in 0.05 M citrate phosphate buffer, pH 5 for 30 minutes. All enzymatic assays were performed in triplicates.

The extracellular β-mannosidase and α-galactosidase activities were determined using 4 mM *p*-nitrophenyl β-D-mannopyranoside (*p*NPM) and *p*NPGal, respectively, in 50 mM citrate buffer pH 3.4. Assays were performed as follows: 50 µl supernatant was added to 50 µl *p*NPM and incubated for 30 minutes at 50°C. Reactions were terminated by the addition of 200 µl of 1 M sodium carbonate. Colour release was measured at OD_{400} on a microtitre plate reader (as above) and converted to nkat/ml using *p*-nitrophenol as standard. When necessary, appropriate dilutions of the enzymes were made. All assays were performed in triplicate. Values were expressed in nkat/ml, where 1 kat equals 1 mol of mannose released per second.

Supernatant, intracellular fractions (cell lysis performed using a TissueLyser LT (QIAGEN)) and whole cell fractions of *E. coli* strains were incubated with *p*NPM in 50 mM citrate buffer

pH 5 at 50°C for 30 minutes. Assays were performed and measured as above. All assays were performed in triplicates.

3.3.9. DETERMINATION OF pH AND TEMPERATURE OPTIMA

Secreted β-mannanase and α-galactosidase from *S. cerevisiae* Y294[man1] *S. cerevisiae* Y294[Agal] and *S. cerevisiae* Y294[Agal-man1] and β-mannosidase from *A. niger* D15[cAnmndA] and *A. niger* D15[CmMan5A] were harvested and lyophilised prior to characterisation. The whole cell fractions of *E. coli* DH5α[XYNSEC-CmMan5A] were used for pH and temperature enzyme characterisation determinations. The pH and temperature optimum was determined using the recommended substrate for the respective enzymes. The LBG, *p*NPGal and *p*NPM substrates were buffered at pH 2.37, 3.4, 4.48, 5.47, 6.6 and 7.2 (with the addition of pH 1.72 for *p*NPGal), respectively, using 50 mM citrate phosphate buffer. The temperature optimum was determined using 0.5% LBG (w/v) in 50 mM citrate phosphate buffer (pH 5.47) for β-mannanase activity, 4 mM *p*NPGal (pH 1.72) for α-galactosidase activity and 4 mM *p*NPM (pH 5.47) for β-mannosidase activity from *E. coli* DH5α[XYNSEC-CmMan5A] and pH 3.4 for *A. niger* D15[cAnmndA] and *A. niger* D15[CmMan5A] strains. Activity was measured at 30, 40, 50, 60, 70 and 80°C, respectively.

3.3.10. PURIFICATION OF THE β-MANNOSIDASE

Supernatant of *A. niger* strains cultivated for 72 hours were collected and filtered through a 0.22 µm Whatman® paper. Solid ammonium sulphate was added to the filtrate with slow stirring overnight at 4°C to obtain 20, 40, 60 and 80% saturation, respectively. The precipitates were collected at each interval by centrifugation at 12 000 rpm at 4°C for 1 hour. The pellet was dissolved in 1 ml of sodium citrate buffer (pH 5) and subsequently dialysed overnight using an 8 kDa Spectra/Por® molecular porous membrane tubing (Spectrum

Medical Industries, Inc). The fractions were lyophilised and stored at -20°C for further analysis.

3.3.11. PROTEIN DEGLYCOSYLATION

The supernatant of *S. cerevisiae* Y294[man1], *S. cerevisiae* Y294[Agal], *S. cerevisiae* Y294[Agal-man1], *A. niger* D15[cAnmndA] and *A. niger* D15[CmMan5A] were collected after 48 hours and 72 hours, respectively. The supernatants were filtered through a 0.22 µm Whatman® filter paper and subsequently lyophilised prior to analysis.

The lyophilised protein from *S. cerevisiae* Y294[man1] *S. cerevisiae* Y294[Agal] and *S. cerevisiae* Y294[Agal-man1] was prepared at a concentration of 5 mg/100 µl deionised water and subjected to acetone precipitation (ratio of 1:2) overnight at -20°C. The protein pellet was harvested by centrifugation at 13000 rpm for 20 minutes at 4°C. The supernatant was removed and the pellet dissolved in 100 µl deionised water. The enzyme was subject to *N*-deglycosylation reactions using the PNGase H kit (New England Biolabs) according to the supplier's specifications.

3.3.12. SDS-PAGE ANALYSIS

Protein samples were subjected to denaturation at 100°C for 2 minutes in denaturing loading buffer (200 mM Tris–HCl pH 6.8, 8% (w/v) SDS, 0.4% bromophenol blue, 40% (v/v) glycerol and 400 mM DTT). A total of 5 mg/ml denatured protein were separated by 8% SDS-PAGE (Laemmli 1970). Silver staining was used to visualise the protein species according to O'Connell and Stults (1997). A protein ladder, Page Ruler^{TM} (Fermentas Inc), was used as a size marker.

3.3.13. LOCUST BEAN GUM RHEOLOGY

A 0.5 % (w/v) Locust bean gum solution was prepared in 50 mM citrate buffer pH 5 and used as substrate for viscosity analysis. Lyophilised Man1, CmMan5A and Agal enzymes were used in combination and individually for viscosity analysis. Viscosity measurements were performed on a Physica MCR 501 (Anton Paar, Germany) using a double gap configuration and heated at 50°C with a Peltier system (C-PTD200). Flow curves were analysed using the Rheoplus software and measurements taken at intervals of 10 sec (60 points) for 10 minutes per sample at a shear rate of 61.9/s. Initial viscosity was determined without enzyme, after which respective enzymes were added to a final concentration of 0.2 nkat/ml LBG for Man1 and 2 nkat/ml LBG for Agal and CmMan5A. All samples were analysed in triplicates.

3.4. RESULTS

3.4.1. STRAIN SELECTION AND CONFIRMATION

Saccharomyces cerevisiae Y294 and *Aspergillus niger* D15 were used as hosts for the heterologous production of the: *Aspergillus aculeatus* β-mannanase (*man1*) and *Talaromyces emersonii* α-galactosidase (*Agal*); and *Aspergillus niger* β-mannosidase (*AnmndA*) and *Cellvibrio mixtus* β-mannosidase (*CmMan5A*) enzymes, respectively. Different expression hosts were used due to the unsuccessful expression of the β-mannosidase in *S. cerevisiae*. The *man1* and *Agal* genes were cloned onto multi-copy, episomal vectors under transcriptional control of the constitutive *S. cerevisiae* enolase I gene (*ENOI*) promoter and terminator. Successful transformation was achieved in *S. cerevisiae* Y294 with the plasmids pBBH1-man1, pBBH1-Agal, pBBH1-Agal-man1 and pBBH1, respectively.

The cDNA copy of the *A. niger* β-mannosidase (*cAnmndA*) gene was obtained using primers based on the GenBank sequence (Accession number XM_001394595) of the *AnmndA* gene from *A. niger* (Ademark et al. 2001). The ~3 kb fragment encodes a 930 amino acid peptide, with a theoretical pI of 4.84 and molecular mass of 104 kDa. The gene was ligated into

pTZS7R. The DNA sequence revealed a 96.86% homology with the sequence of the *A. niger* (Ademark et al. 2001) (Fig. 1). The 1403 bp synthetic *C. mixtus CmMan5A* gene codes for a protein with 100% homology with the *Man5A* (Accession number AY526725) reported by Dias et al. (2004), with a theoretical pI of 4.73 and a molecular mass of 51 kDa. The *cAnmndA* and *CmMan5A* genes (containing their native secretion signals) were cloned into pGTP2, an integrative vector that theoretically yields multi-copies within the genome. The transcription is controlled by the *A. niger* glyceraldehyde-3-phosphate dehydrogenase (gpd_P) gene promoter and *A. awamori* glucoamylase ($glaA_T$) gene terminator. The truncated *C. mixtus CmMan5A* gene (no secretion signal sequence) was also ligated onto pBBH4 (containing the *XYNSEC* secretion signal). XYNSEC-CmMan5A was obtained and ligated into pBluescript to yield pBluescript-XYNSEC-CmMan5A. Successful transformation of *A. niger* D15 occurred using plasmids pGTP2-cAnmndA and pGTP2-CmMan5A, respectively and *E. coli* with plasmid pBluescript-XYNSEC-CmMan5A. The *S. cerevisiae* Y294[BBH1], *A. niger* D15[GTP2] and *E. coli* DH5α[Bluescript] were used as negative control strains.

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1     MRHSIGLAAALLAPTLPVALGQYIRDLSTEKWTLSSRALNRTVPAQFPSQVHLDLLRAGV
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1     MRHSIGLAAALLAPTLPVALGQYIRDLSTEKWTLSSRALNRTVPAQFPSQVHLDLLRAGV

61    IDDPYHGLNDFNLRWIAAANWTYTSQPIKGLLDNYDSTWLVFDGLDTFATISFCGQQIAS
      |   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61    IGE.YHGLNDFNLRWIAAANWTYTSQPIKGLLDNYDSTWLVFDGLDTFATISFCGQQIAS

121   TDNQFRQYAFDVSTALGSCKGDPVLSINFGSAPNIVDAIAQDSNSQKWPDDVQLTYEYPN
      |||||||||| |||||||||||||||||||||||||||| ||||||||||||||||
120   TDNQFRQYAFGVSTALGSCKGDPVLSINFGSAPNIVDAIAQDSNTQKWPDDVQLTYEYPN

181   RWFMRKEQSDFGWDWGPAFAPAGPWKPAYIVQLDKKESVYVLNTDLDIYRKGQINYLPPD
      |||||||||||||||||||||||||||||||||||||||||||||||| ||||| ||
180   RWFMRKEQSDFGWDWGPAFAPAGPWKPAYIVQLDKKESVYVLNTDLDIYRKSQINYLSPD

241   QSQPWVVNASIDILGPLPTKPTMSIEVRDTHSGTILTSRTLNNVSVAGNAITGVTVLDGL
      |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
240   QSQPWVVNASIDILGPLPAKPTMSIEVRDTHSGTILTSRTLNNVSVAGNAITGVTVLDGL

301   TPKLWWPQGLGDQNLYNVSITVQSRGNQTVASVNKRTGFRTIFLNQRNITEAQRAQGIAP
      |||||||||||||||||||||| |||||||||||||||||||||||| ||||||||
300   TPKLWWPQGLGDQNLYNVSITVQRRGNQTVASVNKRTGFRTIFLNQRNITEVQRAQGIAP

361   GANWHFEVNGHEFYAKGSNLIPPDSFWTRVTEEKMSRLFDAVVVGNQNMLRVWSSGAYLH
      |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
360   GANWHFEVNGHEFYAKGSNLIPPDSFWTRVTEERMSRLFDAVVVGNQNMLRVWSSGAYLH

421   DYIYDLADEKGILLWSEFEFSDALYPSDDAFLENVAAEIVYNVRRVNHHPSLALWAGGNE
      |||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
420   DYIYDLADEKDILLWSEFEFSDALYPSDDAFLENVAAEIVYNVRRVNHHPSLALWAGGNE
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481   IESLMLPRVKDAAPSSYSYYVGEYEKMYISLFLPLVYENTRSISYSPSSTTEGYLYIDLS
      |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
480   IESLMLPRVKDAAPSSYSYYVDEYEKMYISLFLPLVYENTRSISYSPSSTTEGYLYIDLS

541   APVPMAERYDNTTSGSYYGDTDHYDYDTSVAFDYGSYPVGRFANEFGFHSMPSLQTWQQA
      |||||||||| |||||||||||||||||| |||||||||||||||||||||||||||||
540   APVPMAERYDSTTSGSYYGDTDHYDYDTSVASDYGSYPVGRFANEFGFHSMPSLQTWQQA

601   VDTEDLYFNSSVVMLRNHHDPAGGLMTDNYANSATGMGEMTMGVVSYYPIPSKSDHISNF
      |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
600   VDTEDLYFNSSVVMLRNHHDPAGGLMTDNYANSATGMGEMTMGVISYYPIPSKSDHISNF

661   SAWCHATQLFQADMYKSQIQFYRRGSGMPERQLGSLYWQLEDIWQAPSWAGIEYGGRWKV
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
660   SAWCHATQLFQADMYKSQIQFYRRGSGMPERQLGSLYWQLEDIWQAPSWAGIEYGGRWKV

721   LHHVMRDIYQPVIVSPFWNYTTGSLDVYVTSDLWSPAAGTVDLTWLDLSGRPIAGNAGTP
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
720   LHHVMRDIYQPVIVSPFWNYTTGSLDVYVTSDLWSPAAGTVDLTWLDLSGRPIAGNAGTP

781   KSVPFTVGGLNSTRIYGTNVSSLGLPDTKDAVLILSLSAHGRLPNSDRTTNLTHENYATL
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
780   KSVPFTVGGLNSTRIYGTNVSSLGLPDTKDAVLILSLSAHGRLPNSDRTTNLTHENYATL


841   SWPKDLKIVDPGLKIGHSSKKTTVTVEATSGVSLYTWLDYPEGVVGYFEENAFVLAPGEK
      |||||||||||||| | | | ||||||||||||||||||||||||||||||||||||||
840   SWPKDLKIVDPGLKLGYSPKRTTVTVEATSGVSLYTWLDYPEGVVGYFEENAFVLAPGEK

901   KEISFTVLEDTTDGAWVRNITVQSLWDQKVRG
      ||| |||| ||||||||||||||||||||||
900   KEIGFTVLDDTTDGAWVRNITVQSLWDQKVRG
```

Fig. 1: Amino acid sequence alignment of the cAnmndA (upper line) and mAnmndA gene (lower line) revealed a 97.74% homology. A three base pair deletion (TCC) at position 197 was present in the cDNA sequence.

3.4.2. PLATE ASSAY

Recombinant yeast strains *S. cerevisiae* Y294[man1], [Agal], [Agal-man1] and [BBH1] were cultured overnight in SC^{-URA} broth and transferred (spotted) to OBR-mannan plates and screened for secretion of β-mannanase activity. Hydrolysis was detected by the appearance of a clear zone around the colonies (Fig. 2). Zones were apparent for recombinant strains *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[Agal-man1]. No zones were visible for the control strain and *S. cerevisiae* Y294[Agal] exhibiting no endo-mannanase activity.

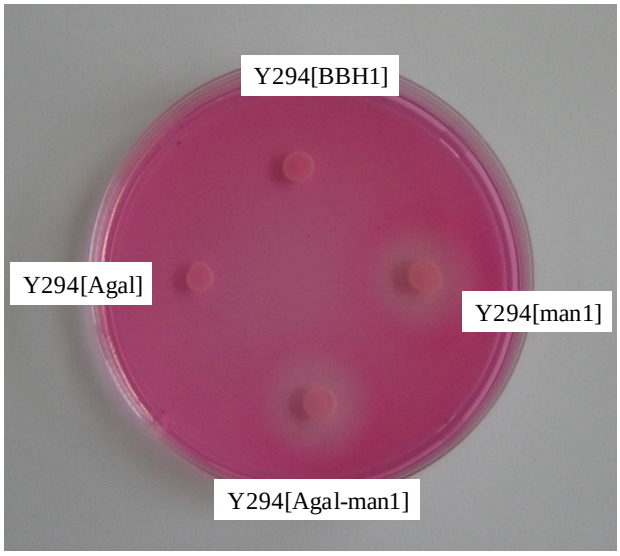

Fig. 2: Recombinant *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[Agal-man1] strains displaying extracellular β-mannanase activity on SC^{-URA} agar plates containing 0.5% (w/v) OBR-mannan after 24 hours of incubation at 30°C.

3.4.3. LIQUID ASSAY

S. cerevisiae Y294 strains were grown for 48 hours in SC^{-URA} media with supernatant samples taken at regular intervals (Fig. 3B) and enzyme activities determined against Locust bean gum and *p*NPGal. Strains *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[Agal] secreted active Man1 and Agal into the medium, respectively, while *S. cerevisiae* Y294[Agal-man1] co-expressed and secreted active Man1 and Agal into the medium. The highest level of activity for Man1 was 36.08 nkat/ml after 48 hours, which is 1.2 times more than that of the co-expressing *S. cerevisiae* Y294[Agal-man1] strain (28.30 nkat/ml), while Agal gave maximum activity of 253.83 nkat/ml after 48 hours, 1.3 times that of the co-expressing *S. cerevisiae* Y294[Agal-man1] (185.118 nkat/ml).

The production of heterologous cAnmndA, CmMan5A and XYNSEC-CmMan5A by *A. niger* D15[cAnmndA], *A. niger* D15[CmMan5A] and *E. coli* DH5α[XYNSEC-CmMan5A], respectively, were monitored over time. The fungal strains were cultivated over a period of 180 hours in 2xMM. Highest levels of activity on *p*NPM was observed for cAnmndA at 11.61 nkat/ml after 156 hours (Fig. 4A), 1.5 times

greater than that of CmMan5A (7.58 nkat/ml after 168 hours). The *E. coli* DH5α strains were cultivated for 144 hours in TB media. Whole cell, extracellular and intracellular fractions were tested for activity against *p*NPM. Maximum activity was obtained with whole cells at 2.14 nkat/ml and 1.67 nkat/ml for intracellular fractions after 144 hours (Fig. 4B).

3.4.4. DETERMINING pH AND TEMPERATURE OPTIMA

The pH and temperature optima was determined for Man1, Agal, cAnmndA and CmMan5A using lyophilized protein (5 mg/ 100 ml), whereas the intact whole cell cultures of bacterial strain *E. coli* DH5α[XYNSEC-CmMan5A] was used for the XYNSEC-CmMan5A. The recombinant Man1, Agal, cAnmndA, CmMan5A and XYNSEC-CmMan5A activity peaked at pH 5.47, 2.37, 3.4, 3.4 and 5.47, respectively (Figs. 3C and 4C, respectively). The strain *S. cerevisiae* Y294[Agal-man1] strain displayed similar pH optima for each of the respective enzymes, Agal and Man1, showing no significant influence on each others' activity when co-expressed. Strains *S. cerevisiae* Y294[Agal], *S. cerevisiae* Y294[man1], *A. niger* D15[cAnmndA] and *A. niger* D15[CmMan5A] displayed optimal activity at 70°C (Figs. 3D and 4D, respectively). XYNSEC-CmMan5A displayed optimal activity at 50°C (Fig. 4D).

3.4.5. GROWTH DETERMINATION CURVE

The recombinant strains *S. cerevisiae* Y294[man1], *S. cerevisiae* Y294[Agal-man1], *S. cerevisiae* Y294[Agal] were cultured in SC^{-URA} medium and recombinant strain *E. coli* DH5α[XYNSEC-CmMan5A] in TB medium supplemented with 100 mg/ml ampicillin every 24 hours. Samples were taken regularly and biomass production was measured at OD_{600} (Figs. 3A and 4B, respectively). Strains *S. cerevisiae* Y294[BBH1] and *E. coli* DH5α[pBluescript] were used as negative control strains. The activities displayed in Figs. 3B and 4A and B had been normalised against the respective negative control strains (values obtained for the negative control strains had been deduced), hence no data is

displayed for the control strains. From the growth curves it is evident that the addition of foreign genes had no significant effect or metabolic burden on the biomass production of the strains.

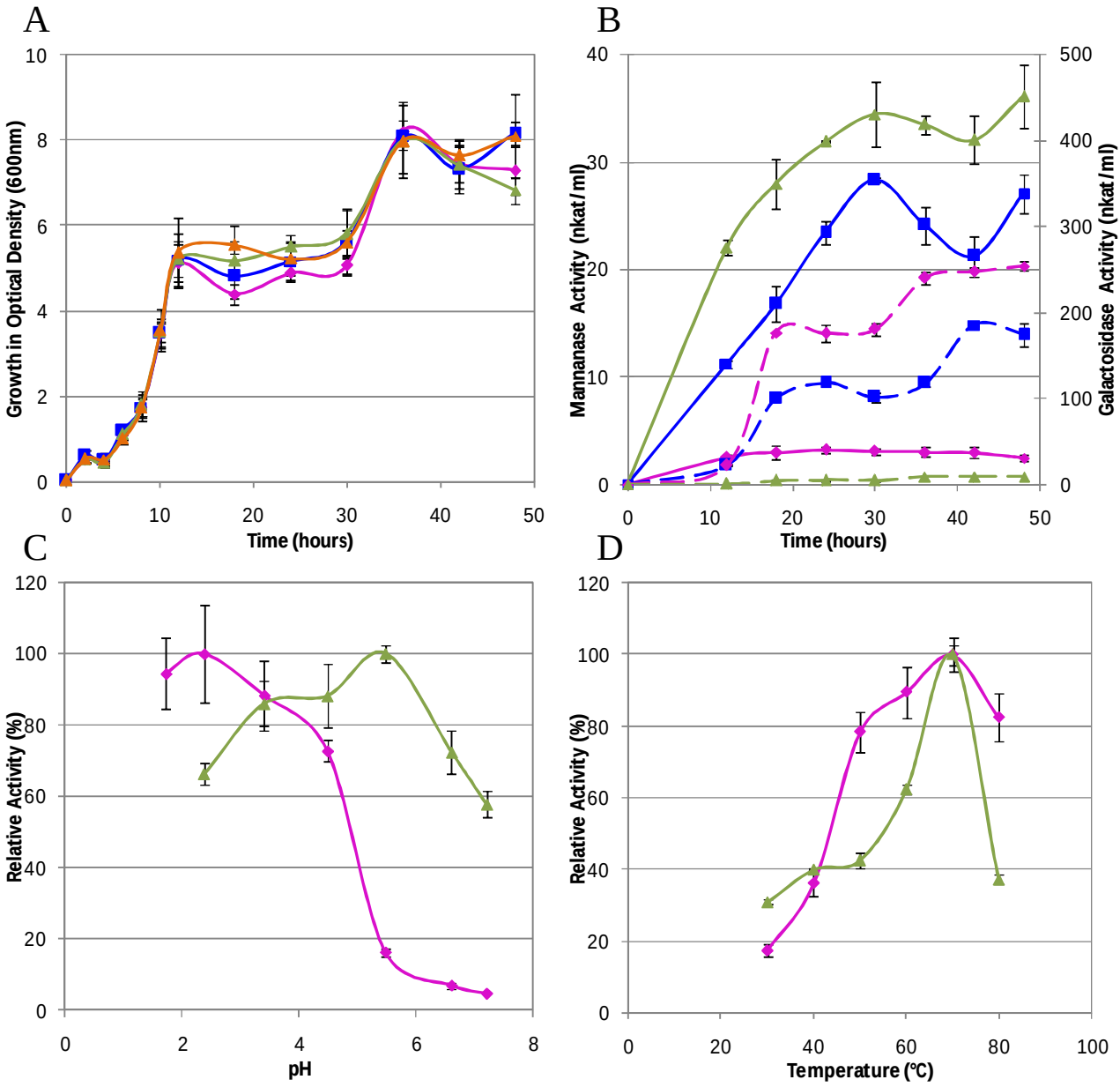

Fig. 3: The recombinant strains —▲— *S. cerevisiae* Y294[BBH1] —▲— *S. cerevisiae* Y294[man1], —♦— *S. cerevisiae* Y294[Agal] and -■-*S. cerevisiae* Y294[Agal-man1] were cultured in SC^{-URA} media and simultaneously monitored for: (A) biomass production in optical density (OD_{600}), and (B) enzyme activity on 0.5% (w/v) LBG for Man1 activity (solid lines) and 4 mM *p*NPGal for Agal activity (dashed lines). Values have been normalised against the values obtained for *S. cerevisiae* Y294[BBH1]. The optimal pH (C) and temperature (D) of lyophilised protein from —▲— *S. cerevisiae* Y294[man1] and —♦— *S. cerevisiae* Y294[Agal] incubated for 30 mins at 50°C on 0.5% (w/v) LBG and 4 mM *p*NPGal, respectively. Error bars indicate the standard deviation from the mean value.

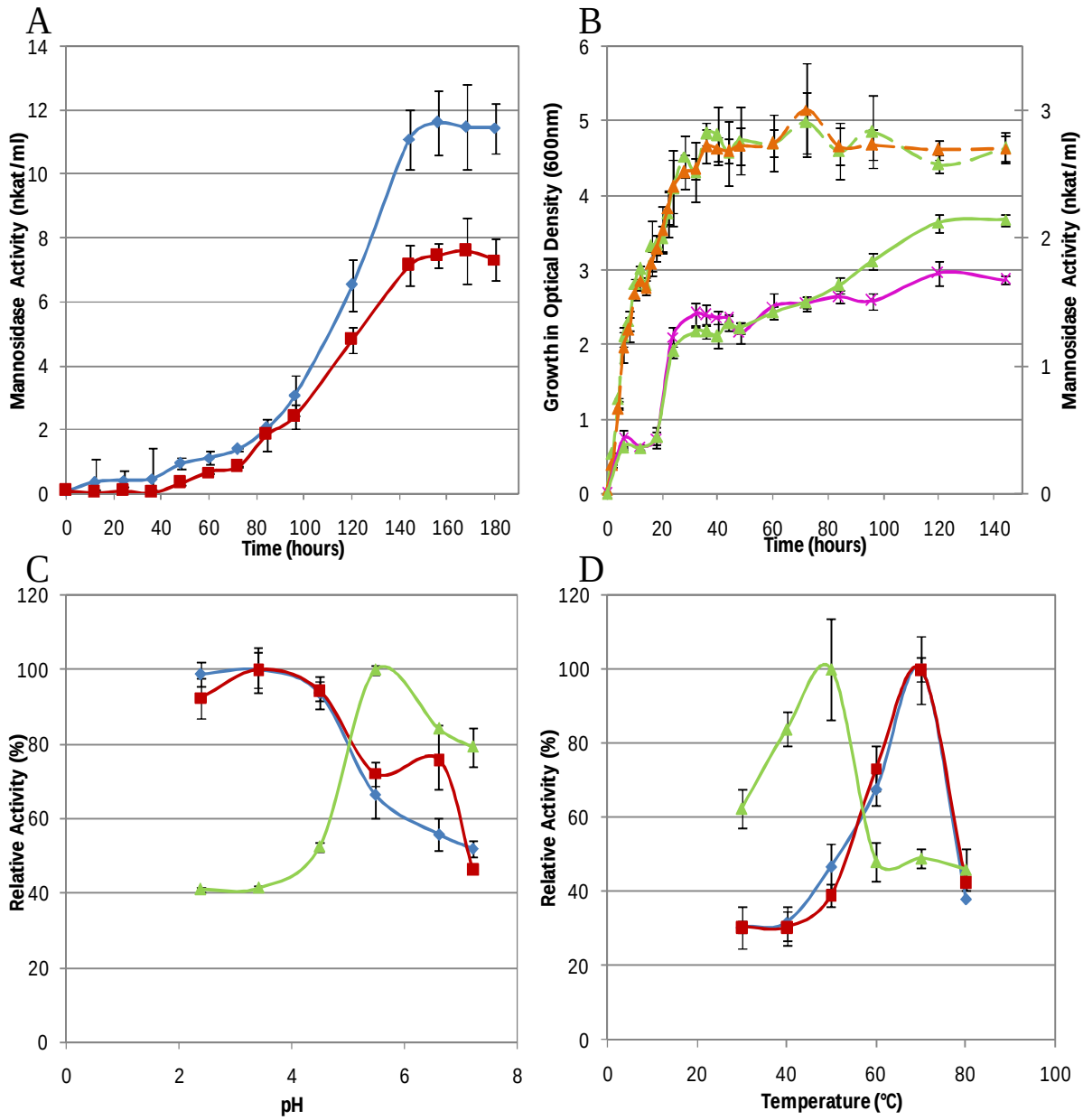

Fig. 4: The Recombinant strains —◆— *A. niger* D15[cAnmndA] and —■— *A. niger* D15[CmMan5A] were cultured on 2xMM for 180 hours and monitored for enzyme activity (A). Bacterial strain —▲— *E. coli* DH5α[XYNSEC-CmMan5A] and —▲— *E. coli* DH5α[Bluescript] was cultured in TB media supplemented with 100 µg/ml ampicillin for 144 hours and monitored for (B) biomass production in optical density (OD_{600}) (dashed lines) and enzyme activity (solid lines), where —▲— whole cell and —✕— intracellular fractions were investigated for XYNSEC-CmMan5A activity. The optimal pH (C) and temperature (D) of lyophilized protein from —◆— *A. niger* D15[cAnmndA], —■— *A. niger* D15[CmMan5A] and intact bacterial strain —▲— *E. coli* DH5α[XYNSEC-CmMan5A] incubated for 30 mins at 50°C on 4 mM *p*NPM. Error bars indicate the standard deviation from the mean value.

3.4.6. SDS-PAGE ANALYSIS

Analysis with 8% SDS-PAGE revealed a protein species for strains *S. cerevisiae* Y294[man1], *S. cerevisiae* Y294[Agal-man1] and *S. cerevisiae* Y294[Agal]. Fig. 5A shows the deglycosylated and glycosylated protein species of lyophilised (5 mg/100 µl) supernatant for strains *S. cerevisiae* Y294[Agal], *S. cerevisiae* Y294[Agal-man1] and *S. cerevisiae* Y294[man1], respectively. The deglycosylated Agal and Man1 were shown to be 60 kDa (lane 1 and 2) and 40 kDa (lane 2 and 3), respectively. The band size of Man1 correspond to the size previously reported by Setati et al. (2001), whereas Agal yielded a band slightly smaller than the native Agal produced by *T. emersonii* (Similä et al. 2010). These species do not occur in the control strain *S. cerevisiae* Y294[BBH1] (lane 4). Glycosylation is prevalent in protein species Agal (Fig. 5B lane 1) and Man1 (Fig. 5B lane 3) yielding slightly bigger diffuse bands than that of the deglycosylated protein counterparts.

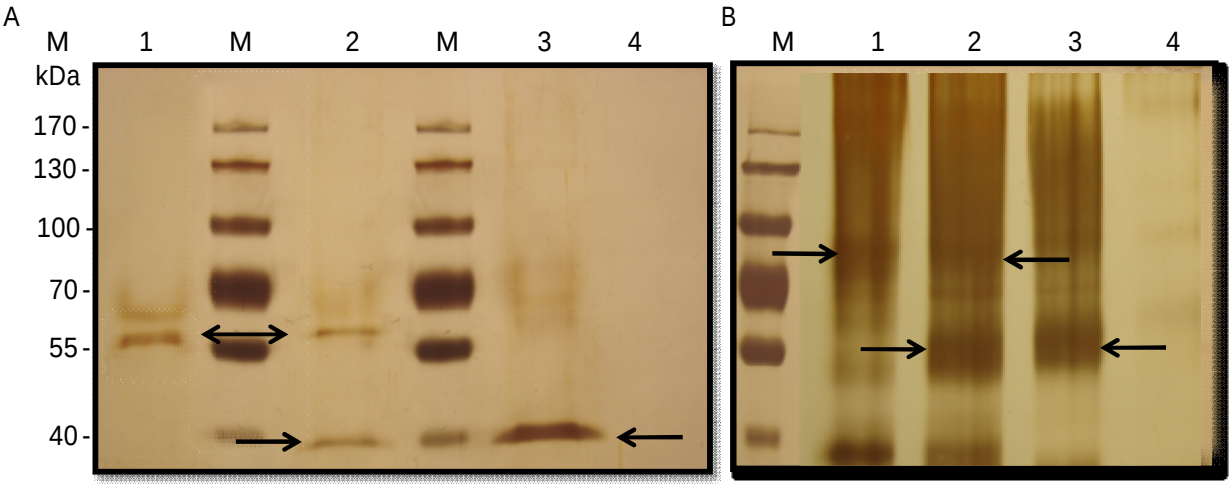

Fig. 5: Deglycosylated (A) and glycosylated (B) supernatants from *S. cerevisiae* Y294[Agal] (lanes 1), *S. cerevisiae* Y294[Agal-man1] (lanes 2), *S. cerevisiae* Y294[man1] (lanes 3) and *S. cerevisiae* Y294[BBH1] (lanes 4) were separated by SDS-PAGE. Molecular weight marker (Page Ruler^{TM}) was used with sizes depicted on the left hand side.

Ammonium sulphate purified (5 mg/100 µl) cAnmndA and CmMan5A from *A. niger* D15[cAnmndA] and *A. niger* D15[CmMan5A], respectively, was seperated by 10% SDS PAGE (Fig. 6A and B). The CmMan5A protein present in the 40% ammonium sulphate

precipitate was present as a single prominent species (Fig. 6A lane 2) displaying an apparent molecular weight of ~58 kDa. The theoretical size of the unglycosylated CmMan5A is 51 kDa as determined by DNAMAN (version 4.1, from Lynnon BioSoft). The cAnmndA protein present in the 60% ammonium sulfate precipitate yielded a distinct band of ~120 kDa (Fig. 6B lane 2). This species is larger than its theoretical size, as well as being larger than previously reported for native secretion of AnmndA from *A. niger* (Ademark et al. 2001). Lanes 1 in Fig. 6A and B indicate negative control *A. niger* D15[GTP2].

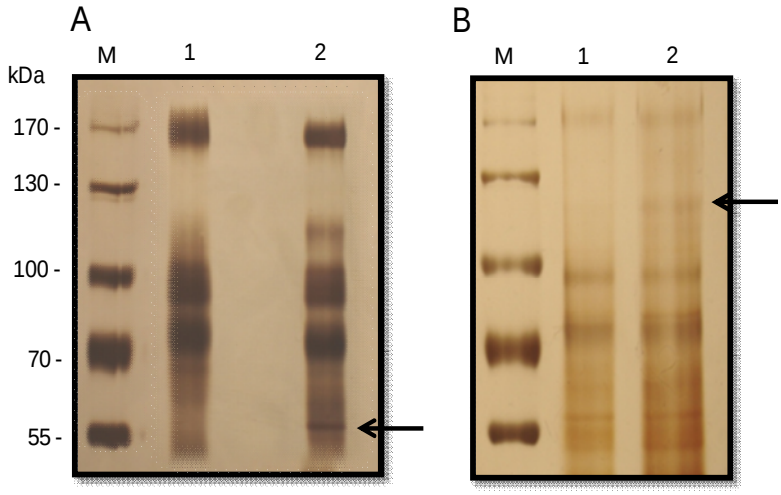

Fig. 6: Separation of the 40% (A) and 60% (B) ammonium sulphate purified protein (5 mg/100 µl) fractions by 10% SDS-PAGE. The cAnmndA from strain *A. niger* D15[cAnmndA], yielded a band size of ~120 kDa (Gel B lane 2), while the CmMan5A from strain *A. niger* D15[CmMan5A], yielded a band size of ~58 kDa protein species (Gel A lane 2). The negative control *A. niger* D15[GTP2] is found in lane 1. Molecular weight marker (Page Ruler^{TM}) was used with sizes depicted on the left hand side.

3.4.7. SYNERGISTIC ACTIVITY ON LBG

Locust bean gum (0.5%) was incubated with lyophilized Man1, Agal and CmMan5A obtained from recombinant strains *S. cerevisiae* Y294[man1], *S. cerevisiae* Y294[Agal] and *A. niger* D15[CmMan5A], respectively. The change in viscosity of the galactomannan was monitored continuously over a period of 10 min at 50°C. A gradual decrease in viscosity is observed for substrate incubated without enzyme indicating the influence of temperature and sheering by the apparatus itself (Fig. 7A). These values were taken into account and the effect of the enzyme treatments normalised accordingly. The most effective synergy of enzymes

was obtained from the combination of Man1, Agal and CmMan5A (Fig. 7B). Little change in viscosity occurred with incubation of the mannan with Agal. CmMan5A had a significant influence on the viscosity of the substrate, although not to the extent of Man1. These results indicate that there is a mutual rapid decrease in the average molecular weight of Locust bean gum, which is followed by a plateau.

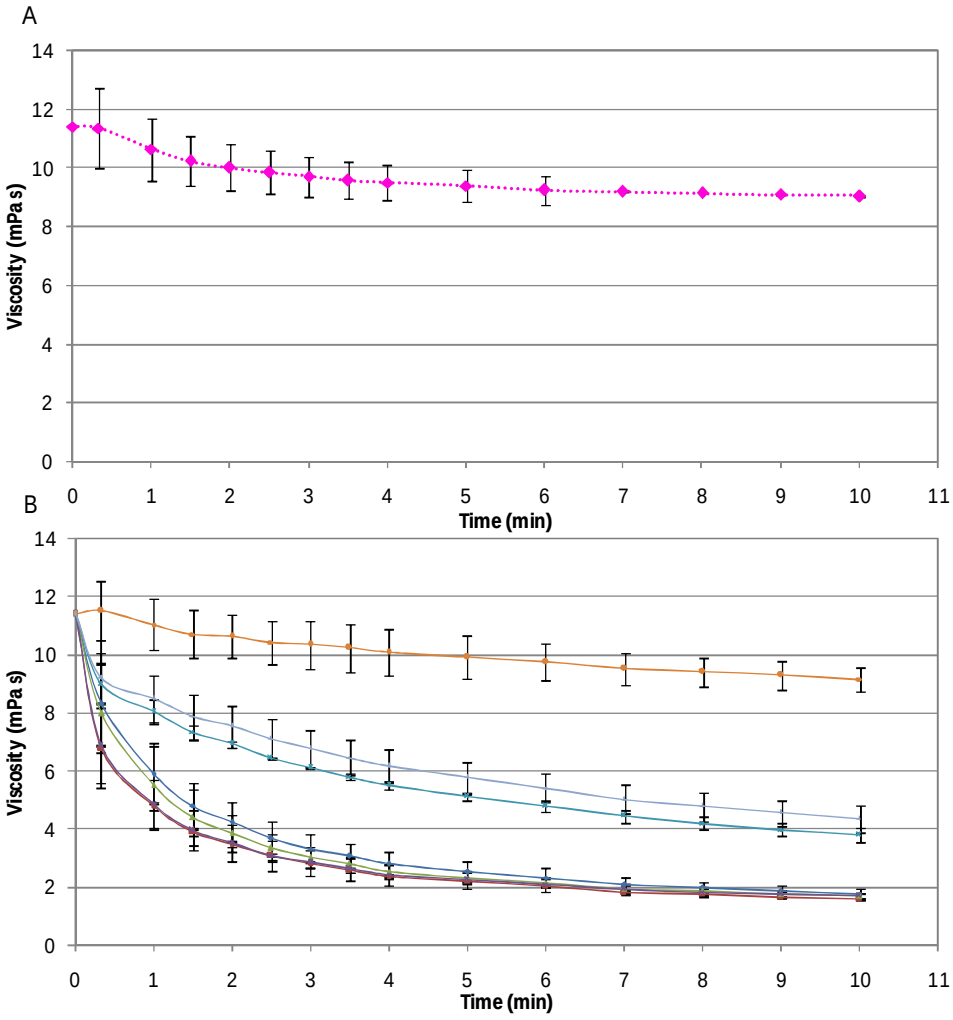

Fig. 7: (A) Temperature and shearing effect on LBG. (B) Analysis of the hydrolytic effect of Man1, Agal and CmMan5A from recombinant strains *S. cerevisiae* Y294[man1], *S. cerevisiae* Y294[Agal] and *A. niger* D15[CmMan5A] on 0.5% (w/v) LBG. Enzymes are used individually and in synergy. The greatest influence on viscosity is given by the combination of all enzymes, whereas Agal had the least effect. Man1, Man1 and Agal, Man1 and CmMan5A, Man1, Agal and CmMan5A, Agal, Agal and CmMan5A, CmMan5A. Values had been normalised to take into account the effect of the temperature on the viscosity. Error bars indicate the standard deviation from the mean value.

3.5. DISCUSSION

S. cerevisiae strains were engineered to secrete the β-mannanase (*man1*) of *A. aculeatus* and α-galactosidase (*Agal*) of *T. emersonii*. The *A. niger* strains were engineered to secrete a cDNA copy of β-mannosidase (*cAnmndA*) from *A. niger* and a synthetic β-mannosidase (*CmMan5A*) from *C. mixtus.* These enzymes were used synergistically to determine the effect on galactomannan viscosity.

The *man1* gene from *A. aculeatus* has previously been cloned and expressed in *S. cerevisiae* using the *PGK1* promoter and terminator expression cassette (Setati et al. 2001). The *Agal* gene from *T. emersonii* has previously been cloned and expressed in *Pichia pastoris* (Similä et al. 2010). In this study the *man1* and *Agal* genes were successfully cloned (both individually and simultaneously) and expressed in *S. cerevisiae*, using the pBBH1 expression vector. This vector allows for consecutive cloning of a number of gene cassettes by making use of the compatibility of the *Bam*HI and *Bgl*II restriction sites flanking the expression cassette. The *Bam*HI-*Bgl*II gene cassette can be sub-cloned into either the *Bam*HI or *Bgl*II sites (provided all internal *Bam*HI and *Bgl*II sites have been eliminated). The *AnmndA* from *A. niger* has been extensively characterised (Ademark et al. 2001, Ademark et al. 1999, Bouquelet et al. 1978, Do et al. 2009, Elbein et al. 1977). The *CmMan5A* from *C. mixtus* has previously been characterized and expressed in *E. coli* (Centeno et al. 2006, Dias et al. 2004). This is the first report where *CmMan5A* from *C. mixtus* has been successfully expressed in *A. niger*. In this study *S. cerevisiae* expressing *man1* and *Agal*, and *A. niger* expressing *cAnmndA* and *CmMan5A*, and *E. coli* expressing *XYNSEC-CmMan5A* was evaluated on the basis of enzyme secretion capacity and characterization in terms of optimal pH and temperature, as well as protein characterisation in terms of size and glycosylation, as well as the effect of these enzymes on the viscosity of galactomannan.

The pH and temperature optimum for the heterologous Man1 and Agal expressed by *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[Agal] were determined (Fig. 3A and B). The Man1 and Agal displayed optimum temperature of 70°C and 80°C, respectively, and pH optimum of 5.47 and 2.37, respectively. The results obtained for Man1 vary slightly from that obtained by Setati et al. (2001) who reported a temperature and pH optima of 50°C and 3, respectively. Similar differences were reported by van Zyl et al. (2009) and Christgau et al. (1994), who expressed the *A. aculeatus* β-mannanase in *A. niger* and *A. oryzae*, respectively. Similiä et al. (2010) reported an optimal pH and temperature for Agal

of 4.5 and 70°C, respectively. Such a variation may be due to host and media differences, where Similiä et al. (2010) expressed the *Agal* in the methylotrophic yeast *Pichia pastoris*. A similar change in these characteristics has been observed previously with the expression of the *Cel7A* of *T. reesei* in *S. cerevisiae*, where it was found that the glycosylation pattern varied when cultivation took place under different conditions (du Plessis et al. 2010, Stals et al. 2004). *N*-glycosylation of the Cel7A occurred at all three glycosylation sites when isolated from minimal medium, whereas cultivation in rich medium resulted less sites containing high-mannose chains (Stals et al. 2004). *S. cerevisiae* generally elongates the mannose chain in heterologous proteins by adding more mannose residues (Cregg et al. 2000, Romanos et al. 1992). Based on this it was speculated that the expression of Man1 in *S. cerevisiae* Y294 cultivated in YPD medium (Setati et al. 2001), the medium could have impacted the glycosylation patterns of the Man1, resulting in a change in the activity and characterization of the enzyme.

The extracellular β-mannanase and α-galactosidase activity produced by the yeast transformants, as well as optical growth (OD_{600}) was monitored over a period of 48 hours. The transformants were cultivated in SC^{-URA} media (Fig. 3C and D). No significant difference in optical density was detected between the control strain *S. cerevisiae* Y294[BBH1] and the transfromants *S. cerevisiae* Y294[man1], *S. cerevisiae* Y294[Agal] and *S. cerevisiae* Y294[Agal-man1], indicating that the foreign genes presented no adverse effects on the recombinant strains growth. Relatively high cell densities were obtained and are comparable to those achieved by Setati et al. (2001). A maximum activity on LBG of 36.08 nkat/ml was achieved for Man1 after 48 hours, whereas a maximum for the Man1 co-expressed with Agal yielded 28.30 nkat/ml. The maximum activity of Man1 was 1.2 times greater than the co-expressed Man1, and the maximum activity of Agal was 1.3 times greater than the co-expressed Agal. Due to the vector size being larger with the addition of an extra gene, it can lead to a lower copy number and consequently less protein production. Although the cell densities observed in this study are comparable to those achieved previously, low levels of activity of the Man1 protein compared to those previously reported (Setati et al. 2001) can be, as stated previously, ascribed to the different culture media used. The maximum Agal activity achieved in this study was significantly greater (2.2 times) than that achieved by expression in *P. pastoris* (Similä et al. 2010). This may also be due to the difference in culture media and vector size, as well as being expressed in a different host.

The protein species produced by *S. cerevisiae* Y294[man1], *S. cerevisiae* Y294[Agal] and *S. cerevisiae* Y294[Agal-man1] displayed molecular masses slightly larger than those obtained following deglycosylation (Fig. 5A and B). The deglycosylated proteins for the Agal and Man1 yielded a 60 kDa and a 40 kDa species, respectively. The band size of Man1 corresponds to the size previously reported by Setati et al. (2001), whereas Agal yielded a band slightly smaller than the native Agal produced by *T. emersonii* (Similä et al. 2010). Based on the size difference between the secreted and deglycosylated proteins, it is clear that the yeast *S. cerevisiae* Y294 hyperglycosylates these heterologous proteins.

The cultivation conditions of *Aspergillus* in submerged cultures significantly determines the mycelium morphology and affects the production of extracellular proteins (Galbraith and Smith 1969, Wosten et al. 1991). The *A. niger* D15 strain has previously been shown to be an excellent host for the production of heterologous proteins (Rose and Van Zyl 2002). The construction of an expression system has previously been described by Rose and van Zyl (2002) where successful expression of the *xyn2* and *egI* genes of *T. reesei* under the transcriptional control of the glyceraldehyde-6-phosphate dehydrogenase (*gpdA*) promoter from *A. niger* and the *glaA* terminator of *A. awamori* was reported. The strong glycolytic promoter was chosen to enable heterologous expression of the β-mannosidase from *A. niger* (Ademark et al. 2001) and the synthetic β-mannosidase of *C. mixtus* (Dias et al. 2004) in *A. niger* D15. The genes were cloned with their native secretion signals intact allowing successful secretion of the heterologous enzymes.

Obtaining the *cAnmndA* from the host *A. niger* requires induction by the addition of mannan to the growth medium. Cultivation on mannan in liquid culture is difficult due to the gelling characteristics of LBG at concentrations of 0.5%. Therefore the genomic copy of *AnmndA* was cloned and over-expressed in *A. niger* using the constitutive *gpd* promoter. This *A. niger* D15[gAnmndA] strain can be cultivated on glucose and was used to obtain the *cAnmndA* which was cloned for expression in *S. cerevisiae*. No activity was detected with the expression of *cAnmndA* or *CmMan5A* in *S. cerevisiae* (Table 1, Appendix).

Analysis of the cDNA copy of the *AnmndA* gene showed that the gene consisted of an ORF of 2793 bp that encodes a protein of 930 amino acids with a theoretical molecular mass of 104 kDa. The sequenced gene showed a 96.86% identity with the sequence of the *A. niger* as reported by Ademark et al (2001). Also noted from the alignment is the presence of three introns (237-354; 434-487; and 720-775), whereas only two introns were detected by

Ademark et al. (2001). A three base pair deletion was present in the cDNA sequence (TCC) at position 197. Since activity was evident on *p*-nitrophenyl β-D-mannopyranoside (*p*NPM) it can be assumed that these regions are not crucial for its function. However, greater activity has been reported for native β-mannosidase expressed by *A. niger* (Ademark et al. 1999) and it therefore cannot be assumed that the absence of three base pairs does not affect protein structure.

The activity of the cAnmndA and CmMan5A proteins expressed by *A. niger* D15[cAnmndA] and *A. niger* D15[CmMan5A] and XYNSEC-CmMan5A expressed by *E. coli* DH5α[XYNSEC-CmMan5A] were monitored over time (Fig. 4C and D, respectively). Maximum activity was observed for cAnmndA at 11.61 nkat/ml after 156 hours, 1.5 times greater than that observed for CmMan5A, which reached a maximum activity of 7.58 nkat/ml after 156 hours. These values are significantly lower than those obtained previously (Ademark et al. 2001, Ademark et al. 1999). This may be due to a combination of the high copy numbers (up to 25) of the over-expressed vectors achieved by Ademark et al. (2001) and the absence of three base pairs in the cDNA copy of AnmndA, which may have led to sub-optimal protein folding or functionality. Maximum activity of XYNSEC-CmMan5A was observed at 2.14 nkat/ml from whole cell fractions, whereas intracellular fractions displayed maximum activity of 1.67 nkat/ml. This difference in activity between whole cell fractions and intracellular fractions implies that the enzyme is mostly cell-wall bound, indicating that the *XYNSEC* secretion signal was ineffective in secreting the protein extracellularly or that the protein was too large to pass through the cell wall.

The pH and temperature optimum for the heterologous cAnmndA, CmMan5A and XYNSEC-CmMan5A expressed by *A. niger* D15[cAnmndA], *A. niger* D15[CmMan5A] and *E. coli* DH5α[XYNSEC-CmMan5A] were determined (Fig. 4A and B). The cAnmndA and CmMan5A both displayed optimum activity at temperature 70°C and pH 3.4, respectively, whereas the XYNSEC-CmMan5A displayed optimal activity at 50°C and pH 5.47, a significantly lower temperature than CmMan5A expressed by *A. niger* D15[CmMan5A]. The result obtained for cAnmndA fall within the range reported by Ademark et al. (1999) which is expected when expressing the *AnmndA* in its native host. Although no previous data is available for optimum temperature for CmMan5A, the optimum pH for CmMan5A obtained in this study falls well below the optimal pH 7.0 reported by Dias et al. (2004). This change in characteristic may be a result of the codon optimized state of the CmMan5A, and the fact

that the gene was expressed in *A. niger*, which generally exhibits optimum activity at a lower pH (Table 1, van Zyl et al. 2010).

The AnmndA protein produced by *A. niger* displayed a molecular mass of 264 kDa on a non-reducing SDS-PAGE and 135 kDa under reducing conditions, suggesting that the mndA is a dimer composed of two 135 kDa subunits (Ademark et al. 1999), whereas over-expression of the *AnmndA* gene in *A. niger* displayed a protein species of 112 kDa following deglycosylation (Ademark et al. 2001). The protein cAnmndA present in the 60% ammonium sulphate precipitate yielded a distinct species of 120 kDa (Fig. 6A and B), which falls within the range reported previously (Ademark et al. 2001, Ademark et al. 1999). The *C. mixtus CmMan5A* protein produced by *E. coli* displayed a protein species with molecular mass of 51 kDa (Centeno et al. 2006). In this study, ammonium sulphate purified cAnmndA and CmMan5A from *A. niger* D15[cAnmndA] and *A. niger* D15[CmMan5A] were separated by 10% SDS-PAGE (Fig. 6A and B). The CmMan5A species was present in the 40% ammonium sulphate precipitate and displayed an apparent molecular mass of 55 kDa, similar to that reported by Centeno et al. (2006).

Synergistic profiles on galactomannan have previously been reported for β-mannanases and β-mannosidases (Gübitz et al. 1996, Kurakake and Komaki 2001), as well as β-mannanases, β-mannosidases and α-galactosidases (Duffaud et al. 1997). When grown on guar-gum based media, the β-mannanase expressed by *Thermotoga neopolitana* 5068 was found mostly in the supernatant, whereas β-mannosidase and α-galactosidase were localised within the cell, suggesting that secretion of the β-mannanase into the culture media initially hydrolyses galactomannan into smaller polysaccharides, which are subsequently transported into the cell and further hydrolysed by β-mannosidase and α-galactosidase (Duffaud et al. 1997). On the other hand, *Sclerotium rolfsii* produces two β-mannosidases which liberate monomers directly from galactomannan. Enhanced hydrolysis was shown by the addition of the β-mannanases, where the enzyme randomly cleaved fragments larger than mannobiose from mannan (Gübitz et al. 1996). Halstead et al. (2000) showed that the sequential addition of an α-galactosidase and β-mannanase (from *Pseudomonas fluorescens* subsp. *cellulosa*) significantly increased the hydrolysis of galactomannan, indicating that the catalytic efficiency of β-mannanase on galactomannan is enhanced by pre-incubation with α-galactosidase. In this study the use of recombinant proteins Man1 and Agal expressed by *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[Agal] strains, respectively, and CmMan5A expressed by *A. niger* D15[CmMan5A] were assessed for their ability to synergistically

degrade galactomannan (Locust bean gum). A gradual decrease was observed for substrate incubated without enzyme, indicating an influence of temperature and shearing by the apparatus itself (Fig. 7A). The greatest synergistic effect of the enzymes was observed when the substrate was incubated with all three enzymes (Man1, Agal and CmMan5A) (Fig. 7B). The least effect on viscosity was observed with incubation of galactomannan with Agal, due to the enzymes inability to hydrolyse the mannan backbone. Interestingly the enzyme CmMan5A had a significantly greater effect on the viscosity than expected. This change in viscosity can only be due to the enzymes ability to release mannose from the non-reducing end resulting in a decrease in molecular weight, since no endo-mannanase activity was detected (Dias et al. 2004). The combination of Man1 and Agal resulted in increased degradation by Man1, attributed to the removal of galactose side-chains by Agal, resulting in more accessible cleavage sites.

In this study β-mannanase and α-galactosidase were expressed in *S. cerevisiae*, and the β-mannosidase in *A. niger* and *E. coli*, respectively. The characteristics of the enzymes were similar to that previously reported (Ademark et al. 2001, Ademark et al. 1999, Setati et al. 2001). High levels of α-galactosidase activity were observed, indicating the host *S. cerevisiae* may be a better suited host for the heterologous expression of α-galactosidase than *P. pastoris*. This is the first report of the *Agal* of *T. emersonii* being expressed in *S. cerevisiae*. The *cAnmndA* and *CmMan5A* could not be functionally expressed in *S. cerevisiae* (Table 1, Appendix). Therefore, *A. niger* was used as expression system. To our knowledge, this is also the first report of the expression of the *CmMan5A* in *A. niger*. This study concluded that the synergistic effect of β-mannanase, β-mannosidase and α-galactosidase had a significant effect on the viscosity of galactomannan when compared to individual incubation of the enzymes with the substrate. In terms of consolidated bioprocessing the ideal scenario would be to express these enzymes in a single host, such as *S. cerevisiae*, to obtain the complete hydrolysis of mannan.

3.6. ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to the National Research Foundation (NRF) for financial support.

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GENERAL DISCUSSION AND CONCLUSIONS

4.1. DISCUSSION

Green house gasses and the depleting fossil fuels have led to the need to develop alternative energy sources. The complete hydrolysis and conversion of lignocellulosic material to biofuels could provide the solution to cost effective and sustainable energy with low levels of green house emissions (Aristidou and Penttilä 2000, Lynd et al. 2002). Due to the complex structure of lignocellulose, several enzymes are required for its complete degradation (Ademark et al. 1998, McCutchen et al. 1996, Stoll et al. 2000). Research is currently focused on the development of a recombinant microorganism capable of utilising lignocelluloses while converting the released sugars to ethanol (or other commodities) in a single step (Consolidated Bioprocessing) (Gírio et al. 2010).

The yeast *S. cerevisiae* is currently the preferred host considered for consolidated bioprocessing (CBP) due to its ability to produce high levels of ethanol (Lin and Tanaka 2006). Other advantages of this yeast include its ability to grow on both simple hexose sugars and disaccharide sugars, has GRAS (**G**enerally **R**egarded **A**s **S**afe) status, has the ability to perform posttranslational modifications of heterologous proteins and relatively good tolerance to lignocelluloses-derived inhibitors and osmotic pressure (Gírio et al. 2010). However, the major inconvenience with using *S. cerevisiae* with regard to lignocellulosic fermentation is its lack of native cellulases and hemicellulases.

Filamentous fungi generally have a greater secretion capacity than yeast and can be easily cultivated on inexpensive media. The diverse natural environments and the competition with other organisms present in these habitats probably underlie the extraordinary metabolic diversity of many of the filamentous fungi (Verdoes et al. 1995). *A. niger* is a commonly used host for foreign gene expression due to its GRAS status and high secretion capacity. The large range of enzymes produced by *Aspergillus* for the degradation of plant cell wall polysaccharides is of particular importance to the food and feed industries. However, a major disadvantage of fungi is their inability to produce or tolerate high levels of ethanol.

Combining the advantageous and positive characteristics of *S. cerevisiae* and *A. niger* resulted in construction of galactomannan-hydrolysing enzyme strains. Ideally these strains would be cultivated on lignocelluloses while producing commercially important commodities such as biofuels.

In this study the following milestones had been reached:

- The *A. aculeatus man1* and *T. emersonii Agal* genes were successfully expressed in *S. cerevisiae* Y294 (laboratory strain) under transcriptional control of the *ENO1* promoter and terminator sequences. The *ENO1* promoter resulted in constitutive expression of *man1* and *Agal*.
- The cDNA generated *A. niger cAnmndA* and synthetically generated *C. mixtus CmMan5A* genes were successfully expressed in *A. niger* D15 (laboratory strain) under the transcriptional control of the *gpd* promoter and *glaA* terminator sequences. The *gpd* promoter resulted in constitutive expression of the *cAnmndA* and *CmMan5A*.
- The native secretion signal of the *CmMan5A* gene was removed and replaced with the *XYNSEC* DNA sequence coding for the secretion signal of *xyn2* of *T. reesei*. XYNSEC-CmMan5A was successfully expressed in *E. coli* DH5α.
- Investigation of the *cAnmndA* gene sequence compared to the sequence obtained from GenBank (Accession number XM_001394595) revealed a three base pair deletion. However, activity was detected on *p*-nitrophenyl β-D-mannopyranoside, indicating a functional protein. The three base pair deletion may only be present in some strains.
- Recombinant Man1 showed activity on Locust bean gum. Hydrolysis zones were observed on plates containing 0.5% Locust bean gum indicating extracellular endo-mannanase activity.
- The *S. cerevisiae* strain expressing the Agal produced higher levels of activity than what had been reported with expression in *Pichia pastoris* (Similä et al. 2010). Host, media and vector type differences may attribute to the lower activities reported previously. The *Agal* used in this study had been codon optimized for expression in *S. cerevisiae*, which might also have benefited expression.
- Co-expression of *man1* and *Agal* in *S. cerevisiae* Y294[Agal-man1] showed lower levels of activities on the respective substrates when compared to *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[Agal]. Saturation of the secretion capacity of the yeast might lead to less protein being produced. This is also the first report of the co-expression of a β-mannanase and an α-galactosidase in *S. cerevisiae*.
- Growth of *S. cerevisiae* and *E. coli* strains showed no significant difference to those of the control strain, respectively, indicating no adverse effects on cell growth (in terms of cell density) created by the addition of foreign genes.

- Hyperglycosylation of Man1 and Agal by *S. cerevisiae* was confirmed when treatment with PNGase H resulted in smaller protein species present on an SDS-PAGE. This result suggests further evidence that *S. cerevisiae* hyperglycosylates heterologous proteins.
- pH and temperature preferences for Man1, Agal, cAnmndA, CmMan5A and XYNSEC-CmMan5A indicated similarities as well as significant differences to previously reported data, possibly due to differences in hosts, cultivation media as well as the impact of glycosylation.
- Both cAnmndA and CmMan5A displayed activity against *p*-nitrophenyl β-D-mannopyranoside, however relatively low levels of activity were observed for cAnmndA and CmMan5A compared to those previously reported. Such low activity may be due to differences in vectors, cultivation conditions and relative copy numbers, or, in the case of cAnmndA, the absence of the three base pairs in the cDNA copy which may have led to incorrect protein folding.
- This is the first report of the expression of *CmMan5A* (native and synthetic) in *A. niger*.
- The synergistic action of the three proteins (Man1, Agal and cAnmndA) essential for the complete hydrolysis of galactomannan displayed significant effects on the viscosity of Locust bean gum when used in combination, indicating hydrolysis of the Locust bean gum.

4.2. UNSUCCESSFUL GENE EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

Expression of the *CmMan5A*, *XYNSEC-CmMan5A* and the *cAnmndA* genes in *S. cerevisiae* was unsuccessful. No extracellular activity could be detected on *p*NPM indicating that these enzymes are not active on the chemical substrate or might be trapped inside the cell. In order to elucidate the problem, several additional vector constructs and *S. cerevisiae* strains had been constructed (Table 1 of Appendix). Difference scenarios might explain why no visible activity was observed:

Scenario 1: The enzymes should be active on LBG, because it is a native substrate. Therefore co-expression of the *man1* (which produces mannobiose and mannotriose as end-product) with *cAnmndA* should result in growth on LBG. No growth was detected for

S. cerevisiae Y294[cAnmndA-man1], indicating either no active AnmndA or no secretion of AnmndA (thus intracellular AnmndA).

Scenario 2: The lactose permease (*lac12*) of *Kluyveromyces lactis* had been proven to be a disaccharide transporter (Njokweni et al. 2012) and should therefore also be able to transport mannobiose. The *lac12* was therefore co-expressed with the CmMan5A and the cAnmndA. If the CmMan5A or the cAnmndA was active (but intracellular), then *S. cerevisiae* Y294[YIplac128-Agal-man1-lac12-CmMan5A/cAnmndA] should be able to grow on mannan. No visible growth was detected on LBG, indicating that the AnmndA and the CmMan5A were either inactive or not being produced.

Scenario 3: The enzymes (cAnmndA and CmMan5A) might not be compatible with the host (*S. cerevisiae*). The genomic copy of the β-mannosidase (*AtmndA*) gene of *Aspergillus tubingensis* was cloned and expressed in *A. niger* and *S. cerevisiae,* but no activity could be detected. No introns could be detected when aligned with the AnmndA DNA sequence.

These findings lead us to conclude that *S. cerevisiae* may not be an ideal host for the expression of β-mannosidases. Evidence has suggested that the smaller the protein species the more efficient *S. cerevisiae* is at producing it (La Grange et al. 2000). The open reading frame of *cAnmndA* is 2.8 kb, double the average size of the *S. cerevisiae* open reading frame (Hauser et al. 1999). Additionally, this enzyme is produced as a dimer when expressed in its native host (Ademark et al. 1999). Expression of foreign genes in *S. cerevisiae* can result in incorrect folding of proteins, hindering competent assemblage of the dimer. Monomers are hyperglycosylated individually which may also affect dimer assembly. This may be the reasons why *S. cerevisiae* was unsuccessful in secreting a functional β-mannosidase.

4.3. FUTURE WORK SUGGESTED

This study paved the way towards the construction of a mannan degrading *S. cerevisiae* strain. The following recommendations are made based on the conclusions deduced from this study:

- Clone and express other β-mannosidases in *S. cerevisiae* in an attempt to find one that is functional and does not have a negative impact on the host.
- Over-express the *man1, Agal* and β-mannosidase in *S. cerevisiae*.

- Analyse the products of viscosity study to determine the rate limiting steps in mannan degradation.
- Optimise the ratio of the enzymes required for optimal degradation (HPLC) using different promoters and vectors.
- Determine if growth on mannan as sole carbon source is possible.
- Perform anaerobic fermentation to determine ethanol yield.

In conclusion, the *man1* and *Agal* genes were successfully expressed in *S. cerevisiae* Y294 whereas the *CmMan5A* and *cAnmndA* genes were successfully expressed in *A. niger* D15. All enzymes were partially characterised and levels of activity determined over time. Their effects on Locust bean gum had been demonstrated.

4.4. REFERENCES

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Table 1: List of plasmids and strains constructed that led to the conclusion that *S. cerevisiae* cannot be used as host to produce β-mannosidase.

Strains and Plasmids	**Relevant Genotype**	**Comment and Reference**
S. cerevisiae strains*:*		
Y294[CmMan5A]	*URA3 ENO1$_P$-CmMan5A-ENO1$_T$*	native secretion signal used
Y294[XYNSEC-CmMan5A]	*URA3 ENO1$_P$-XYNSEC-CmMan5A-ENO1$_T$*	native secretion signal replaced by *XYNSEC*
Y294[cAnmndA]	*URA3 ENO1$_P$-cAnmndA-ENO1$_T$*	cDNA copy with native secretion signal – PCR confirmed gene presence – no activity detected
Y294[AtmndA]	*URA3 ENO1$_P$-AtmndA-ENO1$_T$*	No introns detected in genomic copy
Y294[cAnmndA-man1]	*URA3 ENO1$_P$-cAnmndA-ENO1$_T$; ENO1$_P$-man1-ENO1$_T$*	expected growth if AnmndA is functional might be trapped intracellularly
Y294[YIplac128-man1-lac12]	*LEU2 ENO1$_P$-man1-ENO1$_T$; ENO1$_P$-lac12-ENO1$_T$*	no growth on LBG, as expected; lac12 can transport disaccharides
Y294[YIplac128-Agal-man1-lac12]	*LEU2 ENO1$_P$-Agal-ENO1$_T$; ENO1$_P$-man1-ENO1$_T$; ENO1$_P$-lac12-ENO1$_T$*	no growth on LBG, as expected
Y294[YIplac128-Agal-man1-lac12-CmMan5A]	*LEU2 ENO1$_P$-Agal-ENO1$_T$; ENO1$_P$-man1-ENO1$_T$; ENO1$_P$-lac12-ENO1$_T$ URA3 ENO1$_P$-CmMan5A-ENO1$_T$*	no growth on LBG, no active CmMan5A, might be trapped intracellular
Y294[YIplac128-Agal-man1-lac12-cAnmndA]	*LEU2 ENO1$_P$-Agal-ENO1$_T$; ENO1$_P$-man1-ENO1$_T$; ENO1$_P$-lac12-ENO1$_T$ URA3 ENO1$_P$-cAnmndA-ENO1$_T$*	no growth on LBG, no active cAnmndA intracellular
A. niger strains:		
A. niger D15[AtmndA]	*pyrG$^+$ gpd$_P$-AtmndA-glaA$_T$*	
Plasmids:		
pBBH1-CmMan5A	*bla URA3 ENO1$_P$-CmMan5A-ENO1$_T$*	native secretion signal used
pBBH4-CmMan5A	*bla URA3 ENO1$_P$-XYNSEC-CmMan5A-ENO1$_T$*	native secretion signal replaced by *XYNSEC*
pBBH1-cAnmndA	*bla URA3 ENO1$_P$-cAnmndA-ENO1$_T$*	cDNA copy of AnmndA
pBBH1-AtmndA	*bla URA3 ENO1$_P$-AtmndA-ENO1$_T$*	genomic copy of AtmndA, yet no introns detected
pBBH1-AnmndA-man1	*bla URA3 ENO1$_P$-AnmndA-ENO1$_T$; ENO1$_P$-man1-ENO1$_T$*	required for the final construct
YIplac128-lac12	*bla LEU2 ENO1$_P$-lac12-ENO1$_T$*	disaccharide transporter (Njokweni et al. 2012)
YIplac128-Agal-man1-lac12	*bla LEU2 ENO1$_P$-Agal-ENO1$_T$; ENO1$_P$-man1-ENO1$_T$; ENO1$_P$-lac12-ENO1$_T$*	final construct; to be co-transformed with either pBBH1-CmMan5A or pBBH1-cAnmndA