EXPRESSION OF MANNANASES IN FERMENTATIVE YEASTS

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Declaration

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SUMMARY

The search for a cost-effective, environmentally friendly replacement for fossil fuels resulted in bio-ethanol production receiving a lot of attention. Lignocellulose, is considered to be the most abundant renewable source on earth, and consists of cellulose, hemicellulose and lignin. Exploitation thereof as a substrate for ethanol production, can serve as solution in producing bio-ethanol as an adequate replacement for fossil fuels. Hemicelluloses, contributing up to a third of the lignocellulosic substrate, consists mainly of xylan and mannan and can be degraded by hemicellulolytic enzymes that are produced by plant cell wall degrading organisms. Galactoglucomannan is the most complex form of mannan and requires a consortium of enzymes for complete hydrolysis. These enzymes include β-mannanase, β-mannosidase, α-galactosidase, β-glucosidase and galactomannan acetylesterases.

Saccharomyces cerevisiae is a well-known fermentative organism that has been used in various industrial processes and is able to produce ethanol from hexose sugars. Although this organism is unable to utilize complex lignocellulosic structures, DNA manipulation techniques and recombinant technology can be implemented to overcome this obstacle. Strains of *S. cerevisiae* pose other shortcomings like hyperglycosylation and therefore other non-conventional yeasts (such as *Kluyveromyces lactis*) are now also being considered for heterologous protein production.

The mannanase gene (*manI*) of *Aspergillus aculeatus* was expressed in *K. lactis* GG799 and *S. cerevisiae* Y294. *K. lactis* transformants were stable for two weeks in consecutive subcultures and secreted a Man1 of 55 kDa. The recombinant Man1 displayed an optimum temperature of 70˚C and a pH optimum of 5 when produced by *K. lactis*. Activity levels of about 160 – 180 nkat/ml was obtained after 86 hours of cultivation, which was similar to the activity observed with *S. cerevisiae* under the same conditions. Disruption of the *ku80* gene did not contribute to the stability of the cultures and a heterogeneous culture developed for 10 days of consecutive subculturing.

The mannosidase gene (*man1*) from *A. niger* and mannanase gene (*manI*) from *A. aculeatus* were constitutively expressed in *S. cerevisiae* Y294 and *S. cerevisiae* NI-C-D4. The MndA and Man1 proteins appeared as a 140 kDa and 58 kDa species on the SDS-PAGE analysis

when expressed in *S. cerevisiae* Y294, respectively. MndA had an optimum temperature of 50˚C and optimum pH 5. Man1 produced by *S. cerevisiae* Y294 indicated a pH optimum of 6 and temperature optimum of 70˚C. The MndA displayed low levels of endomannanase activity and no β-mannosidase activity could be detected. Co-expression of *man1* and *mndA* in either *S. cerevisiae* Y294 and *S. cerevisiae* NI-C-D4, resulted in less hydrolysis of galactoglucomannan. An increase in the size of the plasmid generally results in a decrease in the copy number, leading to a decrease in the amount of ManI protein being produced. The co-expression of ManI and MndA could also have resulted in a higher metabolic burden on the cell, hence the amount of ManI are produced.

This study confirms that more research should be done on the evaluation of alternative hosts for expression of foreign proteins. Furthermore, producing enzymes cocktails for industrial application should be considered rather than co-expression of various enzymes in one host.

OPSOMMING

'n Behoefte na 'n koste-effektiewe en omgewingsvriendelike vervoer brandstof is besig om toe te neem. Lignosellulose word beskou as die volopste hernubare bron vir biobrandstof en lignosellulose bestaan uit sellulose, hemisellulose en lignien. Die gebruik daarvan vir die produksie van bio-etanol kan 'n voldoende alternatief vir fossielbrandstowwe bied. Verbruik van lignosellulose as bron vir die produksie van biobrandstof bied 'n oplossing vir die energie krises. Hemisellulose vorm 'n derde van lignosellulose substraat en bestaan uit xilaan en mannaan en word deur hemisellolitiese ensieme afgebreek wat algemeen by plantselwand-verterende organismes voorkom. Galaktoglukomannaan is die mees komplekse vorm van mannaan en benodig verskeie ensieme vir volkome hidroliese. Hierdie ensieme sluit in β-mannanase, β-mannosidase, α-galaktosidase, β-glukosidase en galaktomanaan asetielesterases.

Saccharomyces cerevisiae is 'n bekende fermenterende organisme wat gereeld in verskeie industriële prosesse gebruik word en kan etanol van heksose suikers produseer. Die organisme beskik nie oor die vermoë om komplekse polisakkarides wat in lignosellulose voorkom te hidroliseer nie maar. DNS-manipuleringstegnieke en rekombinante tegnologie maak dit egter moontlik die probellm te oorbrug. *S. cerevisiae* het nogtans tekortkominge soos hiperglikosilering en daarom word ander nie-konvensionele giste (soos *Kluyveromyces lactis*) tans ook vir die produksie van rekombinante proteine ondersoek.

Die mannanase geen (*manI*) vanaf *Aspergillus aculeatus* is in *K. lactis* GG799 en *S. cerevisiae* Y294 uitgedruk. *K. lactis* transformante was stabiel vir twee weke in opeenvolgende subkluture en het 'n Man1 van 55 kDa geproduseer. Die rekombinante Man1 ensiem het 'n temperatuur optimum van 70˚C en pH optimum van 5.0 getoon in *K. Lactis*. Aktiwiteitsvlakke van 160 – 180 nkat/ml was bereik na 86 uur klutivering, In vergelyking met *S. cerevisiae* was aktiwiteitsvlakke eenders oor 'n periode Die disrupsie van die *ku80* geen het geen effek op die stabiliteit van die transformante in 10 dae opeenvolgende sub-kulture getoon nie.

Die mannosidase geen (*mndA*) vanaf *Aspergillus niger* en die mannanase geen (*man1*) van *Aspergillus aculeatus* is konstitutief in *S. cerevisiae* Y294 en *S. cerevisiae* NI-C-D4 uitgedruk. Uitdrukking van die MndA en Man1 proteïen in *S. cerevisiae* Y294 het onderskeidelik 'n 140 kDa en 58 kDa spesie getoon met SDS-PAGE analisering. Die MndA ensiem het 'n

temperatuur optimum van 50˚C and pH optimum van 5.0 getoon. Man1 het 'n pH optimum van 6.0 en 'n temperatuur optimum van 70˚C getoon. MndA het lae hidrolitiese aktiwiteit op galaktoglukomannaan, maar geen β-mannosidase aktiwiteit getoon nie. Wanneer *man1* and *mndA* saam in *S. cerevisiae* Y294 en *S. cerevisiae* NI-C-D4 uitgedruk is, het die hidroliese van galaktoglukomannan dramaties afgeneem. 'n Toename in die grootte van 'n plasmied veroorsaak dikwels 'n afname in kopiegetal wat die produksie van ManI verlaag. Die ko-uitdrukking van ManI en MndA kan ook tot 'n hoër metaboliese las lei en dus die laer produksie van ManI.

Resultate in hierdie studie wys daarop dat meer navorsing benodig word in die soeke na alternatiewe gashere vir uitdrukking van mannanases. Ensiem mengsels vir industriële toepassings behoort eerder gebruik te word as die ko-ekspressie van verskeie ensieme in 'n enkel gasheer.

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GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RESEARCH.

1.1. INTRODUCTION

Plant cell walls consist of complex polymers which are mainly cellulose, hemicellulose and lignin (Zabel and Morrell 1992). Hemicellulose is second to cellulose, the most abundant renewable carbon source on earth and consists mainly of mannan and xylan (Lynd *et al*. 2002, Petkowicz *et al*. 2001). The different forms of mannan include glucomannan, galactomannan and galactoglucomannan (Moreira and Filho 2008). Recalcitrance of biomass is a current impediment in the quest to produce bioethanol from lignocellulose and can be overcome by utilisation of microbial hydrolytic enzymes (Demain *et al*. 2005, Monique *et al*. 2003, Mosier *et al*. 2005). Certain fungi and bacteria, mostly soil-living, produce hydrolytic enzymes that are able to degrade lignocellulosic substrates (Jose and Demain 2003, Polizeli *et al*. 2005). The hydrolysis of mannan requires enzymes like β-mannanases (1,4-β-D-mannan mannohydrolases, EC 3.2.178), β-mannosidases (1,4-β-D-mannopyranoside hydrolases, EC 3.2.1.25), α-galactosidases (1,6-α-D-galactoside galactohydrolases, EC 3.2.1.22), β-glucosidases (1,4-β-D-glucoside glucohydrolases, EC 3.2.1.21) and galactomannan acetylesterases (Moreira and Filho 2008). Mannanases have several industrial applications such as in food technology where they are used in coffee, fruit juices and oil extraction processes for the hydrolysis of high-molecular-weight mannans (Dhawan and Kaur 2007). Mannanases produce manno-oligosaccharides that are used as prebiotics in foodstuffs and pharmaceutical applications (Dhawan and Kaur 2007). The paper and pulp industry uses mannanases in combination with xylanases as biological prebleaching agents enhancing the extractability of lignin and reducing environmental pollution by avoiding traditional chlorine methods (Montiel *et al*. 1999, Tenkanen *et al*. 1997). In most cases, the industries require only one or two enzymes to obtain the desired effect in the final product. Enzymes can be produced by recombinant technology using host strains such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis*, etc. (Dujon *et al*. 2004, La Grange *et al*. 2001, van Rooyen *et al*. 2005). Recombinant DNA technology provides us with tools to study the individual enzymes and the expression systems of hosts in order to select for such systems that are well suited for industrial processes.

Effective production of mannanases for industrial application and insight into different host systems need to be further explored. Since *S. cerevisiae* does not produce hemicellulytic or cellulytic enzymes required to degrade plant cell walls, considerable research is dedicated to expression of enzymes in this organism. Construction of hemicellulolytic yeasts would allow better insight into the realisation of the one-step conversion of lignocellulose for ethanol production. This thesis focuses on the hemicellulolytic enzymes, endo-1,4-β-mannanase and β-mannosidase from *Aspergillus* sp. and their expression in *S. cerevisiae* and *K. lactis*. Production of the recombinant enzymes was evaluated using different gene combinations with their hydrolytic properties evaluated on Locust bean gum as substrate.

1.2. AIMS OF THE STUDY

The aims of this study were:
- to clone and express the β-mannanase encoding gene (*man1*) of *Aspergillus aculaetus* on an episomal plasmid under the transcriptional control of the *S. cerevisiae* phosphoglycerate kinase I (*PGK1*) promoter and terminator;
- to determine the β-mannanase activity produced by *S. cerevisiae* and compare with previously reported data on expression of the same gene;
- to clone and express the β-mannanase gene (*man1*) from *A. aculeatus* in *K. lactis*;
- to determine the optimum pH and temperature of the Man1 enzyme expressed in *K. lactis* and *S. cerevisiae*;
- to compare the levels of activity of the Man1 produced by *K. lactis* and *S. cerevisiae*;
- to compare the biomass production (in mg Cell Dry Weight/ml) between *K. lactis* and *S. cerevisiae*;
- to investigate the impact of the disruption of the *ku80* gene on the plasmid stability in the cultures under conditions of continuous subcultivation;
- to synthetically design the codon optimized 2.8 kb β-mannosidase (*mndA*) from *Aspergillus niger*;

- to clone and express the β-mannosidase encoding gene (*mndA*) on an episomal plasmid under the transcriptional control of the enolase I gene (*ENO1*) promoter and terminator in *S. cerevisiae*;

- to purify and characterize the recombinant β-mannosidase (MndA) and determine the levels of activity thereof on *p*-nitrophenyl β-D-mannopyranoside;

- to co-express the β-mannanase encoding gene (*man1*) and β-mannosidase encoding gene (*mndA*) on one episomal plasmid in *S. cerevisiae* and

- to determine if a synergistic effect between the Man1 and MndA enzymes could be established when they were co-produced in *S. cerevisiae*.

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2.1. INTRODUCTION

Alternative energy is of great interest for the future as energy conversion, usage and access have brought about a global challenge (Lynd *et al*. 2002). Energy sources that might create solutions include solar wind, hydrogen as well as biomass conversion. In combination, these sources might result in a powerful solution to cost-effective and sustainable energy. Bioethanol development has gained considerable interest due to the use of abundant and renewable resources such as lignocellulose (Lynd *et al*. 1999).

The constituents of lignocellulose, namely cellulose, hemicellulose and lignin are the most abundant polymers on earth (Colvin 1980). Lignocellulosic biomass is a well-suited resource, because it has the advantages of being inexpensive, sustainable, greenhouse-gas neutral and it is not a food commodity (Monique *et al*. 2003, Sticklen 2007). The four categories of lignocellulosic biomass includes wood residues, which is the largest source, followed by municipal solid wastes, agricultural residues and energy crops grown for this specific application (Lin and Tanaka 2006). Recalcitrance of biomass is, however, a current impediment that needs to be overcome before energy production from these sources can become a reality (Demain *et al*. 2005, Mosier *et al*. 2005). Certain fungi and bacteria, mostly soil-living, produce hydrolytic enzymes that are able to degrade lignocellulosic substrates. These enzymes can be used effectively to convert the recalcitrant lignocellulose to fermentable sugars resulting in significant cost reductions and yield improvements (Lynd *et al*. 2002).

Ethanol can be produced from biomass by a hydrolysis and sugar fermentation process (Lin and Tanaka 2006). This conversion requires the release of cellulose and hemicellulose from lignin in a delignification step. Hereafter the carbohydrate polymers are depolymerised resulting in free sugars (hexose and pentose sugars) which can subsequently be fermented to ethanol (Yu and Zhang 2004). The current process of lignocellulose conversion to bioethanol is well established and involves size reduction pre-treatment and enzymatic hydrolysis of plant material (Lynd *et al*.

2002). Currently, the process is not economically feasible and needs an improvement (process design) to make the concept more cost-effective.

Biomass conversion may be categorized according to the level to which processes are consolidated (Fig. 1). Separate hydrolysis and fermentation (SHF) consists of four separate steps and requires up to four biocatalysts. Simultaneous saccharification and fermentation (SSF) combines the enzymatic saccharification and fermentation of hydrolysis products and has already shown cost reductions in the process (Lynd *et al*. 2002). Simultaneous saccharification and co-fermentation (SSCF) is a two-step process: cellulases are produced where after both cellulose and hemicellulose are fermented (Bollók *et al*. 2000, Stënberg *et al*. 2000). Based on results from simultaneous saccharification and fermentation (SSF), a new design was proposed termed consolidated bioprocessing (CBP). CBP envisions a one-step conversion process with a recombinant microorganism that has the ability to hydrolyze pretreated substrate and ferment released sugars to desired product, simultaneously, as seen in Fig. 1 (Lynd *et al*. 2002).

Saccharomyces cerevisiae is currently the organism of choice considered for CBP development. This yeast is widely used in the industry for ethanol production and has advantages such as high ethanol production and tolerance and well-developed gene expression systems (Lynd *et al*. 2002). *S. cerevisiae* is however non-cellulolytic and therefore enzymes such as endoglucanases and exoglucanases need to be introduced to efficiently hydrolyse the cellulose and hemicellulose portion of the plant material to fermentable sugars (Henrissat 1994, Lynd *et al*. 2002).

2.2. PLANT BIOMASS

Lignocellulosic biomass is a renewable energy source and is therefore considered as an ideal resource for the production of bio-ethanol (Lynd *et al*. 2002). Forest material (hard and softwood) is considered as the largest sources of lignocellulose, but other sources include agricultural waste, grasses, water plants (algae) and various plant material (Lynd *et al*. 2002). Biomass comprises of three main components. The cellulose portion can contribute to 35 - 50%, hemicellulose to 20 - 30% and lignin to

10% of plant dry weight (Coughlan and Hazlewood 1993). These components have complex molecular structures that can be degraded by various fungi and bacteria.
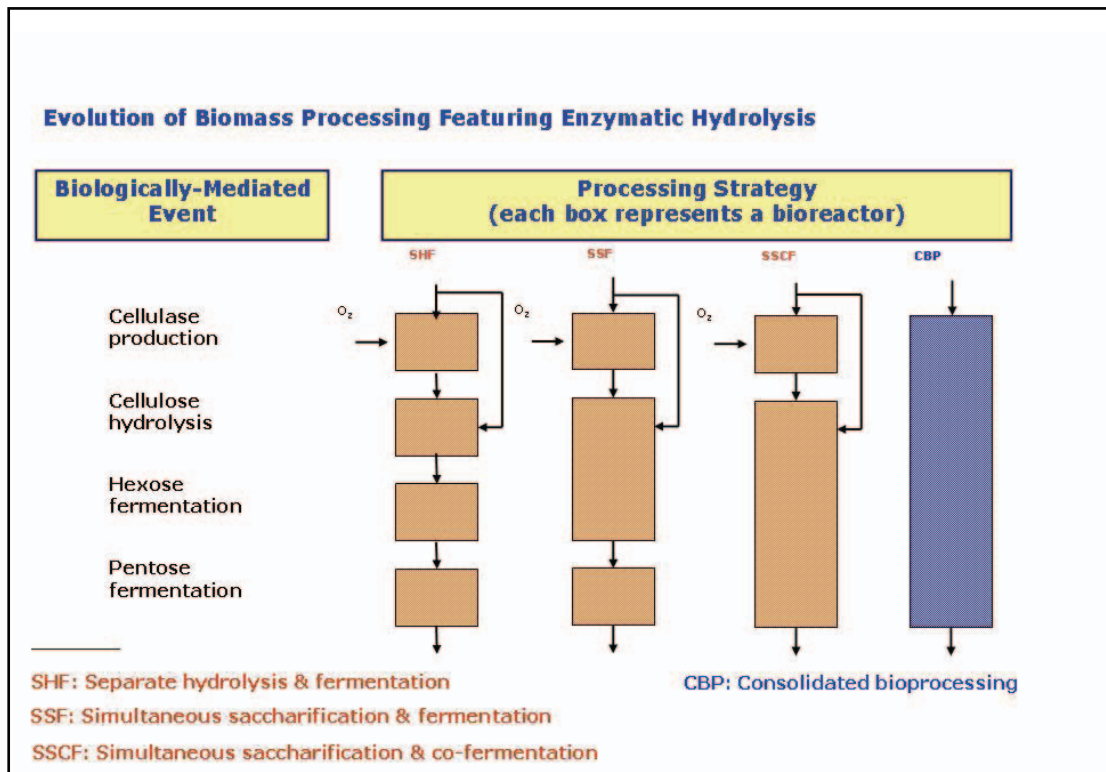
Fig. 1. A schematic representation of the evolution of biomass processing illustrating differences between separate hydrolysis and fermentation, simultaneous saccharification and fermentation, simultaneous saccharification and co-fermentation and lastly consolidated bioprocessing (the one-step process) (Lynd *et al.* 2002).

2.2.1. CELLULOSE

Cellulose is a homopolymer consisting of β-D-glucopyranose units that are linked with β-1,4 glycosidic bonds (Fig. 2) (Lynd *et al.* 1999, Vincent 1999). Cellobiose molecules are bound through intra- and intermolecular hydrogen bonds, which assemble to form elemental fibrils and consequently form microfibrils (Kudlicka and Brown 1996, Brown 1999, Lynd *et al.* 2002). Microfibril sheets are packed together and linked with weak van der Waals forces to form fibrils. Fibrils eventually form the cellulose fibres which appear in highly ordered (crystalline) or less ordered

(amorphous) formation (Brown 1999). Cellulose molecules are insoluble in water and the degree of polymerization (DP) ranges from 300 to 26 000. Even though cellulose is quite recalcitrant, various cellulolytic organisms (such as *Aspergillus* sp. and *Cellulomonas* sp.) produce glycoside hydrolases that are able to cleave the glycosidic linkages in cellulose (Warren 1996).
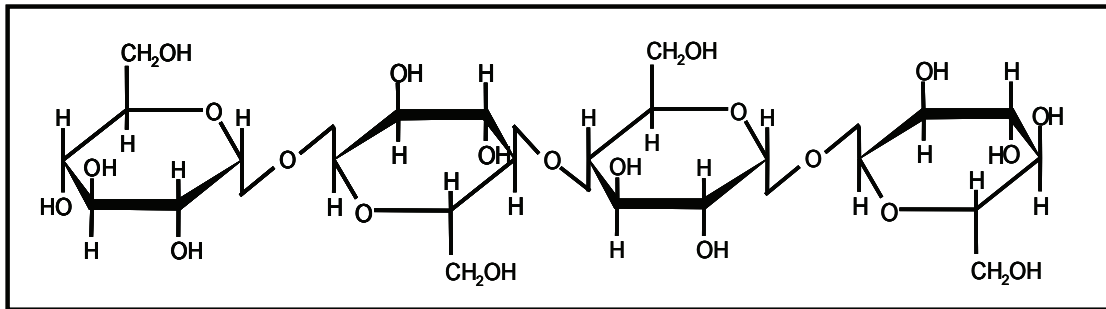
Fig. 2. A portion of a cellulose chain, β-D-glucopyranose units are linked with β-1,4-glycosidic bonds (Coughlan and Hazlewood 1993).

2.2.2. HEMICELLULOSE

Hemicelluloses are non-covalently, cross-linked to cellulose microfibrils. Together, these polymers perform a structural role (Liepman *et al*. 2007). The hemicelluloses are linear or branched polymers that are formed with hexoses (D-glucose, D-mannose and D-galactose) and pentoses (D-xylose, L-arabinose) and can have monosaccharides and acetyl side-groups (Erikkson *et al*.1990, Filho 1998). The main hemicelluloses include mannan, xylan, galactan and arabinan and are characterized by their insolubility in chelating agents and water, and solubility in aqueous alkali (O'Neill and York 2003).

Different types and concentrations of hemicellulose occur in softwood (gymnosperms) and hardwood (angiosperm). The main forms of hemicellulose are mannan and xylan (Erikkson *et al*. 1990) with occurrence in softwood varying

between 5 - 15%, compared to 20 - 30% in hardwood (Coughlan and Hazlewood 1993). Softwood hemicelluloses are mostly galactoglucomannan, glucomannan and arabinoglucoronoxylans (7 - 8%) whereas acetylglucuronoxylan (15 - 30%) and glucomannan (3 - 5%) predominantly occur in hardwood (Coughlan and Hazlewood 1993).

2.2.2.1. VARIOUS FORMS OF MANNAN

Mannan can appear in several different forms but has a general β-1,4-linked D-mannopyranosyl unit backbone from which the different forms originate (Coughlan and Hazlewood 1993). Different forms include a linear homopolymer of mannan, glucomannan, galactomannan and galactoglucomannan.

Mannan present in the endosperm of Palmae (*Phytelephas macrocarpa*), green coffee beans (*Coffea arabica*) and dates (*Phoenix dactylifera*) consist of a backbone of β-1,4-linked mannose residues containing less than 5% galactose (Aspinall 1959, Meier 1958, Petkowicz *et al*. 2001). Ivory nut mannan can be separated into Mannan I and Mannan II structures, the former being a polysaccharide with a DP of ~ 15 and the latter with a DP of ~ 80. Mannan I represents a crystalline and granular form that can be extracted with alkali methods, whereas Mannan II is a microfibrilar form that is less crystalline and cannot be extracted directly (Meier 1958, Aspinall 1959, Petkowicz *et al*. 2001).

Glucomannan comprises a heterogeneous polymer of β-1,4 linked D-mannose and D-glucose molecules in a molar ration of 3:1 with no acetic acid or galactose side groups present (Fig. 3) (Popa and Spiridon 1998). Glucomannan has a DP of less than 200 and chains are loosely packed in a paracrystalline array between cellulose microfibrils. They occur in most hardwood in a glucose/mannose ratio of 1:1.5 - 2 at concentrations of up to 2 – 5% (Coughlan and Hazlewood 1993, Hongshu *et al.* 2002). Glucomannan is found to a lesser extent in softwood and may contain α-1,6-linked galactose branches in a mannose: glucose: galactose ratio of 3:1:0.1 (Coughlan and Hazlewood 1993).
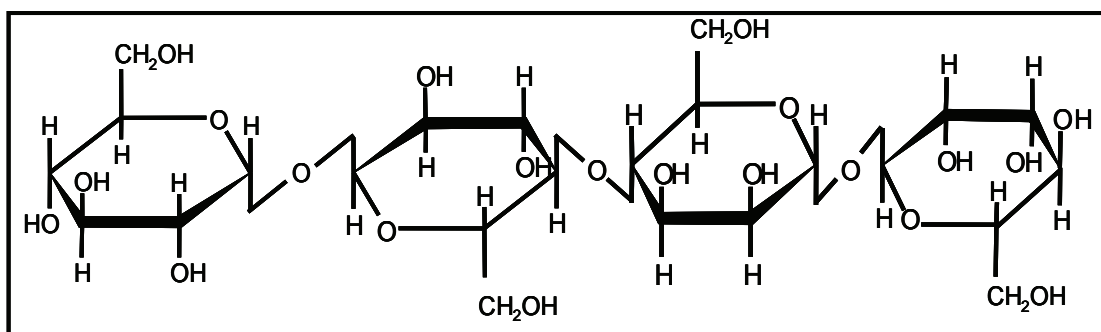
Fig. 3. A portion of the structure of glucomannan - a heterogeneous polymer consisting of β-1,4 linked D-mannose and D-glucose molecules (Coughlan and Hazlewood 1993).

Galactomannan consists of water soluble 1,4-linked β-D-mannopyranosyl chains substituted at position *O*-6 by a single α-D-galactosopyranosyl side chain (Fig. 4) (Eriksson *et al*. 1990, Parvathy *et al*. 2005). The distribution and abundance of the D-galactosyl unit varies among different plant species, but is usually more than 20 galactose units per mannose unit (Coughlan and Hazlewood 1993, Eriksson *et al*. 1990). Species like *Mimosa scabrella* can have galactose to mannose ratios as high as 1:1 whereas species like *Ceratonia silique* has a ratio of 1:4 (Moreira and Filho 2008). Galactomannan is mainly found in the endospermic part of seeds of the Leguminoseae family but have also been found in Annonaceae, Convolvulaceae, Ebenaceae, Loganiaceae and Palmae (Moreira and Filho 2008).

Acetylated galactoglucomannan is the main hemicellulose in cell walls of higher plants. It has the same basic structure as glucomannan, but contains α-1,6- linked galactose residues and acetyl groups substituted on both the D-mannosyl and D-glucosyl units in the backbone chain (Popa and Spiridon 1998, Sjöström 1993). Solubility in water is due to the D-galactose units preventing macromolecules from aligning. Mannosyl units can be partially substituted by *O*-acetyl groups between postitions C-2 and C-3 with an average of 1 group per 3 to 4 hexose units (Coughlan and Hazlewood 1993, Eriksson *et al*. 1990, Popa and Spiridon 1998).
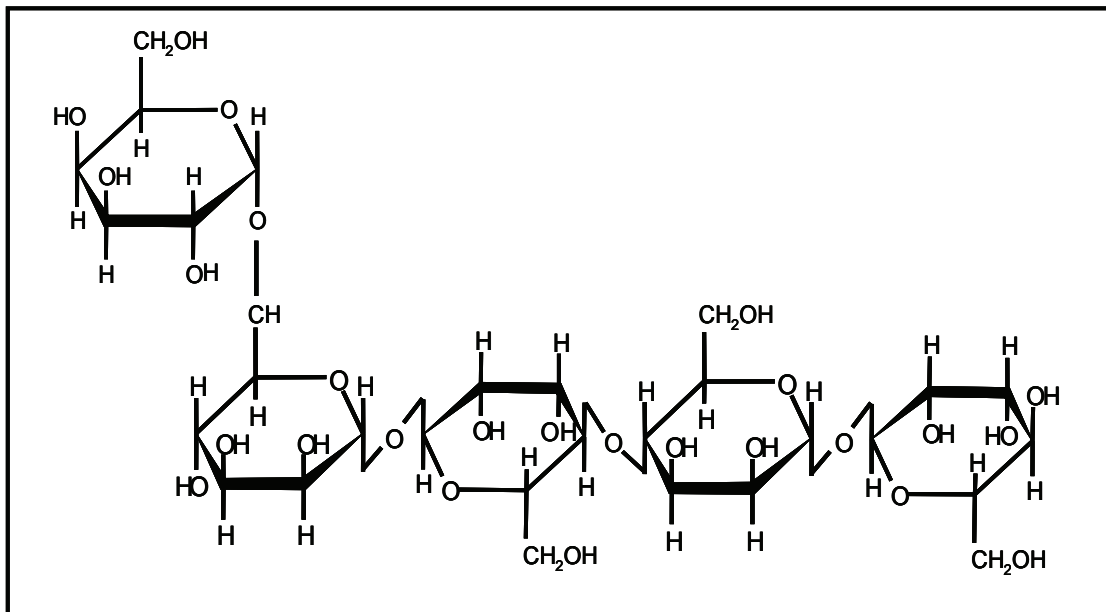
Fig. 4. Galactomannan structure consisting of 1,4 linked β-D-mannopyranosyl chains substituted at position *O*-6 by a single α-D-galactosopyranosyl side chain (Coughlan and Hazlewood 1993).

Acetylated galactoglucomannan are predominant found in softwood and the types of the galactomannan are classified based to the galactose content. The galactoglucomannan type with a high galactose content (5 – 8% wood dry weight) consists of glucose, mannose and galactose in a ratio of 1:4:0.1 while the galactoglucomannan type with a lower galactose content 10 - 15% has a 1:3:1 ratio (Sjöström 1993).

Unusual forms of mannan are present in some species such as *Siphonales* (green algae), where the linear mannan seems to replace cellulose (Preston 1979). *Retama raetum*, a wild plant from the Fabaceae family has β-D-mannopyranosyl units linked in a 1,3- and a 1,4- manner with galactopyranosyl units attached to the *O*-6 position (Ishurd *et al*. 2004). Red seaweed (*Nothogenia fastigiata*) contains a complex sulfated xylo-mannan structure (Erra-Balsells *et al*. 2000). The sulfated α-1,3- linked D-mannan chain structure is characterized by the ends having a β-1,2 linked D-xylose unit (Erra-Balsells *et al*. 2000). Acemannan is a form of mannan produced by *Aloe vera* where β-1,4- linked D-mannosyl residues form the backbone chain

(Femenia *et al*. 1999). Position C-2 or C-3 is acetylated and position C-6 has a galactose side chain attached to it (Femenia *et al*. 1999).

2.2.2.2. THE FUNCTION, APPLICATIONS AND STRUCTURE OF MANNAN

Mannan is a major hemicellulose in softwood and is widely distributed in all plant tissues, serving different functions (Filho 1998, Petkowicz *et al*. 2001). Mannan functions as a major structural unit in wood and seeds of plants and is responsible for interacting with and keeping cellulose fibres connected (Liepman *et al*. 2007). It also serves a storage function in the plant and acts as a carbohydrate reserve in the walls of endosperms and vacuoles of seeds and vegetative tissues (Meier and Reid 1982). In seeds occurring in areas with high atmospheric pressure, mannan retains water by salvation. This prevents denaturation of seed protein that is crucial in development stages (Dea and Morrison 1975). More functions are being discovered and recent studies indicated mannan to be a signalling molecule involved in plant growth and development (Liepman *et al*. 2007).

Mannan has various applications and is used in various industries. They are produced in large amounts and are 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, Tara gum, and Guar gum. The structures of Locust bean gum and Tara gum is shown in Fig. 5 (Moreira and Filho 2008). 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 stabilizers in low-fat and non-fat dairy products and have many fat-replacement applications acting as fat-imitator (Fernández *et al*. 2007, Hsu and Chung 1999). Gum extraction is inexpensive, non-toxic and has GRAS (Generally Regarded As Safe) status (Moreira and Filho 2008). Other mannans like linear mannan from *Aloe vera* have immunopharmacological and therapeutic properties (Aspinall 1959, Mehavr

2003). Glucomannans have a variety of applications including being used in weight control agents and preventing chronic disease (Ishurd *et al*. 2006).
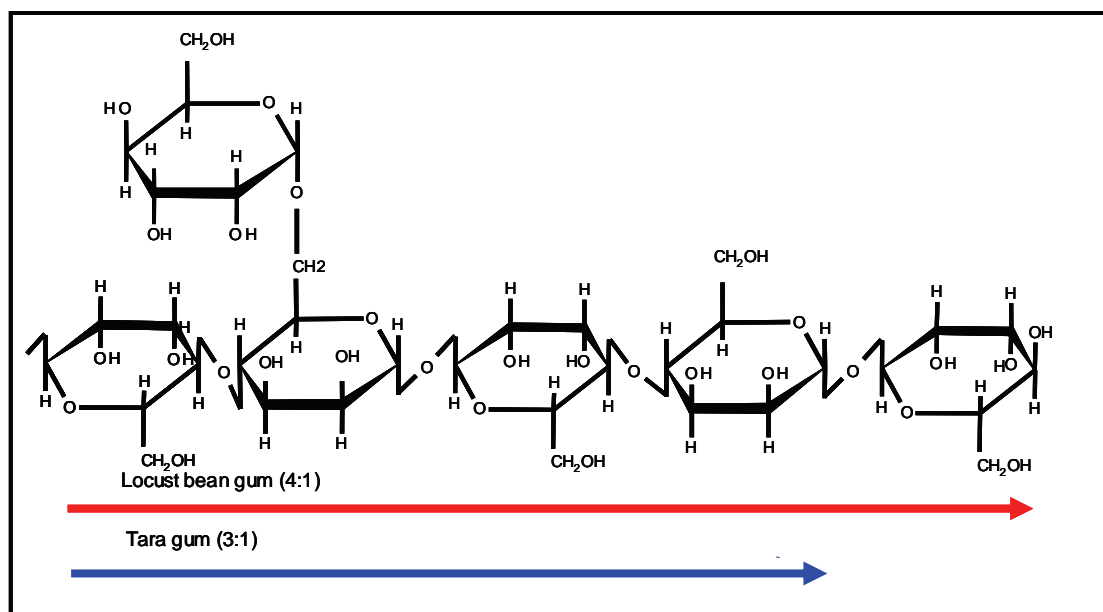
Fig. 5. The repeat units of the two types of gums, consisting of a linear backbone of 1,4-linked β-D-mannose units and a α-D-galactose residue on position C-6 (Sittikijyothin *et al.* 2005). Arrows indicate →(A) The structure of Locust bean gum with a 4:1 ratio of mannose per galactose substitution and → (B) Tara gum with a 3:1 ratio of mannose per galactose substitution.

2.2.2.3. THE STRUCTURE OF XYLAN

Xylan is the second most abundant polysaccharide in nature and the main hemicellulose found in plant cell walls, forming 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. The xylan heteropolysaccharide consists of β-1,4- linked D-xylose and various substituents that can be linked to the main chain (Coughlan and Hazlewood 1993, Eriksson *et al*. 1990). The xylan chain forms a helical three-fold, left handed structure and is versatile in its conformation due to the lack of additional inter and intra molecular hydrogen bonds between the pentose sugars (Atkins 1992). Substituents of the homopolysaccharide backbone chain

determine the viscosity, solubility and physiochemical properties. Frequency and substituent composition differs between plant species. Softwood xylan includes 4-*O*-methyl-D-glucuronosyl (Fig. 6) and L-arabinofuranosyl residues whereas hardwood xylan has *O*-acetyl-4-*O*-methyl-D-glucuronosyl and acetic acid estrified D-xylose residues. Arabinosylated xylans can be found in grasses and cereal (Collins *et al*. 2005, Coughlan and Hazlewood 1993, Eriksson *et al*. 1990).
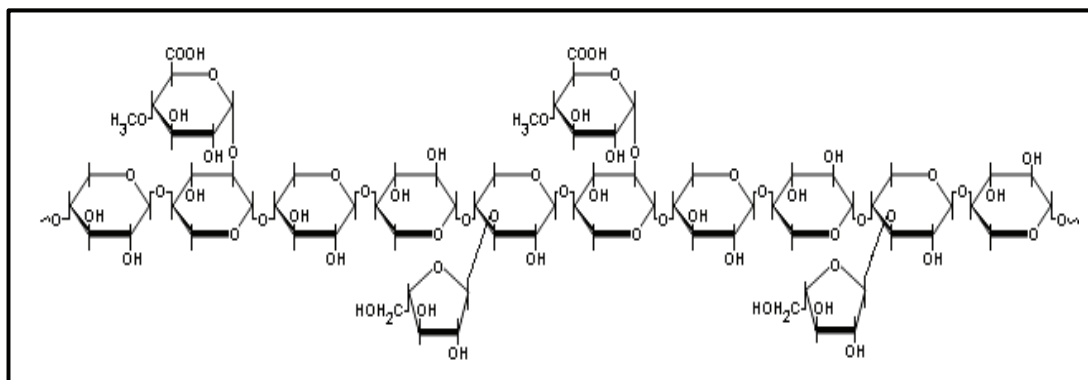
Fig. 6. Structure of softwood xylan, 4-*O*-methyl-D-glucuronosyl xylan (Coughlan and Hazlewood 1993, Eriksson *et al*. 1990).

Arabinoglucuronoxylan is a major hemicellulose in softwood, forming 10 – 15% of wood dry weight (Coughlan and Hazlewood 1993). It consists of a 1,4-linked β-D-xylopyranose backbone where per every tenth xylose unit, two 4-*O*-methyl-α-D-glucuronic acid residues are substituted at the C-2 position (Coughlan and Hazlewood 1993, Eriksson *et al*. 1990). An average of 1.3-α-L-arabinopyranose units is also present per 10 xylose units on position C-3. Reducing-end groups with D-rhamnosyl, D-galacturonosyl and D-xylosyl residues are found in both hardwood and softwood (Coughlan and Hazlewood 1993).

Acetyl-4-*O*-methylglucoronoxylan is the major hemicellulose in hardwood species, forming 10 – 35% of hardwood dry weight (Coughlan and Hazlewood 1993, Eriksson *et al*. 1990). Acetyl groups occur on every seven to ten xylose units on the β-D-1,4-linked xylopyranose backbone at the C-2 or C-3 position. A terminal chain consisting of a single 4-*O*-methyl-α-D-glucoronic acid residue is found at every tenth

xylose unit, linked directly to the C-2 position of the xylose unit. Furthermore, the xylan main chain may also have small amounts of rhamnose and galacturonic acid that are also essential to the structure (Coughlan and Hazlewood 1993).

Arabinoxylan (Fig. 7) consists of the β-1,4-D-xylan backbone chain, but has less 1,2-linked 4-O-methyl-α-D-glucuronic acid residues and is more highly branched. Large proportions of L-arabinofuranosyl units are linked mainly to the C-2 position of xylose. The ratio of xylose and arabinose differ between species and tissues within the same species (Coughlan and Hazlewood 1993). Barley and straw have L-arabinose residues that are esterified with ferulic acid at every 15th residue and one in every 31 residues is esterified with *p*-couamric acid (Puls and Schuseil 1993). Graminaceous plants contain xylan with *O*-acetyl groups. The main chain has β-D-xylanopyranose residue with L-arabinofuranosyl side chains at the C-2 and C-3 postions. Grass cell walls contain 1 - 2% phenolic acid substituents esterified to position 5 of the arabinose substituents (Coughlan and Hazlewood 1993).
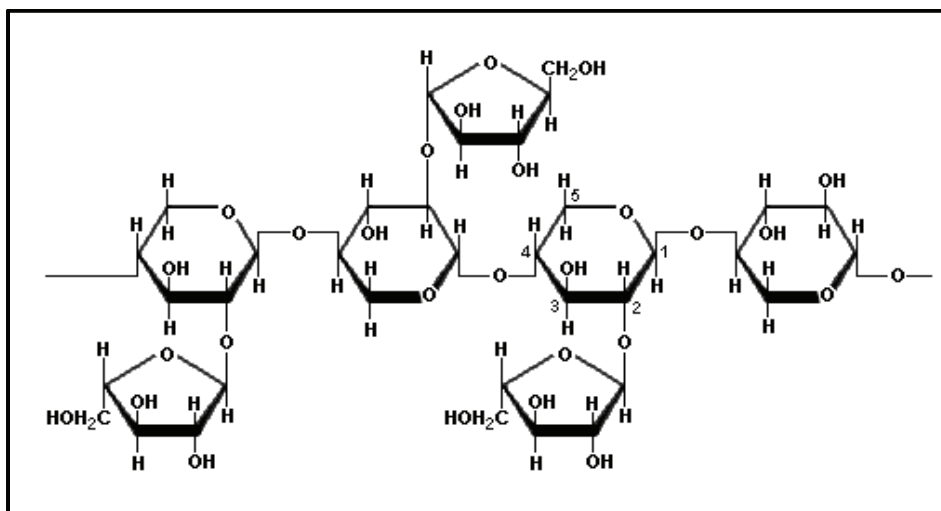
Fig. 7. Arabinoxylan consists of a highly branced β-1,4-D-xylose backbone chain with 1,2-linked 4-*O*-methyl-α-D-glucuronic acid (Coughlan and Hazlewood 1993).

Another form of hemicellulose is arabinogalactans. They are found in larchwood and have a highly branched structure (Coughlan and Hazlewood 1993). The backbone chain consist of two β-1,6-linked D-galactopyranose residues per β-1,3-linked D-galactopyranose unit. Furthermore 3-*O*-β-L-arabinopyranosyl-L-arabinofuranose residues are linked to the galactose units of the main chain. Terminal residues of D-glucuronic acid and L-arabinofuranose are also present (Coughlan and Hazlewood 1993).

2.2.3. LIGNIN

Lignin is a complex polyphenolic compound present in softwood at a concentration of 20 - 30% and in hardwood at 18 – 25% (Eriksson *et al*. 1990). It is responsible for cell wall rigidity and durability occurring mostly in the middle lamella of plants (Mosier *et al*. 2005). Synthesis of lignin takes place via the phenylpropanoid pathway through dehydrogenative polymerization of *p*-hydroxycinnamyl alcohols. The *p*-coumaryl alcohol, *p*-coniferyl alcohol and *p*-sinapyl alcohol are polyphenolic precursors that are linked in an irregular pattern through polymerization by peroxidase and laccase enzymes resulting in an aromatic polymer (Boudet *et al*. 1995, Perez *et al*. 2002). Three major groups of lignin can be distinguished. Coniferyl alcohol is the main precursor in gymnosperms in which case dehydrogenation forms guaiacyl lignin. In angiosperms dehydrogenation of sinapyl alcohol and *p*-coumaryl alcohol forms guaiacyl-syringyl lignin. Grasses contain guaiacyl-syringyl-*p*-hydroxyphenyl-lignin (Eriksson *et al*. 1990, 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 (Perez *et al*. 2002, Mosier *et al*. 2005). A reduction in the concentration, hydrophobicity and cross-linking of lignin enhances enzymatic hydrolysis of the structural polysaccharides in cell walls (Grabber 2005).

2.3. DEGRADATION OF MANNANS

Fungi form a crucial part of the ecosystem (Jose and Demain 2003, Polizeli *et al*. 2005). Fungi decompose polysaccharides and recycle inorganic and organic material.

For many decomposers the plant cell wall polysaccharides are their primary carbon energy source. Degradation of the plant cell wall is achieved by the production of enzymes, usually secreted, to degrade the polysaccharides into mono- and oligosaccharides (Jose and Demain 2003, Polizeli *et al*. 2005). Fungi have different enzymatic affinities for the three different cell wall components (cellulose, hemicellulose and lignin), and therefore different relative proportions of the components are seen during the decay process (Eriksson *et al*. 1990, Rayner and Boddy 1988). These fungi are traditionally termed white rot, brown rot or soft rot fungi (Irbe *et al*. 2001, Rayner and Boddy 1988). Fungi that primarily degrades the lignin component of wood and the cellulose and hemicellulose components to a lesser extent gives the wood a bleached appearance and the fungi involved are therefore termed white rot fungi. Fungal decay in which the wood is discoloured brown (from which hemicelluloses and cellulose have been selectively removed), leaving mostly modified lignin, are caused by brown rot fungi (Irbe *et al*. 2001, Rayner and Boddy 1988). Fungi that degrade cellulose and hemicelluloses, but not lignin, are termed soft rot fungi (Irbe *et al*. 2001, Rayner and Boddy 1988).

Fungi from the genera *Aspergillus* and *Trichoderma* are soft rot fungi. The ability of these soil-living fungi to produce polysaccharide-hydrolysing extracellular enzymes makes them attractive for several industrial applications. Enzymes produced by these strains for the degradation of hemicelluloses, especially mannan, include endo-β-mannanases, β-mannosidases, α-galactosidases and β-glucosidases (Jose and Demain 2003, Moreira and Filho 2008, Polizeli *et al*. 2005).

2.3.1. GLYCOSYL HYDROLASES

Glycoside hydrolases are the main group of enzymes involved in degradation of plant polysaccharides (Davies and Henrissat 1995). The hydrolysis of mannans requires a large variety of enzymes to act cooperatively. The enzymes involved are either endohydrolases or exohydrolases. Endohydrolytic enzymes cleave internal bonds randomly while exohydrolytic enzymes cleave the terminal glycosidic linkages (Crout and Vic 1998, Davies and Henrissat 1995).

The β-glycoside hydrolases are grouped into enzyme families according to amino acid sequence similarities and hydrophobic cluster analysis (Henrissatt and Bairoch 1993, Lemesle-Varloot 1990). New families and addition to different existing families is made as new sequences become available (Henrissatt and Bairoch 1993, Henrissatt and Davies 1997, Henrissat *et al*. 1998, Henrissat and Bairoch 2008). A continuously updated list of the glycoside hydrolase families can be found on the Carbohydrate-Active Enzyme database (CAZY) (http://www.cazy.org). Data gathered over time showed families with the same basic enzyme folds, pointing to the direct relation between family classification and tertiary structure (Henrissat 1991). Glycosyl hydrolases can be further grouped into clans based on the tertiary structure at the active site (Henrissat *et al*. 1995, Juers *et al*. 1999). GH-A is a major clan consisting of at least 16 families (Jenkins *et al*. 1995, Juers *et al*. 1999). The protein fold of this clan is the $(\beta/\alpha)_8$-barrel and the positions of the catalytic residues are conserved, with the catalytic nucleophile and acid/base both situated on the C-termini of β-strands 7 and 4, respectively (Jenkins *et al*. 1995).

In glycosyl hydrolases, that are endo- and exo-acting enzymes, the two major cleavage preferences seems to be correlated to active site architecture (Dominguez *et al*. 1995, Sabini *et al*. 2000a, b). Endo-acting enzymes, like endoglucanases and β-mannanases, frequently have cleft_shaped active sites. Exo-acting enzymes like β-galactosidases and β-mannosidases frequently have pocket-shaped active site architecture (Aleshin *et al*. 1994, Juers *et al*. 1999). However, enzymes with exo-activity sometimes display endo-activity and enzymes with endo-activity can similarly display exo-activity. Therefore, architecture of the active site does not necessarily give an indication of the cleavage preferences (Ståhlberg *et al*. 1993, Tomme *et al*. 1996).

The tertiary structure gives an indication of the mechanism which can either be inverting or retaining (Davies and Henrissat 1995, Withers 2001). A single or double-displacement reaction involves participation of two amino acid residues acting by a general acid catalysis mechanism. It occurs at the anomeric carbon atom of the glycoside hydrolase as a result of inversion or retention of the configuration (Davies

and Henrissat 1995, Withers 2001). Inverting enzymes use direct displacements (Fig. 8) with a general base-catalysed attack of water through an oxocarbenium ion-like transition state. General-acid-catalytic assistance occurs by means of two carboxylic acids. One carboxylic acid provides base catalytic support to the attack of water and the other provides acid catalytic support in order for the glycosidic bond to be cleaved (Withers 2001).
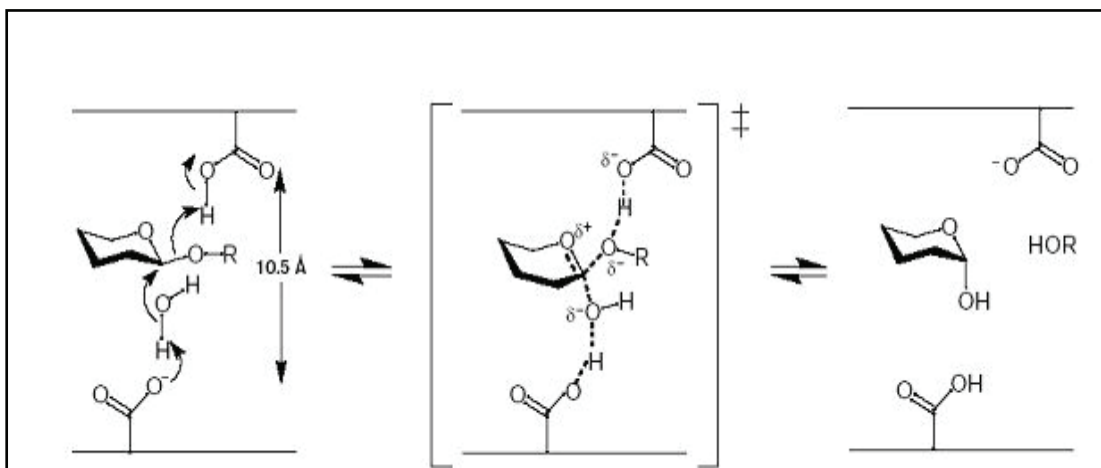
Fig. 8. Inverting glycosyl hydrolases: inversion of the anomeric configuration through a single nucleophilic displacement (Davies and Henrissat 1995).

Retaining enzymes operate via a double-displacement or two-step mechanism where a covalent glycosyl-enzyme intermediate occurs (Fig. 9). Two catalytic carboxylates (aspartate or glutamate) on opposite sides of the sugar plane performs two separate chemical steps (Davies and Henrissat 1995, Withers 2001). In the first glycosylation reaction, the anomeric centre is attacked by a nucleophile and the acid-base catalyst promotes the aglycone removal. This allows the formation of a covalent glycosyl-enzyme intermediate which is stable enough for the aglycone to be removed and replaced with water. The second deglycosylation reaction involves the attack of water on the opposite side of the anomeric centre and is promoted by the base-acid catalyst. The nucleophile is displaced and the aglycone is released having the same anomeric configuration as the substrate (Withers 2001).
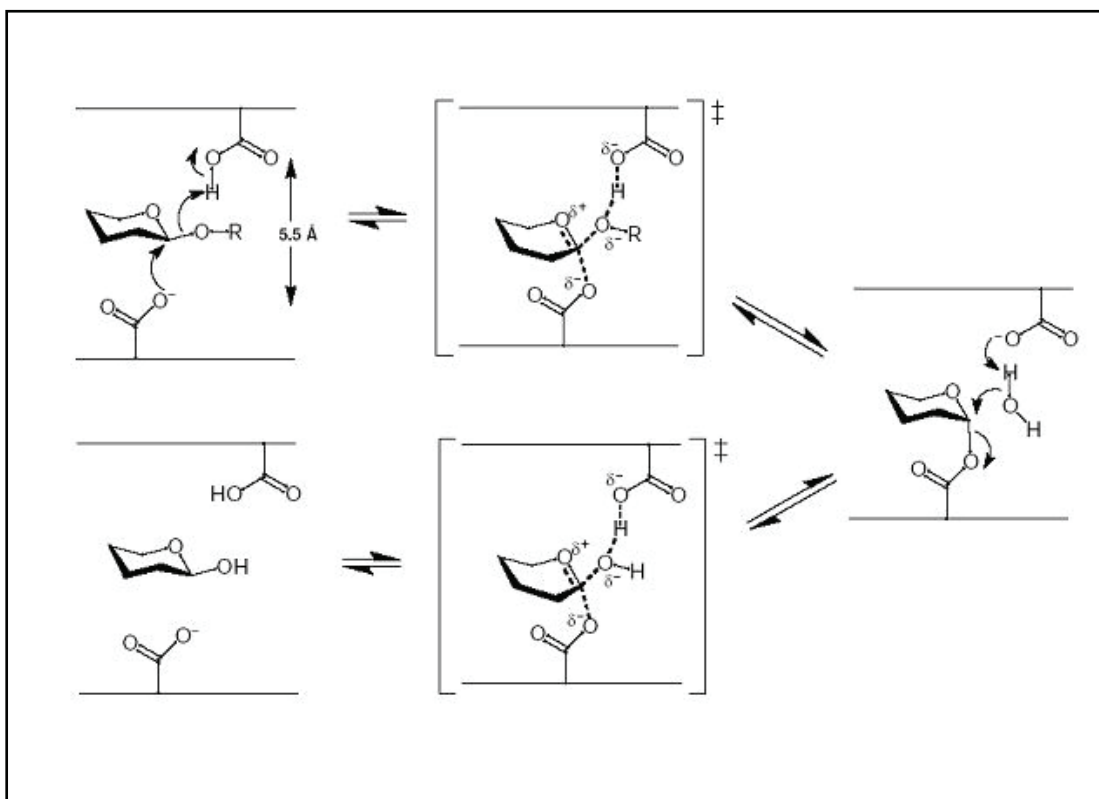
Fig. 9. Retaining glycosyl hydrolases: retaining of anomeric configuration through double displacement (Davies and Henrissat 1995).

2.3.2. MANNAN DEGRADING ENZYMES

Due to the complex structure of hemicelluloses, various enzymes are needed for its complete degradation (Fig. 10). Complete degradation of *O*-acetyl-galactoglucomannan begins with endo-β-1,4 mannanases, endohydrolases that cleave the mannan backbone resulting in oligosaccharides of various lengths (Stoll *et al*. 2000). At the same time, α-galactosidases remove the galactose units present as side chains on the mannan backbone (McCutchen *et al*. 1996). β-mannosidases, exohydrolases, hydrolyze the oligomannans released by β-mannanase activity resulting in single mannose molecules (Moreira and Filho 2008). The β-glucosidases, and esterases are additional enzymes that catalyze the

removal of glucose and acetic acid, respectively, from the main mannan chain (Moreira and Filho 2008).
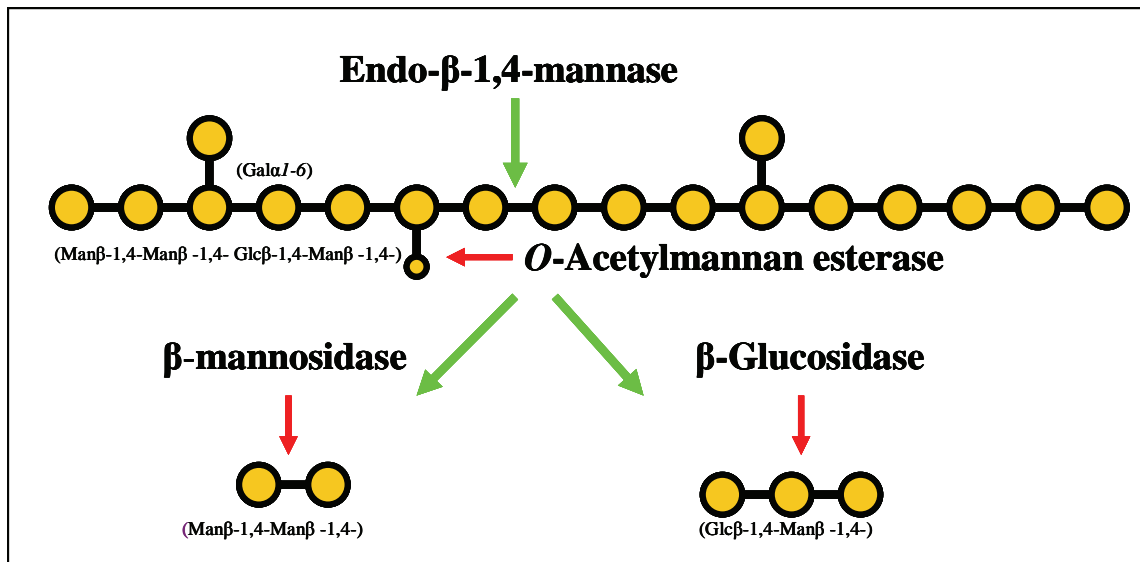
Fig. 10. Schematic representation of the degradation of *O*-acetyl-galactoglucomannan (Moreira and Filho 2008).

2.3.3. ENDO β-1,4-MANNANASE

2.3.3.1. OCCURRENCE

β-mannanase (1,4-β-D-mannan mannohydrolase EC 3.2.178) randomly cleave the β-1,4-mannopyranosyl linkages in mannans, glucomannan and galactomannan, resulting in new chain ends (Stoll *et al*. 2000). β-mannanases have been isolated and characterized from various fungi (Ademark *et al*. 1998, Christgau *et al*. 1994) bacteria (Akino *et al*. 1989, Braithwaite *et al*. 1995), plants (Bewley *et al*. 1997, Marraccini *et al*. 2001) and animals (Xu *et al*. 2002, Yamaura *et al*. 1996) β-mannanases from bacteria include sources such as aerobes, anaerobes and extremophiles (thermophiles, halophiles and psychrophiles) (Parker *et al*. 2001,

Waino and Ingvorsen 1999, Zakaria *et al*. 1998). β-mannanases are mostly produced extracellularly, but cell wall bound mannanases have also been reported. β-mannanases from plant origin, are involved in seed germination and fruit ripening (Nonogaki and Morohashi 1999, Nonogaki *et al*. 2000).

2.3.3.2. HYDROLYSIS AND SUBSTRATE BINDING

A chain length of four sugar residues is required for the binding of β-mannanases to ensure hydrolysis (McCleary and Matheson 1983, Sabini *et al*. 2000a, b). The substrate binding surface can be split into different subsites. Subsites are numbered from –n to +n (n being 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 subsite +1 and -1 (McCleary and Matheson 1983).
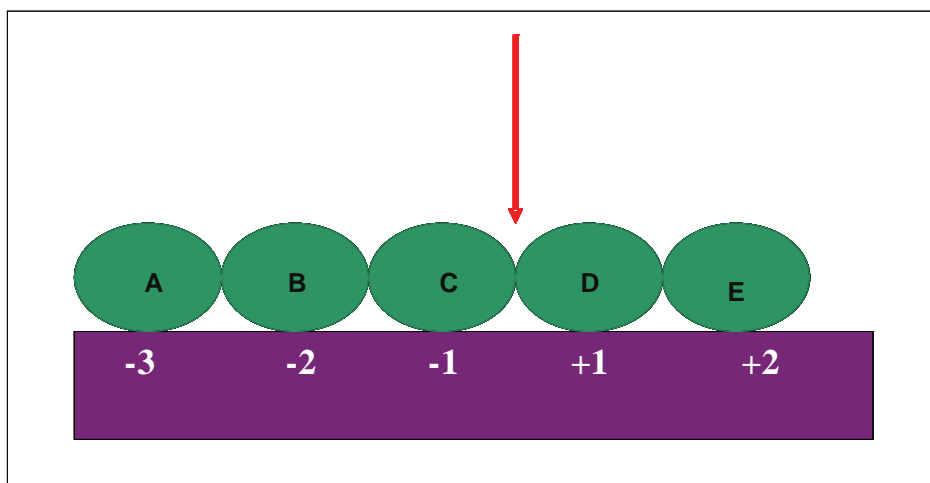
Fig. 11. Representation of the enzyme-substrate interaction and subsite binding of β-mannanase enzyme and the substrate, a β-1,4-mannan chain. Binding subsites are labeled -3 to +2 and mannose residues A-E (McCleary and Matheson 1983).

Most β-mannanases hydrolyse manno-oligosaccharides up to a DP of 4 (Biely and Kremnický 1998, McCleary 1988). Activity on mannotriose has been observed but at a much lower rate, signifying that at least 4 subsites are present in β-mannanases (Akino *et al*. 1989, Harjunpää *et al*. 1995). Hydrolysis by β-mannanases usually

results in mannobiose and mannotriose as end products (Ademark *et al*. 1998, Civas *et al*. 1984). Mannanases from *A. tamarii* (Civas *et al*. 1984), *Trichoderma reesei* (Stålbrand *et al*. 1993) and *A. niger* (Ademark *et al*. 1998) all produced mainly mannobiose, mannotriose and higher oligosaccharides. When side chains occur their distribution pattern can affect hydrolysis efficiency (Ademark *et al*. 2001, Stålbrand *et al*. 1995). Hydrolysis is blocked when the D-galactosyl and D-glucosyl side chains are bound to residue B and D or when C or E is replaced with a D-glucosyl in Fig. 11 (McCleary 1988, McCleary and Matheson 1983, Sabini *et al*. 2000). β-mannanases are seldom able to cleave mannobiose as is the case with Man5D from *Phanerochaete chrysosporium* (Benech *et al*. 2007), however, *A. aculeatus* β-mannanases not only released mannotriose and mannobiose but also mannose when hydrolyzing ivory nut mannan (Setati *et al*. 2001). Torto *et al*. (1996) also found some mannotetraose in hydrolysis experiments using ivory nut mannan and an endomannanase produced by *A. niger* (Torto *et al*. 1996).

Some organisms produce more than one enzyme (of similar function) often showing different specificities. *T. reesei* produces two β-mannanases that hydrolyze ivory nut mannan to mannotriose and mannobiose (Stålbrand *et al*. 1993). *Sclerotium rolfsii* produced a 42kDa, 58kDa and 61kDa β-mannanase (Gübitz *et al*. 1996 a, b, Sachslehner *et al*. 2000). The first hydrolyzed smaller fragments from mannan and was found to be active against mannotetraose and mannotriose. The 58kDa β-mannanase showed activity on mannotetraose, mannotriose and mannobiose, whereas the 61kDa β-mannanase from the same source showed a random-type breakdown of the mannan, with a slower decrease in the viscosity of mannan solutions (Gübitz *et al*. 1996 b, Sachslehner *et al*. 2000).

Apart from hydrolysis, some β-mannanases were also found to perform transglycosylation reactions (Coulombel *et al*. 1981, Gübitz *et al*. 1996 b, Harjunpää *et al*. 1999). A β-mannanase from *T. reesei* was shown to produce transglycosylation products with mannose or mannobiose as glycosidic bond receptors (Harjunpää *et al*. 1999).

2.3.3.3. FAMILY CLASSIFICATION AND STRUCTURE

β-mannanases are classified as glycosyl hydrolase family 5 and 26 based on amino acid sequence similarity (Henrissat 1991, Henrissat and Bairoch 1993). Family 5 represent mannan-degrading enzymes from bacterial organisms (*Caldocellum saccharolyticum, Cladibacillus, Vibrio* species), fungi (*Aspergillus aculaetus, Trichoderma reesei, Agaricus bisporus*) and eukaryotic (*Lycopersicon esculentum* and *Mytilus edulis*) origin (Dhawan and Kaur 2007, Larsson *et al*. 2006, Ximenes *et al*. 2005). Family 26 enzymes are mostly from bacterial origin (*Bacillus* sp*., Cellvibrio japonicus, Pseudomonas fluorescens, Rhodothermus marinus*), but also contain mannanases of the anaerobic fungus (*Piromyces* sp.) (CAZY, Dhawan and Kaur 2007). 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*. 1995 a, b).

Families 5 and 26 (http://www.cazy.org) include endoglucanases and mannanases which are in the GH-A clan. They have a characteristic (βα)$_8$-barrel fold of the enzyme catalytic domain and both families use the retaining mechanism (Ademark *et al*. 1998, Arai *et al*. 1995, Bolam *et al* 1996). Three dimensional structures and X-ray crystallography for β-mannanases from *T. reesei* and *Thermobufida fusca* have been determined and is shown in Fig. 12. The active site can be visualised as a cleft and has eight conserved amino acids for *T. fusca* (Arg50, His86, Asn127, Glu128, His196, Tyr198, Glu225, Trp254) (Hilge *et al*. 1998). With the exception of family 26, the GH-A clan enzymes all have three conserved active site residues (one asparagine and two glutamates) (Bolam *et al*. 1996, Hilge *et al*. 1998, Hogg *et al*. 2001). For *T. fusca* the Glu128 acts as catalytic proton donor, Glu225 as catalytic nucleophile and Asn127 is involved in stabilizing the active site environment. Asparagine (Asn127) is strictly conserved among glycanases and mutation in this region causes complete loss of activity (Hilge *et al*. 1998).
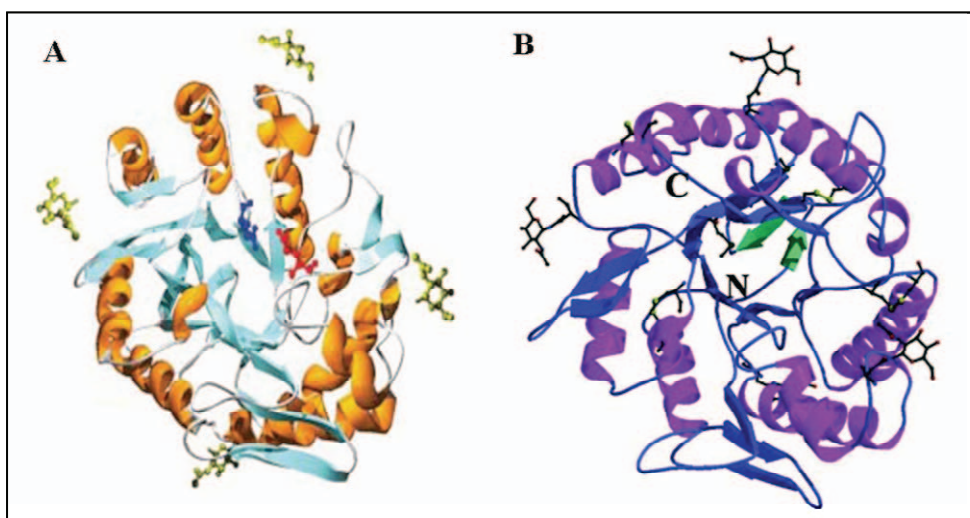
Fig. 12. The structure of the catalytic module of family 5 β-mannanase from (A) *T. fusca* (http://afmb.cnrs-mrs.fr/~CAZY/index.html). (B) *T. reesei* β-mannanase secondary structure. In the *T. fusca* mannanase, two short β-strands (sky blue) at the N-terminus form the bottom of the barrel. A three-stranded and a two-stranded β-sheet (both in blue) that lie close to the C-terminus is also shown (Sabini *et al*. 2000).

2.3.3.4. BIOCHEMICAL PROPERTIES

Several β-mannanases from families 5 and 26 are shown in Table 1. The optimal pH of β-mannanases are mostly neutral or acidic. The temperature optima of β-mannanases range between 40°C – 70°C. However, β-mannanases from thermophiles have a higher temperature optima (Gibbs *et al*. 1999, Politz *et al*. 2000, Parker *et al*. 2001, Sunna *et al.* 2000). Most β-mannanases have molecular weights of 30 – 80kDa, but the modular enzymes can be up to 100kDa (Cann *et al*. 1999, Stoll *et al*. 1999, Sunna *et al*. 2000). Most β-mannanases have isoelectric points between 4 and 8. In some organisms, multiple β-mannanases with different isoelectric points and molecular weights are produced (Akino *et al*. 1989, Marraccini *et al*. 2001, Millward-Sadler *et al*. 1995, Stålbrand *et al*. 1993). These enzymes can 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 different genes (Millward-Sadler *et al*. 1994, Millward-Sadler *et al*. 1996).

2.3.4. β-1,4-MANNOSIDASE

2.3.4.1. OCCURRENCE

β-Mannosidases (β–D-mannoside mannohydrolase, EC 3.2.1.25) are essential for the complete hydrolysis of β-mannans and hydrolyze manno-oligosaccharides to mannose (Moreira and Filho 2008). β-Mannosidases have been isolated and characterized from fungi (Ademark *et al*. 1998, Arai *et al*. 1995), bacteria (Bauer *et al*. 1996, Duffaud *et al*. 1997, Stoll *et al*. 2000), plants (McCleary *et al*. 1982, Mo and Bewley 2002) and animals (Charrier and Rouland 2001). β-Mannosidases from bacteria include sources such as eubacteria and archaebacteria (*T. fusca*) (Béki *et al*. 2003). β-Mannosidases can have different functions depending on the organism of origin. Bacteria and fungi normally produce β-mannosidases that degrade mannan or heteromannan from plants. 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, produces β-mannosidases that hydrolyse terminal non-reducing mannopyranoside linkages of glycoproteins (Chen *et al*. 1995). Lack of a functional β-mannosidase in humans leads to deleterious storage of Man-β-1,4-GlcNAc and is known as β-mannosidosis, a congenital disorder (Alkhayat *et al*. 1998).

2.3.4.2. HYDROLYSIS AND SUBSTRATE INTERACTION

β-Mannosidases can be active on glucosides and mannosides (Bauer *et al*. 1996). β-Mannosidases that are capable of cleaving manno-oligosaccharides with a DP of over 4 have also been found (Akino *et al*. 1988, Arai *et al*. 1995). β-Mannosidase from *Aspergillus niger* was found to cleave up to a DP of 6 with the rate of hydrolysis shown to be dependent on the degree and pattern of the side-chain substitutions (Ademark *et al*. 1999). β-Mannosidases are essential for the complete hydrolyisis of plant heteromannans and convert manno-oligosaccharides to mannose (Moreira and Filho 2008). Fungal β-mannosidases (*A. niger, A. awamori, T. reesei*) were shown to hydrolyse shorter manno-oligosaccharides (Neustroev *et al*. 1991). β -Mannosidases

from eukaryotes (human, bovine, caprine) cleaved the N-linked oligosaccharides of glycoproteins (Alkhayat *et al*. 1998, Chen *et al*. 1995). Hydrolysis of Ivory nut mannan requires only β-mannanase and β-mannosidase whereas the hydrolysis of Locust bean gum (galactoglucomannan) requires α-galactosidases in addition to the β-mannanase and β-mannosidase activities (Stoll *et al*. 1999).

A chromogenic substrate (*p*-nitophenyl β-D-mannopyranoside) is commonly used to determine β-mannosidase activity and 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). These enzymes can be either secreted or intracellular. *A. niger*, *T. reesei* and *A. awamori* produce β-mannosidases that are secreted (Stoll *et al*. 2000). *A. pullulans* and *C. fimi* produces β-mannosidases when β-1,4-mannobiose is present in the medium, but production is repressed in the presence of mannose. The enzyme remains intracellular and a membrane-embedded mannobiose permease transports the dissacharide into the cells (Kremnický and Biely 1997, Stoll *et al*. 1999). It is speculated that the extracellular mannosidase producing organisms lack a mannobiose permease and therefore enzymes are secreted.

2.3.4.3. FAMILY CLASSIFICATION AND STRUCTURE

Most β-mannosidases are classified as glycosyl hydrolase family 2, with the exception of the enzyme produced by *Pyrococcus furiosus* which was placed in family 1 of the glycosyl hydrolases (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). Family 2 also includes β-glucuronidase and β-galactosidase enzymes. In some cases some enzymes have functional differences and they do not correspond to the family consensus pattern but they are none the less still confirmed as family 2 members. Glu-519 was shown as 33 conserved catalytic nucleophile in a β-mannosidase 2A from *Cellumonas fimi* (Stoll *et al*. 2000). This is the same residue that was identified within a β-galactosidase (*E. coli* β-galactosidase) and β-glucuronidase (Human 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 family 2.

2.3.4.4. BIOCHEMICAL PROPERTIES

β-mannosidases possess molecular weights of between 50 – 130kDa and they can consist of several subunits (Bauer *et al*. 1996, Parker *et al*. 2001). The optimum temperature can range between 40˚C - 70˚C and pH optima from 4 - 5.5 (Table 2). Bacterial β-mannosidases have neutral pI but most isoelectric points are acidic.

2.3.5. α-GALACTOSIDASE

α-Galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) hydrolyse 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 exist. Some enzymes cleave α-1,6-linked galactose units linked to the inner mannose residues of the galacto(gluco)mannan substrates 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). α-Galactosidases have been placed in families 4, 27, 36 and 57 of the glycoside hydrolases (Henrissat 1991). Bacterial α-galactosidases are mostly present in families 4 and 36, while eukaryotic enzymes are grouped into family 27. Most of the enzymes in families 4 and 27 can release galactose from polymeric substrates, whereas 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.

Table 1. A summary of biochemical properties of β-1,4-mannanases from bacterial, fungal, animal and plant sources.

Organism name	Protein name	Family	MW (kDa)	pH opt.	Temp opt.(˚C)	pI	Reference
<u>Bacterial</u>							
Bacertiodes ovatus	Man	26	61/190	6.2-6.5	37	4.8-6.9	Gherardini and Salyers 1987
Bacillus circulans K-1	Man1	5	62	6.9	64-65	5.4-6.2	Yosida *et al*. 1997; 1998
Bacillus sp. 5H	Man	26	37	-	-	-	Khanongnuch *et al*. 1999
Bacillus sp. strain AM-001	ManA	26	58	9	60	5.9	Akino *et al*. 1989
Bacillus stearothermophilus	ManF	5	73/162	5.5-7.5	70	-	Talbot *et al*. 1990
Bacillus subtilis NM-39	Man1	26	38	5	55	4.8	Mendoza *et al*.1994; 1995a
Caldicellulosiruptor. saccharolyticus Rt8B.4	ManA	26		6-6.5	60-65		Gibbs *et al*. 1996
Clostridiumcellulovorans	ManA3	5	30.7	6	85	-	Tamaru *et al*. 2000
Cellulomonas fimi	Man26A	26	50	5.5	42		Stoll *et al*. 1999
Dictyoglomus thermophilum	manA	26	40	5.0	80		Gibbs *et al*. 1999
Flavobacterium	ManA	26	46	7	35		Zakaria *et al*. 1998
Pseudomonas fluorescens	ManA	26	46	7			Braithwaite *et al*. 1995
Rhodothermus marinus	ManA	26	60	5.4	85	-	Politz *et al*. 2000
Thermoanaerobacterium polysaccharolyticum	ManA	5	116	5.8	65/75	-	Cann *et al*. 1999
Thermotoga maritima	ManB	5	76.9	7	90	-	Parker *et al*. 2001
Thermotoga neopolitana	Man5	5	55	7.1	92	-	McCutchen *et al*. 1996
Streptomyces lividans	Man5A	5	36	6.8	58	3.5	Arcand *et al*. 1993
Thermobifida fusca KW3	ManA	5	38	Broad	80	-	Hilge *et al*. 1998

Table 1. A summary of biochemical properties of β-1,4-mannanases from bacterial, fungal, animal and plant sources (continue).

Organism name	Protein name	Family	MW (kDa)	pH opt.	Temp opt.(°C)	pI	Reference
Fungal							
Agaricus bisporus D469	Cel2; cel4;	5	44.9	-	-	-	Yagüe *et al*. 1997
Aspergillus aculeatus	AaMan1	5	45	5	60	4.5	Christgau *et al*. 1994
Aspergillus niger	Man1	5	40	3.5	-	3.7	Ademark *et al*. 1998
Orpinomyces sp. Strain PC-2	ManA	26	-	-	50	-	Ximenes *et al*. 2005
Trichoderma reesei	ManA	5	51-53	Several	70	3.6-6.5	Stålbrand *et al* 1993/1995
Sclerotium rolfsii	Man	5	61	2.9	74	3.5	Gübitz *et al*. 1996b
Animals							
Littorina brevicula	Man	-	42	6.5	50		Yamaura *et al*. 1996
Mytilus edulis	Man5A	5	39	5.2	50-55	7.8	Larsson *et al*. 2000
Pomacea insularus	Man	-	44	5.5	50		Yamaura and Matsumoto 1993
Plants							
Trifolium repens	-	5	38-43	5.1-5.6	37	9.3	Villarroya *et al*. 1978
Lycopersicon esculentum	LeMan1,2	5	43	4.8	-	-	Bewley *et al*. 1997

Table 2. A summary of biochemical properties of β-mannosidases from bacterial, fungal, animal and plant sources.

Organism name	Protein name	Family	MW (kDa)	pH opt.	Temp opt.(°C)	pI	Reference
Bacterial							
Bacillus sp. AM-100	Man	2	94	6	50	5.5	Akino *et al*. 1988
Cellulomonas fimi ATCC 484	Man2A	2	103	7	55	-	Stoll *et al*. 1999
Pyrococcus furiosus	Mnd	1	59	7.4	105	6.9	Bauer *et al*. 1996
Thermobufida fusca	ManB	2	94	7.7	105	4.8	Béki *et al*. 2003
Fungal							
Aspergillus aculeatus	ManB	2	130	2	70	4	Arai *et al*. 1995
Aspergillus niger	MndA	2	135	2.5-5	70	5	Ademark *et al*. 2001
Sclerotium rolfsii	Mnd		57.5	2.5	55	4.5	Gübitz *et al*. 1996a
Thermobifida fusca TM51	Man2	2	94	7.17	53°C	4.87	Béki *et al*. 2003
Thermotoga neapolitana 506	Man2	2	95	7.7	87	5.6	Parker *et al*. 2001
Trichoderma reesei	Mnd	-	105	3.5	-	4.8	Kulminskaya *et al*. 1999
Animal							
Helix aspera Müller	-	-	77.8	3.3	37-42	-	Charrier and Rouland 2001
Homo sapiens	ManBA	2	110	4	55	4.7	Alkhayat *et al*. 1998
Pomacea canaliculata	-	-	90	5	45	4.3	Hirata *et al*. 1998
Plant							
Cyamopsis tetragonolobus	-	2	59	5-6	52	9.4	McCleary 1988

2.3.5. β-GLUCOSIDASE

β-Glucosidases (EC 3.2.1.21) are exo-acting glycosyl hydrolases that catalyse the hydrolysis of non-reducing terminal glucose residues (Lin *et al*. 1999). Most β-glucosidases are inhibited by glucose or cellobiose and are not able to hydrolyse long β-1,4-chains (Bauer *et al*. 1996, Lin *et al*. 1999). β-glucosidases are grouped into families 1 and 3 of glycosyl hydrolases (Henrissat and Bairoch 1993). In the degradation of hemicelluloses, β-glucosidases will release glucose from the non-reducing end of oligosaccharides that have already been cleaved by β-mannanases (Bauer *et al*. 1996, Lin *et al*. 1999).

2.3.6. ACETYL-MANNAN ESTERASES

Acetyl esterases are able to hydrolyse acetyl groups from various substrates (Tenkanen *et al*. 1993). Esterases from fungal sources like *A. niger* and *T. reesei* hydrolyse acetic acid from polymeric acetyl galactoglucomannan (Tenkanen *et al*. 1993, Tenkanen *et al*. 1995). *A. oryzae* has been shown to produce an esterase that can liberate phenolic side groups from xylan (Tenakanen *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).

2.4. INDUSTRIAL APPLICATIONS OF MANNANASES

The negative effect that chemical industrial processes have on the environment, has encouraged the use of biological processes such as enzymes applications (Dhawan and Kaur 2007, Wong and Saddler 1993). Mannanases have various applications and can be used in the food and feed industry, in the pulp and paper industry, in the detergent industry as well as in textile and cellulosic fibre processing (Dhawan and Kuar 2007).

Several mannanase cocktails are used for hydrolysing the mannan present in coffee. Mannan is the main polysaccharide of *Arabica* and *Robusta* coffee beans and galactomannan constitutes 20 – 30% of its dry weight (Sachslehner *et al*. 2000). Mannan is mostly responsible for the viscosity in instant coffee and causes gel formation during spray- and freeze-drying. Consequently, immobilised β-mannanases are used to hydrolyse galactomannan in coffee

extract, and when viscosity is lowered, coffee extract concentration is more cost effective and can easily be carried out with evaporation processes. Mannanases that have been successfully used are those from *S. rolfsii* which were shown to hydrolyse coffee mannan and reduce viscosity.

Poultry and livestock feed contain a large amount of galactomannan (Dhawan and Kuar 2007). Meal from soybean, palm kernel, copra, guar gum and sesame seeds are used as animal feed. They all have a high fibre content, low palatability, have few amino acids and anti-nutritional properties, and have a limited intestinal utilization (Chandrasekariah *et al*. 2001, Moss and Givens 1994). Mannans negatively affect animal performance having strong anti-nutritive effect on monogastric animals, swine and humans causing reduced nitrogen retention and fat absorption, having a negative effect on the growth of chickens (Jackson *et al*. 1999, Swapna *et al*. 1982). Incorporation of β-mannanases into chicken corn-soybean feed diets showed decreased intestinal viscosity and resulted in weight gain.

Alkaline treatment of wood pulp poses an environmental pollution problem (Hongpattarakere 2002). Lignin extraction from wood fibres is crucial for bleaching dissolving pulp. Removal of lignin involves pulp treatment under alkaline conditions that allow hemicelluloses to be separated from lignin (Hongpattarakere 2002). A biological solution involves enzymatic bleaching of softwood pulp using mannanases. Lignin is removed from pulp resulting in comparable yields to alkaline pre-treatment whilst reducing environmental pollution by avoiding the ultra-hot alkaline extraction step (Clarke *et al*. 2000, Cuevas *et al*. 1996). Chlorine-free bleaching and avoiding the use of hydrogen peroxide in paper bleaching can also be achieved by using mannanases for increased brightness (Tenkanen *et al*. 1997).

Carbohydrases like amylases and cellulases are well known enzymes in the detergent industry (Dhawan and Kuar 2007). Alkaline mannanases are currently also used for stain removal (Wong and Saddler 1993). Thickeners and stabilisers in household products like shampoo, toothpaste, ice-cream and sauces contain gums (galactomannan, glucomannan and guar gum). The mannan in the gum absorb to cellulose fibres (in clothes) binding cotton textiles, making them difficult to remove in the wash. They also have a gluing effect, which causes a binding to particulate soils, which are then transferred in the washing cycle to clean fabric having a greying effect on fabrics (Dhawan and Kuar 2007). Mannanases cleave the β-1,4- linkages and prevent redeposition of soil. In a likewise manner, compositions can be formulated with

mannanases for sanitization products, dishwashing, surface cleaners and health care products (Bettiol *et al*. 2000). Other applications of mannanases include oil extraction of coconut meats (Chen and Diosady 2003), hydrolysis of guar gum in hydraulic fracturing of gas wells (McCutchen *et al*. 1996), textile dyeing operations (Dhawan and Kuar 2007) and clarification of juices (Dhawan and Kuar 2007).

2.5. PRODUCTION OF HETEROLOGOUS ENZYMES IN YEASTS

S. cerevisiae is one of the main hosts considered for bioethanol production and application in CBP, mainly due its long and successful history with ethanol production for the beer and wine industries (Lynd *et al*. 2002). The yeast, however, is unable to natively produce enzymes such as cellulases and hemicellulases. Yet, the successful production of these enzymes has been obtained through DNA manipulation techniques (La Grange *et al*. 2002, Van Rooyen *et al*. 2000). Many factors need to be considered in the attempt to produce an organism that can efficiently degrade cellulose and hemicellulose through heterologous enzyme production (Lynd *et al*. 2002).

2.6. *SACCHAROMYCES CEREVISIAE* AS HOST FOR HETEROLOGOUS GENE EXPRESSION

Several yeast species are used in the industry to produce heterologous proteins. The yeast *S. cerevisiae* is an attractive host system and is regarded as being the most well characterized eukaryotic expression system (Buckholtz and Gleeson 1991, Mattanovich *et al*. 2004, Romanos *et al*. 1992). This is in part due to the availability of the complete genome sequence - genetic maps, physical maps, functional analysis and biological information - found on the *Saccharomyces* Genome Database (SGD) (www.yeastgenome.org). The complete genomic sequence was made public in 1996 (Dujon 1996, Grivell 1993) and the MIPS Yeast Genome Project (http://mips.gsf.de/proj/yeast/) release information regarding yeast genomic sequences as well as protein information regularly. Physiologically the eukaryotic subcellular organization allows post-translational folding and modification of large proteins from various origins and proteins produced can be secreted into the extracellular environment (Mattanovich *et al*. 2004). Further advantages like robustness, the fact that the yeast is non-pyrogenic and has GRAS (Generally Regarded As Safe) status, makes it a preferred choice in various

industrial applications as well as in research (Cregg *et al*. 2000). Several factors can however influence the productivity and affectivity of the expression system. Codon usage of the expressed gene can cause potential bottlenecks. Furthermore translation signals, translocation determined by secretion of signal peptide and processing and folding in the ER and golgi are some of the factors that can influence successful protein production (Agaphonov *et al*. 2003, Kowalski *et al*. 1998, Mattonovich *et al*. 2004).

2.6.1. CODON OPTIMISATION

The genetic code is degenerate implying that various codons code for the same amino acid (synonymous codons) (Grantham *et al*. 1980, Sharp *et al*. 1993). This implies that various nucleic acid sequences can be translated by ribosomes with differently charged tRNAs to encode the same protein. Some codons occur more frequently in gene sequences of certain species than in others, indicating that organisms have a preferred set of codons shaping its genome (Grantham *et al*. 1980, Sharp *et al*. 1993). Genes show a statistically significant bias towards choice of codons used, to code for a particular amino acid (Carbone *et al*. 2003). This may differ from one gene to another, but within the same genome a related nucleotide-triplet preference exists. An algorithm was designed to predict codon adaptation index (CAI) detecting the most dominant codon bias in a genome. This is based on the "housekeeping genes", i.e. the genes that are highly expressed. The CAI of a given gene correlates with gene expression in a recombinant host and the codon usage frequencies in mRNA translation (Friberg *et al*. 2004). The abundance of optimal codons increase over the length of the gene preventing the occurrence of nonsense errors (Salim and Cavalcanti 2008). As the cost of translating a gene becomes greater as the length of the gene increases, a greater pressure exists for the selection of most accurate codons to avoid unnecessary errors (Salim and Cavalcanti 2008).

Several factors could have an affect on the codon bias (Salim and Cavalcanti 2008). Codon bias seems to be related to selection for optimized translation, expression, location within genes, rate of evolution, secondary structure, nucleotide composition protein length, the environment and evolution of translation. Translation is an energetically expensive process and the efficiency therefore is under selective pressure in exponentially growing cells (Anderson and Kurland 1991). The rate at which polypeptide chain elongation takes place is

limited by the diffusion of the cognate ternary complex to the A-site of the ribosome. Thus the most abundant amino acid-tRNA for given amino acid is predominantly used by the codons of highly expressed genes (Dong *et al*. 1996). The diversity of isoacceptor tRNAs have an influence on an organisms metabolic load and therefore a reduction in diversity could be beneficial for fast growing organisms (Anderson and Kurland 1990). tRNA pools that are more numerous but less diverse have evolved due to the codon preferences of highly expressed genes (Rocha 2004). Codons mostly favoured are the ones that require less energy to bind, or binds more tightly to mRNA. Codons can also have different translational rates and some are less prone to misreading or drop-off events (Rocha 2004, Rodnina and Wintermeyer 2001). Due to adjacent codon-pair bias it was proposed that translational machinery is sensitive to the nature of the codon-pair present in the ribosome A and P decoding sites (Moura *et al*. 2005). Like codon usage, codon context may also be species specific. This bias seems more linked to decoding accuracy than to translational speed (Moura *et al*. 2005).

When proteins are produced heterologously, the difference in codon bias of the host and the donor species might be the cause of low levels of expression (Wu *et al*. 2006). Low protein expression levels are often due to the presence of rare codons in the target gene that appear in clusters or at the N-terminal part of the gene (Gustafsson *et al*. 2004). Altering the gene sequence in such a way that it more closely reflects the codon usage of the host, without affecting the amino acid sequence of the protein, yielded improvement in expression levels. Codons can be altered with site-directed mutagenesis or by synthetically constructing an entire gene (Gustafson 2004, Wu *et al*. 2006).

Synthetic codon optimisation is a relatively new field with a lot of uncertainties as to which algorithm and reference template of codon usage to use (Wu *et al*. 2006). Dramatic increases in protein expression levels were observed upon optimisation and yet some studies reported less protein production than with the native DNA sequence (Wu *et al*. 2004, Wu *et al*. 2006). Many reasons may exist for this failure and codon optimisation is far from being fully understood. When a sequence is altered though codon optimisation, it can result in translational errors because the tRNA pool is imbalanced. Repetitive elements and mRNA secondary structures might occur that inhibit the ribosomal efficiency. Wu *et al*. (2006) concluded that the exclusive use of optimal codons seem to be unnecessary, since too many optimal codons might have an adverse effect on protein expression. Furthermore, non-optimal codons seem to be important in the correct folding of the emerging translated polypeptide and

efficient translation does not necessarily guarantee functional protein (Zalucki and Jennings 2007).

2.6.2. HETEROLOGOUS PROTEIN SECRETION

Heterologous proteins undergo various processes in various membrane-enclosed compartments before being secreted into the periplasmic space or the surrounding medium (Fig. 13) (Walker 1999). In the endoplasmic reticulum (ER)-polysomes, proteins are synthesised and folded in their correct three dimensional conformations. Proteins are discharged into the ER lumen and a protein destined to be secreted undergoes signal peptide cleavage and primary glycosylation (Herscovics and Orlean 1993, Lyman and Schekman 1996). The correctly folded polypeptide is then transported to the Golgi apparatus where post-translational modification such as proteolytic processing and further maturation of the glycan chain, takes place (Lewis and Pelham 1996). In the trans-Golgi network, the protein is packed in secretory vesicles that fuse with the plasma membrane. The processed protein is released through the bud region through an actin filaments-directed process (Govindan *et al*. 1995, Griffiths and Simons 1986, Lupashin *et al*. 1992). Heterologous expression via the yeast secretory pathway has advantages in that downstream purification and isolation is simplified, and secretion avoids toxicity of intracellular accumulation of proteins (Walker 1999).

Proteins exported via the general secretory pathway all have a signal peptide at the N-terminus (Walker 1999). The relative hydrophobicity of the signal peptide determines whether the export of the protein happens post- or co-translationally (Huber *et al*. 2005). Co-translational export involves a signal-recognition particle (SRP). The SRP binds the nascent peptide to facilitate translocation to the inner membrane of the ER and the export apparatus. During post-translational export, molecular chaperones SecB and DnaK bind the pre-protein to maintain an export competent state, which is loosened or unfolded (Kumamoto *et al*. 1985, Phillips and Silhavy 1990).
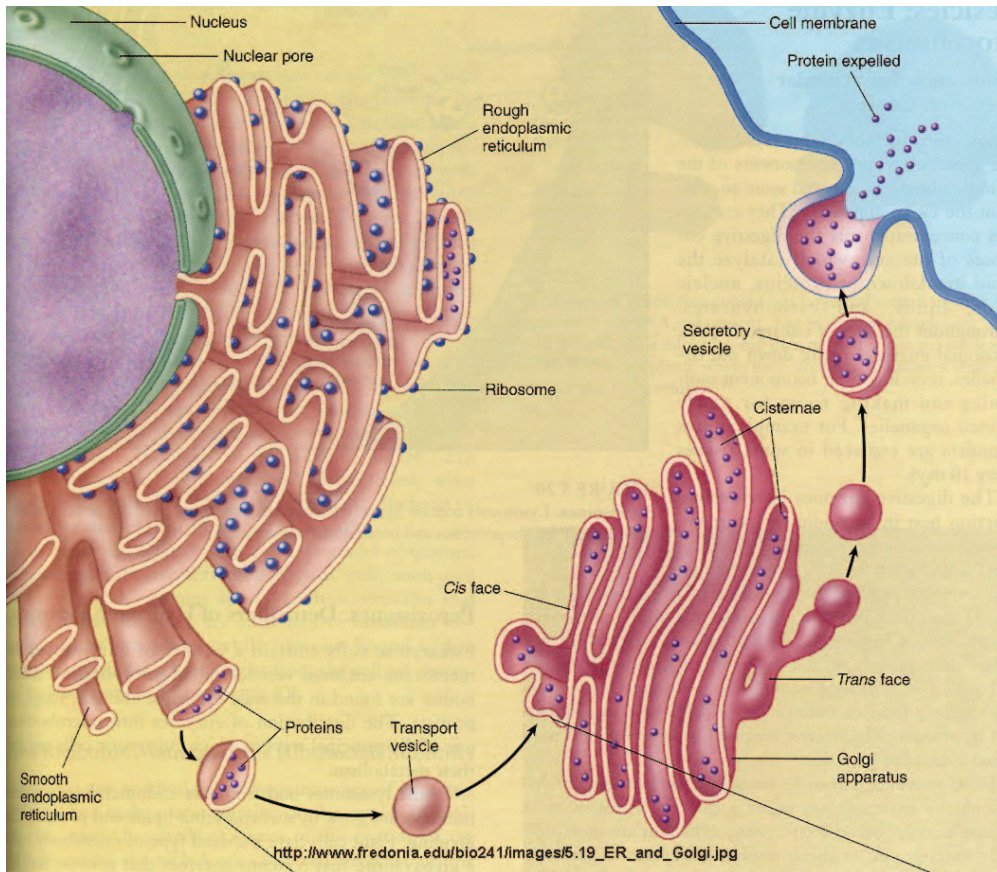
Fig. 13. The protein secretion pathway in *S. cerevisiae*. The newly synthesised protein occurring in the endoplasmic reticulum (ER) becomes packaged into a vesicle and is transported to the golgi. The protein undergo several modifications and becomes enclosed in the endosomes. The vesicles containing the protein are destined for the plasma membrane and will be transported to the cell surface (http://www.fredonia.edu/bio241/images/6.19_ER_and_Golgi.jpg).

Heterologous protein secretion by *S. cerevisiae* can be directed by leader (signal) peptide sequences (Walker 1999). Such signal peptides occur in naturally secreted proteins like mating factors, killer toxins or invertase enzymes and direct post-translational transport of proteins (Romanos *et al*. 1992, Walker 1999). Signal peptides are hydrophobic amino-terminal peptides of 19-30 amino acids long and consist of a hydrophobic core and consensus sequence. The hydrophobic central region (6-15 aa) is involved in the attachment of the ribosome to the ER membrane and movement of the nascent protein into the ER cisterna (Romanos *et al*. 1992). The consensus sequence (commonly Lys-Arg) becomes cleaved by ER cisternal proteases (signal peptidase) from the mature protein during which export across the ER is accomplished. Hydrophobicity and length can determine the amount of protein secreted as

well as the translocation mode (Romanos *et al.* 1992). Heterologous signal peptides or homologous signal peptides can be used to secrete heterologous proteins in *S. cerevisiae* since there is not a high specificity for signal sequences (Hadfield *et al.* 1993). Therefore, most fungal proteins can be secreted successfully using their native signal peptides (Han *et al.* 1999, Lang and Looman 1995).

Native *S. cerevisiae* signal peptides include that of the α-mating pheromone (*MFα1*), invertase (*SUC2*), acid phosphatase (*PHO5*) and killer toxin (Walker 1999). For the removal of the invertase signal, only signal peptidase seems necessary. However, *MFα1* requires KEX2 and STE13 exopeptidases (Walker 1999). Signal peptidase removes the pre-peptide that directed secretion into the ER. In the golgi, KEX2 endopeptidase cleaves the pro-peptide on the COOH at Lys-Arg. This leaves two Glu-Ala dipeptides attached to the N-terminus of the mature protein (Kjeldsen 2000, Romanos *et al.* 1992, Walker 1999). STE13 exopeptide digestion removes the initial Glu-Ala-Glu-Ala or Val-Ala segment from the N-terminus generating mature peptides (Kjeldsen 2000, Romanos *et al.* 1992). Exopeptidases are specific for the sequences on N-termini and using *α*-factor secretion can create secreted protein with authentic N-termini.

2.6.3. POST-TRANSLATIONAL MODIFICATIONS AND GLYCOSYLATION

Protein secretion in eukaryotic cells entails the movement of newly produced proteins through the endoplasmic reticulum (ER) as well as the golgi apparatus (Walker 1999). The first step involves the removal of the initiator methionine by a methionyl aminopeptidase (Romanos *et al.* 1992). Thereafter, the proteins will undergo processes like glycosylation, formation of disulphide bonds and eventually fold into an active state (Romanos *et al.* 1992, Walker 1999). These procedures are of vital importance to cellular functions and the proteins stability, localisation and secretion. Proteins can be *O*-glycosylated and, or *N*-glycosylated (Goto 2007, Walker 1999).

N-glycosylation starts in the endoplasmic reticulum but can be continued in the golgi apparatus (Goto 2007, Walker 1999). *N*-glycosylation occurs by means of a membrane lipid carrier, which is a hydrophobic polyisoprenoid lipid on the rough ER. The first step is the synthesis of the lipid-linked oligosaccharide precursor which is $GlnNAc_2Man_9Glc_3$.

N-acetylglucosamine (GlcNAc) is transferred from UDP-GlcNAc onto dolichol phosphate, on the cytoplasmic face of the endoplasmic reticulum (ER). This is followed by the addition of GlcNAc and mannose (Man), resulting in $Man_5GlcNAc_2$-p-dolichol. A flipase enzyme translocates the structure to the luminal face of the ER membrane. Inside the ER it becomes $Glc_3Man_9GlcNAc_2$-p-dolichol and from here it is transferred to an asparagine residue in the sequence Asn-Xaa-Ser/Thr (Xaa can be any amino acid except proline) as the polypeptide is translocated into the ER lumen (Herscovics and Orlean 1993). The process is catalyzed by oligosaccharide protein transferase. The three glucose and one mannose residues are then removed to produce $Man_8GlcNAc_2$. This is a three-step process catalyzed by three different enzymes, initiating transport into the golgi for further processing. In yeast, the structure then sometimes becomes extended with more mannose sugars to produce a hyper-mannosylated glycan (Goto 2007).

O-glycosylation is a golgi-associated process in which sugar residues are linked onto the polypeptide in a step-wise manner (Fig. 14) (Goto 2007). O-glycans are the sugars attached to the β-hydroxyl group of serine or threonine. An enzyme N-acetylgalactosaminyltransferase catalyzes the transfer of N-acetylgalactosamine from UDP-GalNac to the hydroxylgroup of the Ser or Thr residue. UDP-glucose is utilized to form UDP-GalNAc in the cytosol and in the trans-golgi vesicles the carbohydrate chain of the protein is extended. Glycosyltransferases catalyze the addition of galactose to GalNac. Finally two N-acetylneuramic acid-(sialic acid) residues are added in the trans-golgi reticulum as to complete the O-glycosylation process. In *S. cerevisiae,* the sugars attached are small manno-oligosaccharides. O-glycans are linear chains of up to five mannose residues with the structure Manα1-3Manα1-3Manα1-2Manα1-2Man1-O (Herscovics 1999, Orlean *et al*. 1997). In the ER, the first reaction is catalyzed by a family of seven integral membrane proteins with multiple transmembrane domains. The protein O-mannosyltransferases (*Sc*Pmtl-7p) catalyze the covalent attachment of mannosyl residues to hydroxy amino acids with dolichyl phosphate mannose (Dol-P-Man) acting as mannosyl donor. From here the protein moves to the golgi apparatus and the glycosyl chain is extended by α-1,2- mannosyltransferases (MNT). GDP-Man acts as mannosyl donor and mannosyltransferase catalyzes the addition of sugar to seryl-/threonyl-residues. Six mannosyltransferase genes have been identified namely *PMT1-6. PMT1* mutants showed a reduction of O-mannosylation in vivo by 50% (Goto 2007, Herscovics and Orlean 1993).

Protein glycosylation can be problematic for heterologous protein production as non-identical glycoproteins result when heterologous secretory proteins are glycosylated (Wang *et al*. 2001). It affects the holding, stability, activity and immunogenicity of proteins (Jenkins and Curling 1994). Even though *S. cerevisiae* has been a successful heterologous protein producer, hyperglycosylation can be a major draw back, especially in the pharmaceutical industry (Jose and Demain 2003). The presence of a α-1,3-linked mannose residue can cause an antigenic response in patients and hyperglycosylation may also have an effect on biochemical properties. In some instances hyperglycosylation does not seem to have an effect. A recombinant Man5A in Setati *et al*. (2001) showed a molecular mass of 10 kDa higher than the calculated molecular mass and 5 kDa higher than the native enzyme. In spite of this, the biochemical properties seemed similar (Setati *et al*. 2001). A recent study reported an oversecreting mutant of *S. cerevisiae* that doesn't glycosylate proteins (Wang *et al*. 2001). The oversecretion might be due to an unusual UPR response in the ER. Genetic examination proposed the mutation accountable was in some way dominant and the oversecretion and low-glycosylation phenotypes were the result of a single mutation on one chromosome (Wang *et al*. 2001).

2.7. ALTERNATIVE HOSTS FOR HETEROLOGOUS EXPRESSION

Although *S. cerevisiae* has many advantages regarding the expression of recombinant proteins, limitations associated with poor expression capacity and low product yield is still unresolved and consequently other hosts are being considered. Alternative foreign protein expression hosts include yeasts like *Hansenula polymorpha*, *Pichia pastoris*, *Kluyveromyces lactis*, *Pichia stipitis* and *Schizosaccharomyces pombe* (Mayer *et al*. 1999, Romanos *et al*. 1992).

Pichia pastoris and *H. polymorpha* are facultative methylotrophs capable of obtaining very high cell densities. Expression is based on the *AOX1* gene in *P. pastoris* and the *MOX1* and *FMD* genes in *H. polymorpha*, respectively coding for the alcohol oxidase, methanol oxidase and formate dehydrogenase enzymes (Gellissen and Hollenberg 2001, Mayer *et al*. 1999, Sreekrishna *et al* 1997). Stringent regulation of these promoters allows separation of growth and production phases resulting in biomass of up to 133 g/l (Mayer *et al*. 1999).
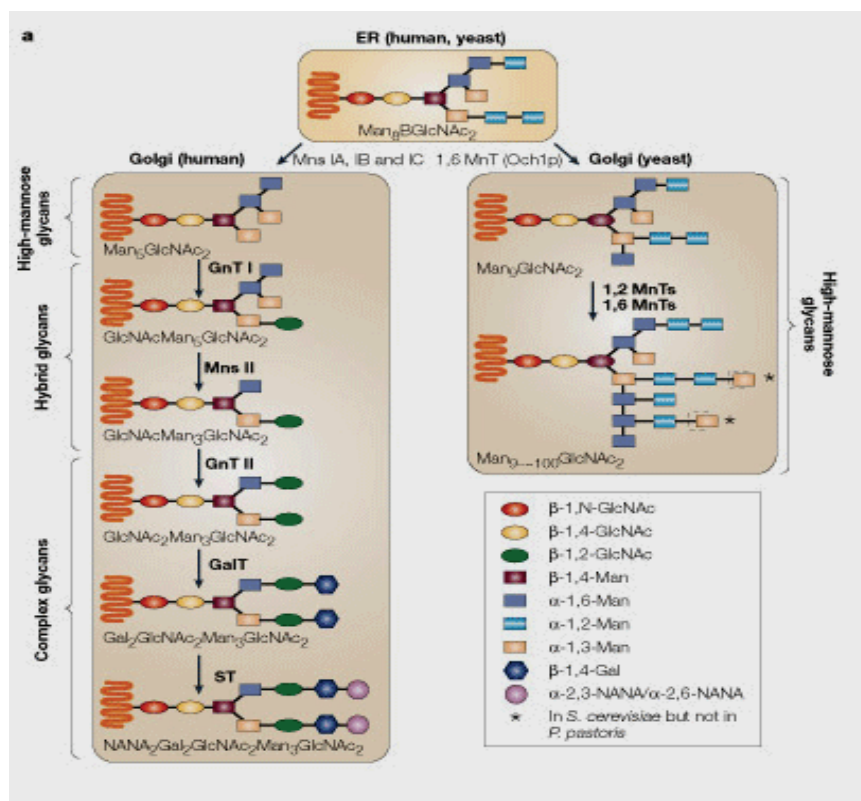
Fig. 14. The pathway of N-linked protein glycosylation in humans (left) and the model organism *S. cerevisiae* (right)*.* N-linked protein glycosylation starts on the cytoplasmic side of the endoplasmic reticulum (ER) membrane with the assembly of an oligosaccharide on a lipid-carrier that is catalyzed by a series of specific glycosyltransferases. The intermediate is flipped into the ER lumen. Hexose residues are added by a distinct set of glycosyltransferases. The oligosaccharide is finally transferred from the lipid to selected asparagine residues of secretory proteins by oligosaccharyltransferase (OST) (Wildt and Gerngross 2005).

The low quantity of native proteins produced by these secretory systems has become well known (Gellissen and Hollenberg 2001, Mayer *et al*. 1999, Sreekrishna *et al* 1997). However, drawbacks include operational complications when methanol is used due to its flammable nature. Glycerol is required for promoter activation, but is an expensive source of carbon and adds substantially to the production cost of the enzymes (Mayer *et al*. 1999).

Kluyveromyces. lactis and *P. stipitis* are also being exploited for their unique substrate utilization pathways. *P. stipitis* can grow on xylose and *K. lactis* is able to grow on lactose as sole carbon source (Dujon *et al*. 2004). Like *S. cerevisiae, K. lactis* is easily genetically manipulated, has commercially available vectors, has a fully sequenced genome and has excellent protein synthesizing capabilities (Dujon *et al*. 2004). The promoters, selection

marker genes and secretion signals can be interchanged between *S. cerevisiae* and *K. lactis* (van Ooyen *et al*. 2006). As with *S. cerevisiae* commonly used auxotrophic markers (*ura3*, *leu2*, *trp1*) have been used in *K. lactis* and autoselection systems were developed to enhance plasmid stability (Hsieh and Da Silva 1998). Other markers that have been used include the dominant bacterial marker which allow for resistance to hygromycin B, as well as dominant nitrogen source selection (Selten *et al*. 1997). Acetamide selection allows the recycling of the *amdS* marker gene using counterselection with fluoroacetimide, a process similar to the generation on *ura3*⁻ mutants using FOA plates (van Oooyen *et al*. 2006). Unlike methylotrophic yeasts *K. lactis* does not require explosion-proof fermentation equipment and the organism has GRAS status (van Ooyen *et al*. 2006). Additional advantages of *K. lactis* include little or no catabolite repression, reduced hyperglycosylation and an efficient secretion system (Romanos *et al*. 1992, van Ooyen *et al*. 2006). *K. lactis* has been used in the therapeutic protein production industry with much success with the only drawback being non-human glycosylation patterns (van Ooyen *et al*. 2006). *K. lactis* even though it is able to ferment, is unable to grow in fully anaerobic conditions (Kiers *et al*. 1998). This limits its efficiency to produce ethanol from components such as whey, containing lactose as the main sugar component (Snoek and Steensma 2006).

Even though *S. cerevisiae* has low secretion capacity, the abundance of vector systems and wide range of transcriptional promoters can be exploited to improve gene expression and is still a preferred choice for heterologous protein production and the main organism considered in the CBP process (Lynd *et al*. 2002).

2.8. THIS STUDY

The yeast *S. cerevisiae* has a long and successful history in the baking industry as well as the wine and beer industries due to its fermentation ability. The ease of cultivation, genetic manipulation and product purification have contributed to the development of *S. cerevisiae* as the model organism for the heterologous production of recombinant proteins. Yet, limitations such as low product yield and hyperglycosylation, associated with expression in *S. cerevisiae,* is yet to be addressed. These limitations have emphasised the need for the evaluation of alternative hosts for foreign gene expression.

Mannanases have various industrial applications and therefore need to be heterologously produced to meet the demand. Therefore, this study focused on the expression of the *man1* gene (native to *A. aculeatus*) and the characterisation of the recombinant Man1 enzymes produced by *S. cerevisiae* and *K. lactis*. The production of the Man1 acted as the basis for comparing the two yeast expression systems. The effect of continuous sub-culturing on the *K. lactis* transformants has also been evaluated. The benefit of using codon optimised genes was investigated through the cloning and expression of the synthetically designed β-mannosidase (*mndA*) of *A. niger*. The effect of co-expression of genes was evaluated in *S. cerevisiae* using the *man1* and *mndA* genes.

The increasing pressure placed on the economy due to the escalating energy demand has encouraged the interest in the production of alternative forms of energy. The ideal would be to provide bioethanol cost effectively, while using primarily agricultural residues and municipal lignocellulosic waste. In order to reach this objective, the ideal host would need to be able to degrade and utilise cellulose, xylan and mannan as carbon sources. This study paves the way towards the construction of a mannan-degrading yeast strain.

2.9. REFERENCES

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COMPARING THE EXPRESSION OF MANNANASES BY THE YEAST *S. CEREVISIAE* AND *K. LACTIS* BY EVALUATING THEIR ABILITY TO PRODUCE RECOMBINANT MAN1 ENZYME

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3.1. ABSTRACT

The mannanase gene, *man1*, of *Aspergillus aculeatus* was successfully cloned and expressed in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Man1 displayed a temperature optimum of 70˚C in both species and a pH optimum of 5 and 6 in *K. lactis* and *S. cerevisiae*, respectively. Activity levels of 160 - 180 nkat.ml^{-1} were obtained in both strains after 86 hours of cultivation. The recombinant *K. lactis* obtained a cell density of 5-fold more than the *S. cerevisiae* strain. The *K. lactis* secreted the Man1 as a 55kDa protein compared to the 58kDa protein produced by *S. cerevisiae* transformantss. *K. lactis* transformants were stable during consecutive sub-cultures for a period of 2 weeks. Disruption of the *ku80* gene did not have a positive impact on the stability of the *K. lactis* transformants.

Keywords: *K. lactis*, *A. aculeatus*, mannanase, heterologous expression

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3.2. INTRODUCTION

Plant biomass contains a variety of compounds of which cellulose and hemicellulose are the most prominent (Whitney *et al*. 1998, Schwarz 2001). Hemicelluloses are low molecular weight heterogenous chemical compounds with a wide variation in both structure and composition. Commonly occurring hemicellulases include the 1,3- and 1,4-β-galactans, mannans and xylans (Aristidou and Penttilä 2000). Xylan and mannan are the main forms of hemicelluloses present in plant cell walls and can constitute up to 35% and 25% of the dry weight of plants, respectively (Puls and Schuseil 1993). Mannan can occur as either a homo- or a heteropolysaccharide in the form of glucomannan, galactomannan and galactoglucomannan (Jeffries 1994). Glucomannan consists of β-1,4-linked D-mannose and D-glucose molecules, whereas galactomannan consists of a mannose backbone with galactose substituents on C-6. Galactoglucomannan has the same backbone chain as glucomannan, but also contain α-1,6-linked galactose residues and may be acetylated at C-2 or C-3. In nature, galactoglucomannans predominantly occur in softwoods, whereas galactomannans are mostly found in seeds of leguminous plants and carob beans (Setati *et al*. 2001). The hydrolysis of these substrates is accomplished through the action of endo-β-1,4-mannanase (EC 3.2.1.80), which randomly cleaves the β-mannosidic linkages within the main chain together with the exo-enzymes β-mannosidase (EC 3.2.1.25) and α-galactosidase (EC 3.2.1.22) (Ademark *et al*. 1998, Aristidou and Penttilä 2000).

Mannanases are widely distributed in nature and have been isolated, characterized and sequenced from various sources (Bewley *et al*. 1997, Braithwaite *et al*. 1995, Christgau *et al*. 1994, Yamaura *et al*. 1996). Endo-β-1,4-mannanase (mannanase EC 3.2.1.78) from fungal sources (*Aspergillus* and *Trichoderma*) are classified into glycosyl hydrolase family GH-5 (Christgau *et al*. 1994, Eriksen *et al*. 1998, Stålbrand *et al*. 1995) and genes sequenced from the anaerobic fungus (*Piromyces*) were classified as GH-6 (Millward-Sadler *et al*. 1996). Some genes encode multidomain proteins implying that in addition to the mannanase catalytic domain, it contain either one or two discrete noncatalytic cellulose-binding domains (CBDs) or both a CBD and an endoglucanase (Millward-Sadler *et al*. 1996, Stålbrand *et al*. 1995). The three dimensional structure of β-mannanases from *Trichoderma reesei* have been resolved (Sabini *et al*. 2000a, b). Enzymes display a prominent cleft in which the active site is situated. This architecture allows cleavage of substrate in the middle of the galacto(gluco)mannan chain, releasing oligosaccharides of various lengths that together with

other hemicellulytic enzymes (β-mannosidases and α-galactosidases) can be converted to monosaccharides (Harjunpää. *et al*. 1999, Setati *et al*. 2001).

β-mannanases have several potential applications in industrial processes. In the manufacturing of instant coffee, the reduction of viscosity in coffee extracts are obtained by application of β-mannanases (Sachslehner *et al*. 2000). These enzymes can also be used for biobleaching of softwood kraft pulps to enhance the extractability of lignin (Montiel *et al*. 1999, Suurinaki *et al*. 1997). Legume seeds in poultry feeds contain high concentrations of galactomannan that results in reduced nitrogen retention, fat absorption and amino acid uptake in chickens. Application of β-mannanases to animal feed has shown an improvement of chicken growth and development (Jackson *et al*. 1999, Swapna *et al*. 1982). Recombinant endo-β-mannanases are important for these and other biotechnological applications. Efficient application often requires that various enzymes can be utilized in different ratios in enzyme cocktails (Dhawan and Kaur 2007).

Saccharomyces cerevisiae and *Kluyveromyces lactis* are well known hosts for heterologous protein production and have been used extensively for the production of various proteins (La Grange *et al*. 2001, van Ooyen *et al*. 2006, van Rooyen *et al*. 2002). Both yeasts have various advantages including efficient vector expression systems (Buckholtz and Gleeson 1991, Romanos *et al*. 1992, Mattanovich *et al*. 2004), easy genetic manipulation, the ability to use both integrative and episomal expression vectors and the availability of a fully sequenced genome (Dujon 1996). Strains of *S. cerevisiae* and *K. lactis* can be cultivated in standard yeast media and does not require the explosion-proof fermentation equipment necessary for large-scale growth of methylotrophic yeasts such as *Pichia pastoris* (Mayer *et al*. 1999). Strains of *S. cerevisiae* and *K. lactis* have GRAS (G̲enerally R̲egarded A̲s S̲afe) status and FDA approval, permitting their use in various food and feed applications (Cregg *et al*. 2000, Dujon 1996). In contrast to *S. cerevisiae*, *K. lactis* displays Crabtree-negative growth, reduced hyperglycosylation, reduced carbon catabolite repression and a wider substrate range (Hsieh and Da Silva 1998, Romanos *et al*. 1992, van Ooyen *et al*. 2006). Even though methylotrophic yeast strains have become important host organisms for foreign gene expression, *S. cerevisiae* and *K. lactis* are still preferred hosts for heterologous protein production (Romanos *et al*. 1992, van Ooyen *et al*. 2006).

Targeted gene integration is supported by the cellular machinery that accomplishes recombination and DNA repair (Daley *et al*. 2005). Homologous recombination (HR) involves retrieving genetic information from homologous sequences and results in targeted

integration of exogenous DNA at homologous sites. *S. cerevisiae* mainly uses the HR system while *K. lactis* can perform homologous recombination as well as non-homologous end joining (Daley *et al.* 2005, Morlino *et al.* 1999). Non-homologous end joining enables the host to integrate foreign DNA at random requiring functional KU70 and KU80 proteins (Kooistra *et al.* 2004). In this study, created a *K. lactis ku80* mutant to investigate its effect on stability of a plasmid lacking *Kluyveromyces* sequences (except for the origin of replication). The expression of endo-β-mannanase from *A. aculeatus* was characterized in *S. cerevisiae* and in *K. lactis* strains. The secretion efficiency of both strains was evaluated and the production levels of recombinant Man1 protein were compared.

3.3. MATERIALS AND METHODS

3.3.1. STRAINS AND PLASMIDS

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

3.3.2. MEDIA AND CULTIVATION

All chemicals used were of analytical grade. *Escherichia coli* DH 5α (*supE44 ΔlacU169 (∅80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1)* was used as the host strain for the recombinant DNA manipulations and plasmid propagation. Bacterial cultivation (37°C) took place in Terrific Broth (12 g.l^{-1} tryptone, 24 g.l^{-1} yeast extract, 4 ml.l^{-1} glycerol, 100 ml.l^{-1} of phosphate buffer) containing 100 μg.ml^{-1} ampicillin (Sambrook *et al.* 1989). Strains of *K. lactis* and *S. cerevisiae* strains were aerobically cultivated on a rotary shaker (160 rpm) at 26°C in 125 ml Erlenmeyer flasks containing 20 ml of double strength synthetic complete (SC) medium (3.4 g.l^{-1} yeast nitrogen base without amino acids and ammonium sulphate (Difco laboratories), 10 g.l^{-1} $(NH_4)_2SO_4$, 40 g.l^{-1} glucose/lactose and supplemented with appropriate amino acids) buffered at pH 6 with 20 mM citrate phosphate buffer. Yeast strains were maintained on agar (20 g.l^{-1}) plates of the same composition. For enzymatic assays, stationary phase pre-cultures were used to inoculate the fresh medium to approximately 1 x 10^5 cells.ml^{-1}. Samples were taken periodically to determine the optical density at OD$_{600}$. FOA plates were prepared according to Ausubel *et al.* (1998).

Table 1. The genotypes of relevant strains and plasmids used in this study.

Strains	Genotype	Reference
S. cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-289*	ATCC 201160
S. cerevisiae Y294 [YEpENO-BBH]	*URA3 ENO1$_P$-ENO1$_T$*	This study
S. cerevisiae Y294 [YEpENO-BBH-Man1]	*URA3 ENO1$_P$-Man1-ENO1$_T$*	This study
K. lactis GG799	Wild type	New England Biolab
K. lactis GG799-ura	*ura3*	This study
K. lactis GG799 [YEpENO-BBH]	*URA3 ENO1$_P$-ENO1$_T$*	This study
K. lactis GG799 [YEpENO-BBH-man1]	*URA3 ENO1$_P$-Man1-ENO1$_T$*	This study
K. lactis SHR7	*ku80-gfp-URA3-gfp-ku80*	This study
K. lactis SHR8	*ku80-gfp-ku80*	This study
K. lactis SHR8 [YEpENO-BBH.Kl]	*URA3 ku80-gfp-ku80 ENO1$_P$-ENO1$_T$*	This study
K. lactis SHR8 [YEpENO-BBH.Kl-man1]	*URA3 ku80-gfp-ku80 ENO1$_P$-Man1-ENO1$_T$*	This study
Plasmids		
pTZ57R	*Bla*	Inqaba Biotech, South Africa
Yep352	*bla URA3*	ATCC
pGFP	*bla gfp*	Clontech Laboratories, USA
yENO1	*bla URA3 ENO1$_P$-ENO1$_T$*	Den Haan *et al*. 2007
YEpENO-BBH	*bla URA3 ENO1$_P$-ENO1$_T$*	This study
YEpENO-BBH.Kl	*bla URA3 ENO1$_P$-ENO1$_T$*	This study
YEpENO-BBH-man	*bla URA3 ENO1$_P$-man1-ENO1$_T$*	This study
YEpENO-BBH.Kl-man	*bla URA3 ENO1$_P$-man1-ENO1$_T$*	This study
pTZ-URA3-blaster	*bla gfp-URA3-gfp*	This study
pTZ-KU80	*bla KU80*	This study
pTZ-ku80-URA3-blaster	*bla ku80-gfp-URA3-gfp-ku80*	This study
pMes2	*bla URA3 PGK1$_P$-man1-PGK1$_T$*	Setati *et al*. 2001

3.3.3. CONSTRUCTION OF PLASMIDS

Standard protocols were followed for DNA manipulations (Sambrook *et al*. 1989). The enzymes used for restriction digests and ligations were purchased from Inqaba Biotec, South

Africa and used as recommended by the supplier. Digested DNA was eluted from agarose gels using the Zymoclean TM Gel Recovery Kit (Zymo Research Corporation, USA). PCR reactions were carried out with a Perkin Elmer GeneAmp® PCR System 2400 using *Taq* DNA polymerase (Inqaba Biotec, South Africa) according to the suppliers specifications. Table 2 contains a list of all the primers (Whitehead Scientific, South Africa) used in the study is provided in Table 2.

Table 2. DNA sequence of the primers used in this study. Restriction sites are underlined.

Primer name	Sequence
ENOnew left	5'-GATCGGATCCCAATTAATGTGAGTTACCTCA-3'
ENOnew right	5'-GTACAAGCTTAGATCTCCTATGCGGTGTGAAATA-3'
KlKU-left	5'-TATTAGCTCTAAGACCCGAT-3'
KlKU-right	5'-ACGATGAACTTCTGATGAAC-3'
GFP-left	5'-GATCGGATCCGAAGAACTTTTCACTGGAGT-3'
GFP-right	5'-GTACATCGATAGATCTGGGTATCACCTTCAAACTT-3'
Ura Bam-left	5'-GATCGGATCCGACGTCTAAGAAACCATTAT-3'
Ura Bgl-right	5'-GTACAGATCTGATAAGCTGTCAAACATGAG-3'

3.3.4. YEAST TRANSFORMATIONS

Yeast strain *S. cerevisiae* was transformed with recombinant plasmids according to the lithium acetate/DMSO method described by Hill *et al*. (1991). Transformation of *K. lactis* was performed through electrotransformation method described by Cho *et al*. (1999). Transformants were selected for growth on SC^{-URA} plates.

3.3.5. PLATE ENZYME ASSAYS

The recombinant yeast strains were screened for β-mannanase activity. *K. lactis* SHR8[YEpENO-BBH.Kl], *K. lactis* SHR8[YEpENO-BBH.Kl-man1] *S. cerevisiae* Y294[YEpENO-BBH] and *S. cerevisiae* Y294[YEpENO-BBH-man1] were grown at 30˚C on selective synthetic complete (SC) agar plates supplemented with amino acid pool lacking uracil (SC^{-URA}) and containing 0.1% Locust bean gum (Sigma, Sweden). After 2 days the cultures were washed from the plates and plates stained with a 0.1% Congo Red solution for 30 min. A 1.2 M NaCl solution was used to destain the plates and visualise hydrolysis zones

(Stålbrand *et al*. 1993). Plates were treated with 1 M HCl after Congo Red staining, resulting in a colour change.

3.3.6. CHARACTERIZATION OF MAN1

The activity of the Man1 enzyme was determined using 0.25% galactoglucomannan (Locust bean gum) (Sigma, Sweden). The amount of reducing sugars released during the hydrolysis of mannan, was determined by the dinitrosalicylic acid method using mannose as the standard (Miller *et al*. 1960). One unit of enzyme was defined as the activity producing 1 μmol reducing sugar per minute in mannose equivalents under the optimal assay conditions. The temperature and pH optima of the Man1 enzyme was determined (using 0.05 M citrate phosphate buffer) as described by Bailey *et al*. (1992).

3.3.7. GROWTH OPTICAL DENSITY AND DRY CELL WEIGTH (DCW)

The different transformants were cultivated in double strength SC medium, buffered at pH 6 with citrate phosphate buffer. *K. lactis* and *S. cerevisiae* strains were grown for up to 100 hours and the optical density was measured at OD_{600} using a Pharmacia LKB Ultrospec III spectrometer. Dry cell weight was measured according to den Haan *et al*. (2007). All dry weight estimations and growth curves were done in triplicate.

3.3.8. PLASMID STABILITY DETERMINATION

Transformants of *K. lactis* GG799-ura[YEpENO-BBH.Kl] and *K. lactis* SHR8[YEpENO-BBH.Kl] transformants were cultivated in liquid SC^{-URA} and transferred to fresh medium every second day. Periodically the transformants were also transferred to 10 ml YPD medium. After cultivation in YPD for 48 hours, the cultures were transferred to YPD and SC^{-URA} plates respectively.

### 3.3.9. PROTEIN ISOLATION AND GEL ELECTROPHORESIS

The supernatant of the different strains were collected after 3 days of cultivation in double strength medium and freeze dried before determining the specific activity. Two milligrams of

freeze dried material (approximately 50 μg of total extracellular protein) were dialysed and denatured for 3 minutes at 100˚C in SDS-denaturing buffer containing 0.5 M DDT (NOVEX, Novel Experimental Technology, San Diego, CA) before separation on a 8% SDS-PAGE gel. A Page Ruler™ from Fermentas Inc. (Maryland, USA) premixed protein molecular weight marker were used to estimate the size of the proteins. Silver staining was used to visualize the protein species (Ausubel *et al*. 1998).

3.4. RESULTS

3.4.1. PLASMID CONSTRUCTION

Plasmid yENO1 contains the YEp352 backbone (ATCC) with the *S. cerevisiae ENO1* gene promoter and terminator sequences cloned into the *Bam*HI and *Hin*dIII sites. The plasmid was digested with *Bam*HI and the overhang filled in with Klenow polymerase and dNTPs. The plasmid was re-ligated to generate YEpENO-B. Using the same method, the *Bgl*II site was subsequently destroyed, generating YEpENO-BB after which the *Hin*dIII site was destroyed, generating YEpENO-BBH template. YEpENO-BBH template was used as template for the PCR reaction in which the *ENO1* expression cassette was amplified flanked by 150-bp region (spacer) upstream of the promoter and 220-bp downstream of the terminator. The PCR product was cloned into pTZ57R for further amplification. The *ENO1* expression cassette was retrieved by digestion with *Bam*HI and *Hin*dIII and the over hangs filled in by treatment with Klenow. The product was cloned between the two *Pvu*II sites on Yep352 generating YEpENO-BBH (Fig. 1). The *K. lactis* replication of origin (*Kl*. ori) was synthetically made (Genescript) based on the sequence of pKD1 (Accession number X03961). It was cloned as a *Bgl*II-*Bam*HI fragment into the *Bgl*II site on YEpENO-BBH, generating YEpENO-BBH.Kl. The *Man1* of *A. aculeatus* was retrieved from pJC1 (Setati *et al*. 2001) and cloned onto this plasmid as an *Eco*RI–*Xho*I fragment generating YEpenoBBH.Kl-Man1 (Fig. 1). A *URA3*-blaster cassette was constructed using the *URA3* of *S. cerevisiae* flanked on both sides with direct repeats of a portion of the *gfp* gene (Acc nr XXU17997). This truncated gfp' does not result in a functional GFP, but merely acts as a spacer region in the disruption strategy. The partial *gfp* sequence and the complete *URA3* gene were amplified by PCR and have been cloned into the cloning vector pTZ57R (Inqaba Biotech, South Africa) generating plasmids pTZ-B-gfp-B and pTZ-B-URA3-B.
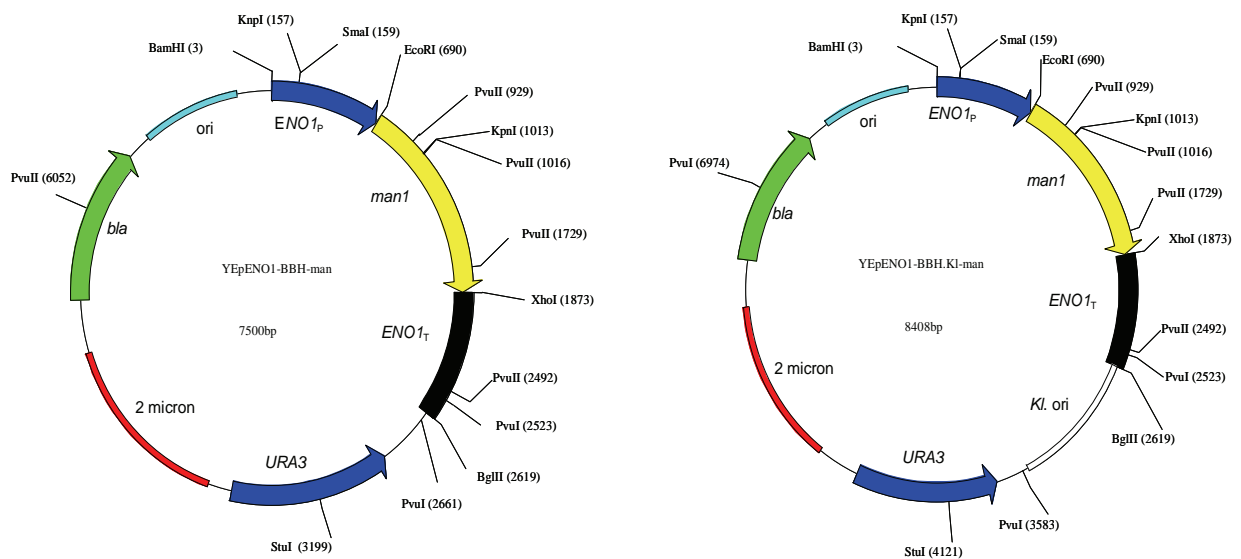
Fig. 1. The relevant plasmid maps of the vectors used in this study. The plasmids used to construct the reference strains, did not contain the *man1* gene. The *Kl*. ori. contains the DNA sequence of the replication of origin that is present on the native pKD1 plasmid (Accession number X03961).

The *URA3* was retrieved as a *Bam*HI-*Bgl*II fragment and cloned into the *Bgl*II site of pTZ-B-gfp-B, generating pTZ-gfp-URA3. Similarly, the *gfp* was retrieved as a *Bam*HI-*Bgl*II fragment and cloned into the *Bgl*II site of pTZ-gfp-URA3, generating pTZ-URA3-blaster. A *URA3*-blaster cassette (Fig.2) has been constructed using the *URA3* of *S. cerevisiae* flanked on both sides with direct repeats of a portion of the *gfp* gene (Acc nr XXU17997). This truncated gfp' does not result in a functional GFP, but merely acts as a spacer region in the disruption strategy. The partial *gfp* sequence and the complete *URA3* gene have been amplified through PCR and have been cloned into the cloning vector pTZ57R (Inqaba Biotech, South Africa) generating plasmids pTZ-B-gfp-B and pTZ-B-URA3-B. The *URA3* was retrieved as a *Bam*HI-*Bgl*II fragment and cloned into the *Bgl*II site of pTZ-B-gfp-B, generating pTZ-gfp-URA3. Similarly, the *gfp* was retrieved as a *Bam*HI-*Bgl*II fragment and cloned into the *Bgl*II site of pTZ-gfp-URA3, generating pTZ-URA3-blaster.
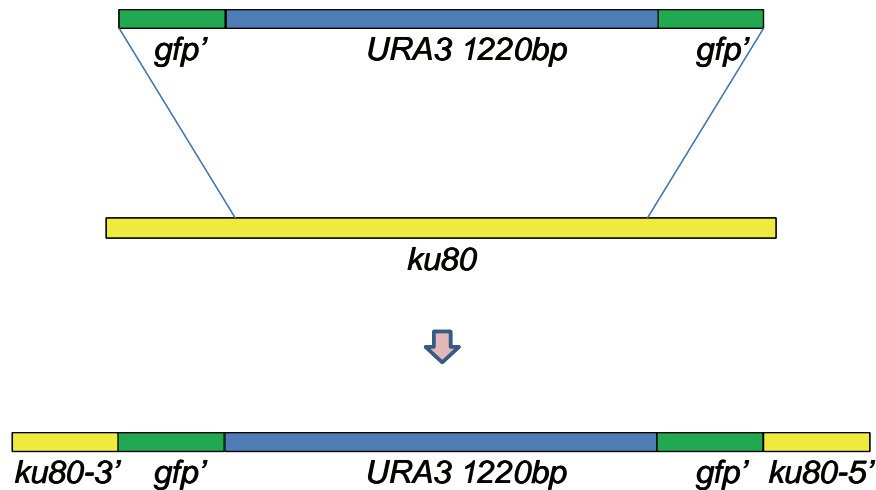
Fig. 2. Schematic representation of the *URA3*-blaster cassette and the disruption strategy of the *ku80* gene of *K. lactis*. The gfp′ are non-functional, direct repeats of a portion of the *gfp* gene. The *ku80-3′* and *ku80-5′* refers to the 3′ and 5' ends of the *ku80* gene.

3.4.2. PLATE ASSAY

The various cultures were cultivated overnight in SC^{-URA} broth and transferred to the SC^{-URA} plates containing 0.1% Locust bean gum. Hydrolysis zones were observed for the recombinant strains of *K. lactis* SHR8[YEpENO-BBH.Kl-man1] and the *S. cerevisiae* Y294[YEpENO-BBH-man1] displaying Man1 activity (Fig.3. B, C and E, F, respectively). Both reference strains, *K. lactis* SHR8[YEpENO-BBH.Kl] and *S. cerevisiae* Y294[YEpENO-BBH, showed no clearing zone formation signifying no hydrolysis on the Locust bean gum plates (Fig. 3. B and E, respectively). Cultivation of *K. lactis* SHR8[YEpENO-BBH.Kl-man1] caused an indent in the agar surrounding the colony whereas no indent was observed with *S. cerevisiae* Y294[YEpENO-BBH-man1] (Fig. 3. A and D, respectively).
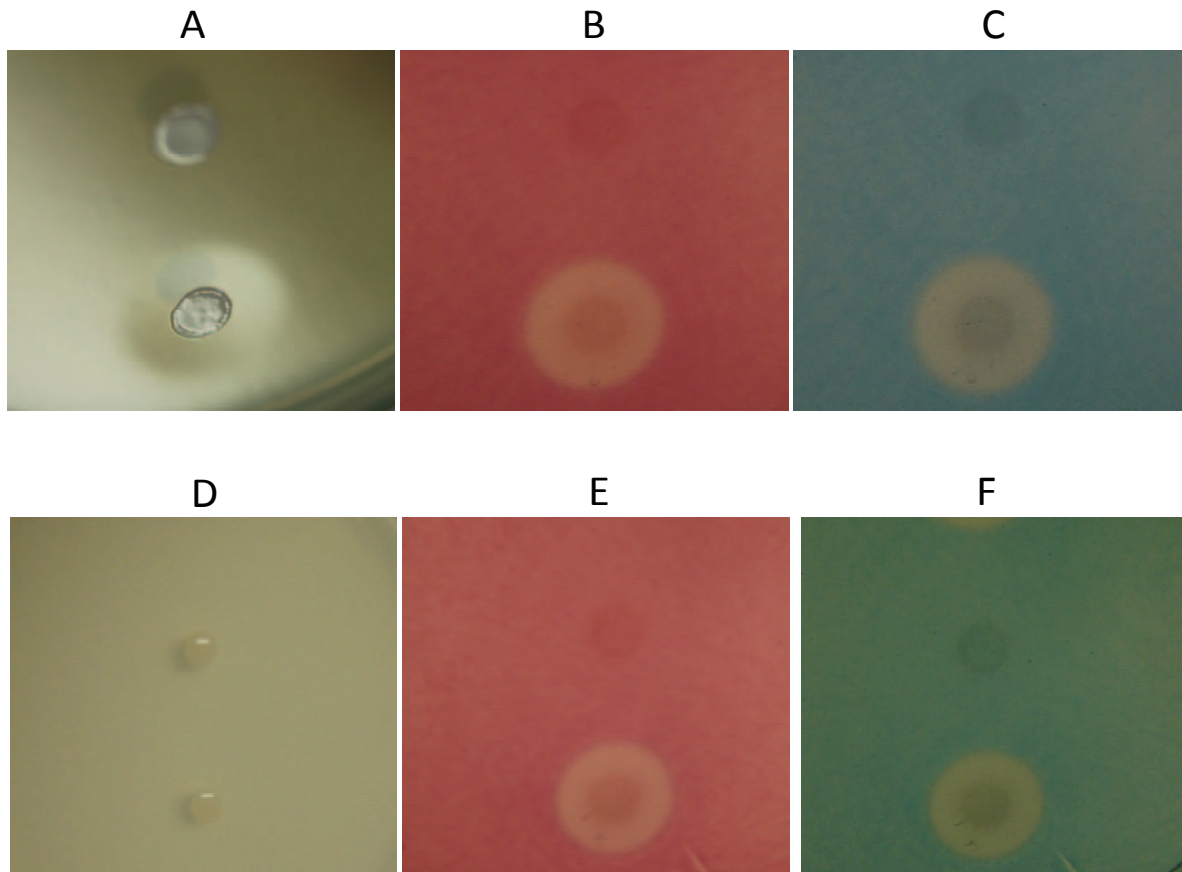
Fig. 3. Man1 activity displayed by *K. lactis* SHR8[YEpENO-BBH.Kl] (top of plates A, B, C), *K. lactis* SHR8[YEpENO-BBH.Kl-man1] (below on A ,B, C), *S. cerevisiae* Y294[YEpENO-BBH] (above on D, E, F) and *S. cerevisiae* Y294[YEpENO-BBH-man1] (below on D, E, F) produced after 48 hours of growth on SC^{-URA} plates containing 0.1% Locust bean gum at 30°C. In (A, D) the plates are unstained, (B, E) the same plates were stained with Congo Red and in (C, F) the plates had been treated with 1 M HCl after Congo Red staining.

3.4.3. DETERMINATION OF ENZYME ACTIVITY, OPTIMUM pH AND TEMPERATURE

The effect of pH and temperature on the activity of the secreted Man1 was investigated (Fig. 4). The Man1 produced by *K. lactis* SHR8[YEpENO-BBH.Kl-man1] displayed a pH optimum of 5 while *S. cerevisiae* Y294[YEpENO-BBH-man1] had a pH optimum of 6. Both strains displayed a temperature optimum of 70°C. *K. lactis* and *S. cerevisiae* strains produced enzyme activity of 160 – 180 nkat/ml.
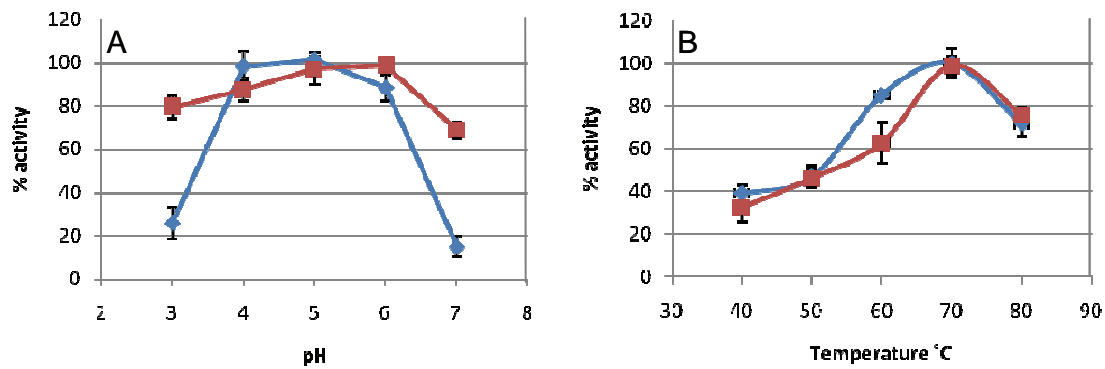
Fig. 4. Effect of (A) pH and (B) temperature on the activity of the Man1 produced by ◆ *K. lactis* SHR8[YEpENO-BBH.Kl-man1] and ■ *S. cerevisiae* Y294[YEpENO-BBH-man1]. Activity is expressed as a percentage of the highest value. Assays were performed in triplicate with the error bars representing the standard deviation.

3.4.4 GROWTH ON LACTOSE AND GLUCOSE

The different transformants were cultivated in double strength SC medium, buffered at pH 6 with citrate phosphate buffer. The *K. lactis* strains were able to grow to a DCW of about 23 mg/ml using lactose as carbon source compared to a DCW of 25 mg/ml on the same glucose molar equivalent (Fig. 5). The DCW is almost 5-fold more than that obtained with the *S. cerevisiae* cultivated on glucose.

3.4.5. PLASMID STABILITY DETERMINATION

The plasmid stability was determined for *K. lactis* GG799-ura[YEpENO-BBH.Kl] and *K. lactis* SHR8[YEpENO-BBH.Kl] over a period of two weeks (Table 3). The cultivation in liquid YPD would result in loss of plasmid, hence no colonies on the SC^(-URA) plates. Transformants able to grow on SC^(-URA) plates, after incubation in YPD medium, still contained episomal plasmids. For the first 7 days of continuous cultivation, an almost 100% loss of plasmid was observed. After 14 days of cultivation in liquid SC^(-URA), the strain either started to integrate the plasmid (or at least the marker gene) or rectified the point mutation originally induced by the FOA mutagenesis on *K. lactis* GG799 (used for the construction of *K. lactis* GG799-ura).
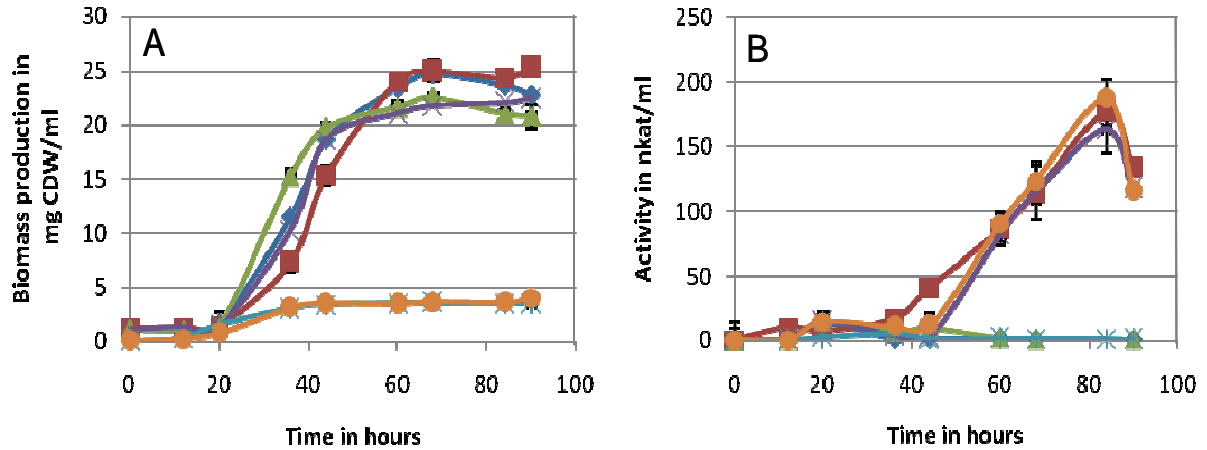
Fig. 5. (A) Biomass production in DCW (Dry Cell Weight) and (B) the levels of extracellular mannanase activity by strains ◆ *S. cerevisiae* Y294[YEpENO-BBH], ● *S. cerevisiae* Y294[YEpENO-BBH-man1]. ✕ *K. lactis* SHR8[YEpENO-BBH.Kl-man1] and ▲ *K. lactis* SHR8[YEpENO-BBH.Kl],, when cultivated on glucose as carbon source whereas ■ *K. lactis* SHR8[YEpENO-BBH.Kl-man1] and ✳ *K. lactis* SHR8[YEpENO-BBH.Kl] where cultivated on lactose. Assays were performed in triplicate with the error bars representing the standard deviation.

Table 3. The stability of the *K. lactis* GG799-ura[YEpENO-BBH.Kl-man1] and *K. lactis* SHR8[YEpENO-BBH.Kl-man1] strains increase after several sub-cultures over a period of 14 days.

K. lactis **GG799-ura[YEpENO-BBH.Kl-man]**			*K. lactis* **SHR8[YEpENO-BBH.Kl-man]**		
Days	% CFU* on SC^{-URA}	Std dev	Days	% CFU* on SC^{-URA}	Std dev
5	0	0	5	0	0
7	0.348	±0.032	7	0.555	±0.120
10	0.541	±0.072	10	1.617	±0.691
14	11.559	±1.872	14	25.972	±1.959

*- the % CFU refers to the number of colonies on the SC^{-URA} plates depicted as a percentage of the number of colonies present on the YPD-plates at the same time interval.

The experiment was performed in triplicate with the error bars representing the standard deviation.

3.4.6. SDS-PAGE ANALSIS

Analysis with SDS-PAGE indicated a protein species of 55kDa in the *K. lactis* SHR8[YEpENO-BBH.Kl-man1] and *S. cerevisiae* Y294[YEpENO-BBH-man1] had a 58kDa protein species (Fig. 6 lane 2 and 3 respectively). This protein species are not present in the lanes containing proteins samples of the *K. lactis* SHR8[YEpENO-BBH.Kl] and *S. cerevisiae* Y294[YEpENO-BBH] (Fig. 6 lane 4 and 5 respectively).
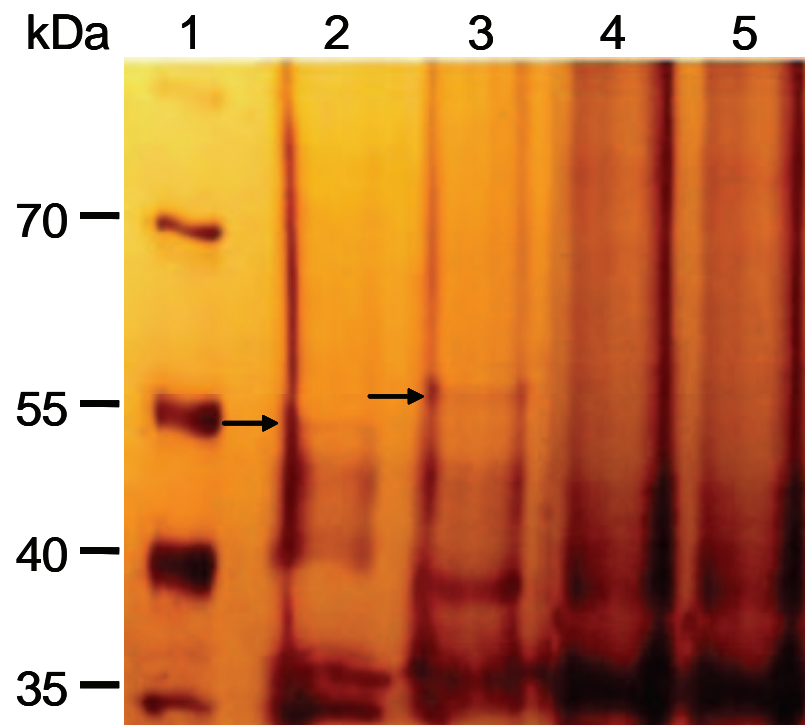
Fig. 6. Supernatant of recombinant yeast containingSeparation of the (1) Protein ladder Page Ruler ™ Fermentas Inc. (Maryland, USA), and the total extracellular protein fraction of (2) *K. lactis* SHR8[YEpENO-BBH.Kl-man1], (3) *S. cerevisiae* Y294[YEpENO-BBH-man1], (4) *K. lactis* SHR8[YEpENO-BBH.Kl] and (5) *S. cerevisiae* Y294[YEpENO-BBH] by means of SDS-PAGE. The sizes of the protein ladder is depicted on the left hand side of the SDS-PAGE

3.5. DISCUSSION

Mannanases have a wide range of industrial applications including the pulp and paper industry, food and feed industries as well as for the bioconversion of lignocellulosic residues for bioethanol production (Moreira and Filho 2008). Through recombinant technology and

heterologous expression, more efficient industrial exploitation of these enzymes is made possible. Host strains that efficiently secrete these enzymes avoid the need for expensive purification processes, thereby reducing the production costs (Dhawan and Kaur 2007).

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). In this study, the *man1* gene of *A. aculeatus* were cloned and expressed in both *S. cerevisiae* and *K. lactis*. The different host strains was evaluated on the bases of their secretion capacity as well as the size of the Man1 protein secreted (glycosylation). A *K. lactis* strain containing the deletion of the native *ku80* gene had been constructed (using the *URA3*-blaster technology) to investigate its impact on the stability of the transformants under continuous cultivation conditions.

The use of a blaster cassette such as the *URA3*-blaster (Fig. 2), have been demonstrated before (Alani *et al*. 1987, Wang *et al*. 2003). Making use of the *URA3*-blaster technology enables the recycling of the *URA3* marker gene, making it possible to (1) create several knock-out mutations or (2) introduce several constructs into the genome (multiple integrations) of the same strain. In this study recycling of the marker was required due to the presence of the *URA3* marker gene on the vectors used. *K. lactis* can perform both homologous recombination as well as non-homologous end joining (Morlino *et al*. 1999). Non homologous end joining enables the host to integrate foreign DNA at random requiring functional KU70 and KU80 proteins (Kooistra *et al*. 2004). The *ku80-URA3*-blaster cassette PCR product was transformed to *K. lactis* GG799-ura to disrupt the *ku80* gene and generate the knocking-out strain, *K. lactis* SHR7. Strains *K. lactis* SHR7 and SHR8 is therefore only able to perform homologous recombination (targeted integration) (Daley *et al*. 2005). In this study we constructed a plasmid YEpENO-BBH.Kl which contains the *K. lactis* replication of origin, while the rest of the plasmid contains either *E. coli* or *S. cerevisiae* DNA, thereby limiting the possibility of homologous recombination taking place.

The YEpENO1-BBH and YEpENO-BBH.Kl vectors constructed in this study are similar to the yENO1 vector previously constructed by Den Haan *et al*. (2007). However, these vectors had been modified in such a way as to allow for consecutive integration of a number of gene cassettes by making use of the compatibility of the *Bam*HI and *Bgl*II restriction sites. This strategy makes it possible to retrieve a gene cassette by digestion of the vector with *Bam*HI and *Bgl*II, provided all internal *Bam*HI and *Bgl*II sites had been

eliminated (elimination by means of PCR or by using synthetically designed genes). This strategy had been successfully employed for the ligation of the *Kl*. ori. onto YEpENO1-BBH. Although the addition of another gene cassette was not required for this study, the construction of these vectors will be helpful for future work where the mannosidase gene *mndA,* will be co-expressed in the same yeast.

Several attempts had been made in the past to obtain stable episomal vectors for *K. lactis*, but with limited success (Hsieh and Da Silva 1998, Kooistra *et al*. 2004). Plasmid integration seems to be inevitable. The best vector stability had been obtained with vectors using the complete pKD1 sequence, making the vectors quite large (Hsieh and Da Silva 1998, Romanos *et al*. 1992). By using strains that can only perform homologous recombination, we aimed to improve the stability of episomal vectors, yet keeping the size of the vector as small as possible. Smaller vectors generally results in a higher copy number of the plasmid, hence a higher level of expression (Morlino *et al*. 1999).

The *man1* gene was subcloned onto plasmid YEpENO-BBH with the *ENO1* promoter and terminator directing its expression (Fig. 1). YEpENO-BBH and YEpENO-BBH.Kl therefore has the same DNA sequence, with the exception of the *Kl*. ori fragment being present on the latter plasmid. *K. lactis* SHR8[YEpENO-BBH.Kl-man1] transformants were able to secrete the Man1 effectively and degrade the mannan present in the agar plates (Fig. 3), leaving an indent around the transformants. Clearing halos were produced upon staining of the plates with Congo Red solution.

The pH and temperature optimum of the heterologous Man1, expressed by *K. lactis* SHR8[YEpENO-BBH-man1] and *S. cerevisiae* Y294[YEpENO-BBH-man1] were determined (Fig. 4). The Man1 displayed a temperature optimum of 70˚C for both species and a pH optimum of approximately 5 and 6 for *K. lactis* SHR8[YEpENO-BBH-man1] and *S. cerevisiae* Y294[YEpENO-BBH-man1], respectively. The results obtained in this study differ slightly from that of Setati *et al*. (2001) who reported a pH optimum of 3 and temperature optimum of 50˚C. A similar change in characteristics was observed by two independent groups with the expression of the Cel7A of *T. reesei*. Both groups found that the glycosylation pattern varied when cultivation took place under different conditions (Du Plessis *et al*. 2009, Stals *et al*. 2004). The Cel7A was *N*-glycosylated at all three glycosylation sites when isolated from minimal medium, whereas cultivation in rich medium, resulted in only one or two sites contained high-mannose chains (Stals *et al*. 2004). A

difference in the *O*-glycosylation pattern in the linker region was also observed. In general *S. cerevisiae* elongates the mannose chain in heterologous proteins by adding even more mannose residues (Romanos *et al.* 1992). Based on this, we speculate that, when *S. cerevisiae* Y294[Man1] was cultivated in YPD medium (Setati *et al*. 2001), the medium could have had an impact on the glycosylation patterns of the Man1 resulting in a change on the activity/characterization of the enzyme.

The extracellular mannanase activity produced by the various transformants was monitored over a period of 96 hours. The *K. lactis* transformants were cultivated in medium containing either glucose or lactose as carbon source (Fig. 5), whereas the *S. cerevisiae* transformants were only cultivated on glucose due to the inability of the yeast to grow on lactose. The glucose and lactose containing medium yielded similar results for the biomass of the *K. lactis* transformants and their levels of mannanase activity (160-180 nkat.ml^{-1}). Although *S. cerevisiae* Y294[YEpENO-BBH-man1] produced similar levels of activity, the cultures reached 5-fold less biomass compared to that obtained by the *K. lactis* transformants. The lower DCW could be explained by the high levels of glucose in the medium (40 g.l^{-1}) which would have resulted in the *S. cerevisiae* strains fermenting the glucose despite the aeration of the culture. This phenomenon is due to the *S. cerevisiae* strains being Crabtree positive and the cultures losing carbon due to the release of CO_2. *K. lactis,* on the other hand, is Crabtree negative, does not ferment when exposed to high levels of glucose and therefore can channel more carbon towards biomass production. The difference in DCW implies that the Man1 production per cell of *S. cerevisiae* Y294[YEpENO-BBH-man1] is approximately 5-fold more than that of *K. lactis* SHR8[YEpENO-BBH.Kl-man1].

A noticeable difference in the size of the Man1 could be detected by SDS-PAGE when expressed by the different hosts. The *K. lactis* SHR8[YEpENO-BBH-man1] strain produced the Man1 as a 55kDa protein compared to the 58kDa protein secreted by *S. cerevisiae* Y294[YEpENO-BBH-man1] (Fig. 5). Indicating that *K. lactis* clearly does not glycosylate the Man1 as much as the *S. cerevisiae* strain.

The stability of the YEpENO-BBH.Kl vector in *K. lactis* GG799-ura and *K. lactis* SHR8 strains seems to have had anegative effect on the disruption of the *ku80* gene (Table3). Eighty percent of the CFU on the SC^{-URA} plates indicated mannanase activity confirming integration of the plasmid (data not shown). Although the stability of the transformants was

not improved a heterogeneous culture started to develop after ten days which would have a negative effect on the production levels of the heterologous enzyme.

The extracellular mannanase production levels per cell of *S. cerevisiae* Y294[YEpENO-BBH.Kl-man1] has been established at being almost five times more than that obtained with *K. lactis* SHR8[YEpENO-BBH.Kl-man1], yet similar extracellular levels of mannanase activity was obtained. The increase in biomass demonstrates a more effective utilisation of the carbon source by *K. lactis*. *K. lactis* has the additional advantage of being able to use a broader substrate range than *S. cerevisiae*. Although episomal vectors have been successfully constructed for use in *K. lactis,* the stability of the transformants could not be guaranteed for prolonged periods of time due to the tendency of the plasmids to integrate after a number of generations (Kooistra *et al*. 2004, van Ooyen *et al*. 2006). This study concluded that the disruption of the non homologous end joining complex does not contribute to the stability of the transformants. However, high levels of activity can still be accomplished through multi-copy integration of the gene cassette which has been proven to yield stable transformants (Morlino et al. 1999).

3.6. ACKNOWLEDGEMENTS

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4. EXPRESSION OF THE *A. NIGER* β-MANNOSIDASE AND *A. ACULEATUS* ENDO-β-1,4-MANNANASE IN *S. CEREVISIAE.*

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4.1. ABSTRACT

The endo-β-1,4-mannanase (*man1*) of *Aspergillus aculeatus* was cloned under the transcriptional control of the truncated phosphoglycerate kinase ($PGK1_{PT}$) promoter and terminator. The *Aspergillus niger* β-mannosidase gene, *mndA,* was codon optimized for expression in *Saccharomyces cerevisiae* and synthetically produced. The β-mannosidase gene was cloned under the control of the enolase1 promoter and terminator ($ENO1_{PT}$) and was co-expressed with the *A. aculeatus* endo-β-1,4-mannanase *man1* gene on the same episomal plasmid. The β-mannosidase protein, MndA, occured as a 140 kDa protein species on the SDS-PAGE analysis. However, no activity was detected on *p*-nitrophenyl β-D-mannopyranoside as substrate. *S. cerevisiae* Y294[man1, mndA] showed a 4-fold lower endomannanse activity than the *S. cerevisiae* Y294[man1]. Hydrolysis of 0.5% Locust bean gum showed the same profile in *S. cerevisiae* Y294[man1] and Y294 [man1, mndA] indicating that the β-mannosidase had no effect on hydrolysis. Expression was also evaluated in *S. cerevisiae* strain NI-C-D4, known to produce unglycosylated heterologous proteins at higher expression levels. The *S. cerevisiae* NI-C-D4[man1] strain showed the highest mannanase activity on day 5 at 251.39 nkat/ml while *S. cerevisiae* NI-C-D4[man1, mndA] produced 69.69 nkat/ml, almost double that of *S. cerevisiae* Y294[man1, mndA].

Keywords:
Endomannanase
β-mannosidase
Saccharomyces cerevisiae
Heterologous protein
Aspergillus

4.2. INTRODUCTION

Rising oil prices and the negative environmental impact of fossil fuel emissions have led to the search for an alternative form of transportation fuel. Alternative energy sources, such as bioethanol, is already used as gasoline replacement worldwide (Sun and Cheng 2002). Bioethanol can in principle be produced from lignocellulose through consolidated bioprocessing, a one step fermentation involving a single organism capable of hydrolysing the substrate and produce ethanol as fermented product (Lynd *et al*. 2002). Development of these recombinant microorganisms will affect the future overall performance and success of biomass conversion in future and will allow more process steps in one reactor (Hamelinck *et al*. 2005, Lynd *et al*. 2002). *Saccharomyces cerevisiae* has proven to be the most efficient microorganism for the production of ethanol from monomers such as glucose and mannose. This yeast has also been used extensively in industrial fermentation processes, making it the ideal organism for the production of bioethanol (Gray *et al*. 2006, Yu and Zhang 2004).

Lignocellulose is an abundant renewable resource and sources include agricultural and forestry waste material (Lynd *et al*. 2002). Hemicelluloses are structural polysaccharides found in close association with cellulose and lignin in plant cell walls (Moreira and Filho 2008). Mannan, together with xylan, constitutes the major hemicellulose components contributing to as much as a third of the lignocellulose portion depending on the plant source. Mannan can be classified as linear mannan, glucomannan, galactomannan or as galactoglucomannan. Mannan is composed of a linear chain of β-D-1,4-linked mannose and glucose units which can be substituted with α-1,6-linked galactose side chains (Moreira and Filho 2008). Hydrolysis of galacto(gluco)mannan is achieved through various enzymes cooperatively acting together. These enzymes include β-mannanases (1,4-β-D-mannan mannohydrolases, EC 3.2.178), β-mannosidases (1,4-β-D-mannopyranoside hydrolases, EC 3.2.1.25), α-galactosidases (1,6-α-D-galactoside galactohydrolases, EC 3.2.1.22), β-glucosidases (1,4-β-D-glucoside glucohydrolases, EC 3.2.1.21) and galactomannan acetylesterases (Moreira and Filho 2008). Endo-β-1,4-mannanases are endohydrolases that catalyze the cleavage of β-1,4 linkages releasing mainly mannobiose and mannotriose as end products. β-mannosidases hydrolyse the 1,4-β-D-mannopyranose and β-glucosidases the 1,4-β-D-glucopyranose linkages at the non-reducing end of the main galacto(gluco)mannan chain, releasing D-mannose and D-glucose. Both enzymes readily convert the manno-oligosaccharides produced by β-mannanase to mannose and glucose units (Ademark et al. 1999). α-Galactosidases and acetyl mannan esterases are debranching enzymes

catalyzing the hydrolysis of α-1,6-linked D-galactopyranosyl side chains and acetyl groups respectively from galactoglucomannan (Ademark *et al*. 2001).

Mannan degrading enzymes are produced by various fungi such as *Aspergillus* sp. (Ademark *et al*. 1998, Christgau *et al*. 1994) and *Trichoderma* sp. (Harjunpjää *et al*. 1995), bacteria such as *Cellumonas* sp. (Stoll *et al*. 1999) and *Bacillus* sp. (Mendoza *et al*. 1995, Yosida *et al*. 1997) and have even been isolated from animals (Chen *et al*. 1995). In plants, these enzymes are involved in growth and maturation (Mo and Bewley 2002). Mannanases have a wide range of industrial applications including the pulp and paper industry, food and feed industries as well as for bioconversion of lignocellulosic residues for bioethanol production (Moreira and Filho 2008).

The inability of *S. cerevisiae* to grow on the complex sugars present in lignocellulose has to be overcome in order for it to be used in the CBP process. Various cellulolytic and hemicellulolytic enzymes from fungal and bacterial origin have been successfully expressed in *S. cerevisiae* enabling partial hydrolysis of lignocellulosic substrates (Lynd *et al*. 2002). Developing *S. cerevisiae* for mannan degradation would contribute to the development of a CBP organism resulting in a more economical production of bioethanol (Lynd *et al*. 2002). In this study, a β-mannosidase has been codon optimized for *S. cerevisiae* and produced synthetically using the amino acid sequence from *A. niger mndA* (Ademark *et al*. 1999). This enzyme was co-expressed with the β-mannanase of *A. aculeatus* in *S. cerevisiae* Y294 and *S. cerevisiae* NI-C-D4, a super-secretor strain (Wang *et al*. 2001).

4.3. MATERIALS AND METHODS

4.3.1. MICROBIAL STRAINS AND PLASMIDS

Genotypes and sources of yeast and bacterial strains, as well as plasmids used in this study are listed in Table 1.

4.3.2. MEDIA AND CULTIVATION CONDITIONS

All chemicals, media components and supplements were of analytical grade standard. *Escherichia coli* XL1-Blue (Stratagene, USA) was used for the amplification and construction of recombinant plasmids. Bacterial strains were grown at 37°C in Luria Bertani (LB) medium or Terrific Broth (TB) supplemented with ampicillin to a final concentration of 100 μg.ml^{-1} (Sambrook *et al*. 1989). Strains of *S. cerevisiae* Y294 were cultivated at 30°C on a rotary shaker set at 100 rpm. Strains were grown in either YPD (1% yeast extract, 2% peptone and 2% glucose) or synthetic

complete (SC) medium (2% glucose, 0.17% yeast nitrogen base without amino acids and 0.5% $(NH_4)_2SO_4$)) supplemented with appropriate amino acids, but lacking uracil (SC^{-URA}) or leucine (SC^{-LEU}).

Table 1. A list of strains and plasmids used in this study

Strains or Plasmids	Relevant genotype	Source or reference
Strains:		
E. coli XL1-Blue	MRF *endA1 supE44 thi-1 RecA1 gyrA96 relA1 lac [F' proAB lacq Z(Δ M15 Tn10 (tet)]*	Stratagene (La Jolla, CA, USA)
S. cerevisiae Y294	MATα *leu2-3,122 ura3-52 his3 trp1-289*	ATCC 201160
S. cerevisiae NI-C-D4	*trp1 ura3 pep4*	Wang *et al.* 2001
S. cerevisiae Y294[mndA]	*bla ura3/URA3 ENO1$_P$ –mndA-ENO1$_T$*	This study
S. cerevisiae Y294[man1]	*bla ura3/URA3 PGK1$_P$-man1-PGK1$_T$*	This study
S. cerevisiae Y294[man1, mndA]	*bla ura3/URA3 PGK1$_P$-man1-PGK1$_T$ ENO1$_P$ –mndA-ENO1$_T$*	This study
S. cerevisiae Y294[pJC1]	*bla ura3/URA3 PGK1$_P$-PGK1$_T$*	This study
S. cerevisiae NI-C-D4[mndA]	*bla ura3/URA3 ENO1$_P$ –mndA-ENO1$_T$*	This study
S. cerevisiae NI-C-D4[man1]	*bla ura3/URA3 PGK1$_P$-man1-PGK1$_T$*	This study
S. cerevisiae NI-C-D4[man1, mndA]	*bla ura3/URA3 PGK1$_P$-man1-PGK1$_T$ ENO1$_P$–mndA-ENO1$_T$*	This study
S. cerevisiae NI-C-D4[YEpENO-BBH]	*bla ura3/URA3 ENO1$_P$ ENO1$_T$*	This study
S. cerevisiae Y294[Bgl1]	*bla ura3/URA3 PGK1$_P$-bgl1-PGK1$_T$*	Van Rooyen *et al.* 2005
Plasmids:		
pBluescript SK$^+$	*bla*	Stratagene
pJC1	*bla URA3 PGK1$_P$-PGK1$_T$*	Crous *et al.* 1995
YEpENO-BBH	*bla URA3 ENO1$_P$ ENO1$_T$*	Fouché *et al.* 2009
pNFmndA	*bla URA3 ENO1$_P$ –mndA-ENO1$_T$*	This study
pNFman1	*bla URA3 PGK1$_P$-man1-PGK1$_T$*	This study
pNFman1mndA	*bla URA3 PGK1$_P$-man1-PGK1$_T$ ENO1$_P$–mndA-ENO1$_T$*	This study
pMes2	*bla URA3 PGK1$_P$-man1-PGK1$_T$*	Setati *et al.* 2001
ySFI	*bla URA3 PGK1$_P$-BGL1-PGK1$_T$*	van Rooyen *et al.* 2005

4.3.3. DNA MANIPULATIONS

DNA manipulations were carried out according to standard protocols (Sambrook *et al.* 1989). Restriction endonucleases and T4 DNA ligase were supplied by Fermentas Inc. (USA). DNA fragments were eluted from agarose gels using Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research Corporation, USA).

### 4.3.4. DESIGN OF CODON OPTIMISED SYNTHETIC GENE SEQUENCE

The amino acid sequence of the *A. niger mndA* was used as template to design a DNA sequence containing only codons favoured by *S. cerevisiae* using software available at http://www.evolvingcode.net (Sharp and Cowe 1991). The codon adaptation index (CAI) for the wild type *mndA* gene when expressed in *S. cerevisiae*, was calculated as 0.1 (Sharp and Li 1987), whereas the codon optimised *mndA* gene had a CAI value of 0.774. The codon optimised version of the *mndA* gene contained the native secretion signal and endonuclease restriction sites at the 5'-end (*Eco*RI) and 3'-ends (*Xho*I) were added for cloning purposes. The optimised gene was synthesized by GenScript Corporation, USA.

4.3.5. CONSTRUCTION OF PLASMIDS

Plasmids used in this study are depicted in Fig. 1. The 2.8 kb synthetic β-mannosidase gene (*mndA*) was retrieved from pUC57 (GenScript Corporation, USA) as an *Eco*RI-*Xho*I fragment and ligated into the corresponding sites on YEpENO-BBH, generating pNFman1. A 1.3 kb *Eco*RI-*Xho*I fragment containing the endo-β-mannanase (*man1*) gene was recovered from pMES2 (Setati *et al.* 2001) and cloned into ySFI (van Rooyen *et al.* 2005), replacing the β-glucosidase fragment. The yeast expression plasmid ySFI contains a truncated *PGK1* promoter. Plasmid pNFmndA was digested with *Bam*HI and *Bgl*II, retrieving the $ENO1_P$-*mndA*-$ENO1_T$ cassette which was cloned into the unique *Bam*HI site on pNFman1, generating pNFman1mndA.

4.3.6. YEAST TRANSFORMATION AND PCR CONFIRMATION

Yeast cells were harvested from YPD cultures in logarithmic phase. Transformations were performed using the dimethyl sulfoxide-lithium acetate method (Hill *et al.* 1991). The *S. cerevisiae* strains Y294 and NI-C-D4 were transformed with the individual recombinant yeast expression vectors. Colony PCR reactions were carried out with Taq DNA polymerase (Inqaba Biotec, South Africa) as prescribed by suppliers, using a Perkin Elmer GeneAmp® PCR System 2400. The PCR reaction mixture was as follows: 200 ng template, 100 pmol of each primer, 0.2 mM each of deoxynucleotide triphosphate and reaction buffer supplied by the manufacturer. Products were visualized on a 1% agarose gel and stained with ethidium bromide. The presence of the genes were confirmed by PCR using the primers listed in Table 2.
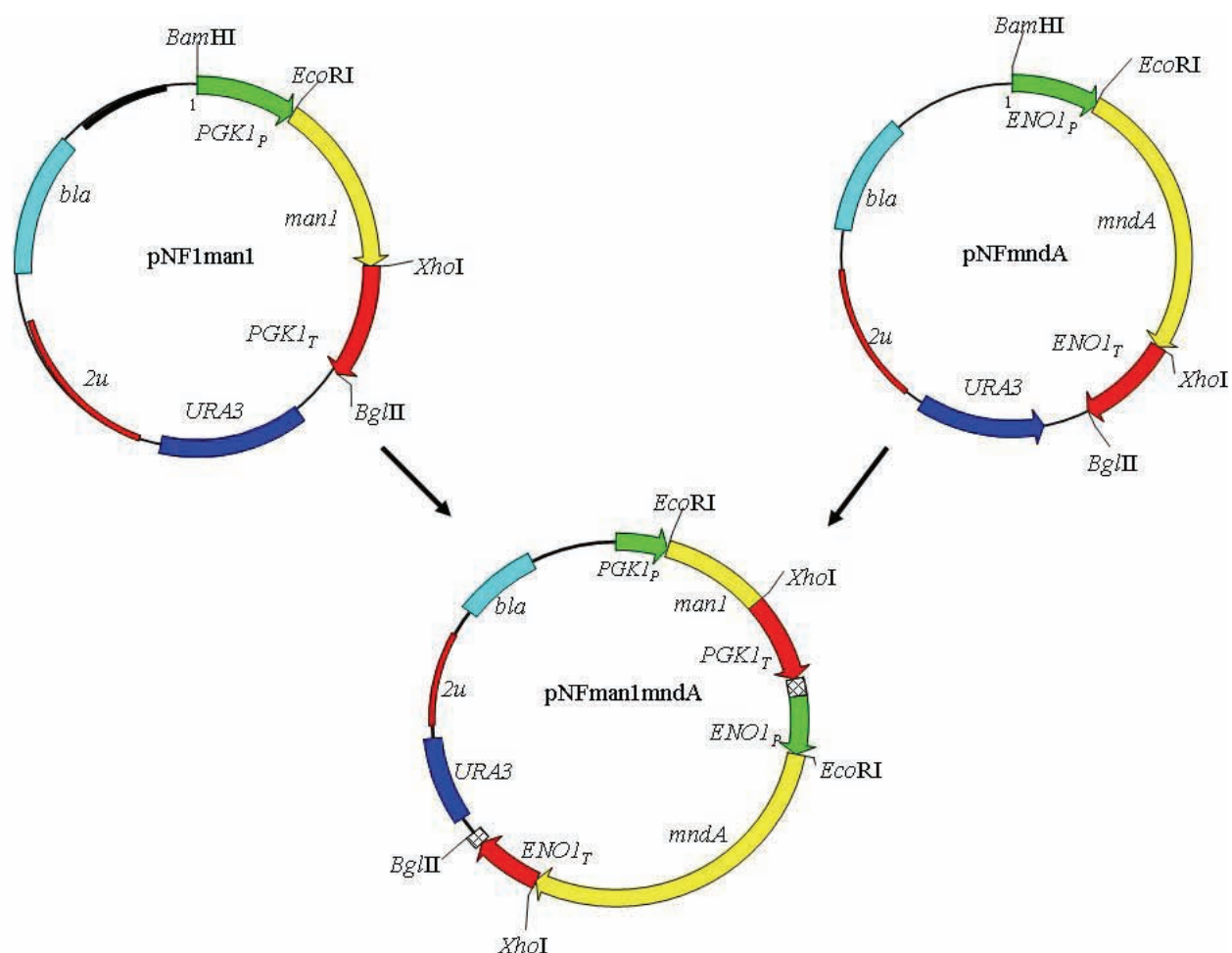
Fig. 1. A schematic representation of the plasmids used in this study. The *S. cerevisiae* 2 micron autonomous replicating sequence (*2μ*) allows for episomal replication of the plasmid. The bacterial β-lactamase (*bla*), and *S. cerevisiae* orotidine-5'-phosphate decarboxylase (*URA3*) were used as selectable markers.

Table 2. Primers used in this study.

Primer name	Sequence
Manoslft	5'-GGTTTCTTCAGAGGTGGCGTTACCG-3'
Manosrght	5'-CGTTACCAGCAGCCCACAAAGCC-3'
Mannanlft	5'-AGATGAAGCTTTCTCACATG-3'
Mannanrght	5'-CACATGATCCGTCACCAG-3'
PrimerA	5'-CTGCTCCATCTTCTTACTCT-3'
Eno prom	5'-GTAACATCTCTCTTGTAATCCCTTATT-3'
Eno term	5'-GCAACCCTATATAGAATCATAAAACA-3'
PGKbeginprom	5'-ACTGAAGCTTGGATCCTTAAAGATGCCG-3'
PGKendterm	5'-ACTGAAGCTTGGCCAAGCTTTAACGAAC-3'

4.3.7. DNA SEQUENCING

The nucleotide sequence of the β-mannosidase gene was determined with the dideoxy chain termination method using fluorescently labelled nucleotides on an ABI PRISM TM 3100 Genetic Analyser. Various primers sets were used to sequence the 2.8 kb fragment (Fig. 2). The internet based BLAST program (www.ncbi.nih.gov/BLAST) was used to analyze sequence data. The software package DNAMAN (version 4.1) (Lynnon Biosoft) was used to predict protein sequences and identify restriction sites.
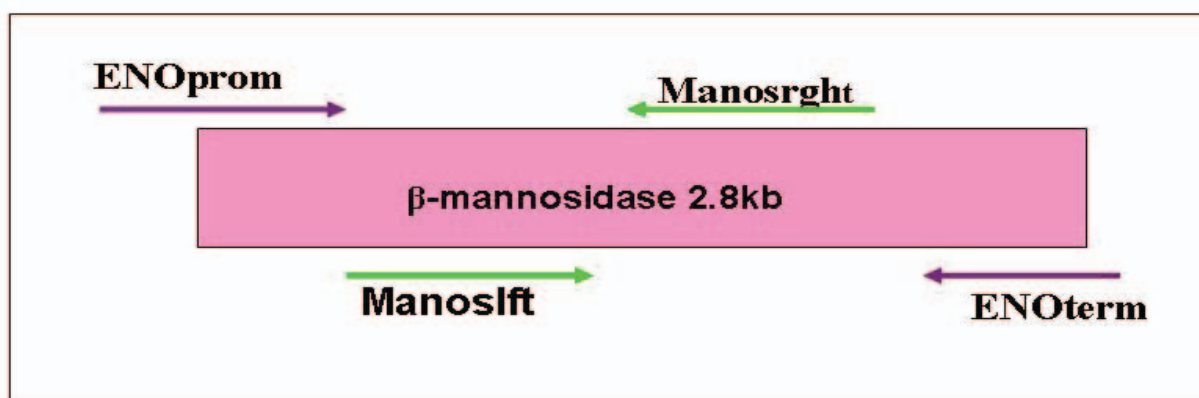
Fig. 2. Schematic representation of the primer annealing positions with the arrows indicating the direction of sequencing. Primers ENOprom and ENOterm was used to determine the sequence at the 3' and 5' of the gene, whereas Manoslft and Manosrght was used to obtain the internal DNA sequence.

4.3.8. SDS-PAGE ANALYSIS

Cultures were grown in (SC^{-URA}) containing 20 mM succinate (pH 6) for five days. Supernatant was obtained by centrifugation for 15 min at 4 000 g. The supernatant was filtered through Whatman filter paper (0.22 μm filters) and concentrated 60-fold (for *S. cerevsiae* Y294 strains) or 5-fold (for *S. cerevisiae* NI-C-D4 strains) through ultrafiltration at 100 kPa using the Minitan system (Millipore Corporation, Bedford, MA, USA) housing a 10 kDa cut-off membrane. Whole cells were resuspended in buffer and protein preparations were performed on ice. The intracellular fractions were prepared by placing 500 μl cells in 500 μl buffer, with 0.3 g acid washed glass beads. The mixture was incubated on ice and vortexed every alternating 20 s for 6 minutes. The mixtures were centrifuged for 3 minutes at 4 000 g and the supernatant used as the intracellular fraction.

Supernatant fractions were further concentrated with TCA as described at http://www.protocol-online.org/prot/Molecular_Biology. Pellets were dried and resuspended in 0.1 M NaOH/1% SDS. Sodium azide (NaN$_3$) was added to a final concentration of 0.02% to inhibit microbial growth.

Protein preparations (10 μg) were denatured for 3 minutes at 100˚C in SDS-denaturing buffer containing 0.5 M DDT (Novel Experimental Technology, San Diego, CA). Non-denatured samples were not boiled and did not contain SDS-denaturing buffer. Protein samples were separated on SDS-PAGE using an 8% (wt/vol) separation gel and were visualized with silver staining (Ausubel *et al*. 1998). A protein ladder, Page Ruler™ from Fermentas Inc. (Maryland, USA), was used as a size marker.

4.3.9. PROTEIN ASSAY

Protein concentrations were determined using the Bradford Assay (Bio-Rad). Absorbancies were measured at 595 nm and bovine serum albumin (BSA) was used as a standard.

4.3.10. PLATE ENZYME ASSAYS

For mannan enzyme activity, *S. cerevisiae* was grown at 30˚C on selective synthetic complete (SC) agar plates supplemented with an amino acids excluding uracil (SC^{-URA}) as well as 0.5% Locust bean gum (Sigma, Sweden). After 3 days, the cultures were washed from the plates and plates stained for 30 minutes with a 0.1% Congo Red solution. A 1.2 M NaCl solution was used to destain the plates and visualise hydrolysis zones. Treatment with 1 M HCl followed the Congo Red staining.

4.3.11. ENZYME ASSAY (*P*-NITROPHENYL β-D-MANNOPYRANOSIDE, *P*-NITROPHENYL β-D-GLUCO-PYRANOSIDE)

The supernatant was harvested from 100 ml *S. cerevisiae* Y294[mndA] and *S. cerevisiae* NI-C-D4[mndA] cultures over a period of 6 days. β-mannosidase activity for both *S. cerevisiae* strains were determined as described by Ademark *et al*. (1999). Supernatant, intracellular fractions and whole cells were incubated with *p*-nitrophenyl β-D-mannopyranoside and *p*-nitrophenyl β-D-glucopyranoside separately, in concentrations ranging from 0.1 to 4 mM at 37˚C–50˚C in 50 mM sodium citrate buffer with pH ranging from pH 4 to pH 6. All assays were performed in triplicate and pure β-mannosidase [EC 3.2.1.78] from *Cellumonas fimi* (Megazyme) was used as a reference.

4.3.12. PROTEIN DEGLYCOSYLATION

The carbohydrate content of recombinant β-mannosidase was determined by treating it with N-glycosidase F (PNGase F, New England Biolabs, USA) as described by the manufacturer. The supernatant of *S. cerevisiae* Y294[mndA] was harvested by centrifugation for 5 minutes at 4 000 g and filtered through a 0.22 μm membrane. Protein was denatured at 100˚C in loading buffer

containing 60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14 mM β-mercaptoethanol, and 0.1% bromophenol blue. The deglycosylation reaction was performed by adding 0.25 units of PNGase F to 40 μl of denatured protein. After an incubation period of 1 hour at 37˚C the samples were denatured at 70˚C in DTT containing buffer. Endoglycosidase F (0.2 U) was added and the reaction was again incubated for 1 hour. Proteins were analyzed using SDS-PAGE. Untreated MndA was prepared in the same manner but without PNGase F being added.

4.3.13. GROWTH ON CELLOBIOSE

S. cerevisiae Y294 and NI-C-D4 strains with the β-mannosidase *mndA* gene were plated onto (SC⁻ URA) medium containing cellobiose as the sole carbon source. Reference strain *S. cerevisiae* Y294[pJC1] and *S. cerevisiae* Y294[Bgl1] was also plated on the medium as controls, negative and positive, respectively.

4.3.14. ENZYME ASSAYS (DNS)

The enzyme activity on galactoglucomannan (Sigma, Sweden) was determined in liquid assays using the reducing sugar assay method (Miller, 1959) with 0.5% Locust bean gum in 50 mM sodium citrate buffer (pH 5) as substrate. The reaction was carried out at 50˚C for 5 minutes and the hydrolysis reaction was terminated by adding the DNS reagent (1% 3.5-dinitro-salicyclic acid, 20% potassium sodium tartrate, 1% NaOH, 0.2% phenol, 0.05% Na_2SO_3) and boiling at 100˚C for 15 minutes. One nanokatal (nkat) of enzyme activity was defined as the amount of enzyme producing 1 nmol of reducing sugars (mannose) per second under the given conditions.

4.3.15. DETERMINATION OF THE OPTIMUM PH AND TEMPERATURE OF MNDA

The *S. cerevisiae* Y294[mndA] was grown for 3 days in double strength SC medium and lyophilised to powder form. The lyophilised material was dissolved in 50 mM citrate phosphate buffer to a concentration of 5 μg/ml and assays were conducted over a period of 2 hours in 0.5% Locust bean gum. The temperature optimum was determined at 20˚C to 80˚C at pH 5 in 50 mM citrate phosphate buffer. The optimal pH was determined using 0.5% Locust bean gum prepared in 50 mM citrate-phosphate buffer with a pH range of pH 3 – pH 7 at 50˚C. Residual activities were calculated as a percentage of the maximum activity displayed at the different temperature or pH conditions.

4.3.16. MEASUREMENT OF GROWTH

The dry cell weight was measured by filtering 20 ml culture through 0.47 µm glass microfibre filter (Whatman ®), which was washed with distilled water and dried in a microwave to constant weight (approximately 15 minutes at 30% power) (Plüddemann and van Zyl 2003). All dry weight estimations and growth curves were done in triplicate. Dry cell weights were calculated at specific time points when assays were performed in order to determine the enzyme activity produced per g of cells.

4.3.17. SYNERGISM AND HYDROLYSIS PRODUCTS OF ENZYMES ON LOCUST BEAN GUM SUBSTRATE

Hydrolysis of 0.5% Locust bean gum in 50 mM sodium acetate pH 5 was carried out for 10 minutes with supernatants of *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[man1, mndA]. Samples were diluted 10 times in deionised water and filtered through 0.22 µm membrane. Hydrolysis products were separated on a Dionex CarboPac PA 100 column (Dionex, Sunnyvale, USA) using 100 mM NaOH as eluent. Analyses were carried out using a Dionex 500 chromatographic system equipped with an ED 40 electrochemical detector and PeakNet® software (Dionex). Stock solutions (50 ug/ml) of D-mannose and mannobiose were used as standards (Megazyme).

4.3.18. HIGH PERFORMACE LIQUID CHROMATOGRAPHY

A 0.5% Locust bean gum solution was centrifuged at 12 000 g to remove the larger mannose chains. The Locust bean gum supernatant was incubated overnight with the supernatant of *S. cerevisiae* Y294[mndA] and *S. cerevisiae* Y294[YepENOBBH]. The samples were centrifuged for 5 minutes at 12 000 g and incubated with up to 2% perchloric acid on ice for 10 minutes. Ninety nine microliters of KOH (7 N) was added to the sample, followed by incubated on ice for 10 minutes and centrifuged for 10 minutes at 12 000 g. Samples were analysed using Finnigan Surveyor high performance liquid chromatography (HPLC) from Thermo Scientific (San Jose, USA) to identify the sugars released by the MndA enzyme. The supernatant was directly filtered into vials using a 0.22 µm membrane. The compounds were separated on an Phenomenex column (Rezex RHM – monosaccharide H+ (8%)), at a column temperature of 45˚C with MilliQ water as mobile phase at a flow rate of 0.6 ml min.$^{-1}$ and subsequently detected with a Finnigan Surveyor refractive index plus detector. D-Mannose, mannobiose and raffinose (a representation of a triose sugar) were used as standards.

4.4. RESULTS

### 4.4.1. SEQUENCE OF THE CLONED β-MANNOSIDASE GENE

The nucleotide and deduced amino acid sequence of the synthetic *A. niger mndA* are presented in Appendix A. The 2.8 kb fragment encoding a 931 amino acid peptide was cloned and expressed in *S. cerevisiae*. The mature protein has a theoretical pI of 5.32 and molecular mass of 104.8 kDa. The cloned fragment showed 72.80% homology with the DNA sequence published for *A. niger mndA*, but the protein sequences were identical (Ademark *et al*. 2001). The amino acid sequence was analysed using neural networks (NN) and hidden Markov models (HMM) on the SignalP 3.0 program (Expasy Proteomics Server, (http://www.cbs.dtu.dk/services/SignalP) which showed the presence of a signal peptide on the amino acid chain position 1 to 21, with a cleavage site between amino acids 21 and 22 (Appendix A).

4.4.2. GLYCOSYLATION PREDICTIONS

N-glycosylation is possible on asparagine residues which occur in the Asn-Xaa-Ser/Thr consensus sequence (Xaa being any amino acid except proline). Sequence analysis of the *A. niger mndA* revealed that the mature peptide contained 11 putative *N*-glycosylation sites. The posttranslation modification tool *NetNGlyc* (http://www.cbs.dtu.dk/services/NetNGlyc) attempts to distinguish truly glycosylated sequences from non-glycosylated ones. When the possible glycosylation sites of synthetic *A. niger mndA* were analysed, ten possible glycosylation sites were found. Fig. 3 shows predicted *N*-glycosylation sites on the protein chain, where the x-axis represents protein length from N- to C-terminal. All the potential sites crossing the threshold at 0.5 are predicted to be glycosylated.

4.4.3. CONFIRMATION OF PRESENCE OF GENE *MNDA* AND *MAN1*

DNA samples used in the PCR were obtained from the different recombinant *S. cerevisiae* Y294 and NI-C-D4 strains (Fig 4). All the constructs that were made were confirmed with PCR sequence specific primers. DNA from *S. cerevisiae* Y294[mndA] and *S. cerevisiae* NI-C-D4[mndA] shows the presence of the *mndA* whereas DNA from *S. cerevisiae* Y294[man1, mndA] and *S. cerevisiae* NI-C-D4[man1, mndA] respectively contained the *man1* and *mndA* genes. *S. cerevisiae* Y294[man1] and *S. cerevisiae* NI-C-D4[man1] indicating the presence of *man1* gene but no *mndA*. The reference strain *S. cerevisiae* NI-C-D4[pJC] displayed no PCR product.
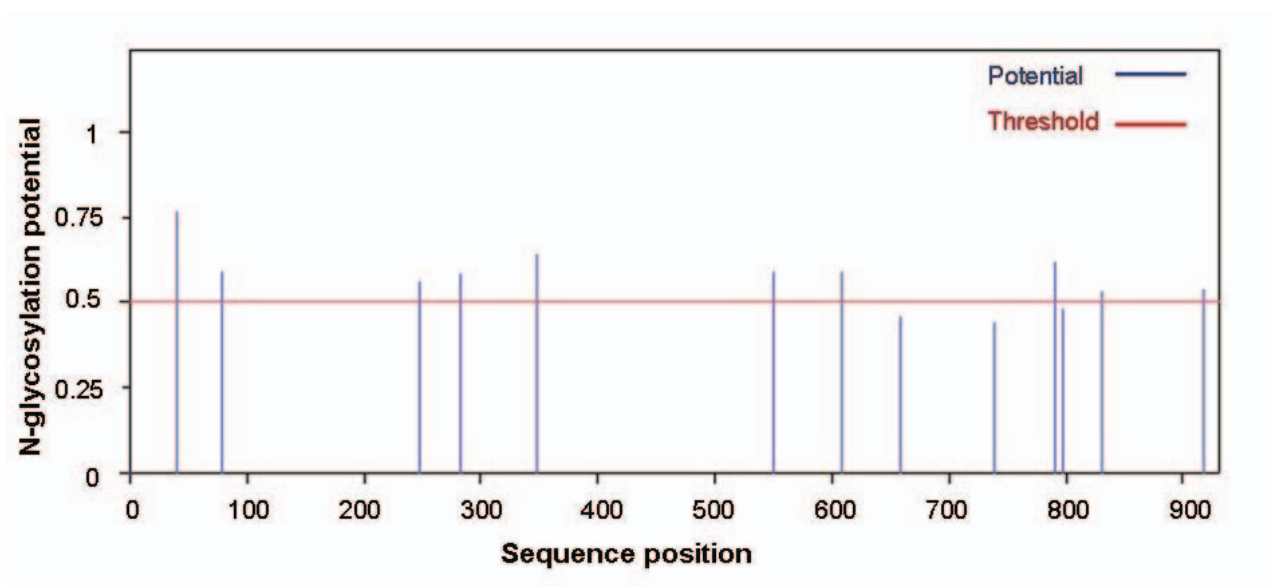
Fig. 3. Predicted *N*-glycosylation sites in the *mndA* sequence. Potential glycosylation sites crossing the 0.5 threshold are predicted to be true glycosylation sites.

s
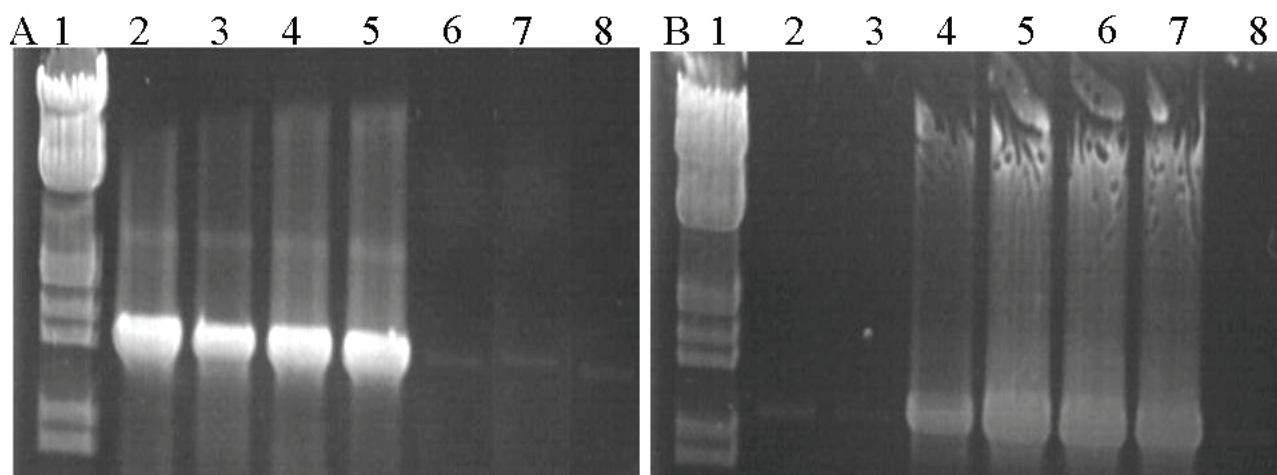
Fig. 4. Identification of the *man1* and *mndA* genes in *S. cerevisiae*. PCR results obtained with (A) primers Manoslft and Manosrght and (B) primers Mannanlft and Mannanrght. Lane 1 shows the lambda DNA (*Eco*RI/*Hin*dIII) marker. PCR results were separated on agarose gels in the following sequence: lane 2 *S. cerevisiae* Y294[mndA]; lane 3 *S. cerevisiae* NI-C-D4[mndA]; lane 4 *S. cerevisiae* Y294[man1, mndA]; lane 5 *S. cerevisiae* NI-C-D4[man1, mndA]; lanes 6 have the *S. cerevisiae* Y294[man1] and 7 *S. cerevisiae* NI-C-D4[man1], and 8, *S. cerevisiae* NI-C-D4[pJC].

4.4.4. PLATE ASSAY RESULTS

The various cultures were grown overnight in (SC^{-URA}) broth and transferred to the (SC^{-URA}) plates with 0.5% Locust bean gum. Hydrolysis zones with dark rings were observed for the *S. cerevisiae* Y294[mndA] strain (Fig. 5A). Whereas the *S. cerevisiae* Y294[man1] strain showed a clear hydrolysis zone (Fig. 5B). The *S. cerevisiae* Y294[man1, mndA] strain produced a light and dark hydrolysis zone (Fig. 5C). The reference strain, *S. cerevisiae* Y294[pJC1], strain showed no hydrolysis on the Locust bean gum plates. *S. cerevisiae* NI-C-D4 strains containing the various plasmids are shown in Fig. 6 A, B and C. The hydrolysis zone of the strain with the *S. cerevisiae* NI-C-D4[mndA] showed no dark ring, whereas the *S. cerevisiae* NI-C-D4[man1] and the *S. cerevisiae* NI-C-D4 [man1, mndA] showed hydrolysis zones of similar sizes as the *S. cerevisiae* Y294 strains. No growth was observed on cellobiose plates except for the reference strain (results not shown).

4.4.5. *P*-NITROPHENYL β-D-MANNOPYRANOSIDE AND *P*-NITROPHENYL β-D-GLUCOPYRANOSIDE ASSAYS

The β-mannosidase producing yeast strains (*S. cerevisiae* Y294[mndA] and *S. cerevisiae* NI-C-D4[mndA]) were analyzed for their ability to cleave the β-1,4-mannopyranoside linkage and β-1,4-glucopyranoside linkage in an exo manner. In contrast to previous results obtained (Ademark *et al*. 1998) for the native enzyme, no activity could be detected for mndA produced in this study. Extracellular, whole cell and intracellular phases all tested negative over periods of 24 hours at temperatures ranging from 37˚C to 70˚C and buffers ranging from pH 3 to pH 7. The commercially available β-mannosidase from *Cellumonas fimi*, used as reference enzyme, showed activity within 5 minutes of incubation at 37˚C and pH 5.
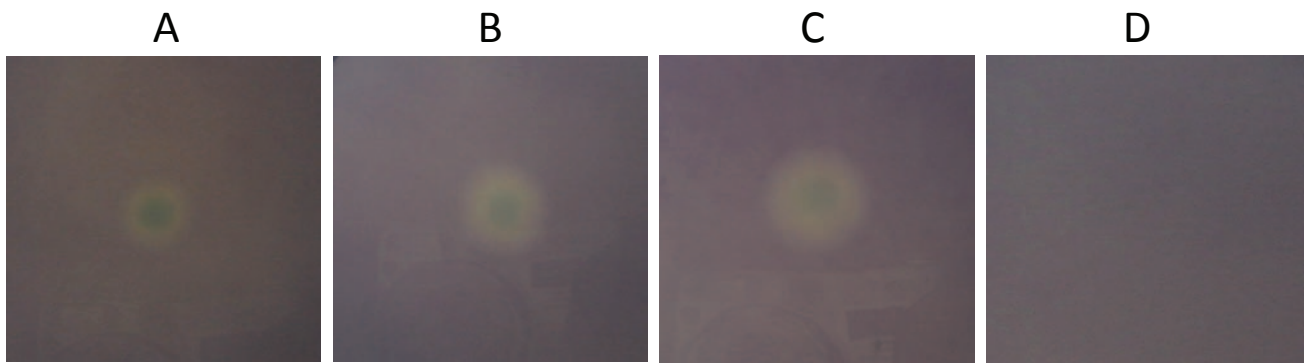
Fig. 5. Hydrolysis zones on (SC^{-URA}) with 0.5% Locust bean gum of (A) *S. cerevisiae* Y294[mndA], (B) *S. cerevisiae* Y294[man1] and (C) *S. cerevisiae* Y294[man1, mndA] (D) *S. cerevisiae* Y294[pJC1].
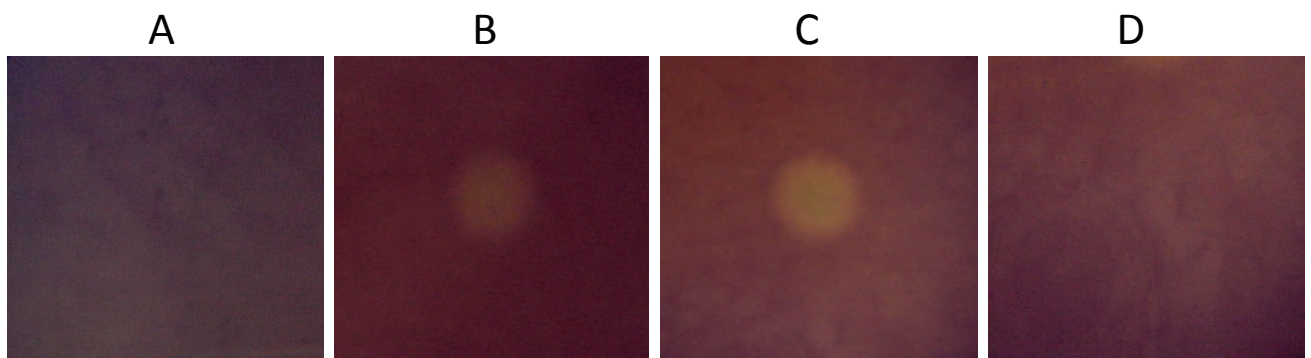
Fig. 6. Hydrolysis zones on (SC^{-URA}) with 0.5% Locust bean gum of (A) *S. cerevisiae* NI-C-D4[mndA], (B) *S. cerevis iae* NI-C-D4[man1], (C) *S. cerevisiae* NI-C-D4[man1, mndA] and (D) *S. cerevisiae* NI-C-D4[YEpENO-BBH].

4.4.6. ENZYME ASSAYS

The recombinant Y294 β-mannosidase and β-mannanase producing strains were analysed for their ability to hydrolyse Locust bean gum as substrate. All strains were grown for 48 hours on (SC^{-URA}) medium before assays started. The yeast strains secreted active Man1 into the medium reaching levels of 251.3 nkat/ml and 163.162 nkat/ml in *S. cerevisiae* NI-C-D4[man1] and Y294[man1] respectively (Fig. 7). Recombinant protein produced by the *S. cerevisiae* NI-C-D4[man1] were 57 mg/L and *S. cerevisiae* Y294[man1] produced 37 mg/L. Recombinant *S. cerevisiae* NI-C-D4[man1, mndA] and *S. cerevisiae* Y294[man1, mndA] produced 3.6-fold (69.69 nkat/ml) and 3.8-fold (42.66 nkat/ml) less enzyme than *S. cerevisiae* NI-C-D4[man1] and *S. cerevisiae* Y294[man1], respectively. The β-mannosidase showed no activity in the time (5 minutes) that the assays were conducted, but did show some activity when incubated over a period of 24 hours at 50˚C (Fig. 8). The pH and temperature optimum were determined with the freeze dried (5 μg/ml) protein indicated optimum conditions at pH 5 and 50˚C. An increase in degradation was observed at 80˚C.
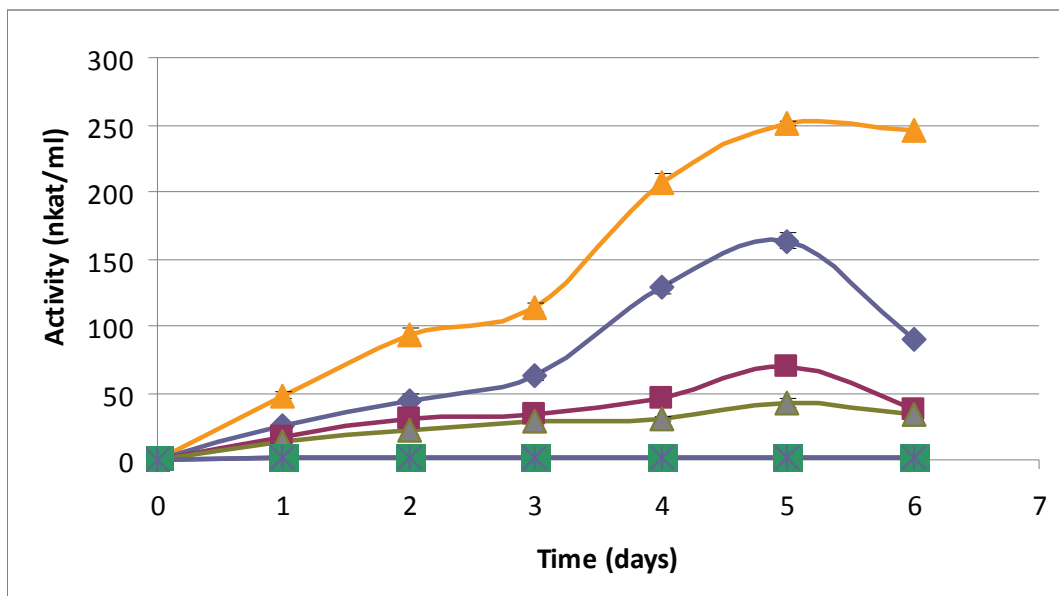
Fig. 7. Enzyme activity produced by strains ▲ *S. cerevisiae* NI-C-D4[man1]; ◆ *S. cerevisiae* Y294[man1] ■ *S. cerevisiae* NI-C-D4[man1, mndA]; ▲ *S. cerevisiae* Y294[man1, mndA]; ■ *S. cerevisiae* NI-C-D4[YEpENO-BBH]; **X** *S. cerevisiae* Y294[pJC1] displayed over time on 0.5% Locust bean gum as substrate at optimum conditions.
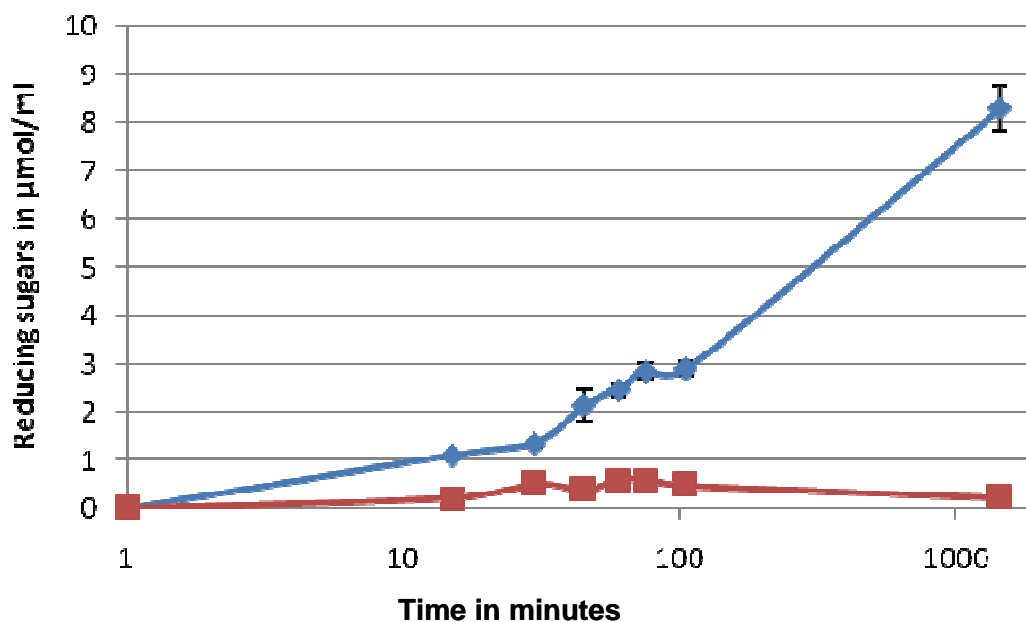
Fig. 8. Reducing sugars released by the degradation of 0.5% Locust bean gum by the supernatant of ◆ *S. cerevisiae* Y294[mndA] and ■ *S. cerevisiae* Y294[pJC1] over a period of 1440 minutes.
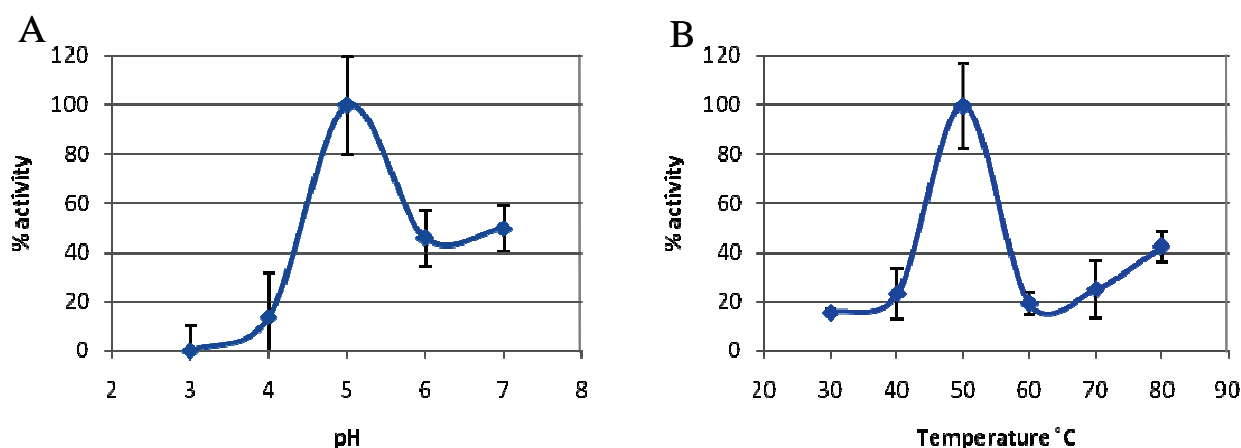
Fig. 9. (A) pH and (B) temperature optimum of *S. cerevisiae* Y294[mndA] incubated on 0.5% Locust bean gum for 2 hours.

4.4.7. SDS-PAGE ANALYSIS

Analysis with SDS-PAGE gave a protein species of 140 kDa in the *S. cerevisiae* Y294[mndA] strain as well as the *S. cerevisiae* Y294[man1, mndA] strain (Fig. 10 Lane 2 and 4 respectively). This protein species is not present in the lanes containing supernatant proteins of the *S. cerevisiae* Y294[man1] or the *S. cerevisiae* Y294[pJC1] protein species (Fig. 10 Lane 2 and 5 respectively). *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[man1, mndA] (Fig. 10 Lane3 and 4) have a 58 kDa protein species, which is the same size as the endo-β-1,4-mannanase protein previously reported in Setati *et al*. (2001).

4.4.8. PROTEIN DEGLYCOSYLATION ANALYSIS

Analysis with SDS-PAGE showed that when the recombinant MndA protein produced by *S. cerevisiae* Y294[mndA] was treated with PNGase F enzyme the 140 kDa protein species disappeared and only a 130 kDa pecies could be seen (lane 1). This protein species was however present for *S. cerevisiae* Y294[mndA] (lane 2), *S. cerevisiae* Y294[mndA] (lane 3) and in *S. cerevisiae* NI-C-D4[mndA] a 130 kDa protein species is seen. Observed previously in Fig. 9 this 140 kDa protein species is absent in the reference strain *S. cerevisiae* Y924 [pJC1] as well (Fig. 11, lane 4).
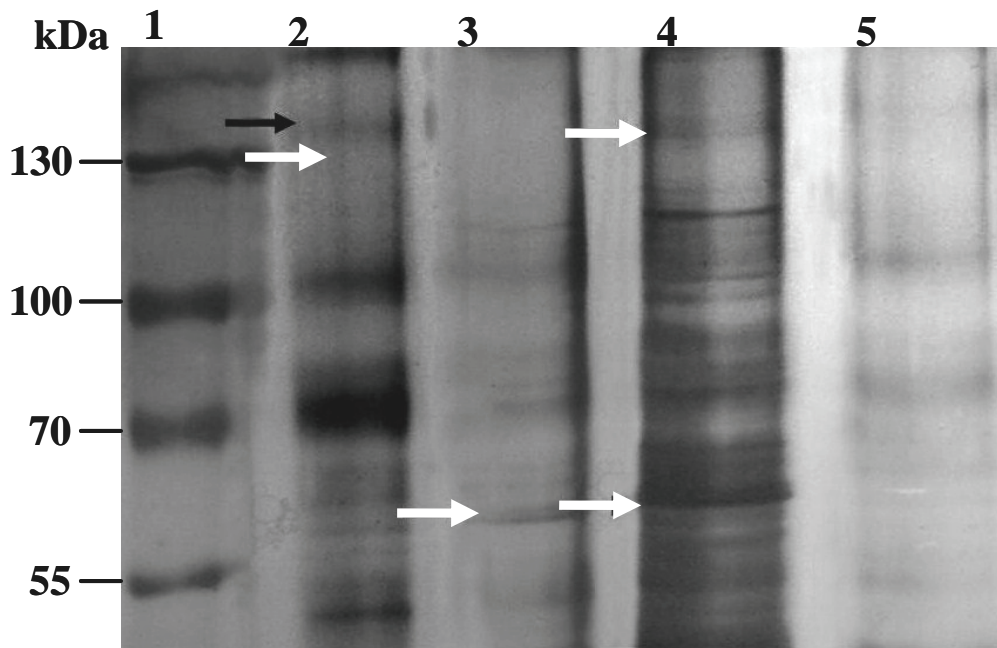
Fig. 10. SDS-PAGE analysis of the secreted recombinant protein produced by various yeast strains (1) Protein ladder Page Ruler ^TM Fermentas Inc. (Maryland, USA), (2) *S. cerevisiae* Y294[mndA], (3) *S. cerevisiae* Y294[man1], (4) *S. cerevisiae* Y294[man1, mndA], (5) *S. cerevisiae* Y294[pJC1]
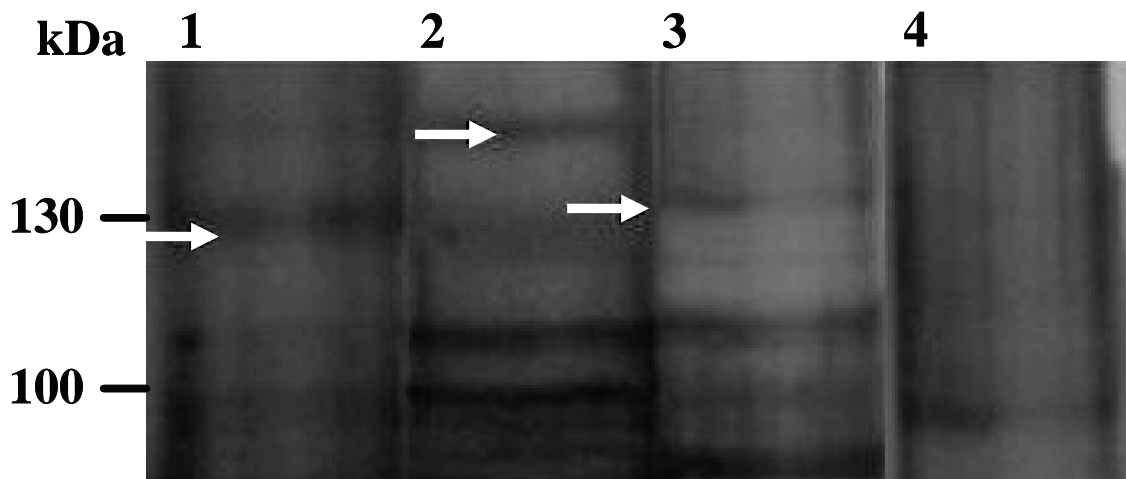
**Fig. 11.** Molecular weight differences of recombinant MndA protein due to hyperglycosylation. (1) *S. cerevisiae* Y294[mndA] deglycosylated, (2) *S. cerevisiae* Y294[mndA], (3) *S. cerevisiae* NI-C-D4[mndA], (4) *S. cerevisiae* Y294[pJC1].
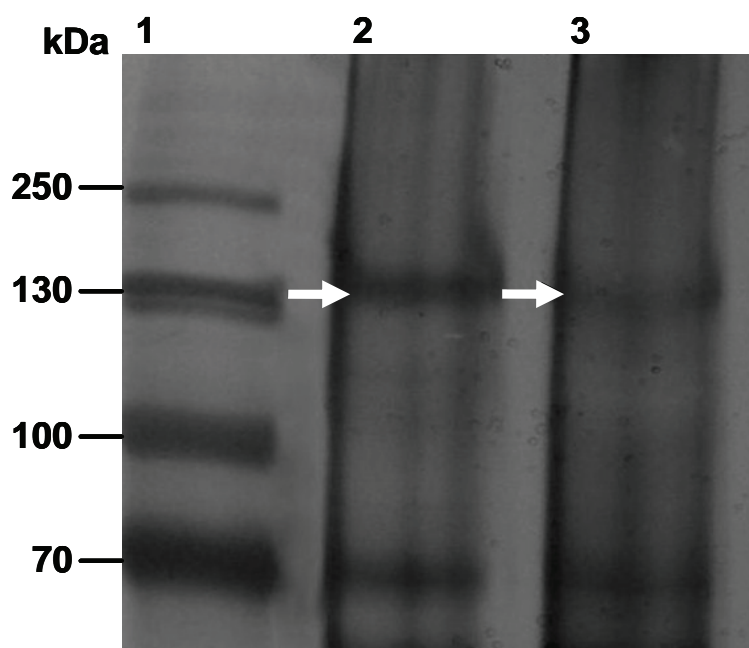
Fig. 12. SDS-PAGE analysis with non-denatured protein. (1) Ladder Page Ruler [TM] Fermentas Inc. (Maryland, USA), (2) *S. cerevisiae* Y294[mndA] unboiled (undenatured) sample, (3) *S. cerevisiae* Y294[mndA] boiled (denatured) sample.

The *S. cerevisiae* Y294[mndA] denatured and undenatured protein (Fig. 12) gave a protein species of 130 kDa each. No protein species could be seen at 240 kDa and thus no dimer formed in the yeast.

4.4.9. SUBSTRATE HYDROLYSIS AND SYNERGISM OF ENZYMES ON LOCUST BEAN GUM SUBSTRATE.

In order to see if the MndA protein had any effect on the hydrolysis when expressed together with Man1 in the same host, hydrolytic properties were determined for *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[man1, mndA]. Locust bean gum was hydrolysed with recombinant Man1 for 10 minutes at 50°C and in comparison with the standards (mannose and mannobiose) it is evident that peaks of similar sizes were released (Fig. 13). Unhydrolysed Locust bean gum substrate did not reveal hydrolysis peaks (Fig. 13). Galactoglucomannan has acetyl side groups as well as galactosyl side groups. Hydrolysis seldom results in only mannose or mannobiose and thus analysis reveals profiles with different peaks. The profiles of the peaks after hydrolysis are the same for both strains, but with *S. cerevisiae* Y924[man1] the peaks are greater, indicating greater mannanase activity (Fig.13 D, E). Hydrolysis of 0.5% Locust bean gum by Man1 and MndA revealed the same peaks as with only the Man1 enzymes. Incubation with both strains caused double the amount of substrate to be released and is probably the sum of the two enzyme products (results not shown).

4.4.10. SUBSTRATE HYDROLYSIS ANALYSIS

The MndA protein was incubated with 0.5% Locust bean gum at 50˚C in 50 mM Sodium Citrate buffer pH 5. HPLC analysis was performed on the supernatant after 18 hours of incubation (Fig. 14). Raffinose (representing a triose sugar peak), mannobiose and mannose sugars were used as standards. The reference reaction (Fig. 14 C) had the 0.5% Locust bean gum supernatant containing shorter galacto(gluco)mannan chains. MndA degradation displayed a lower peak for sugars greater than raffinose in MW when compared to the reference strain. This could indicate very low activity on longer chains but there is no additional mannobiose or mannose released, confirming that this enzyme has no activity at the non-reducing end of the galacto(gluco) chain.
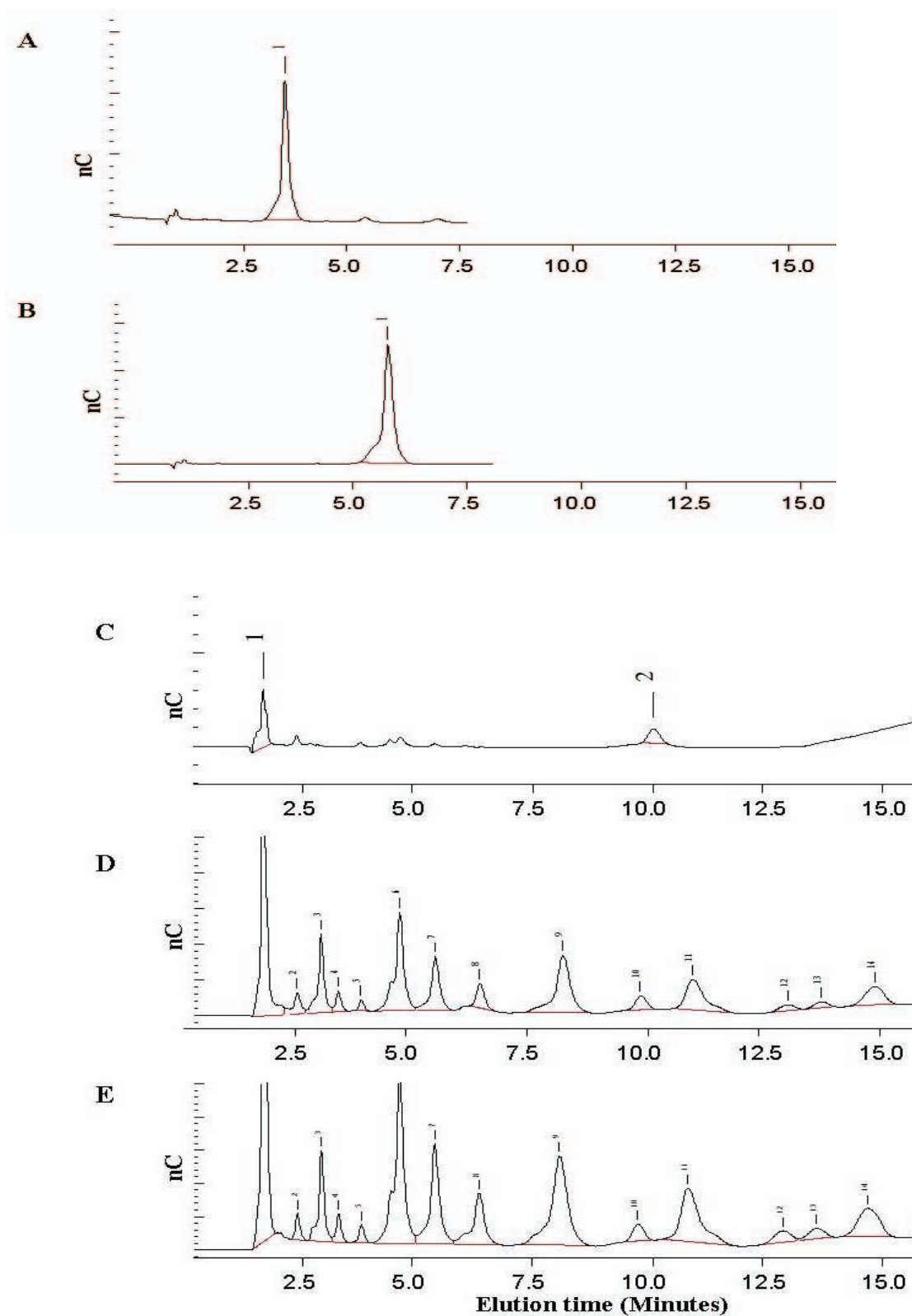
Fig. 13. A chromatographic representation of products detected after hydrolysis of 0.5% Locust bean gum. (A) Mannose standard, (B) Mannobiose standard, (C) Chromatogram obtained from non-hydrolysed Locust bean gum, (D) Hydrolyses of Locust bean gum with *S. cerevisiae* Y294[man1, mndA] and (E) *S. cerevisiae*[man1].
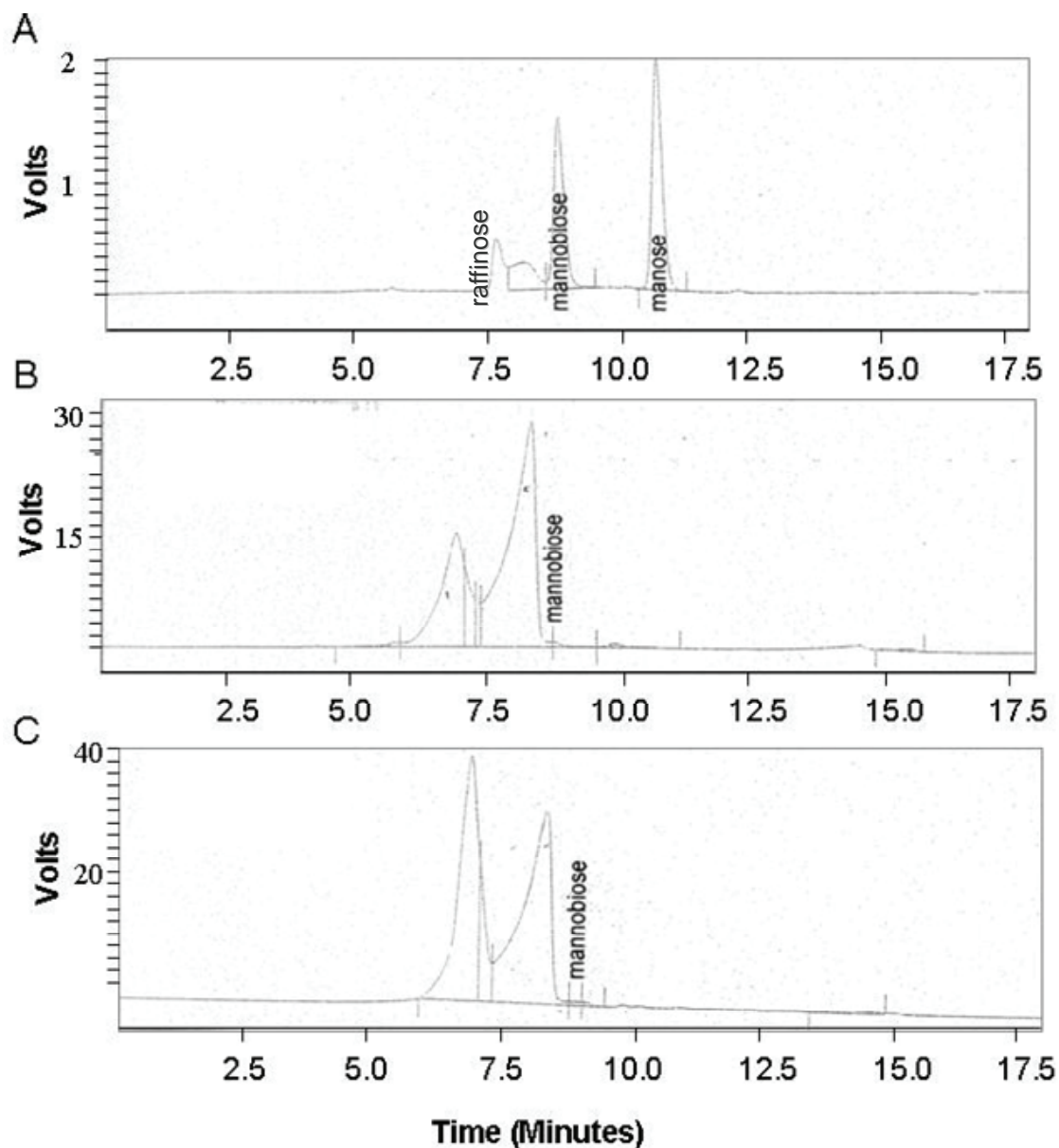
Fig. 14. Sugars released by the hydrolysis of 0.5% Locust bean gum as indicated by HPLC (A) Standards: Raffinose was used as representation of a triose, followed by mannobiose and mannose; (B) Peaks representing sugars released by recombinant MndA and (C) peaks representing the unhydrolysed peaks where *S. cerevisiae* Y294[pJC1] was used as reference strain.

4.5. DISCUSSION

A consortium of enzymes is required for the complete degradation of the galacto(gluco)mannan portion of hemicellulose (Moreira and Filho 2008). Hemicelluloses constitute a third of

lignocellulosic material, the degradation of which is important to achieve efficient and cost-effective conversion to ethanol (Lynd *et al.* 2002). In order to achieve this conversion, an organism producing enzymes that are capable of hydrolysis and producing ethanol in a one-step process is needed. In this study, yeast strains were engineered to secrete a mannanase from *A. aculeatus* and a synthetic, codon optimised β-mannosidase from *A. niger*. These enzymes were also co-expressed to investigate the synergistic effect when produced in the same host.

β-mannosidases (β-D-mannoside mannohydrolases, EC 3.2.1.25) catalyze the successive removal of D-mannose residues from the non-reducing end of various β-1,4-linked manno-oligosaccharides, the end products produced by the action of β-mannanase (EC 3.2.1.78). Currently, a chromogenic substrate (*p*-nitrophenyl β-D-mannopyranoside) is used to quantify β-mannosidase activity and 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).

In this study, the activity of the MndA protein (Ademark *et al.* 1999), was tested on *p*-nitrophenyl β-D-mannopyranoside substrate. β-mannosidases have been reported to be active on glucosides and mannosides (Bauer *et al.* 1996), therefore transformants were evaluated on the basis of *p*-nitrophenyl β-D-glucopyransoside hydrolysis and cellobiose utilization on plates. Even though no activity was detected in the supernatant, the signal peptide should allow secretion of the protein as *S. cerevisiae* does not have high preference for specific signal sequences (Han *et al.* 1999). No activity could be detected with the whole cells or intracellular fraction indicating that no active protein was trapped inside the cell. No growth was observed on the cellobiose plates except for the reference strain *S. cerevisiae* Y294[BglI].

The MndA 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). It was also found that the deglycosylated enzyme had a molecular mass of 112 kDa indicating an N-linked carbohydrate content of 17% by weight (Ademark *et al.* 1999). In this study, a protein species of around 140 kDa was observed on the SDS-PAGE analysis (Fig. 9). Analysis of the protein sequence indicated 11 possible glycosylation sites (Fig. 3). The hyperglycosylation was confirmed when the protein sample was treated with the pNgaseF deglycosylation enzyme, the 140 kDa protein species disappeared and only a 130 kDa protein species could be observed (Fig. 10). As *S. cerevisiae* is known for its hyperglycosylation this could also be a reason for low levels of activity. Hyperglycosylation can cover the active site (depending on the proximity), resulting in unusual activity or preventing entry of the substrate into the active site. Therefore, the *mndA* was also expressed in the *S. cerevisiae* NI-C-D4 strain that was created by Wang *et al.* (2001). This strain was reported to have super

secretion properties (up to 3-fold higher than the parental strains) and was shown not to hyperglycosylate the heterologous proteins produced. SDS-PAGE analysis confirmed the lack of glycosylation since the hyperglycosylated 140 kDa protein species was absent, however no activity was observed for the MndA protein produced by this host. The enzyme is produced as a dimer in the native host (Ademark *et al*. 1999) whereas no dimer could be detected with expression in *S. cerevisiae* likely causing the lack of activity. Foreign gene expression can result in incorrect folding of the protein which can prevent proper assembling of the dimer. The monomers are separately hyperglycosylated which could also affect assembly due to the oligosaccharides acting as hindrance, preventing the formation of di-sulphide bridges.

Some evidence suggest that, the smaller the protein, the more efficient *S. cerevisiae* is at producing it (La Grange *et al*. 2000). When taken into account that the average size of the *S. cerevisiae* open reading frame is 1.4 kb (Hauser *et al*. 1999), compared to the 2.8 kb of *mndA*, difficulty in expression should be expected. It might be that the protein is just too big for the yeast to produce and secrete efficiently.

The presence of rare codons in the target gene can result in low levels of expression (Gustafsson *et al*. 2004). Therefore the β-mannosidase gene was codon optimized for expression in *S. cerevisiae* and the gene sequence was confirmed by automated DNA sequencing. Even though this DNA sequence differed from the native *A. niger* sequence, the protein sequence was unaltered and should yield a functional protein. Codon optimization has been reported to have a positive effect on gene expression, but negative impacts have also been documented (Gustafson 2004, Wu *et al*. 2004, Wu *et al*. 2006). In some studies, optimal codons seemed to be unnecessary when genes are overexpressed and might even have an adverse effect on protein expression (Wu *et al*. 2006). Non-optimal codons were reported to be necessary for translational pausing which is crucial in the correct folding of the proteins. Yet the correct folding of emerging translated polypeptide and efficient translation does not necessarily guarantee a functional protein (Zalucki and Jennings 2007). It was also suggested that translation is sensitive to the nature of the codon-pairs present in ribosomal A and P decoding sites. Software (Anaconda) http://www.bio.ua.pt/genomica/lab) was developed using statistical methodologies based on contingency tables and residual analysis to determine specific codon-pair context patterns. These codon pairs could result in translational errors which could lead to the production of a full length protein but production of very low levels of the mndA (Moura *et al*. 2005). Preferred and rejected pairs of codons are detected by the Anaconda algorithm for specific species and it was found that codon context is highly biased in *S. cerevisiae*. The *mndA* gene sequence was analysed using this information (Appendix B). Which revealed the presence of 21 unfavoured pairs.

The MndA protein did however give unexpected results when transformants were grown for three days on 0.5% Locust bean gum containing plates. Staining with Congo red resulted in a small hydrolysis zone (Fig. 5.A). Even though no activity was detected after 5 minutes by means of the non-reducing assay (*p*-nitrophenyl β-D-mannopyranoside), some sugars seemed to be released when the enzyme was incubated for longer periods (up to 24 hours). HPLC analysis of the sample revealed that the mannotriose peak stayed constant but a small peak containing the larger sugars (probably mannopentose and larger) was observed. The enzyme seemed to require longer chains as substrate but does not release any mannose or mannobiose. β-mannosidases have been shown to cleave manno-oligosaccharides with a DP greater than 4 (Akino *et al*. 1988, Arai *et al*. 1995). β-mannosidase from *Aspergillus niger* was found to cleave up to a DP of 6, but side-chain galactose patterns mostly determined the rate of hydrolyses (Ademark *et al*. 1998).

The affect of the MndA on incubation with 0.5% Locust bean gum was also investigated in a hydrolysis experiment. The HPLC profiles for *S. cerevisiae* Y294[man1] and *S. cerevisiae* Y294[man1, mndA] were similar, but lower levels were detected for the latter, confirming the reducing sugar assay results. The pH and temperature optimum conducted with concentrated (5 μg/ml) protein indicated optima at pH 5 and 50°C respectively. The increase in activity at temperature 80°C (Fig. 9 B) could be ascribed to the 2 hour incubation period at the high temperature causing galactoglucomannan hydrolysis independent of enzyme activity.

β-mannanase (1,4-β-D-mannan mannohydrolase EC 3.2.178) randomly cleaves the β-1,4-mannopyranosyl linkages in the galacto(gluco)mannan backbone, resulting in new chain ends (Stoll *et al*. 2000). Setati *et al*. (2001) reported endomannanase levels of 379 nkat/ml (SC^{-URA}) and 86 mg/L recombinant protein produced upon expression of the *man1* of *A. aculeatus* in *S. cerevisiae*. The recombinant strain *S. cerevisiae* NI-C-D4[man1] yielded a maximum activity of 251,39 nkat/ml while *S. cerevisiae* Y294[man1] produced 163.16 nkat/ml. Protein produced by he recombinant strain *S. cerevisiae* NI-C-D4[man1] were 57 mg/L while *S. cerevisiae* Y294[man1] produced 37.06 mg/L. Different culture conditions and plasmids were used compared to the study in Setati *et al*. (2001) which could explain the difference in activity observed. Cultivation conditions have a noticeable impact as the cell density of yeast in the previous study reached an optical density of up to 9 compared to the 2.8 reached in this study. The cell density directly affects the amount of enzyme that's produced.

The *S. cerevisiae* Y294[man1mndA] strain showed a 4-fold decrease in endomannanase activity compared to *S. cerevisiae* Y294[man1]. This phenomenon was also previously reported by La Grange *et al*. (2000). As the *mndA* gene is 2.8 kb, and the bigger the plasmid, may lead to a lower copy number and consequently less protein is produced. The *S. cerevisiae* NI-C-D4[man1, mndA]

showed a higher endomannanse activity but the activity was still lower than that obtained with *S. cerevisiae* NI-C-D4[man1] and *S. cerevisiae* Y294[man1].

When the two enzymes were incubated together the activity was not more than the sum of the individual activities and no significant synergism could therefore be detected. It is clear that the MndA enzyme produced could not yield in greater hydrolysis of the mannan chain.

In this study a β-mannanase and a β mannosidase were expressed in *S. cerevisiae* to enable this yeast to hydrolse mannan. The recombinant β mannosidase, produced by the expression of a codon optimized gene, yielded a surprising result of not showing activity on low DP substrates but showing a low level of activity on larger chain substrates. The difference in substrate preference can be attributed to the improper folding of the protein allowing entry of larger substrates into the active site. Improper folding can be a direct result of increased translation speed made possible by the elimination of translational pausing sites through codon optimization of the gene. The presence of non-optimal codon pairs results in mistakes in translation which can have a direct effect on protein structure, substrate preference and levels of activity.

4.6. ACKNOWLEDGEMENTS

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5.   http://www.cbs.dtu.dk/services/SignalP
6.   http://www.bio.ua.pt/genomica/lab

APPENDIX A

The synthetic *mndA* gene and deduced protein sequence. Arrow indicate the computed signal protein sequence and point of cleavage (SignalP 3.0)

```
1    ATGAGACACTCTATCGGTTTGGCTGCTGCCTTGTTGGCTCCAACTTTGCCAGTTGCTTTG
1    M   R   H   S   I   G   L   A   A   A   L   L   A   P   T   L   P   V   A   L
```

```
61   GGTCAACACATTAGAGACTTATCCTCTGAAAAGTGGACCTTGTCTTCTAGAGCTTTGAAC
21   G   Q   H   I   R   D   L   S   S   E   K   W   T   L   S   S   R   A   L   N
```

s

```
121  AGAACTGTTCCAGCTCAATTCCCATCCCAAGTTCACTTGGACTTATTGAGAGCTGGTGTT
41   R   T   V   P   A   Q   F   P   S   Q   V   H   L   D   L   L   R   A   G   V
```

```
181  ATCGGGGAATACCACGGTTTGAATGATTTCAACTTGAGATGGATCGCCGCTGCCAACTGG
61   I   G   E   Y   H   G   L   N   D   F   N   L   R   W   I   A   A   A   N   W
```

```
241  ACCTACACCTCTCAACCAATTAAGGGTTTGTTGGATAACTATGGTTCTACTTGGTTGGTT
81   T   Y   T   S   Q   P   I   K   G   L   L   D   N   Y   G   S   T   W   L   V
```

```
301  TTCGACGGTTTGGATACTTTCGCTACCATCTCTATCTTGTGGACCGCTAACAGAGTTCAC
101  F   D   G   L   D   T   F   A   T   I   S   I   L   W   T   A   N   R   V   H
```

```
361  GGTCAATCCGTCTCTCCAGTCTCCGGTTCTATGTATTTGCCAGCTTTAGAAGCCTGTCAA
121  G   Q   S   V   S   P   V   S   G   S   M   Y   L   P   A   L   E   A   C   Q
```

```
421  AGAAGGATCTTGATTAGAAAGGTTTCTTTCAGAGGTGGCGTTACCGCCGAAGTTAACACC
141  R   R   I   L   I   R   K   V   S   F   R   G   G   V   T   A   E   V   N   T
```

```
481  TGTTATTTACACATTGAATGGCCTGACGACGTCCAATTGACTTACGAATACCCAAACAGA
161  C   Y   L   H   I   E   W   P   D   D   V   Q   L   T   Y   E   Y   P   N   R
```

```
541  TGGTTCATGAGAAAGGAACAATCTGATTTCGGTTGGGACTGGGGTCCAGCTTTCGCTCCA
181  W   F   M   R   K   E   Q   S   D   F   G   W   D   W   G   P   A   F   A   P
```

```
601  GCTGGTCCATGGAAGCCAGCTTACATCGTTCAATTGGATAAGAAGGAATCTGTCTACGTT
201  A   G   P   W   K   P   A   Y   I   V   Q   L   D   K   K   E   S   V   Y   V
```

```
661  TTGAACACCGACTTGGACATTTACAGAAAGAACCAAATTAATTACTTGCCACCAGACCAA
221  L   N   T   D   L   D   I   Y   R   K   N   Q   I   N   Y   L   P   P   D   Q
```

```
721  TCTCAACCATGGGTTGTTAATGCTTCTATCGACATTTTGGGTCCATTGCCAGCTAAGCCA
241  S   Q   P   W   V   V   N   A   S   I   D   I   L   G   P   L   P   A   K   P
```

```
781  ACCATGTCCATTGAAGTTAGAGACACTCATTCCGGTACTATCTTGACTTCCAGAACCTTA
261  T   M   S   I   E   V   R   D   T   H   S   G   T   I   L   T   S   R   T   L
```

```
841  AACAATGTTTCCGTCGCCGGTAACGCTATCACCGGTGTTACCGTTTTGGATGGCTTGAAC
281  N   N   V   S   V   A   G   N   A   I   T   G   V   T   V   L   D   G   L   N
```

```
901  CCAAAATTGTGGTGGCCACAATCTTCTGTCATTAGAACCTCTACCATGTTCTTGTCTTTA
301  P   K   L   W   W   P   Q   S   S   V   I   R   T   S   T   M   F   L   S   L
```

```
961  TCTAAGGTTGAAGGTACTAGACCATGGCCAGTGTGGACTAACGGTAGAGCCTCTGCTCCA
321  S   K   V   E   G   T   R   P   W   P   V   W   T   N   G   R   A   S   A   P
```

```
1021  TTCTTCTTGAACCAAAGAAATATCACCGAAGTTCAAAGAGCTCAAGGTATCGCCCCTGGT
```

```
341  F   F   L   N   Q   R   N   I   T   E   V   Q   R   A   Q   G   I   A   P   G

1081 GCCAATTGGCACTTCGAGGTCAATGGTCATGAGTTCTACGCCAAGGGTTCGAACTTGATC
361  A   N   W   H   F   E   V   N   G   H   E   F   Y   A   K   G   S   N   L   I

1141 CCACCAGATTCTTTTTGGACTCGTGTCACTGAAGAACGTATCTCTCGTTTGTTCGACGCC
381  P   P   D   S   F   W   T   R   V   T   E   E   R   I   S   R   L   F   D   A

1201 GTCGTCGTCGGTAACCAAAACATGTTGAGAGTTTGGTCCTCCGGTGCTTACTTGCACGAC
401  V   V   V   G   N   Q   N   M   L   R   V   W   S   S   G   A   Y   L   H   D

1261 TACATTTACGATTTGGCCGACGAAAAGGGTATTTTGTTGTGGTCTGAATTTGAGTTCTCT
421  Y   I   Y   D   L   A   D   E   K   G   I   L   L   W   S   E   F   E   F   S

1321 GATGCTTTATACCCATCCGATGATGCTTTTTTGGAAAACGTCGCTGCTGAAATTGTCTAC
441  D   A   L   Y   P   S   D   D   A   F   L   E   N   V   A   A   E   I   V   Y

1381 AACGTTAGAAGAGTTAACCACCACCCATCTTTGGCTTTGTGGGCTGGTGGTAACGAAATT
461  N   V   R   R   V   N   H   H   P   S   L   A   L   W   A   G   G   N   E   I

1441 GAAAGCTTAATGTTGCCAAGAGTCAAGGACGCTGCTCCATCTTCTTACTCTTATTACGTT
481  E   S   L   M   L   P   R   V   K   D   A   A   P   S   S   Y   S   Y   Y   V

1501 GGTGAATACGAAAAGATGTACATCTCCTTGTTCTTGCCATTGGTTTACGAAAACACTCGT
501  G   E   Y   E   K   M   Y   I   S   L   F   L   P   L   V   Y   E   N   T   R

1561 TCCATTTCTTACTCCCCATCCTCTACCACTGAAGGTTACTTGTATATTGACTTATCCGCC
521  S   I   S   Y   S   P   S   S   T   T   E   G   Y   L   Y   I   D   L   S   A

1621 CCTGTCCCTATGGCTGAACGTTACGACAACACCACCTCTGGTTCCTACTACGGTGACACC
541  P   V   P   M   A   E   R   Y   D   N   T   T   S   G   S   Y   Y   G   D   T

1681 GACCATTACGACTACGATACCTCTGTCGCTTTCGATTACGGTTCTTACCCAGTCGGTCGT
561  D   H   Y   D   Y   D   T   S   V   A   F   D   Y   G   S   Y   P   V   G   R

1741 TTCGCTAACGAGTTCGGTTTCCACTCCATGCCATCCTTGCAAACCTGGCAACAAGCTGTT
581  F   A   N   E   F   G   F   H   S   M   P   S   L   Q   T   W   Q   Q   A   V

1801 GACACTGAAGATTTGTACTTCAACTCTTCTGTTGTTATGTTGAGAAACCACCACGACCCA
601  D   T   E   D   L   Y   F   N   S   S   V   V   M   L   R   N   H   H   D   P

1861 GCTGGTGGCTTGATGACCGATAATTACGCTAACTCTGCTACTGGTATGGGTGAAATGACC
621  A   G   G   L   M   T   D   N   Y   A   N   S   A   T   G   M   G   E   M   T

1921 ATGGGTGTCATTTCCTACTATCCAATCCCAAGCAAGTCTGATCACATTTCCAACTTCTCC
641  M   G   V   I   S   Y   Y   P   I   P   S   K   S   D   H   I   S   N   F   S

1981 GCCTGGTGTCACGCTACTCAATTGTTCCAAGCCGACATGTACAAGTCTCAAATTCAATTC
661  A   W   C   H   A   T   Q   L   F   Q   A   D   M   Y   K   S   Q   I   Q   F

2041 TACAGAAGAGGTTCCGGTATGCCAGAAAGACAATTGGGTTCTTTGTACTGGCAATTGGAA
681  Y   R   R   G   S   G   M   P   E   R   Q   L   G   S   L   Y   W   Q   L   E

2101 GACATTTGGCAAGCTCCATCCTGGGCTGGTATCGAATACGGTGGTAGATGGAAGGTTTTG
701  D   I   W   Q   A   P   S   W   A   G   I   E   Y   G   G   R   W   K   V   L

2161 CACCACGTTATGCGTGACATTTACCAACCAGTCATCGTTTCTCCATTCTGGAACTACACC
721  H   H   V   M   R   D   I   Y   Q   P   V   I   V   S   P   F   W   N   Y   T

2221 ACCGGTTCCTTGGACGTTTACGTTACCTCCGACTTGTGGTCTCCAGCTGCCGGTACTGTC
741  T   G   S   L   D   V   Y   V   T   S   D   L   W   S   P   A   A   G   T   V

2281 GACTTGACCTGGTTAGACTTGTCTGGTAGACCAATCGCTGGGAACGCTGGTACTCCAAAG
761  D   L   T   W   L   D   L   S   G   R   P   I   A   G   N   A   G   T   P   K
```

```
2341  TCCGTCCCATTCACTGTCGGTGGTTTGAACTCCACCAGAATCTACGGTACTAACGTTTCT
781   S   V   P   F   T   V   G   G   L   N   S   T   R   I   Y   G   T   N   V   S

2401  TCTTTGGGTTTGCCAGACACTAAAGACGCTGTCTTGATCTTGTCCTTGTCCGCCCACGGT
801   S   L   G   L   P   D   T   K   D   A   V   L   I   L   S   L   S   A   H   G

2461  AGATTGCCAAACTCCGACAGAACTACTAACTTAACTCACGAAAACTACGCCACTTTGTCT
821   R   L   P   N   S   D   R   T   T   N   L   T   H   E   N   Y   A   T   L   S

2521  TGGCCAAAGGATTTAAAGATTGTTGATCCAGGTTTTGAAGTTGGGTTACTCCTCTAAGAAG
841   W   P   K   D   L   K   I   V   D   P   G   L   K   L   G   Y   S   S   K   K

2581  ACTACTGTTACTGTCGAAGCTACCTCTGGTGTCTCTTTGTACACCTGGTTGGACTACCCA
861   T   T   V   T   V   E   A   T   S   G   V   S   L   Y   T   W   L   D   Y   P

2641  GAAGGTGTCGTTGGTTACTTCGAAGAAAACGCTTTCGTTTTGGCTCCAGGTGAAAAGAAG
881   E   G   V   V   G   Y   F   E   E   N   A   F   V   L   A   P   G   E   K   K

2701  GAAATTGGTTTCACCGTCTTGGACGACACCACTAACGGTGCCTGGGTTAGAAACATTACC
901   E   I   G   F   T   V   L   D   D   T   T   N   G   A   W   V   R   N   I   T

2761  GTCCAATCCTTGTGGGACCAAAAAGTTAGAGGTAA
921   V   Q   S   L   W   D   Q   K   V   R   G   STOP
```

β-mannosidase was analysed for rejected codon pairs (shown in red) according information from the Anaconda algorithm (Zalucki and Jennings 2007).

```
GAATTC-
ATG AGA CAC TCT ATC GGT TTG GCT GCT GCC TTG TTG GCT CCA ACT TTG CCA GTT GCT TTG
GGT CAA CAC ATT AGA GAC TTA TCC TCT GAA AAG TGG ACC TTG TCT TCT AGA GCT TTG AAC
AGA ACT GTT CCA GCT CAA TTC CCA TCC CAA GTT CAC TTG GAC TTA TTG AGA GCT GGT GTT
ATC GGG GAA TAC CAC GGT TTG AAT GAT TTC AAC TTG AGA TGG ATC GCC GCT GCC AAC TGG
ACC TAC ACC TCT CAA CCA ATT AAG GGT TTG TTG GAT AAC TAT GGT TCT ACT TGG TTG GTT
TTC GAC GGT TTG GAT ACT TTC GCT ACC ATC TCT ATC TTG TGG ACC GCT AAC AGA GTT CAC
GGT CAA TCC GTC TCT CCA GTC TCC GGT TCT ATG TAT TTG CCA GCT TTA GAA GCC TGT CAA
AGA AGG ATC TTG ATT AGA AAG GTT TCT TTC AGA GGT GGC GTT ACC GCC GAA GTT AAC ACC
TGT TAT TTA CAC ATT GAA TGG CCT GAC GAC GTC CAA TTG ACT TAC GAA TAC CCA AAC AGA
TGG TTC ATG AGA AAG GAA CAA TCT GAT TTC GGT TGG GAC TGG GGT CCA GCT TTC GCT CCA
GCT GGT CCA TGG AAG CCA GCT TAC ATC GTT CAA TTG GAT AAG AAG GAA TCT GTC TAC GTT
TTG AAC ACC GAC TTG GAC ATT TAC AGA AAG AAC CAA ATT AAT TAC TTG CCA CCA GAC CAA
TCT CAA CCA TGG GTT GTT AAT GCT TCT ATC GAC ATT TTG GGT CCA TTG CCA GCT AAG CCA
ACC ATG TCC ATT GAA GTT AGA GAC ACT CAT TCC GGT ACT ATC TTG ACT TCC AGA ACC TTA
AAC AAT GTT TCC GTC GCC GGT AAC GCT ATC ACC GGT GTT ACC GTT TTG GAT GGC TTG AAC
CCA AAA TTG TGG TGG CCA CAA TCT TCT GTC ATT AGA ACC TCT ACC ATG TTC TTG TCT TTA
TCT AAG GTT GAA GGT ACT AGA CCA TGG CCA GTG TGG ACT AAC GGT AGA GCC TCT GCT CCA
TTC TTC TTG AAC CAA AGA AAT ATC ACC GAA GTT CAA AGA GCT CAA GGT ATC GCC CCT GGT
GCC AAT TGG CAC TTC GAG GTC AAT GGT CAT GAG TTC TAC GCC AAG GGT TCG AAC TTG ATC
CCA CCA GAT TCT TTT TGG ACT CGT GTC ACT GAA GAA CGT ATC TCT CGT TTG TTC GAC GCC
GTC GTC GTC GGT AAC CAA AAC ATG TTG AGA GTT TGG TCC TCC GGT GCT TAC TTG CAC GAC
TAC ATT TAC GAT TTG GCC GAC GAA AAG GGT ATT TTG TTG TGG TCT GAA TTT GAG TTC TCT
GAT GCT TTA TAC CCA TCC GAT GAT GCT TTT TTG GAA AAC GTC GCT GCT GAA ATT GTC TAC
AAC GTT AGA AGA GTT AAC CAC CAC CCA TCT TTG GCT TTG TGG GCT GGT GGT AAC GAA ATT
GAA AGC TTA ATG TTG CCA AGA GTC AAG GAC GCT GCT CCA TCT TCT TAC TCT TAT TAC GTT
GGT GAA TAC GAA AAG ATG TAC ATC TCC TTG TTC TTG CCA TTG GTT TAC GAA AAC ACT CGT
TCC ATT TCT TAC TCC CCA TCC TCT ACC ACT GAA GGT TAC TTG TAT ATT GAC TTA TCC GCC
CCT GTC CCT ATG GCT GAA CGT TAC GAC AAC ACC ACC TCT GGT TCC TAC TAC GGT GAC ACC
GAC CAT TAC GAC TAC GAT ACC TCT GTC GCT TTC GAT TAC GGT TCT TAC CCA GTC GGT CGT
TTC GCT AAC GAG TTC GGT TTC CAC TCC ATG CCA TCC TTG CAA ACC TGG CAA CAA GCT GTT
GAC ACT GAA GAT TTG TAC TTC AAC TCT TCT GTT GTT ATG TTG AGA AAC CAC CAC GAC CCA
GCT GGT GGC TTG ATG ACC GAT AAT TAC GCT AAC TCT GCT ACT GGT ATG GGT GAA ATG ACC
ATG GGT GTC ATT TCC TAC TAT CCA ATC CCA AGC AAG TCT GAT CAC ATT TCC AAC TTC TCC
GCC TGG TGT CAC GCT ACT CAA TTG TTC CAA GCC GAC ATG TAC AAG TCT CAA ATT CAA TTC
TAC AGA AGA GGT TCC GGT ATG CCA GAA AGA CAA TTG GGT TCT TTG TAC TGG CAA TTG GAA
GAC ATT TGG CAA GCT CCA TCC TGG GCT GGT ATC GAA TAC GGT GGT AGA TGG AAG GTT TTG
CAC CAC GTT ATG CGT GAC ATT TAC CAA CCA GTC ATC GTT TCT CCA TTC TGG AAC TAC ACC
ACC GGT TCC TTG GAC GTT TAC GTT ACC TCC GAC TTG TGG TCT CCA GCT GCC GGT ACT GTC
GAC TTG ACC TGG TTA GAC TTG TCT GGT AGA CCA ATC GCT GGG AAC GCT GGT ACT CCA AAG
TCC GTC CCA TTC ACT GTC GGT GGT TTG AAC TCC ACC AGA ATC TAC GGT ACT AAC GTT TCT
TCT TTG GGT TTG CCA GAC ACT AAA GAC GCT GTC TTG ATC TTG TCC TTG TCC GCC CAC GGT
AGA TTG CCA AAC TCC GAC AGA ACT ACT AAC TTA ACT CAC GAA AAC TAC GCC ACT TTG TCT
TGG CCA AAG GAT TTA AAG ATT GTT GAT CCA GGT TTG AAG TTG GGT TAC TCC TCT AAG AAG
ACT ACT GTT ACT GTC GAA GCT ACC TCT GGT GTC TCT TTG TAC ACC TGG TTG GAC TAC CCA
GAA GGT GTC GTT GGT TAC TTC GAA GAA AAC GCT TTC GTT TTG GCT CCA GGT GAA AAG AAG
GAA ATT GGT TTC ACC GTC TTG GAC GAC ACC ACT AAC GGT GCC TGG GTT AGA AAC ATT ACC
GTC CAA TCC TTG TGG GAC CAA AAA GTT AGA GGT TAA −CTCGAG
```

5.1. DISCUSSION

The need to develop alternative energy sources is increasing due to the adverse effects of greenhouse gas emissions on the environment and the depletion of fossil fuel reserves. Biofuels produced from lignocellulose could (in combination with other sustainable energy resources like solar, wind and hydrogen) result in a powerful solution to provide cost-effective and sustainable energy (Lynd *et al.* 2002). Lignocellulosic biomass is a renewable, widely distributed and inexpensive source of fermentable sugars that can be converted to bioethanol. Constituents of lignocellulose, namely cellulose, hemicellulose and lignin are the most abundant polymers on earth. Of these, hemicellulose is the second most abundant and consists of xylan and mannan. Mannan occurs as glucomannan, galactomannan or galactoglucomannan. Due to the complex structure of mannan, various enzymes are needed for its complete degradation. The enzymes include endo-β-1,4-mannanases (endohydrolases, that randomly cleave the mannan backbone), β-mannosidases (exohydrolases, hydrolyse the oligomannans released by endo-β-1,4-mannanases) and α-galactosidases that remove the galactose side chains on the mannan backbone (Ademark *et al*. 1998, McCutchen *et al.* 1996, Stoll *et al.* 2000). The β-glucosidases and esterases are additional enzymes that catalyze the removal of glucose and acetic acid respectively, from the mannan chain (Moreira & Filho 2008).

The use of biological processes such as enzyme applications, is becoming more frequent in various industries (Coughlan & Hazlewood 1993, Dhawan & Kaur 2007). Industries such as the food and feed industry, pulp and paper industry, the detergent industry, etc. use mannanases to replace or limit the use of chemical treatments (Dhawan & Kuar 2007). Mannanases are currently considered for use in consolidated bioprocessing (CBP) where lignocellulosic substrate hydrolysis is a prerequisite for the production of bioethanol. CBP is proposed to be the most cost-effective means of bioethanol production (from cellulosic biomass), but ultimately requires a recombinant microorganism capable of hydrolysing lignocellulose and utilising the resulting sugars through fermenation (Lynd *et al.* 2002).

Saccharomyces cerevisiae is currently the preferred host considered for CBP due to its robust nature and high levels of ethanol production (Lynd *et al.* 2002). Problems

regarding low level of foreign gene expression in *S. cerevisiae*, has led to the search and evaluation of alternative yeasts for heterologous protein production (Buckholtz and Gleeson 1991, Mattanovich *et al.* 2004, Romanos *et al.* 1992). *K. lactis* is well known and has been used extensively for the production of various proteins (van Ooyen *et al.* 2006). Strains of *S. cerevisiae* and *K. lactis* have GRAS (<u>G</u>enerally <u>R</u>egarded <u>As</u> <u>S</u>afe) status and FDA approval, permitting their use in various food and feed applications (Dujon 1996, Cregg *et al.* 2000). In contrast to *S. cerevisiae*, *K. lactis* displays crabtree negative growth, reduced hyperglycosylation, reduced carbon catabolite repression and has a wider substrate range, making *K. lactis* an interesting alternative host (Romanos *et al.* 1992). Neither *S. cerevisiae* nor *K. lactis* natively produce mannanase enzymes. The expression of a mannanase enzyme in *S. cerevisiae* and *K. lactis* creates the opportunity to evaluate and compare the two hosts on aspects such as enzyme expression levels, glycosylation of the foreign mannanase, biomass production, etc.

5.2. CONCLUSIONS

- The following could be concluded from this study:
- The *S. cerevisiae* Y294[YEpENO-BBH-man1] and *K. lactis* SHR8[YEpENO-BBH.Kl-man1] strains successfully produced and secreted Man1 into the culture medium.
- Extracellular mannanase production levels per cell of *S. cerevisiae* Y294[YEpENO-BBH-man1] was significantly more than that obtained with *K. lactis* SHR8[YEpENO-BBH.Kl-man1].
- In *K. lactis,* the stability of the transformants could not be guaranteed for prolonged periods of time due to the tendency of the plasmids to integrate after a number of generations and the subsequent development of a heterogeneous culture.
- The disruption of the non-homologous end-joining complex did not contribute to the stability of the transformants.
- Glucose and lactose containing media yielded similar results regarding the biomass production of the *K. lactis* transformants and their levels of extracellular mannanase activity (160-180 nkat.ml^{-1}).

- Differences in the size of the recombinant Man1 when produced in *K. lactis* and *S. cerevisiae* indicated that the former glycosylated the protein to a lesser extent.

- Recombinant MndA showed no activity on *p*-nitrophenyl β-D-mannopyranoside, *p*-nitrophenyl β-D-glucopyranoside or cellobiose. Slight hydrolysis zones were observed on plates containing 0.5% Locust Bean Gum, indicating endo-mannanase activity.

- Investigation of the MndA protein sequence indicated 11 possible glycosylation sites. The molecular mass of 140 kDa indicated that the protein was hyperglycosylated when expressed in *S. cerevisiae.*

- Hyperglycosylation was confirmed when treatment with PNGase F resulted in a smaller protein species present on SDS-PAGE. Less hyperglycosylation was observed with expression of the MndA in the *N*-glycosylation deficient strain, *S. cerevisiae* NI-C-D4.

- The native MndA (*A. niger*) is produced as a dimer with a molecular mass of 264 kDa on a non-reducing SDS-PAGE (135 kDa monomer under reducing conditions). However, no dimer conformation could be detected (for the MndA produced by *S. cerevisiae*) on a non-denaturing gel.

- Due to the size (2.8 kb) of the *mndA* gene and when taking into consideration that the average *S. cerevisiae* open reading frame is 1.4 kbp (Hauser *et al.* 1999), difficulty in expression and additional metabolic stress is to be expected.

- The information regarding the effect of codon optimisation on translation is incomplete. Fast and efficient translation can be detrimental to the folding of the emerging peptide, resulting in a non-functional protein (Zalucki and Jennings 2007). The analysis of the *mndA* gene sequence revealed the presence of 21 unfavoured codon pairs. These codon pairs can result in translational errors (Moura *et al.* 2005).

- The MndA protein has unexpected activity on longer chains of Locust Bean Gum, which could imply that the enzyme lost the mannosidase activity due to the inability to form dimers.

- The recombinant strain *S. cerevisiae* NI-C-D4[man1] yielded more extracellular activity ($251,39$ nkat.ml^{-1}) than *S. cerevisiae* Y294[man1]

- The *S. cerevisiae* Y294[man1mndA] strain showed a 4 fold decrease in extracellular endomannanase activity compared to *S. cerevisiae* Y294[man1]. This phenomenon was also previously reported by La Grange *et al.* (2000) and is indicative of the additional metabolic burden placed on the cell due to the presence of the *mndA* gene.

5.3. FUTURE RESEARCH

The mannanase which are successfully expressed in *S. cerevisiae* and *K. lactis*, but the yeasts were unable to grow on mannan as sole carbon source. A β-mannosidase is needed for the hydrolysis of the mannan chain to mannose units that can be utilised by the hosts to produce ethanol. In this study, the β-mannosidase of *A. niger* was successfully secreted by *S. cerevisiae*, but yielded a non-functional β-mannosidase. Therefore, other sources of β-mannosidases should be investigated. It is advisable to consider a monomeric β-mannosidase for expression in *S. cerevisiae*.

Co-expression of genes in *S. cerevisiae* have been reported to result in a decrease in levels of activity of the individual enzymes, presumably due to the additional metabolic burden imposed on the cells. Alternatively, two strains of *S. cerevisiae* can be used in co-culture with the one strain secreting the Man1 and the other strain secreting a functional β-mannosidase. Using different vector systems and different promoters, a complementary ratio of enzymes can be obtained that would benefit the production of ethanol by both strains. Co-culturing is not the desired approach, but does provide an alternative short term solution. Furthermore, it would also be interesting to see if similar results (decrease in levels of activity of the individual enzymes) would be observed with co-expression of genes in *K. lactis* as host.

It is further suggested that the *mndA* gene be expressed in *K. lactis* to determine if dimer formation could be observed. Similarly, alternative yeasts like *Yarrowia lipolytica*, *H. polymorpha* and *P. pastoris*, should also be evaluated for use as host organisms for expression of foreign genes using the *man1* as reporter gene (Domínguez *et al.* 1998).

Current processes for lignocellulosic conversion to ethanol, is inefficient and expensive (Rubin 2008). In spite of several successes, further research in this field is required in order to construct ideal organisms for CBP. Extension of substrate and product range, improvement of process performance, improvements in product yield and elimination of toxic by-products is some of the areas that need attention (Ostergaard *et al.* 2000).

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