Heterologous production of family 5 fungal endo-1,4-β-mannanases in *Saccharomyces cerevisiae*

By

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

Mannan polysaccharides occur in the hemicellulose fraction of plant cell walls, either as structural polymers or as reserve carbohydrates. They are found predominantly in the seeds of leguminous plants in the form of galactomannan, and in softwoods as galactoglucomannan. Endo-1,4-β-mannanases hydrolyze mannan polysaccharides to oligosaccharides of various lengths. These enzymes are secreted as single catalytic modules or as part of multi-modular proteins by fungi, bacteria, plants and animals. For example, the β-mannanase of *Aspergillus aculeatus*, designated Aa-Man5A, is secreted as a single catalytic module, whereas that of *Trichoderma reesei*, designated Tr-Man5A, contains a β-mannanase catalytic module linked to a cellulose-binding module by a Pro-Ser-Thr-rich linker.

Heterologous gene expression in yeast provides the opportunity to produce individual hydrolytic enzymes in a host expression system devoid of related activities. *Saccharomyces cerevisiae* has a well-developed expression system and has frequently been used as a model organism for heterologous gene expression. A number of autoselection systems have been devised so that recombinant *S. cerevisiae* strains can be cultivated in any medium of choice without exerting selective pressure. An autoselection system based on defective chromosomal *ura3* and *fur1* genes involved in the pyrimidine biosynthesis pathway of *S. cerevisiae*, and complementation of the *ura3* gene with a multicopy plasmid-borne *URA3* gene, were used in this study.

The *man1* of *A. aculeatus* gene encoding Aa-Man5A was cloned and expressed in autoselective *S. cerevisiae* under the regulation of the alcohol dehydrogenase (*ADH2*<sub>PT</sub>) and the phosphoglycerate kinase (*PGK1*<sub>PT</sub>) promoter and terminator sequences. Expression of *man1* under both promoters resulted in high production levels of Aa-Man5A. The production levels were significantly higher than the levels of endo-1,4-β-mannanases produced by heterologous expression in *Escherichia coli*, and were comparable to the production levels of enzymes produced in *Pichia pastoris*, which presumably has a higher secretion capacity than *S. cerevisiae*. The recombinant yeast
strain expressing man1 under the regulation of the PGK1P promoter displayed stunted biomass formation during the logarithmic phase, which was relieved when the native β-
mannanase secretion signal was replaced with the yeast MFα1S secretion signal. The recombinant Aa-Man5A displayed biochemical properties similar to those of the native Aa-Man5A. The recombinant enzyme hydrolyzed unsubstituted mannan to predominantly mannose, mannobiose, and mannotriose.

The expression of the man1 and man1Δcbd gene constructs of T. reesei in S. cerevisiae fur1::LEU2 strains under the regulation of the PGK1PT promoter and terminator resulted in the production and secretion of Tr-Man5A and Tr-Man5AΔCBD (lacking the cellulose binding module), respectively. However, the production levels of both proteins were approximately 15-fold lower than the production levels of Aa-Man5A. These levels did not improve after replacement of the native secretion signal with the MFα1S secretion signal. Interestingly, reducing the cultivation temperature from 30°C to 20°C led to a five-fold increase in the secreted levels of Tr-Man5A, but a three-fold decrease in the production of Aa-Man5A.

A preliminary investigation was performed to evaluate the possibility of using the recombinant Aa-Man5A in the processing of instant coffee. Arabica coffee extracts treated with Aa-Man5A displayed low viscosity in comparison to the untreated extract and showed better retention of volatile/aromatic compounds than the autoclaved extract. The results indicated that Aa-Man5A is capable of hydrolyzing coffee galactomannan and can be used for processing instant coffee.
OPSOMMING

Mannaanpolisakkariede kom in die hemisellulose fraksie van plantselwande as strukturele polimere of reserwe koolstofbron voor. Mannaan word hoofsaaklik in die sade van peulplante, in die vorm van galaktomannaan, en in sagtehout as galaktoglucomannaan aangetref. Endo-1,4-β-mannanase kan mannaanpolisakkariede na oligosakkariede van verskillende lengtes afbreek. Hierdie ensieme word deur fungi, bakterieë, plante en diere as enkele katalitiese modules of as deel van multi-modulêre proteïene uitgeskei. Die β-mannanase (Aa-Man5A) van Aspergillus aculeatus is byvoorbeeld 'n enkele katalitiese module, maar die β-mannanase (Tr-Man5A) van Trichoderma reesei bestaan uit 'n β-mannanase katalitiese module gekoppel aan 'n sellulose-bindingsmodule deur middel van 'n Pro-Ser-Thr-ryke koppelstuk.

Heteroloë geenuitdrukking in gis bied die geleentheid om individuele hydrolitiese ensieme in 'n gasheer uitdrukkingsisteem sonder verwante aktiwiteite te produseer. Saccharomyces cerevisiae het 'n goed ontwikkelde uitdrukkingsisteem en word as model organisme vir heteroloë geenuitdrukking gebruik. 'n Aantal outoseleksiesisteme is ontwikkeld, waardeur rekombinante S. cerevisiae-rase in enige medium sonder selektiewe druk gekweek kan word. 'n Outoseleksiesisteem, gebaseer op defektiwe chromosomale ura3 en furl gene wat vir ensieme in die pirimidien biosinteseweg kodeer, en komplementasie van die ura3-geen met die wilde-tipe URA3-geen wat op 'n multikopie plasmied teenwoordig is, is vir hierdie studie gebruik.

Die manl-geen, wat vir die Aa-Man5A β-mannanase van A. aculeatus kodeer, is gekloneer en in outoselektiewe S. cerevisiae onder die regulering van die alkoholdehidrogenase 2 (ADH2pt) en fosfogliseraatkinase 1 (PGK1pt) promotor- en terminateerderopeenvolgings uitgedruk. Uitdrukking van die manl-geen onder albei promotors het hoë produksievakke van Aa-Man5A gelewer. Die produksievakke was aansienlik hoër as die endo-1,4-β-mannanase-vakke wat deur heteroloë geenuitdrukking in Escherichia coli geproduseer was, en kon vergelyk word met die produksievakke van
ensieme in *Pichia pastoris*. *P. pastoris* is veronderstel om 'n hoër sekresiekapasiteit as *S. cerevisiae* te hê. Die rekombinante gisras wat die manl-geen onder beheer van die \(PGK1_p\) promotor uitgedruk het, se biomassavorming was belemmer gedurende die laat logaritmiese fase. Die belemmering is opgehef nadat die natuurlike sekresiesein van \(\beta\)-mannananase met die MF\(\alpha1_S\) sekresiesein vervang is. Die rekombinante Aa-Man5A het soortgelyke biochemiese eienskappe as die natuurlike Aa-Man5A getoon. Die rekombinante ensiem het onvertakte mannaan tot hoofsaaklik mannose, mannobiose en mannotriose gehidroliseer.

Die uitdrukking van die *manl*- en \(manl\Deltacbd\)-geenkonstruuke van *T. reesei* in *S. cerevisiae fur1::LEU2-rasse onder regulerings van die \(PGK1_{PT}\) promotor en termineerder het tot die produksie en sekresie van onderskeidelik die Tr-Man5A en Tr-Man5\(\DeltaCBD\) (sonder die sellulose-bindingsdomein) ensieme gelei. Die produksievakke van beide proteïene was egter ongeveer 15-voudig laer as die vlakke van Aa-Man5A. Hierdie vlakke het egter nie verbeter nadat die natuurlike sekresiesein met die MF\(\alpha1_S\) sekresiesein vervang is nie. Interessant is die feit dat 'n afname in opkwekingstemperatuur vanaf 30\(^\circ\)C tot 20\(^\circ\)C tot 'n vyf-voudige toename in sekresievakke van die Tr-Man5A gelei het, maar tot 'n drie-voudige afname in die produksie van Aa-Man5A.

'N Voorlopige ondersoek na die moontlike gebruik van rekombinante Aa-Man5A in kitskoffieprosessering is ondersoek.. *Arabica* koffie-ekstrak wat met Aa-Man5A behandel is, het 'n laer viskositeit in vergelyking met onbehandelde ekstrak getoon, asook beter behoud van vlugtige/aromatiese verbindinges in vergelyking met geoutoklaveerde ekstrak. Hierdie resultate toon dat Aa-Man5A in staat is om koffie galaktomannaan te hidroliseer en dat dit vir die prosessering van kitskoffie gebruik kan word.

vi
Bibliographical Sketch

Mathabatha Evodia Setati was born on the 25th of August 1974 in Seshego, Limpopo Province, South Africa. She started her formal education at Dorothy Langa Lower Primary School (Seshego) and matriculated at Khaiso High School (Seshego) in 1991. Evodia enrolled at the University of the North in 1992 and obtained a B.Sc. degree in Microbiology and Physiology in 1994. In 1995, she completed a B.Sc. Hons. degree in Microbiology at the same institution. She enrolled at the University of Stellenbosch for a Master’s degree in 1996, which was subsequently upgraded to a PhD degree in 1998.
"The roots of education are bitter, but the fruit is sweet" - Aristotle

I dedicate this thesis to my siblings; Raphael, Norah, Flavia and Reneilwe
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PREFACE

This thesis is presented as a compilation of three manuscripts written according to the style of the journals to which they were submitted. Each manuscript constitutes a chapter and is introduced separately.

Chapter 2  “Expression of the *Aspergillus aculeatus* endo-β-1,4-mannanase encoding gene (*man1*) in *Saccharomyces cerevisiae* and characterization of the recombinant enzyme” has been published in *Protein Expression and Purification*: 21, 105-114.

Chapter 3  “The effect of signal peptide replacement and cultivation temperature on the production and secretion of fungal endo-1,4-β-mannanases in *Saccharomyces cerevisiae*” has been submitted to *FEMS Yeast Research*.

Chapter 4  “The effect of endo-1,4-β-mannanase on the properties of coffee extracts”-manuscript in preparation.
# TABLE OF CONTENTS

## 2 INTRODUCTION

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 AIMS AND OBJECTIVES</td>
<td>2</td>
</tr>
</tbody>
</table>

## 3 BACKGROUND LITERATURE

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2 PLANT CELL WALL COMPOSITION</td>
<td>3</td>
</tr>
<tr>
<td>3.2.1 Cellulose</td>
<td>3</td>
</tr>
<tr>
<td>3.2.2 Lignin</td>
<td>3</td>
</tr>
<tr>
<td>3.2.3 Hemicellulose</td>
<td>3</td>
</tr>
<tr>
<td>3.2.3.1 Xylan</td>
<td>3</td>
</tr>
<tr>
<td>3.2.3.2 Mannan</td>
<td>4</td>
</tr>
<tr>
<td>3.3 MANNAN-DEGRADING ENZYMES</td>
<td>5</td>
</tr>
<tr>
<td>3.3.1 Endo-1,4-β-mannanase</td>
<td>5</td>
</tr>
<tr>
<td>3.3.1.1 Enzyme-substrate interaction</td>
<td>6</td>
</tr>
<tr>
<td>3.3.1.2 The three-dimensional structure of β-mannanases</td>
<td>7</td>
</tr>
<tr>
<td>3.3.1.3 Reaction mechanism</td>
<td>11</td>
</tr>
<tr>
<td>3.3.1.4 β-Mannanase domain structures</td>
<td>13</td>
</tr>
<tr>
<td>3.3.1.5 Phylogenetic relationship between β-mannanases</td>
<td>17</td>
</tr>
<tr>
<td>3.3.1.6 Biochemical properties of β-mannanases</td>
<td>20</td>
</tr>
<tr>
<td>3.3.2 β-Mannosidase</td>
<td>22</td>
</tr>
<tr>
<td>3.3.3 α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22)</td>
<td>23</td>
</tr>
<tr>
<td>3.3.4 β-Glucosidase</td>
<td>23</td>
</tr>
<tr>
<td>3.3.5 Acetyl-mannan esterase</td>
<td>24</td>
</tr>
<tr>
<td>3.4 INDUSTRIAL APPLICATIONS OF ENDO-1,4-β-MANNANASES</td>
<td>24</td>
</tr>
<tr>
<td>3.4.1 Poultry feeds</td>
<td>24</td>
</tr>
<tr>
<td>3.4.2 Coffee processing</td>
<td>25</td>
</tr>
<tr>
<td>3.4.3 Production of oligosaccharides</td>
<td>25</td>
</tr>
<tr>
<td>3.5 HETEROLOGOUS GENE EXPRESSION IN SACCHAROMYCES CEREVISIAE</td>
<td>26</td>
</tr>
<tr>
<td>3.5.1 Autoselection</td>
<td>27</td>
</tr>
<tr>
<td>3.5.2 Protein secretion</td>
<td>28</td>
</tr>
<tr>
<td>3.5.3 Glycosylation and the influence of N-linked glycans on enzyme properties</td>
<td>30</td>
</tr>
<tr>
<td>3.6 NON-SACCHAROMYCES YEASTS AS ALTERNATIVE HOSTS FOR HETEROLOGOUS PROTEIN EXPRESSION</td>
<td>31</td>
</tr>
<tr>
<td>3.7 REFERENCES</td>
<td>34</td>
</tr>
</tbody>
</table>
CHAPTER 1
LITERATURE REVIEW
2 INTRODUCTION

Hydrolytic enzymes occur naturally in almost all organisms and are involved mainly in breaking down complex substrates, such as carbohydrates, lipids and polyphenols to simple units that can be assimilated easily. Microbial hydrolases are the most extensively studied and were introduced into commercial industries in the 1960s (26). Most of the commercialized microbial enzymes are produced from just a small number of fungal and bacterial genera clustered in taxonomic ‘hot spots’. The best known of these ‘hot spots’ are primarily bacterial species of Bacillus and Pseudomonas and fungal species of Aspergillus, Fusarium, Trichoderma, Humicola, Mucor and Rhizomucor (27). These and other microorganisms secrete mixtures of hydrolytic enzymes that degrade the polymeric substrates through synergistic action.

Plant cell walls are built up of complex polymers, which are mainly cellulose, hemicellulose and lignin. Cellulose is the most abundant renewable carbon source, followed by hemicellulose. Fungi and bacteria secrete multiple enzymes that degrade these polymers into their constituent sugars. However, for most industrial applications, only selected enzymes are required. For instance, pectinases are used for the clarification of fruit juices, lipases are incorporated in some detergents, and cellulases and hemicellulases are used in the food, textile, and paper and pulp industries. Furthermore, only specific enzymes within these groups are necessary to have a significant effect on the final product in industrial processes. Consequently, most of the enzymes are produced by recombinant technology using host production strains such as Saccharomyces cerevisiae, Aspergillus niger, Pichia pastoris etc. Recombinant DNA technology provides the tools with which individual enzymes can be studied and characterized in isolation, thus creating opportunities to select specific enzymes that are well suited for different industrial applications.

This thesis focuses on the hemicellulolytic enzyme, endo-1,4-β-mannanase, which is secreted by most fungi and bacteria as the main ingredient of an enzyme cocktail that is responsible for the degradation of mannan in the hemicellulose fraction of plant cell walls. Endo-1,4-β-mannanase hydrolyzes the mannosidic bonds in the backbone of mannan polysaccharides, such as galactomannan, glucomannan and galactoglucomannan (116). β-Mannanases have not been studied as extensively as
their counterpart β-xylanase (also a hemicellulase); however, they have great potential in poultry feed production (66,123) and instant coffee processing (109). Interestingly, β-mannanases such as those of the Clostridium butyricum-Clostridium beijerinckii group can also be incorporated in commercial enzyme supplement formulas meant to aid food digestion in the human stomach (94).

So far, only a few β-mannanases have been characterized on both the molecular and biochemical level and their industrial applications need to be explored further. The work reported in this thesis is based on fungal β-mannanases from Trichoderma reesei and Aspergillus aculeatus that were expressed and produced in S. cerevisiae by heterologous expression. Production of the recombinant β-mannanases was evaluated using different gene combinations and cultivation conditions. The recombinant β-mannanase of A. aculeatus was purified and biochemically characterized. The recombinant A. aculeatus β-mannanases were tested for possible use in instant coffee production.

2.2 AIMS AND OBJECTIVES
1. To clone and express the β-mannanase encoding gene (man1) of Aspergillus aculeatus in S. cerevisiae.
2. To purify and characterize the recombinant β-mannanase for comparison with the native fungal β-mannanase of A. aculeatus.
3. To determine the kinetic properties (Km and Vmax) of the recombinant and native β-mannanase on locust bean gum, a polymeric galactomannan substrate.
4. To optimize the production and secretion of the recombinant fungal family-5 β-mannanases of A. aculeatus and T. reesei in S. cerevisiae, with respect to cultivation temperature and the nature of the secretion signal employed.
5. To evaluate the effect of the cellulose binding domain and the linker region on the secretion of the T. reesei β-mannanase in S. cerevisiae.
6. Preliminary evaluation of the effect of the addition of the recombinant A. aculeatus β-mannanase in the instant coffee production process.
3 BACKGROUND LITERATURE

3.2 PLANT CELL WALL COMPOSITION

Cellulose, hemicellulose and lignin are the three main constituents of wood (132,146). These biopolymers are closely associated and represent not only the major organic compounds in the biosphere, but also the principal carbon-sink in terrestrial ecosystems (132,146). Cellulose and hemicellulose are readily digested by many organisms, whereas, due to its recalcitrance lignin can only be degraded by a few groups of specialized fungi and bacteria (146).

3.2.1 Cellulose
Cellulose is a homopolymer of β-1,4-linked D-glucose molecules with the degree of polymerization (DP) ranging between 300 and 26 000 (146). Cellulose molecules are attached to one another through hydrogen bonds, forming sheets of microfibrils that consist of crystalline regions interspersed with amorphous zones (146).

3.2.2 Lignin
Lignin is a heterogeneous polyphenolic biopolymer with both aliphatic and aromatic constituents (146). It is formed from three types of phenyl propane units (p-coumaryl alcohol, p-coniferyl alcohol and p-sinapyl alcohol) and provides plant tissues with strength and durability (146).

3.2.3 Hemicellulose
Hemicelluloses are structural polysaccharides found intertwined with lignin and cellulose in plant cell walls. Depending on the plant species they constitute 20-30% of the dry weight in plants on average (122). The DP has been reported to be about 103 to 200 (132). The two major hemicelluloses found in plant cell walls are xylan and mannann (101).

3.2.3.1 Xylan
Xylan is found predominantly in hardwood in the form of o-acetyl-4-o-methylglucorono-xylan and contributes up to 35% of the total dry weight (24,101,116,122).
Arabino-4-\(\alpha\)-methylglucurono-xylan constitutes 15% of the dry weight of softwoods (24,116).

3.2.3.2 Mannan

Mannan occurs in nature as a linear homopolymer or as a heteropolysaccharide in the form of glucomannan, galactomannan and galactoglucomannan.

Glucomannan is found in hardwoods at a concentration of 3-5% (101). The polysaccharide consists of \(\beta\)-1,4-linked D-mannose and D-glucose molecules. The glucose to mannose ratio ranges from 1:3 in salep to 2:3 in konjac, and no galactose is present (101,132). In contrast, 25% of softwoods is composed of galactoglucomannan (116,132). This complex polymer has the same backbone structure as glucomannan, partially substituted with \(\alpha\)-1→6 linked galactose residues (101,116). The hexose units in the backbone are also frequently acetylated at C-2 or C-3 (78,129,130). Softwood \(\alpha\)-acetylgalactoglucomannan consists of a water-soluble, galactose-rich fraction (mannose:glucose:galactose, 3:1:1) and an alkali-soluble, galactose-poor fraction (mannose:glucose:galactose, 3:1:0.2) (101,102). Figure 1 shows a hypothetical structure of galactoglucomannan and the enzymes involved in the hydrolysis of this polymer.

Galactomannan is the major seed energy reserve polysaccharide in the endosperm of seeds of *Leguminosae, Palmae, Anonaceae, Rubiaceae* and *Convolvulaceae* (29,100,146). It has a mannose backbone substituted with galactose on C-6. The molecular weight varies from species to species, ranging from 50 000 to 300 000, and the galactose content varies from 10 to 50% (29,84,101). The proportion of mannose to galactose units varies widely throughout the legume family and the water solubility of galactomannans increases with the number of side groups (2). The galactose content has a profound effect on the rheological properties of the polymer, for example, locust bean gum (mannose:galactose, 4:1) has superior properties of self-interaction and gelation in mixed polysaccharide systems than guar gum with a lower ratio (mannose:galactose, 2:1) (29).
Fig. 1 A schematic representation of an acetylated galactoglucomannan and the enzymes involved in complete hydrolysis of polymeric mannan substrates.

3.3 MANNAN-DEGRADING ENZYMES

Mannans are complex heteropolysaccharides and their complete hydrolysis involves an enzyme consortium comprising endo-β-1,4-mannanases, β-mannosidases, β-glucosidases, α-galactosidases and, in some cases, acetyl-mannan esterases (40,58,116). β-Mannanase hydrolyzes the mannan backbone to manno-oligosaccharides of various lengths. These are further degraded to single mannose units by β-mannosidase. α-Galactosidase catalyzes the removal of α-1,6-linked galactose residues, whereas other accessory enzymes, such as β-glucosidase and esterase, liberate glucose and acetic acid from the mannan chain, respectively.

3.3.1 Endo-1,4-β-mannanase

β-1,4-Mannanases (1,4-β-D-mannan mannohydrolase; EC 3.2.1.80) are endohydrolases that catalyze random hydrolysis of internal β-1,4-mannopyranosyl linkages in the main chain of mannan polymers (86,106,114). The rate of hydrolysis
of these polysaccharides and the enzyme characteristics ($K_m$ and $V_{max}$) are affected by the degree and pattern of substitution of the main chain by $D$-galactose residues and the distribution of $D$-glucosyl residues within the chain (86). Generally, as the galactose content increases, the amount of hydrolysis by $\beta$-mannanase decreases, the $V_{max}$ decreases and the $K_m$ increases (85). The action of $\beta$-mannanases on substituted mannan polymers is enhanced by the addition of $\alpha$-galactosidase and acetyl-mannan esterase (2).

The expression of $\beta$-mannanase-encoding genes by fungi is affected by carbon catabolite repression, as has also been found for other endoglycanases (63). Easily metabolizable sugars, such as glucose, mannose, and galactose, repress $\beta$-mannanase gene expression, whereas L-sorbose, mannobiose, cellobiose, $\alpha$-cellulose, bacterial cellulose and a variety of mannan polymers act as potent inducers in several fungal systems (6,71,72,108). However, the regulation might differ from species to species. For instance, *Aureobasidium pullulans* exhibit a diauxic growth on a medium containing 0.5% locust bean gum and 0.5% glucose. In contrast, production of $\beta$-mannanase in *Sclerotium rolfsii* is constitutive even in the presence of glucose, although the levels are significantly lower than those reached after glucose consumption (108). The generally accepted mechanism for induction of endoglycanases is that the respective polysaccharide is first partly hydrolyzed by hydrolases, which are constitutively produced in very low amounts. Subsequently, the soluble low molecular weight catabolites can enter the cell, signal the presence of an extracellular substrate and provide the stimulus for the accelerated synthesis of the respective enzymes (35).

3.3.1.1 Enzyme-substrate interaction

In order to depolymerize the mannan chain, $\beta$-mannanase enzymes require a certain number of adjacent substrate monomers for anchorage. Usually sufficient binding occurs across a chain with a degree of polymerization of at least four sugar residues allowing a significant rate of hydrolysis (85,106,107,114). Figure 2 illustrates an interaction between $\beta$-mannanase and mannopentose. The sugar binding subsites within the active sites of the enzyme are labeled from $-n$ to $+n$ (where $n$ is an integer) using the nomenclature proposed by Davies *et al.* (28). The $-n$ subsite binds to the
non-reducing end and the +n subsite binds to the reducing end of the β-mannan substrate, and cleavage occurs between the -1 and +1 subsites. If the enzyme binds to a chain of five mannosyl residues designated A-E, the bond between residues C and D becomes the cleavage site of preference (85).

![Diagram](image)

**Fig.2.** A schematic representation of enzyme-substrate interaction and subsite binding between *Aspergillus niger* β-D-mannanase and the (1→4)-β-D-mannan chain (86). The binding subsites are labeled (-3 → +2) and (A-E) are mannose residues.

(1→4)-β-D-linked mannan chains form a flat ribbon conformation with a two-fold axis, which places neighbouring hydroxymethyl groups on opposite sites of the ribbon (85,86). Therefore, it seems possible that the enzyme binds to alternate edges of the five mannosyl rings, so that, at residues B and D, binding occurs to the hydroxymethyl edge, whilst for A, C, and E binding is to the dihydroxyl edge (86). The hydrolysis is affected by the distribution pattern of D-galactosyl and D-glucosyl residues (2,85,116). This is likely due to an influence on the binding of sugar monomers in the subsites. For the *Aspergillus niger* β-mannanase, it has been shown that α-1,6-D-galactosyl substitution on residues B and D blocks hydrolysis, but not on A, C, and E (85). Replacement of residues C or E with a D-glucosyl residue also blocks hydrolysis (86,106). Four subsites (-4,-3,-2 and -1) were identified in the complexes of *Thermonospora fusca* mannanase with manno-oligomers, and the -1 subsite was proposed as the catalytic subsite (58).

### 3.3.1.2 The three-dimensional structure of β-mannanases

β-glycanases are often modular enzymes composed of discrete catalytic and non-catalytic domains, which are arranged in different combinations in the different
enzymes (92). Glycosyl hydrolases have been classified into families based on amino acid sequence similarities. The classification reflects the structural features of the enzymes and helps to reveal the evolutionary relationships between them (56). The classification of glycosyl hydrolases, is regularly updated and is available at http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html.

Using hydrophobic cluster and primary structure analyses, endo-1,4-β-mannanases were classified into two families (family-5 and -26) of glycosyl hydrolases (12,56). So far, family-26 mainly contains β-mannanases with only two endoglucanases and one xylanase, whereas family-5 contains a wide variety of hydrolases. Both families belong to clan GH-A, in which the overall fold of the catalytic domains is a (β/α)8-barrel architecture. The enzymes in this clan, with the exception of family-26 enzymes, share three conserved active site residues (one asparagine and two catalytic glutamates) (12,56,58,59,106). The structures of family-5 β-mannanase from T. fusca and Trichoderma reesei have been solved and are represented in figure 3.

Fig. 3. (A) shows the catalytic cleft of the T. fusca mannanase in complex with mannohexose (PDB/3D 3MAN), (B) represents the secondary structure of the β-mannanase catalytic domain of T. reesei (PDB/3D 1QNS), both available at http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html
The molecules feature a prominent cleft (Fig. 3A), which is about 15Å deep, 15 Å wide and 30Å long (58). Alignment with other family-5 β-mannanases indicates that there are eight strictly conserved amino acid residues in the catalytic cleft. These residues participate in the binding of the sugar in the −1 subsite or stabilize the position and protonation state of the catalytic glutamates (58,106). In *T. fusca*, the residues were identified as Arg50, His86, Asn127, Glu128, His196, Tyr198, Glu225 and Trp254 (58). Glu128 and Glu225 act as the catalytic proton donor and nucleophile, respectively. His86 facilitates in hydrogen bond-mediated recognition of the hydroxyl group on the third carbon (HO-C(3)) of the sugar, and Trp254 forms a hydrophobic sugar binding platform in subsite −1 (58,106). Arg50, Asn127, His196 and Tyr198 stabilize the active-site environment (58). The asparagine (Asn127) preceding the proton donor is strictly conserved in glycanases of the GH-A clan except (family-26 enzymes) and mutations of this residue lead to complete loss of activity (58). In *T. reesei*, the conserved residues are Arg54, Asn168, Glu169, His241, Tyr243, Glu276, Trp306 and His102 (106). These residues are conserved in all family-5 β-mannanases. Interestingly, bacterial β-mannanases have an additional conserved residue, Val263, in *T. fusca*. This residue is thought to play a role during substrate interaction in the −2 subsite (58). The β-mannanase from *T. reesei* can be used to predict the structure of the β-mannanase of *Aspergillus aculeatus*, since the two enzymes were reported to share 56% identity with the *T. reesei* β-mannanase (18).

Recently, the structure of a family-26 β-mannanase enzyme from *Pseudomonas cellulosa* was solved (Fig. 4). In this molecule, some of the amino acids contributing to the active site or substrate-binding cleft are similar to family-5 enzymes. The conserved residues are Arg208, His211, Glu212, Asp283, Tyr285, Glu320 and Trp360, which correspond to Arg50, Asn127, Glu128, His196, Tyr198, Glu225 and Trp254 of the *T. fusca* enzyme (58,59). The amino acid preceding the putative acid/base catalyst in family-26 enzymes is a histidine (His211), which plays a different role in these enzymes and other clan GH-A enzymes (59). In family-5 enzymes, the asparagine residue (Asn127 of *T. fusca*) preceding the proton donor (Glu128) plays a role in transition-state binding through which the glycosyl ester is formed. This residue forms a hydrogen bond with the catalytic nucleophile and also
interacts with the equatorial hydroxyl group on carbon two (HO-C(2)) of the sugar (58). In family-26, the histidine residue is thought to only play a role in maintaining the position of the catalytic nucleophile via hydrogen bonding between the imidazole ring (of histidine) and the carboxylic group of the glutamate (59).

Fig 4. The structure of a family-26 β-mannanase from *P. cellulosa* (PDB/3D 1J9Y), available at http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html

Family-5 and -26 β-mannanases share similar conserved amino acid residues in the catalytic cleft. Although some of the amino acids may play different roles, the enzymes have similar disposition of the active site residues. It is therefore most likely that the two enzymes could have evolved through convergent/parallel evolution (57). The interaction between the catalytic cleft and the substrate apparently shows discrimination towards gluco-configurated analogues, such as cellulose (106). This substrate selectivity seems to be based on conformational differences between mannan and cellulose (106).
3.3.1.3 Reaction mechanism

Glycosyl hydrolases act by a general acid catalysis mechanism in which two amino acid residues participate in a single-displacement or double-displacement reaction, resulting in either inversion or retention of configuration at the anomeric carbon atom of the hydrolyzed glycoside (57). The enzymes in family-5 and -26 are retaining enzymes and they cleave glycosidic bonds via a double-displacement mechanism (12,54,56). Fig. 5 represents a general double-displacement mechanism as it would occur with the T. reesei β-mannanase.

![Diagram](image)

**Fig. 5.** The double displacement reaction mechanism with which β-mannanases hydrolyze mannosidic bonds. In the first step of the reaction, the nucleophile attacks the anomeric centre of the substrate while the acid-base catalyst protonates the glycosidic oxygen. A covalent glycosyl-enzyme intermediate of inverted stereochemistry is formed at the anomeric centre relative to the substrate. In the second step, the acid-base catalyst promotes attack of a water molecule to displace the nucleophile and release the sugar molecule with the same anomeric configuration as the substrate (12,58).
In this reaction mechanism, the stereochemistry of the cleaved 1,4-β-mannosidic bond is retained; i.e. cleavage of a 1,4-β-mannosidic linkage will result in a β-anomer at the new reducing end (54,56). The reaction involves the two catalytic glutamates, one acting as an acid-base catalyst and the other as a nucleophile. In clan GH-A, these glutamates are generally located near the carboxy-terminal ends of β-strands 4 and 7 (56). In the case of T. reesei, Glu169 acts as a proton donor and Glu276 as the nucleophile (106) (see Fig. 6).

Fig. 6. An interaction of the two catalytic glutamates in the T. reesei β-mannanase active site with a TRIS molecule.

Several β-mannanases were reported to have a subsidiary transferase activity that catalyses a transglycosylation reaction (49,54,86). As the anomic configuration of the cleaved mannosidic linkage is retained, oligosaccharides formed during hydrolysis can serve as acceptors instead of water. Consequently, glycosyl residues are transferred onto manno-oligosaccharides, leading to elongation of the saccharide chain (49,54,86). For example, hydrolysis of mannotetraose by β-mannanase of
*A. niger* liberates two mannobiose molecules; however, in the presence of a β-mannanase with ancillary transferase activity, the products are mannobiose, mannotriose and traces of mannose.

This results from the transfer of mannobiose to the tetraose, forming mannohexose, which in turn is hydrolyzed to mannotriose. The mannotriose is further hydrolyzed to mannobiose and mannose, but the rate of this reaction is very slow (86). Alternatively, the mannose may be produced by a minor attack on mannotetraose at an extremity of the molecule and subsequently transferred to mannobiose to form mannotriose (25).

Family-5 and -26 β-mannanases are endo-acting enzymes and require binding across a certain number of consecutive mannose residues to allow a significant rate of hydrolysis. Mannotetraose is the shortest chain that is favorable for most of the enzymes, as mannose, mannobiose and mannotriose have been reported as products of hydrolysis in cases where extended hydrolysis was performed (14,61,111).

### 3.3.1.4 β-Mannanase domain structures

Endo-1,4-β-mannanase-encoding genes of bacterial, fungal and plant origin have been sequenced. Table 1 represents a list of the genes, their origin, hydrolase family numbers and additional catalytic domains. β-Mannanases occur as single catalytic domains or as part of multi-enzyme complexes including endoglucanases and putative substrate-binding modules (usually cellulose binding modules, CBDs) (17,33,40,92,115,120,139). Some bacterial β-mannanase complexes include S-layer-like modules that are known to anchor proteins to the peptidoglycan in bacterial cell walls (17,117). S-layers are proteinaceous crystalline surface layers that completely cover the cell surface of some eubacteria and archaea. They function as protective coats, molecular sieves, and molecule / ion traps, and can be involved in cell adhesion and surface recognition (15). These subsidiary domains can be either on the C- or N-terminus of the β-mannanase catalytic domain. The different domains are joined by recognizable Pro-Ser-Thr rich spacers, often referred to as PT-boxes that act as flexible hinges between functionally distinct portions of the enzyme complex (40). Figure 7A represents an arrangement of different modules in multi-modular proteins.
Table 1. A summary of prokaryotic and eukaryotic β-mannanases and additional domains that are associated with the mannanase catalytic domains.

<table>
<thead>
<tr>
<th>Name of organism</th>
<th>Family</th>
<th>Protein name</th>
<th>Position of the Man-domain</th>
<th>Additional domains</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus circulans K-1</em></td>
<td>5</td>
<td>G-enzyme</td>
<td>Entire</td>
<td></td>
<td>(144)</td>
</tr>
<tr>
<td><em>Bacillus stearothermophilus</em></td>
<td>5</td>
<td>ManF</td>
<td>N-terminal</td>
<td>Unknown</td>
<td>(32)</td>
</tr>
<tr>
<td><em>Caldibacillus cellulovorans</em></td>
<td>5</td>
<td>ManAd3</td>
<td>C-terminal</td>
<td>CBDs</td>
<td>(120)</td>
</tr>
<tr>
<td><em>Caldocellum</em></td>
<td>5</td>
<td>ManA</td>
<td>N-terminal</td>
<td>CBDs and Eng</td>
<td>(40)</td>
</tr>
<tr>
<td><em>saccharolyticum</em></td>
<td>5</td>
<td>ManB</td>
<td>C-terminal</td>
<td>CBDs and Eng</td>
<td>(35,92)</td>
</tr>
<tr>
<td><em>Caldocellulosiruptor</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>saccharolyticus</em></td>
<td>5</td>
<td>ManA</td>
<td>N-terminal</td>
<td>Dockerin and Eng</td>
<td>(126)</td>
</tr>
<tr>
<td><em>Clostridium cellulovorans</em></td>
<td>5</td>
<td>ManA</td>
<td>Entire</td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em></td>
<td>5</td>
<td>ManA</td>
<td>N-terminal</td>
<td>CBDs and SLH</td>
<td>(17)</td>
</tr>
<tr>
<td><strong>Thermoanaerobacterium polysaccharolyticum</strong></td>
<td>5</td>
<td>ManA</td>
<td>Entire</td>
<td></td>
<td>(125)</td>
</tr>
<tr>
<td><em>Vibrio sp. strain MA-141</em></td>
<td>26</td>
<td>ManA,B</td>
<td>Entire</td>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td><em>Bacillus sp. strain AM-001</em></td>
<td>26</td>
<td>Mannanase</td>
<td>Entire</td>
<td></td>
<td>(88)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>26</td>
<td>ManA</td>
<td>C-terminal</td>
<td>MBD</td>
<td>(41,121)</td>
</tr>
<tr>
<td><em>Caldicellulosiruptor Rt8B.4</em></td>
<td>26</td>
<td>Man26A</td>
<td>N-terminal</td>
<td>SLH and MBD</td>
<td>(117,118)</td>
</tr>
<tr>
<td><em>Cellulomonas fimis</em></td>
<td>26</td>
<td>Man26A</td>
<td>N-terminal</td>
<td>Dockerin and MBD</td>
<td>(51)</td>
</tr>
<tr>
<td><em>Clostridium thermocellum</em></td>
<td>26</td>
<td>ManA</td>
<td>Entire</td>
<td></td>
<td>(42)</td>
</tr>
<tr>
<td><em>Dictyoglomus thermophilum</em></td>
<td>26</td>
<td>Mannanase</td>
<td>Entire</td>
<td></td>
<td>(37)</td>
</tr>
<tr>
<td><em>Prevotella ruminicola</em></td>
<td>26</td>
<td>MANA</td>
<td>Entire</td>
<td></td>
<td>(14)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>26</td>
<td>ManA</td>
<td>C-terminal</td>
<td>Unknown</td>
<td>(99)</td>
</tr>
<tr>
<td><em>Rhodothermus marinus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fungal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Agaricus bisporus</em></td>
<td>5</td>
<td>Cel4</td>
<td>C-terminal</td>
<td>CBD</td>
<td>(128,140)</td>
</tr>
<tr>
<td><em>Aspergillus aculeatus</em></td>
<td>5</td>
<td>Man1</td>
<td>Entire</td>
<td></td>
<td>(18)</td>
</tr>
<tr>
<td><em>Aspergillus aculeatus</em></td>
<td>5</td>
<td>Man1</td>
<td>N-terminal</td>
<td>CBD</td>
<td>(115)</td>
</tr>
<tr>
<td><em>Trichoderma reesei</em></td>
<td>26</td>
<td>ManA,B,C</td>
<td>N-terminal</td>
<td>Protein docking</td>
<td>(33,89)</td>
</tr>
<tr>
<td><strong>Piromyces</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Plant</em></td>
<td>5</td>
<td>LeMan1,2</td>
<td>Entire</td>
<td></td>
<td>(96)</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
of two anaerobic bacteria and an aerobic soft-rot fungus. This modular organization has been observed in cellulases and xylanases, and seems to be a characteristic feature in several enzymes that hydrolyze insoluble substrates (133). The non-catalytic substrate binding domains (now generally termed carbohydrate binding modules (CBMs)) are thought to enhance activity by increasing local enzyme concentration on the substrate surface or by disrupting non-covalent interactions thereby increasing substrate accessibility (122,133). However, among wood degrading enzymes this has mostly been shown for cellulases.

In non-aggregating enzyme systems such as those of *Caldocellum*, *Thermoanaerobacterium*, *Caldibacillus*, *Trichoderma* and *Agaricus*, the CBD forms an integral part of the enzyme (4). The CBDs in these systems seem to play different roles, for instance, removal of the CBD of *Caldocellum saccharolyticus* and *Thermoanaerobacterium polysaccharolyticum* result in loss of activity on galactomannan and carboxymethyl cellulose (17,35). In addition, one of the CBDs of *T. polysaccharolyticum* is required for proper folding of the β-mannanase (17). In contrast, the β-mannanase of *T. reesei* is not affected by removal of the CBD. This CBD mediates binding to mannan/cellulose complexes but has no affinity towards galactomannan (55).

In the aggregating cellulase systems of clostridia (prototype, the cellulosome of *Clostridium thermocellum*), the enzymes do not carry CBDs but they contain reiterated residues that form a protein-docking domain (dockerin). The dockerin mediates formation of tightly associated extracellular cellulase complexes called cellulosome by binding catalytic sub-units to the cohesin domains of the cellulosome-integrating protein (CipA) through their receptors (9,34,51,133). CipA is a non-catalytic scaffolding protein and it promotes cellulose hydrolysis by anchoring enzymes to the cellulose surface (34,133). This protein has nine cohesin domains and a CBD as the tenth domain (4,9,34,133). It is this CBD that mediates adsorption of the entire cellulosome complex for cellulose (see Fig. 7B). The β-mannanase-encoding genes of the anaerobic fungus *Piromyces* has protein-docking domains that are involved in the formation of multi-modular (cellulase/hemicellulase) enzymes (33,89). As in clostridia, the cellulose-binding proteins of *Piromyces* are thought to constitute an aggregating cellulase system with essentially one CBD (4,33,89).
Fig. 7. Microbial enzymes containing β-mannanase catalytic modules, auxiliary catalytic and substrate-binding modules. (A) Shows an arrangement of different domains in non-aggregating enzyme systems, and (B) is a representation of aggregating systems as found in Clostridium thermocellum.

Three bacterial family-26 β-mannanases have been reported to have mannann-binding modules (MBDs) (51,118,121). The MBDs of Cellulomonas fimi and Caldocelluloruptor Rt8B.4 are located on the C-terminus of Man26A and have high affinity towards soluble galactomannan. These modules allow reversible binding to
soluble mannan and do not bind insoluble mannan (118,121). Conversely, the MBD of *C. thermocellum* facilitates binding and enhances activity on recalcitrant insoluble substrates. The domain is thought to provide the cellulosome with a receptor for mannan (51). The MBDs of *C. fimi* and *C. thermocellum* have only 26.4% identity at protein level (51,118).

β-Glycanases are thought to have evolved through the reshuffling of catalytic modules and several binding modules. Consequently, some enzymes may have acquired substrate-binding domains that are responsible for binding insoluble substrates and may have formed complexes with other catalytic domains (35,138). The acquisition of multi-modular enzymes in microorganisms is perhaps a necessary and perpetual evolutionary adaptation that allows access to a wide range of complex substrates as carbon sources.

### 3.3.1.5 Phylogenetic relationship between β-mannanases

DNAMAN 4.13 sequence analysis software (Lynnon Biosoft Copyright© 1994-1999) was used to build multiple alignments and to set up a phylogenetic tree based on the protein sequence alignments of prokaryotic and eukaryotic β-mannanase catalytic modules. The tree was based on distance matrices using the Neighbor-Joining method, and statistical evaluation was performed through bootstrapping. Bacterial and eukaryotic β-mannanases were grouped into three main clusters (Fig. 8).

Cluster 1 comprises family-5 bacterial β-mannanases. In this cluster, the enzymes from the two actinomycetes found in soil (*Streptomyces lividans* and *Thermonospora fusca*), which share 61.8% identity are clustered together. The enzyme from *Vibrio*, an aquatic Gram-negative rod, shows high homology with those of the actinomycetes, with 51% identity shared within this group. The enzymes from low-GC Gram positives, *Caldibacillus cellulovorans*, *C. saccharolyticum*, *Bacillus circulans* and *T. polysaccharolyticum*, cluster close to each other. However, *C. cellulovorans* and *C. saccharolyticum* form a cluster of their own, sharing 65% identity, whereas the enzymes from *B. circulans* and *T. polysacchorolyticum* are slightly distant.
Fig. 8. A phylogenetic tree of prokaryotic and eukaryotic endo-1,4-β-mannanases constructed from the alignment of protein sequences. Prokaryotic strains producing family-5 enzyme are colored (blue), eukaryotic family-5 (red), bacterial family-26 (black) and eukaryotic family-26 (green).

The second cluster is composed mainly of family-5 eukaryotic β-mannanases. The β-mannanases from the two ascomycetous fungi, A. aculeatus and T. reesei, which share 58% identity, and the β-mannanase from Agaricus bisporus, a basidiomycete, cluster close to each other. The β-mannanase from the higher eukaryote L. esculentum is clustered at the bottom of the group and shows low homology to those of the lower eukaryotes. Surprisingly, the β-mannanases from the low-GC Gram positives,
Bacillus stearothermophilus and Clostridium cellulovorans, are clustered with the eukaryotic enzymes. The two enzymes share 18% identity and show high homology to enzymes from the lower eukarotes. The enzyme from B. stearothermophilus shares more than 30% identity with the eukaryotic enzymes and shows only very low (<15%) similarity with β-mannanases from other bacilli. The enzyme from C. cellulovorans shares more than 20% identity with the β-mannanase of A. aculeatus, T. reesei and A. bisporus (126). It is possible that the β-mannanase of B. stearothermophilus and C. cellulovorans may have evolved through horizontal gene transfer from a common ancestor with the eukaryotic β-mannanases.

Bacterial and eukaryotic family-5 β-mannanases are distant from each other and cluster in two distinct groups. This is in agreement with the proposal by Hilge et al (58) to separate family-5 eukaryotic and bacterial β-mannanases into subfamily A7 and A8, respectively. The β-mannanases in subfamily A8 share sequence identities above 43%, whereas the identity between the two subfamilies is below 20% (58).

Cluster 3 comprises bacterial family-26 β-mannanases. In this cluster, the β-mannanase of Cellulomonas fimi, Pseudomonas fluorescens and Bacillus subtilis are grouped together. The β-mannanases from C. fimi (an actinomycete) and P. fluorescens (a Gram-negative rod) share 40% identity. The β-mannanase of B. subtilis shares 26% and 23% identity with the β-mannanase of C. fimi and P. fluorescens, respectively. B. subtilis is known as a hay bacterium (easily isolated from hay, by enrichment), whilst C. fimi and P. fluorescens are known for their ability to utilize cellulose as the sole carbon source. All three organisms are commonly found in soil. The high homology in this group suggests that these organisms could have inherited their genes from related ancestors.

The β-mannanase from P. equi is the only fungal enzyme in family-26 reported so far and it forms a cluster on its own. P. equi is a ruminal fungus. Its glycosyl hydrolase genes have no introns and are believed to be of prokaryotic origin (36). In addition, the β-mannanase of P. equi and other glycosyl hydrolases of this fungus form a
cellulosome-like structure found only in anaerobic bacteria. It has been shown that genes encoding these enzymes were acquired through horizontal gene transfer (36).

The phylogenetic relationship of amongst β-mannanases shows that there is high homology between enzymes in the same family of glycosyl hydrolases, such that family-5 β-mannanases cluster separately from family-26 β-mannanases. Secondly, within the families, prokaryotic and eukaryotic β-mannanases seem to separate into distinct groups. In the various groups, enzymes isolated from microorganisms from common taxonomic groups, or isolated from common habitats cluster close to each other. Based on these observations, we can infer that some of the β-mannanases evolved from common ancestors and thus may have been acquired through horizontal gene transfer.

3.3.1.6 Biochemical properties of β-mannanases

Due to their importance in several industries, β-mannanases have been purified from a variety of sources and characterized. Bacterial and fungal β-mannanases are secreted directly into the cultivation medium, whereas, in molluscs, they are contained in the viscera (141,142). In fruit such as tomato, the enzyme is synthesized in the cells of the aleurone layer and secreted into the endosperm to metabolize galactomannan, which is present as the storage reserve (100,135). β-Mannanase multiplicity has been reported in plants, bacteria and fungi (3,39,100,114,135,144). Some of the isoforms secreted by these organisms show differences in structural specificity and can either be products of the same gene or encoded by different genes (3,96,114). The β-mannanases so far reported have acidic to neutral pH optima and are mainly mesophilic (Table 2). L. esculentum secretes a single germinative and three post-germinative isoforms that can be distinguished by different electrophoretic mobilities. The different isoforms are encoded by different genes and are expressed during different developmental stages of the fruit (96). The germinative isoform is localized to the endosperm cap and is thought to be involved in the weakening of this tissue prior to radicle emergence, whereas the post-germinative isoforms are associated with the mobilization of cell wall mannans reserves in the lateral endosperm during seedling growth (96). ManI and ManII of Trifolium repens have distinct specificities; ManI...
Table 2. A summary of the biochemical properties of β-1,4-mannanases from bacterial, fungal, plant and animal sources.

<table>
<thead>
<tr>
<th>Name of organism</th>
<th>MW (kDa)</th>
<th>pH optima</th>
<th>Temp. optima (°C)</th>
<th>pI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial sources</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus circulans K-I</em></td>
<td>62</td>
<td>6.0</td>
<td>64</td>
<td>5.4 – 6.2</td>
<td>(144)</td>
</tr>
<tr>
<td><em>Flavobacterium sp.</em></td>
<td>46</td>
<td>7.0</td>
<td>35</td>
<td></td>
<td>(147)</td>
</tr>
<tr>
<td><em>Vibrio sp. strain MA-141</em></td>
<td>49</td>
<td>6.5</td>
<td>40</td>
<td>3.8</td>
<td>(127)</td>
</tr>
<tr>
<td><em>Bacteroides ovatus</em></td>
<td>61</td>
<td>6.2 – 6.5</td>
<td>37</td>
<td>4.8 – 6.9</td>
<td>(39)</td>
</tr>
<tr>
<td><em>Thermotoga neapolitana</em></td>
<td>55</td>
<td>7.1</td>
<td>92</td>
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<td>(30,87)</td>
</tr>
<tr>
<td><em>Bacillus stearothermophilus</em></td>
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<td>5.5 – 7.5</td>
<td>70</td>
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<td>(124)</td>
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<tr>
<td><em>Rhodothermus marinus</em></td>
<td>60</td>
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<td>(99)</td>
</tr>
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<td><em>Caldocellum saccharolyticum</em></td>
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<td><em>Pseudomonas fluorescens</em></td>
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<td>7.0</td>
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<td></td>
<td>(14)</td>
</tr>
<tr>
<td><em>Dictyoglomus thermophilum</em></td>
<td>40</td>
<td>5.0</td>
<td>80</td>
<td></td>
<td>(42)</td>
</tr>
<tr>
<td><em>Streptomyces sp.</em></td>
<td>36</td>
<td>6.8 – 7.0</td>
<td>45 – 58</td>
<td>3.5</td>
<td>(7,90)</td>
</tr>
<tr>
<td><em>Cellulomonas fimi</em></td>
<td>50</td>
<td>5.5</td>
<td>42</td>
<td></td>
<td>(117)</td>
</tr>
<tr>
<td><em>Clostridium thermocellum</em></td>
<td>69</td>
<td>6.5</td>
<td>65</td>
<td></td>
<td>(51)</td>
</tr>
<tr>
<td><em>Caldibacillus cellulovorans</em></td>
<td>98</td>
<td>6.0</td>
<td>85</td>
<td></td>
<td>(120)</td>
</tr>
<tr>
<td><em>Clostridium cellulovorans</em></td>
<td>47</td>
<td>7.0</td>
<td>45</td>
<td></td>
<td>(126)</td>
</tr>
<tr>
<td><em>Thermoanaerobacterium polysaccharolyticum</em></td>
<td>97</td>
<td>5.8</td>
<td>75</td>
<td></td>
<td>(17)</td>
</tr>
<tr>
<td><strong>Fungal sources</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thielavia terrestris</em></td>
<td></td>
<td>6.0</td>
<td>50</td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td><em>Thermoascus aurantiacus</em></td>
<td></td>
<td>5.0</td>
<td>50</td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td><em>Aspergillus sp.</em></td>
<td>40 – 53</td>
<td>3.5 – 5.0</td>
<td>45 – 60</td>
<td>3.7 – 4.5</td>
<td>(1,18,19)</td>
</tr>
<tr>
<td><em>Trichoderma reesei</em></td>
<td>51 – 53</td>
<td>3 – 4</td>
<td>70</td>
<td>3.6 – 6.5</td>
<td>(116)</td>
</tr>
<tr>
<td><em>Sclerotium rolfsii</em></td>
<td>61</td>
<td>2.9</td>
<td>74</td>
<td>3.5</td>
<td>(45)</td>
</tr>
<tr>
<td><em>Trichosporon cutaneum</em></td>
<td>50</td>
<td>4.0 – 6.5</td>
<td>50</td>
<td></td>
<td>(97)</td>
</tr>
<tr>
<td><strong>Plant sources</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trifolium repens</em></td>
<td>38 – 43</td>
<td>5.1 – 5.6</td>
<td>37</td>
<td>9.3</td>
<td>(135)</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em></td>
<td>43</td>
<td>4.8</td>
<td></td>
<td></td>
<td>(100)</td>
</tr>
<tr>
<td><strong>Animal sources</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pomacea insularis</em></td>
<td>44</td>
<td>5.5</td>
<td>50</td>
<td></td>
<td>(141)</td>
</tr>
<tr>
<td><em>Littorina brevicula</em></td>
<td>42</td>
<td>6.5</td>
<td>50</td>
<td></td>
<td>(142)</td>
</tr>
</tbody>
</table>
requires a chain length of five mannose residues for its activity, whereas ManII requires at least four (135). *Bacillus* sp. KK01 produces four β-mannanase isoforms with different temperature optima and similar action properties although slight differences in specificity were apparent in some isoforms (5).

In *B. ovatus*, one isoform is soluble and the other one is cell-associated (39). *T. reesei* and *A. aculeatus* were also reported to secrete multiple forms of β-mannanase with different isoelectric points (114,115). β-Mannanases are generally monomeric with molecular weights ranging between 35 and 101 kDa, except for the β-mannanase of *T. polysaccharolyticum*, *B. stearothermophilus* and *B. ovatus*, which are dimeric in the native form and have molecular weights of 123 kDa, 162 kDa, and 192 kDa, respectively (17,39,124). Thermophilic β-mannanases have temperature optima of 66°C-95°C and are stable for 24 hours at 71°C (11,17,30,42,51,87,99). The β-mannanase of *Dictyoglomus thermophilum* is stable at 82°C over 16 hours. However, the β-mannanase of *Thermotoga neapolitana* is by far the most stable, with a half-life of 34 hours at 87°C and 13 hours at 92°C (30,87).

β-Mannanases are secreted by prokaryotes and eukaryotes as single enzyme activities or as multiple isoforms that are either encoded by the same gene or are products of different genes. These isoforms have distinct biochemical properties and, in some cases, different structural specificity, such that they could play complementary hydrolytic functions in the degradation of mannan polymers.

### 3.3.2 β-Mannosidase

β-Mannosidases (β-D-mannoside mannohydrolase, EC 3.2.1.25) have been isolated and characterized from bacteria and fungi (8,30,117,119). The enzymes from both sources catalyze the hydrolysis of terminal, non-reducing mannopyranoside linkages of β-manno-oligosaccharides, releasing single mannose molecules (8,116,117). In higher eukaryotes, β-mannosidases degrade the terminal non-reducing mannopyranoside linkages of glycoproteins (8,117). In humans, the absence of β-mannosidase results in the deleterious storage of the disaccharide Man-β-1,4-GlcNAc (8). β-Mannosidase enzymes are either secreted or intracellular. For example, the β-
mannosidases of *A. pullulans* and *C. fim* are induced by β-1,4-mannobiose and repressed in the presence of mannose. The enzyme remains intracellular and the disaccharide is transported actively into the cells via a membrane-embedded mannobiose permease (71,117). In other systems, such as those of *A. niger*, *T. reesei* and *A. awamori*, β-mannosidase is secreted, which could imply that these organisms lack a β-mannobiose permease, as a result only monomeric units are transported into the cells (117).

### 3.3.3 α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22)

There are currently two groups of α-galactosidases with distinct substrate specificities (52,68,79,82,116). The first group shows preference for oligosaccharides in which galactose is linked to the non-reducing end of the substrate, e.g. melibiose, raffinose, stachyose, verbascose and some galactomanno-oligosaccharides. The second group preferably liberates α-1,6-linked galactose units attached to the inner mannose residues of the main chain of galactomanno-oligosaccharides and intact galacto(gluco)mannan polymers, but is also able to hydrolyze smaller substrates to various extents (52,68,79,116). Some fungal species were reported to secrete a mixture of isoenzymes comprising three α-galactosidases that exhibit different substrate specificities (79,82). The enzymes in the first group show improved hydrolysis efficiency in the presence of β-mannanase and/or β-mannosidase, whereas the second group of α-galactosidases such as the AGLI of *Penicillium simplicissimum* can work efficiently on their own (68,79,82).

### 3.3.4 β-Glucosidase

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) is an exo-acting glycosyl hydrolase that catalyzes the release of terminal, non-reducing β-D-glucose residues in various β-D-glucosides (8,76). β-Glucosidases are not capable of degrading long β-1,4-linked glucose chains. Most purified β-glucosidases are competitively inhibited by glucose and cellobiose (8,43,76,116).
3.3.5 Acetyl-mannan esterase

Acetyl-mannan esterase liberates acetic acid from acetylated mannan substrates \((102,129,130)\). Esterases isolated from fungal sources show different substrate specificities. The esterases from \(T. reesei\) and \(A. niger\) preferably liberate acetic acid from polymeric acetyl galactoglucomannan \((129,130)\). The esterase of \(Aspergillus oryzae\) has broad substrate specificity, as it is able to deacetylate not only galactoglucomannan and oligomers of acetyl galactoglucomannan but can also liberate phenolic side groups from xylan \((129,130)\). The action of esterases is greatly enhanced by the addition of \(\beta\)-mannanase and \(\alpha\)-galactosidase, but not \(\beta\)-glucosidase or \(\beta\)-mannosidase \((130)\). Tenkanen \textit{et al.} \((130)\) reported that the hydrolysis yield of the esterase of \(A. oryzae\) on o-acetyl-galactoglucomannan increased to 87% when the esterase was used in combination with the \(\beta\)-mannanase from \(T. reesei\).

3.4 INDUSTRIAL APPLICATIONS OF ENDO-1,4-\(\beta\)-MANNANASES

Over the years, several industries had to seek biological approaches as alternatives to traditional chemical processing methods, due to concerns over the effect of unwanted chemical loading on the environment. This prompted the quest for appropriate enzymes that could be used in different industrial applications. Endo-1,4-\(\beta\)-mannanases have emerged as alternative tools in several industries.

3.4.1 Poultry feeds

Galactomannan is a major carbohydrate component in poultry and livestock feedstuffs based on legume seeds, such as soybean, guar bean and alfalfa \((123)\). If fed in large quantities, this polysaccharide can have a strong anti-nutritive effect on monogastric animals, swine and humans \((66,123)\). Research has shown that high a galactomannan concentration in chick feed results in reduced nitrogen retention, fat absorption, and amino acid uptake, which ultimately affects growth \((66,123)\). In humans and swine, galactomannan interferes with glucose metabolism and the control of insulin
concentrations, and reduces levels of plasma gastric inhibitor polypeptide in humans only (123). Incorporation of β-mannanase in corn-soybean chicken feed has been shown to alleviate growth depression in chicks and to improve hen-day production and egg weight in laying hens (66,123).

3.4.2 Coffee processing

Galactomannan constitutes 20 – 30% of the dry weight of Arabica and Robusta coffee beans (109). This polysaccharide is the major contributor to the high viscosity of coffee extract when it is concentrated before spray- and freeze-drying (109). Reduction of the viscosity is expected to facilitate the production of instant coffee by improving the effectiveness with which the extracts can be concentrated using low-cost processes such as evaporation (109,139). On the other hand, carbohydrates have the capacity to bind volatile compounds at adsorptive sites and could therefore play a vital role in aroma retention (134). The hydrolysis of carbohydrates at very high temperatures such as those used in conventional processing causes great loss of flavor and aroma (21). Sachslehner et al. (109) have shown that the β-mannanase of S. rolfsii can hydrolyze coffee bean mannan, resulting in a significant reduction in viscosity. Therefore, using β-mannanase in this industry may provide a simple alternative method to conventional processing methods and allow extraction under mild conditions. Controlled enzyme hydrolysis can modify polysaccharides to oligosaccharides, resulting in a reduction in viscosity and, at the same time, still providing enough capacity to bind aromatic compounds.

3.4.3 Production of oligosaccharides

Oligosaccharides form part of membrane-bound proteins, where they serve as recognition signals for hormones, toxins, antibodies, viruses and bacteria (49). They are used in the pharmaceutical industries for therapeutic and diagnostic purposes, e.g. antibodies against cancer-associated carbohydrate antigens are being used in diagnostic kits (95). Endo-1,4-β-mannanases can be used for the production of manno-oligosaccharides from cheap agricultural by products such as copra or konjac mannan. These oligosaccharides are thought to be excellent prebiotics for stimulating
growth of beneficial intestinal microorganisms and can therefore be used in the pharmaceutical and food industries (44). Endo-1,4-β-mannanases with transglycosylation activity can be used to synthesize manno-oligosaccharides. These enzymes hydrolyze the glycosidic bond with retention of the anomeric configuration of the cleaved bond, and are able to transfer monomers to oligosaccharides (49,54,85).

Other potential applications include, the clarification of fruit juices (90,116), hydrolysis of residual coconut waste after the extraction of coconut oil (88), bio-bleaching in the paper and pulp industry (20,47,122,125) and hydrolysis of guar gum during hydraulic fracturing of oil and gas wells (87).

3.5 HETEROLOGOUS GENE EXPRESSION IN SACCHAROMYCES CEREVISIAE

Over the years, yeast has been used as a host for the production of foreign proteins. Saccharomyces cerevisiae, in particular, has been developed as a eukaryotic model organism after Escherichia coli (50,105). In contrast to E. coli, S. cerevisiae is non-pyrogenic (does not produce toxins), has the ability to process other eukaryotic proteins post-translationally and, most importantly, is able to secrete certain products into the extracellular environment in the same way as higher animal and plant cells (50,105). Heterologous expression in yeast uses autonomously replicating, high copy expression vectors based on the yeast 2μ-plasmid. The plasmid is transformed into an auxotrophic strain with the auxotrophic mutation complemented by a plasmid-borne wild-type gene. Selection requires growth of the transformants in a defined medium lacking the relevant nutrient. In many instances low expression levels and product yields presented some limitations. With increased understanding of the secretion processes, mutant strains in which relevant steps of protein synthesis and secretion were altered, were created (105). This helped in solving problems related to secretion, such as retention in the endoplasmic reticulum and the periplasm, but had little effect on product yields. Several researchers have shown that low yields are often a result of plasmid instability, low gene dosage (copy number) and the use of minimal media (23,103,149). Consequently, autoselection systems were developed to eliminate some
of these factors. In such systems, plasmid maintenance is obligatory for cell viability and is independent of the cultivation medium (22, 23, 77, 103).

3.5.1 Autoselection

Loison et al. (77) developed a system by which autoselective ura3 furl double mutants were created by mating a furl strain with a ura3 strain containing a plasmid-borne URA3 gene. This system is based on the fact that a yeast lacking the uracil phosphoribosyl transferase activity (in the uracil salvage pathway), encoded by the FUR1 gene, and the orotidine-5-phosphate decarboxylase activity (in the pyrimidine synthesis pathway), encoded by the URA3 gene is not viable (77). Hence, introducing the plasmid-borne URA3 gene, allows de novo synthesis of pyrimidines and the recombinant yeast retains the expression plasmid, as it needs the URA3 gene for survival. This system has been used in S. cerevisiae and Kluyveromyces lactis (62, 77).

In a different approach, La Grange et al. (73) used a gene replacement method to replace the functional chromosomal FUR1 gene with a defective fur1::LEU2 allele. The foreign gene was first introduced into a ura3 auxotrophic recipient strain on a plasmid vector that contained a functional URA3 gene. Subsequently, the chromosomal FUR1 gene of the recombinant strain was replaced with a disruptive fur1::LEU2 allele (73). This method was used to construct autoselective S. cerevisiae strains producing recombinant β-mannanase from A. aculeatus in this study (chapter 2).

Other autoselective systems include disruption of the chromosomal fructose-1,6-bisphosphate (FBA1) gene and replacing it with a functional plasmid-borne gene (22, 23). The system reported by Rech et al. (103) used S. cerevisiae srb1-1 fragile mutants as recipients of expression vectors carrying a foreign gene and a functional SRB1-1 gene to complement the mutation. All autoselective systems share two common features: they are stable in batch and continuous cultivation, and they allow flexible choices of cultivation medium.
3.5.2 **Protein secretion**

In yeast, protein secretion is a sequential process that involves the transfer of proteins through various membrane-enclosed compartments constituting the secretory route. The process consists of translocation into the endoplasmic reticulum (ER) during translation (synthesis of the protein), transport of the polypeptide chain from the ER to the Golgi apparatus for post-translational modification, transport by secretory vesicles from the Golgi to the cell membrane and, finally, exocytosis (70,105,148). Secretory proteins feature a special signal sequence at the N-terminus. The signal sequence mediates the attachment of the ribosome to the ER membrane, movement of the nascent protein into the ER cisterna and export of the mature protein from the ER (70,105,110,136,146). Generally, signal sequences are 19-30 amino acid long peptides with a charged N-terminus, a central hydrophobic core and a consensus sequence, at which the signal sequence is cleaved from the mature protein once export of the growing protein chain across the ER is initiated (105,110,136). The essential feature of the signal peptide is the hydrophobic core (6-15 amino acids), which is responsible for export across the ER membrane. Non-hydrophobic residues occasionally interrupt the core (105,136). In yeast, the nature of the signal peptide, i.e. its length and hydrophobicity, has direct correlation with the quantity of the protein secreted and therefore plays a pivotal role in the secretion competence of the cell (74,75,105). The classical cleavage site is a sequence of two basic amino acids; however, a number of monobasic cleavage sites have been identified (110). All possible combinations of lysine (Lys) and arginine (Arg) are encountered in dibasic sites, but the Lys-Arg sequence is the most common (110). Cleavage of the signal peptide from the translation product is executed by a signal peptidase that cleaves on the carboxy-terminal side of the two basic residues, i.e. after the last basic residue (105,110,136).

Signal sequences are recognized with low specificity in yeast; consequently, most fungal proteins were successfully produced in yeast using their native signal peptides (53,65,74,75,105). Nevertheless, the structural gene of the yeast mating pheromone α-factor, the *MFα1*, has been used extensively for heterologous protein secretion from *S. cerevisiae* (10,70,74,75,105,112). *MFα1* codes for a 166 amino acid prepro-polypeptide featuring a leader that consists of a 19 amino acid signal (pre-) sequence.
and a 67 amino acid pro-sequence, followed by four tandem repeats of the mature 13 amino acid α-factor sequence. Each repeat is preceded by a short spacer peptide with the structure Lys-Arg-(Glu/Asp-Ala)₂₃ (70,105). The structure of the MFα1 gene and the enzymes involved in the processing of the prepro region is represented in Fig. 9. The pre- region is cleaved by a signal peptidase between amino acids 19 and 20, whereas the repeating α-factor peptide units are excised from the precursor by a KEX2 endoprotease, which cleaves the glycosylated pro-α-factor, after the pro-peptide, and between the α-factor repeats. Cleavage by the KEX2 endoprotease occurs on the carboxyl terminal side of the dibasic sequence Lys-Arg in the pro-peptide (70,105,148). In addition, STE13, a dipetidyl amino peptidase, removes the spacer residues at the amino terminus of each repeat before secretion by the sequential removal of the Glu-Ala dipeptides (69,70,105). The KEX1 carboxypeptidase serine protease, removes the Lys and Arg residues at the carboxyl terminus of the first three repeats (70,105).

Fig. 9. A schematic representation of the structure and processing of the yeast MFα1 secretion signal peptide. The glycosylation sites are indicated by shaded circles.
The β-mannanase from *A. aculeatus* and *T. reesei* are naturally secreted enzymes preceded by secretion signal peptides. Both enzymes appear to undergo a two-step proteolytic processing. In the first step, the pre-peptide is released by a signal peptidase that cleaves between Ala-18 and Leu-19 of the *A. aculeatus* enzyme and between Ala-19 and -20 of the *T. reesei* enzyme. The pro-peptide in both enzymes is significantly shorter than the pro-peptide of the yeast MFα1. The *T. reesei* β-mannanase pro-peptide seems to be cleaved by a proline-directed arginyl protease at the carboxyl terminus of Arg-27 (115). This protease is specific for monobasic cleavage site processing (110). The pro-petide of the *A. aculeatus* mannanase is cleaved at the COOH terminus of Ala-28. The two fungal mannanase-encoding genes were cloned and expressed in *S. cerevisiae*. Protein secretion was successfully directed by the native secretion signals. However, the production levels of the *T. reesei* enzyme in *S. cerevisiae* were relatively low (111,115). In chapter 3, it was observed that secretion of the *A. aculeatus* β-mannanase with the native secretion signal resulted in stunted growth. Replacement of the native *A. aculeatus* β-mannanase secretion signal with the yeast MFα1S secretion signal alleviated the growth defect, but resulted in a two-fold reduction in production levels. In contrast, replacement of the native *T. reesei* β-mannanase secretion signal with the yeast MFα1S secretion signal improved the production levels 1.5-fold.

3.5.3 Glycosylation and the influence of N-linked glycans on enzyme properties

As the nascent protein enters the ER, it undergoes other co-translational modifications, including glycosylation, formation of disulfide bonds and protein folding (105). Using carboxypeptidase Y in *S. cerevisiae* as a model system, Holst *et al.* (60) demonstrated that there is an intimate interplay between these processes and that glycosylation does not necessarily precede folding. Glycosylation is one of the ubiquitous features of the eukaryotic secretory pathway (60). There are two forms of glycosylation: N-linked and O-linked glycosylation. N-glycosylation involves the transfer of the core oligosaccharide GlnNAC₂Man₉Glc₃ from the membrane lipid carrier, dolichol, to the asparagine residue in the sequon Asp-Xaa-Set/Thr (Xaa can be
any amino acid other than proline) (60,143). During O-glycosylation, the oligosaccharide is attached to the hydroxy groups of Thr and/or Ser residues (16). In yeasts, the mannose residue that is α-glycosidically linked to Thr or Ser is derived from a dolichol phosphate mannose in the ER, whereas, in mammalian processes, it is derived from a sugar nucleotide in the Golgi complex (16). O-glycans synthesized by yeasts are composed only of mannose residues. Conversely, mammalian proteins have sialylated O-linked chains of the mucin type (105). Protein glycosylation is believed to play a common role in facilitating the efficiency of folding of the nascent polypeptide chain and in conformational stabilization of the mature glycoprotein (104,137,143). In addition, carbohydrate moieties have been shown to improve the solubility of proteins in the denatured or partially denatured states and to prevent aggregation of the newly synthesized polypeptide chains (137). Substitution of the Asn molecules in the glycosylation site of rice α-amylase with Gln resulted in decreased thermal stability and cleaving efficiency of soluble starch hydrolysis, and caused changes in the temperature dependence of $K_m$ (131). Similar results were observed when an Asn of the SWA2 amylase of S. occidentalis was replaced with Ala, suggesting that carbohydrate moieties have an influence on the dynamic stability and functional activity of the enzyme. However, these effects could be protein dependent. For instance, in chapter 2, the recombinant Aa-Man5A was hyperglycosylated and exhibited a molecular mass 10 kDa higher than the calculated molecular weight and 5 kDa higher than the secreted native enzyme. However, the recombinant Aa-Man5A exhibited similar biochemical properties to the native Aa-Man5A.

3.6 NON-SACCHAROMYCES YEASTS AS ALTERNATIVE HOSTS FOR HETEROLOGOUS PROTEIN EXPRESSION

*S. cerevisiae* offers an attractive tool for the expression of recombinant proteins. However, some of the limitations associated with poor expression capacity and low product yields could not be overcome. This has led to a quest for alternative non-Saccharomyces yeast strains with better expression and secretion capacities. Yeasts such as *Pichia pastoris, Hansenula polymorpha, Kluveromyces lactis, Yarrowia lipolytica, Pichia stipitis* and *Schizosaccharomyces pombe* were explored and are now
used for heterologous protein production (83,93,98,105). Amongst these yeasts, the facultative methylotrophic ascomycetes *P. pastoris* and *H. polymorpha* have emerged as the most potent systems for heterologous protein production. The expression strategies are based on promoter elements of the *AOXI* gene of *P. pastoris*, the *MOXI* of *H. polymorpha* and the *FMD* gene, which encode alcohol oxidase, methanol oxidase, and formate dehydrogenase, respectively (38,83,113). The *AOXI* gene is tightly regulated by induction/repression, whilst the *MOXI* gene is regulated by repression/derepression, and catabolite repression of the two genes follows the series: methanol>glycerol>glucose>ethanol (31,83). The stringent regulation of the two promoters allows separation of the growth and production phases. Thus, the recombinant yeast can be grown to very high cell densities (103-133 g/l), resulting in high volumetric yields of the secreted heterologous protein (105). Like *S. cerevisiae*, these methylotrophs secrete low quantities of their native proteins, making purification of the recombinant protein simple and less tedious (38). Both yeasts are capable of post-transcriptional and post-translational modifications, such as glycosylation and signal peptide processing (38,113). In *P. pastoris*, fewer mannose units are added to the glycosylated proteins, whereas *H. polymorpha* tends to resemble *S. cerevisiae* in that it hyper-glycosylates secreted proteins (50). Maturation of the pre-pro structures in the secretory apparatus is believed to follow a universal eukaryotic scheme (38). Therefore, processing of the secretion signal is executed efficiently in these yeasts and the recombinant proteins can be directed by their natural signal sequences or as fusions with other secretion signals, such as the MFα1 secretion signal from *S. cerevisiae* (38,113). Alternatively, intracellular recombinant proteins can be targeted to the peroxisomes by fusing a universal C-terminal tripeptide motif of the consensus sequence S/A/C-K/R/H-L and subsequently purified by cell fractionation (38). Recombinant proteins have been produced in these yeasts in grams per liter quantities. The highest extracellular product concentration reported so far is 13.5 g/l of an *A. fumigatus* phytase secreted by *H. polymorpha* (84). As in other systems, expression in methylotrophs has bottlenecks, some of which are protein dependent. For instance, the proteolytic instability of some secreted proteins in *P. pastoris* requires constant pH monitoring. On the other hand, limitations in the secretory apparatus result in the formation of inclusion bodies and the intracellular accumulation of other proteins (50). The high costs of the carbon source glycerol and
operational problems related to the use of methanol as substrate, especially on an industrial scale, have been cited as some of the limitations associated with the use of expression systems based on the methanol utilization pathway (83).

The dimorphic non-pathogenic yeast, *Y. lipolytica*, has significant capacities for high molecular weight protein secretion, and the ability to grow to high cell densities (50,67). This alkane-utilizing yeast is also known for its efficient degradation of hydrophobic substrates (67). These recognizable features have rendered *Y. lipolytica* as an attractive host for heterologous gene expression. Expression vectors based on strongly regulated promoters of the *XPR2* and the *IC11* genes have been developed (67,93). *XPR2* encodes alkaline protease and its expression is tightly regulated by pH, carbon and nitrogen sources (50). Expression is only inducible in rich protein-containing media, but not in minimal media (67). The *IC11* gene encodes the glyoxisomal isocitrate lyase of the anaplerotic glyoxylate cycle. It is induced when carbon sources such as n-alkanes, fatty acids, ethanol and acetate are used, and is repressed to a basal level when cells are grown in the presence of glucose (67). Similarly, *K. lactis* and *P. stipitis* are being exploited for their unique substrate utilization pathways. *K. lactis* features the ability to grow on lactose as a sole energy and carbon source, while *P. stipitis* grows on xylose (38,98). These special features have provided a platform for dedicated, intensive studies that have led to the development of an array of expression vectors for heterologous protein expression in the yeasts discussed above. This, in turn, provides a variety of tools that can be used to relieve some of the limitations encountered when *S. cerevisiae* is used as a host.

Although *S. cerevisiae* has a low secretion capacity, it continues to be used as a host for heterologous protein production mainly because of the abundance of vector systems and transcriptional promoters that can be exploited to improve gene expression. A combination of factors, such as copy number, strong promoters and autoselection systems, can promote increased heterologous protein production in rich growth media. In chapter 2, this combination was used to produce the recombinant β-mannanase (Aa-Man5A) in *S. cerevisiae*. The production levels achieved were comparable to the levels reported for other heterologous proteins produced in *P. pastoris*. Therefore, depending on the intended application of the recombinant
enzyme, *S. cerevisiae* can still be used as preferred host for heterologous protein production.

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We have a habit in writing articles published in scientific journals to make the work as finished as possible, to cover up all the tracks, to not worry about the blind alleys or describe how you had the wrong idea first, and so on. So there isn't any place to publish, in a dignified manner, what you actually did in order to get to do the work

-Richard Phillips Feynman (1918-1988)
CHAPTER 2

Expression of the *Aspergillus aculeatus* endo-β-1,4-mannanase encoding gene (*man1*) in *Saccharomyces cerevisiae* and characterization of the recombinant enzyme

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EXPRESSION OF THE *ASPERGILLUS ACULEATUS* ENDO-\(\beta\)-1,4-MANNANASE ENCODING GENE (*MANI*) IN *SACCHAROMYCES CEREVISIAE* AND CHARACTERISATION OF THE RECOMBINANT ENZYME

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**ABSTRACT**

The endo-\(\beta\)-1,4-mannanase encoding gene *manI* of *Aspergillus aculeatus* MRC11624 was amplified from mRNA by polymerase chain reaction (PCR) using sequence specific primers designed from the published sequence of *manI* from *A. aculeatus* KSM510. The amplified fragment was cloned and expressed in *Saccharomyces cerevisiae* under the gene regulation of the alcohol dehydrogenase (*ADH2\(_{PT}\)*) and phosphoglycerate kinase (*PGK1\(_{PT}\)*) promoters and terminators, respectively. The *manI* gene product was designated Man5A. Subsequently, the FUR\(_I\) gene of the recombinant yeast strains was disrupted to create auto-selective strains: *S. cerevisiae* Man5ADH2 and *S. cerevisiae* Man5PGK1. The strains secreted 521 nkat/ml and 379 nkat/ml of active Man5A after 96 h of growth in a complex medium. These levels were equivalent to 118 mg/l and 86 mg/l of Man5A protein produced, respectively. The properties of the native and recombinant Man5A were investigated and found to be similar. The apparent molecular mass of the recombinant enzyme was 50 kDa compared to 45 kDa of the native enzyme due to glycosylation. The determined *K_m* and *V_{max}* values were 0.3 mg/ml and 82 \(\mu\)mol/min/mg for the recombinant, and 0.15 mg/ml and 180 \(\mu\)mol/min/mg for the native Man5A respectively. The maximum pH and thermal stability was observed within the range of pH 4 - 6, and 50°C and below. The pH and temperature optima and stability were relatively similar for recombinant and native Man5A. Hydrolysis of an unbranched \(\beta\)-1,4-linked mannan polymer released mannose, mannobiose and mannotriose as the main products.
INTRODUCTION

Endo-β-1,4-mannanase (β-mannanase EC 3.2.1.78) encoding genes of bacterial (2,3,5,6,7,14,27,28,48), and fungal (8,33,45) origin have been cloned and sequenced. Some of these genes encode multidomain proteins that in addition to the mannanase catalytic domain contain either one or two discrete non-catalytic cellulose-binding domains (CBDs) or both a CBD and an endoglucanase (7,14,33,45). The fungal β-mannanases from Aspergillus aculeatus and Trichoderma reesei belong to glycosyl hydrolase family 5 and the protein sequence revealed 58% identity (8,13,45). Three genes sequenced from the anaerobic fungus Piromyces were assigned to family 26 (33).

β-Mannanases are useful in several industrial processes, such as the extraction of vegetable oils from leguminous seeds and the reduction of viscosity in coffee extracts during the manufacturing of instant coffee (52). Recent developments indicate that the Aspergillus niger β-mannanase is potentially useful for the hydrolysis of galactomannan-based water-soluble polymers used in hydraulic fracturing of oil and gas wells (12,30). β-Mannanases can also be used for biobleaching of softwood kraft pulps to enhance the extractability of lignin (34,47). In nature galactoglucomannans predominantly occur in softwoods, whereas galactomannans are mostly found in seeds of leguminous plants and carob beans (3,13,27). The hydrolysis of these substrates is accomplished through the action of endo-β-1,4-mannanase, which randomly cleaves the β-mannosidic linkages within the main chain together with the exo-enzymes β-mannosidase and α-galactosidase.

Efficient industrial exploitation of these enzymes requires that different enzyme ratios can easily be obtained to formulate a range of enzyme cocktails aimed at specific applications. Conventional microbial strains used in industries usually produce a wide spectrum of extracellular enzymes that interfere with each other in specific applications, e.g. A. aculeatus produces cellulases that interfere with the use β-mannanases in pulp bleaching (47). Gene technology provides the possibility to express individual enzymes in host strains that do not produce such related enzyme activities thus avoiding the need for extensive purification for certain applications. Saccharomyces cerevisiae, which by itself does not produce cellulases or hemicellulases, has long been used as a host for heterologous protein production.
including fungal hydrolases (11,15,25,45,53). However, other non-conventional yeast strains have become important host organisms for foreign gene expression (16,40). Methylotrophic yeast, particularly *Pichia pastoris*, are currently used in preference to *S. cerevisiae* due to the ease of high-density growth and scale-up without any reduction of specific productivity (16,40). In *Pichia*, abundant concentrations up to 3 g/l of heterologous proteins are secreted into the medium following induction by methanol (21,23,39). However, the need to use defined media and methanol may present limitations for the choice of cultivation media in different industries. Efficient vector expression systems have been developed for heterologous protein production in *S. cerevisiae* (16,40). In addition, autoselection systems that ensure plasmid maintenance were devised to avert limitations such as low expression levels resulting from the use of selective minimal media (9,22,26,38). Such systems have proved to be advantageous as selection occurs independently of the growth medium, hence, they allow use of relatively inexpensive complex media of choice (10,26). Autoselection systems based on double mutations of the chromosomal *ura3* and *fur1* genes in the pyrimidine biosynthesis pathways, and complementation of *ura3* with a plasmid-borne *URA3* gene have been reported in *S. cerevisiae* and *Kluyveromyces lactis*. In both host strains improved volumetric activities were reported using complex media as compared to minimal media (11,22,25,26).

The aim of the current study was to efficiently produce the *A. aculeatus* β-mannanase in *S. cerevisiae* and to compare the properties of the purified native and recombinant enzyme in order to evaluate the applicability of this expression strategy. We describe the production of the *A. aculeatus* MRC11624 β-mannanase in *S. cerevisiae* under auto-selective conditions that permits cultivation of the recombinant strains in complex non-selective media. This resulted in high production levels of the recombinant protein. The recombinant enzyme was characterised and compared to the native fungal enzyme with regard to biochemical properties and catalytic activity.
MATERIALS AND METHODS

Microbial strains and plasmids
Detailed descriptions of the strains and plasmids used in this study are summarised in Table 1.

Media and cultivation conditions

*Aspergillus aculeatus* MRC11624 characterised by Dr. R.A. Sampson (Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands) as *Aspergillus aculeatus* Iizuka, was maintained on potato dextrose agar plates and cultivated at 30°C in a basal medium containing 0.5% locust bean gum (LBG)\(^4\) (Sigma, Stockholm, Sweden). *S. cerevisiae* Y294 was cultivated on either complex yeast

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

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
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<td><strong>Strains:</strong></td>
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<tr>
<td><em>S. cerevisiae</em> Y294</td>
<td>MATα leu2-3,112 ura3-52 his3 trp1-289</td>
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<td>Wild type strain</td>
<td>This laboratory</td>
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<td><em>E. coli</em> XL1-Blue</td>
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<table>
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<td>pBluescript SK(^+)</td>
<td>bla</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pDLG1</td>
<td>bla URA3 ADH2(_{PT})</td>
<td>25</td>
</tr>
<tr>
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<td>bla URA3 PGK1(_{PT})</td>
<td>11</td>
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<td>24</td>
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<td>bla URA3 ADH2(_{P-man1-ADH2})</td>
<td>This study</td>
</tr>
<tr>
<td>pMES2</td>
<td>bla URA3 PGK1(_{P-man1-PGK1})</td>
<td>This study</td>
</tr>
</tbody>
</table>
peptone glucose (YPD) or a selective synthetic complete (SC) medium supplemented with the appropriate amino acids (41) at 30°C. *E. coli* XL1-Blue (Stratagene, California, USA) was cultivated at 37°C in Luria Bertani (LB) medium supplemented with 70 μg/ml ampicillin for plasmid selection (43).

**PCR amplification of the man1 gene**

Total RNA was prepared from the mycelia of *A. aculeatus* MRC11624 as described by Crous *et al* (11). The poly (A)* mRNA was purified from total RNA using the Oligotex™ mRNA isolation kit (Qiagen, Hilden, Germany). First strand cDNA was synthesised from 116 ng of mRNA using a first strand cDNA synthesis kit (Roche Molecular Biochemicals, Ottweiler, Germany), and used as template for amplification of the β-mannanase encoding gene *man1* by PCR on a Biometra Trio Thermoblock TB1 (Biometra Biomedizinische Analytik, Göttingen, Germany). The primers used were designed based on the sequence of the *man1* gene of *A. aculeatus* strain KSM510 (Accession. No. L35487) (8):

MANR (*XhoI* restriction site; 28-mer)

5′-CTAGCTCGAGCGCCAACAGTCTACTTCG

MANL (*EcoRI* restriction site; 28-mer)

5′-GATCGAATTCCACCACCACACAACAG

The amplification reaction was carried out using an Expand™ High Fidelity PCR system (Roche Molecular Biochemicals, Ottweiler, Germany). The reaction yielded a 1265 bp fragment, which was digested with *EcoRI* and *XhoI*, and ligated to plasmid pBluescript. The sequence was analysed on an automated sequencing system using theromesequenase fluorescent-labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, UK). For expression in *S. cerevisiae* the fragment was ligated into plasmids pDLGI and pJC1 resulting in plasmids pMES1 (containing the alcohol dehydrogenase, *ADH2pt*), and pMES2 (with the phosphoglycerate kinase, *PGK1pt*) promoter and terminator, respectively. The latter plasmids were transformed into *S. cerevisiae* Y294 using the dimethyl sulfoxide-enhanced lithium acetate method (19).
Construction of auto-selective strains

The FUR1 gene encoding UPRTase⁴ that catalyzes the conversion of uracil to UMP⁴ in the pyrimidine salvage pathway of S. cerevisiae, was disrupted with the fur1:: LEU2 allele as previously described by La Grange et al (25). Integration of the linear fur1::LEU2 employed a one-step gene disruption method demonstrated by Rothstein (42). This resulted in replacement of the FUR1 gene on chromosome VIII of the two transformants: S. cerevisiae Y294 (pMES1) and S. cerevisiae Y294 (pMES2), generating S. cerevisiae Y294 (fur1::LEU2 pMES1) and S. cerevisiae Y294 (fur1::LEU2 pMES2), respectively. The auto-selective strains were then designated S. cerevisiae Man5ADH2 and S. cerevisiae Man5PGK1. These strains both expressed the same man1 gene, however, gene regulation was under different promoters (ADH2 and PGK1). The β-mannanase product formed from the expression of man1 was named Man5A in this study following the scheme proposed by Henrissat et al (17).

Southern- and Northern-blot analysis

Total DNA from the yeast strains was digested with NsiI and NcoI, separated on a 1% agarose gel and transferred onto a Hybond™-N nylon membrane (Amersham International, Buckinghamshire, England). The membrane was pre-hybridised for 4 h at 60°C and probed with an α²³P-labelled 1330 bp FUR1 fragment isolated from plasmid pPE (24). Total RNA was isolated from 24-h old cultures grown in YPD medium, 5 μg samples of RNA was separated on a 0.8% agarose/formaldehyde denaturing gel and transferred onto Hybond-N nylon membrane. The membrane was pre-hybridised and probed with the α-²³P-labelled man1 cDNA fragment at 42°C. All the hybridisation and post-hybridisation procedures were done as described by Sambrook et al (43). The DNA probes were labelled using a random-primed labelling kit following the manufacturers’ recommendations (Roche Molecular Biochemicals, Ottweiler, Germany).

Enzyme assays

The Man5A secreting yeast strains were cultivated in 100 ml YPD medium in 500 ml conical flasks and incubated on a rotary shaker at 150 rpm. Production of active Man5A by the yeast strains was analysed as described by Stålbrand et al. (46) using 0.5% LBG as a substrate. The cell-free culture filtrates used as enzyme sources
were collected by centrifugation at 3,000 × g and appropriately diluted in 50 mM citrate buffer at pH 5.3. The reactions were carried out at 50°C for 10 min after which the release of reducing sugars was determined using the modified DNS method (32). Absorbency was measured at 540 nm and Man5A activity was expressed in katals (1 kat = 1mol s⁻¹) (4).

Purification of Man5A

*S. cerevisiae* Man5ADH2 was chosen for biochemical characterisation of the recombinant enzyme. The cultures (100 ml) were grown in SC medium in 500 ml shake flasks for 60 hrs at 30°C. Cells were removed by centrifugation at 3,000 × g and the extract (500 ml) was precipitated with ice cold acetone in a ratio of 1:1.5. The precipitate was collected by centrifugation and re-suspended in cold 50 mM citrate buffer at pH 5.3. The protein was purified by anion exchange chromatography and gel filtration on an FPLC® system (Pharmacia, Uppsala, Sweden) using the method adopted from Adermark *et al* (1). The anion exchange column ResourceQ 6 ml (Pharmacia, Uppsala, Sweden) was equilibrated with 20 mM TEA-HCI buffer pH 7.5. Proteins were eluted with a linear salt gradient (0 - 45%) of 1 M NaCl in the same buffer at a flow rate of 1 ml/min. Further purification by gel filtration was carried out using a 100 mM phosphate buffer pH 6.5 containing 200 mM NaCl. The native protein from *A. aculeatus* MRC11624, was purified as described by Adermark *et al* (1). The cultivation was carried on for 120 hrs at 30°C. The level of purity of proteins was analysed by SDS-PAGE gels stained with a silver express staining kit (NOVEX, Novel Experimental Technology, San Diego, USA). Protein concentrations were measured using Micro-BCA protein assay reagent (PIERCE, Illinois, USA).

Protein preparation and Western blot analysis

The culture filtrates were concentrated using Microsep filtron centrifugal concentrators (Filtron Scandinavia, Bjarred, Sweden) with a molecular weight cut-off of 10 kDa. Five microgram of protein was denatured at 70°C in SDS-denaturing buffer containing 0.5 M DTT (NOVEX, Novel Experimental Technology, San Diego, USA) before separation on SDS-PAGE. Two microgram of protein was denatured at 100°C in 0.5% SDS/1% β-mercapto-ethanol denaturing buffer, and treated with 0.25 U PNGase F (NewEngland BioLabs, Hertfordshire, England) for 1 h
at 37°C. The reaction mixture was denatured again at 70°C in DTT containing buffer. Proteins were separated on a (4 - 12%) Tris-Glycine SDS-PAGE pre-cast gel following the manufacturers' instructions (NOVEX). Following transfer to a nitrocellulose membrane using a Semi dry blotter II (KEM-EN-TEC, Copenhagen, Denmark), the membranes were exposed to β-mannanase antiserum (Antibody AB, Lund, Sweden) raised against the *A. niger* β-mannanase (1). The formed antigen-antibody complex was detected with a goat anti-rabbit IgG [H+L] alkaline phosphatase conjugate (Bio-Rad, California, USA). The membrane was developed in a BCIP^4^/NBT^4^ colour development reagent (BioRad, California, USA).

**The effect of pH and temperature on the activity of Man5A**

The optimal pH of the enzyme was determined using 0.5% LBG prepared in 50 mM citrate-phosphate buffer (pH 2.6 - 6) and 50 mM phosphate buffer (pH 7 - 8). The enzyme diluted to 1.2 μg was incubated for 10 min with the substrate and subsequently assayed for mannanase activity using standard assay methods. The stability of the enzyme was measured after a 24-h incubation of the enzyme at 50°C within the same pH ranges. Thermal optima and stability studies were carried out at temperatures ranging from 4°C to 70°C at pH 5.3 in 50 mM citrate buffer. The same protein concentration was used as for the pH experiments. For stability studies, the enzyme was incubated in the absence of the substrate for 24 h and 100 μg/ml of BSA^4^ (Sigma) was included in the reaction mixtures. After the 24-h incubation, the standard enzyme-substrate reaction was performed for 10 min. Residual activities were calculated as a percentage of the initial activities detected before incubation for 24 h at various temperature or pH conditions.

**Hydrolysis of Ivory nut mannan (Phytelephas macrocarpa)**

A 5-mg/ml stock solution of ivory nut mannan (Megazyme, Australia) was prepared in 20 mM sodium acetate buffer pH 4.5 as previously described (1). The hydrolysis was carried out for 48 h at 40°C with a final concentration of 2 nkat Man5A/mg substrate. The reaction was terminated by inactivating the enzyme at 100°C for 5 min. Samples were diluted 50 times in deionised water before loading, and the substrate incubated under the same conditions without the enzyme was used as a control. Hydrolysis products were isocratically separated on a Dionex CarboPac
PA 100 column (Dionex, Sunnyvale, USA) using 100 mM NaOH as an eluent. The analyses were performed on the Dionex 500 chromatographic system equipped with an ED40 electrochemical detector and was controlled using PeakNet® software (Dionex).

**Determination of kinetic properties**

The substrate (LBG) used for kinetic experiments was pre-treated with ethanol to remove the water insoluble material as described by Arcand *et al.* (3). Experiments were run in triplicates and the averages were used for the determination of initial hydrolysis rates of the enzyme at substrate concentrations ranging from 0.2 to 2.5 mg/ml. The reactions were carried out at pH 5 and 50°C in 50 mM acetate buffer. Samples were collected from 2 to 10 min and assayed for activity. The Michaelis-Menten kinetic parameters, $K_m$ and $V_{max}$ were calculated from the Lineweaver-Burk and Hanes-Woolf plots.

**RESULTS**

**Amplification of the manl gene from *A. aculeatus* MRC11624**

The endo-β-1,4-mannanase encoding gene, *manl* of *A. aculeatus* MRC11624 was amplified by PCR from a cDNA-mRNA hybrid as a 1180 bp fragment, using primers based on the sequence of the *manl* gene of *A. aculeatus* KSM510 (8). The gene was ligated to pBluescript and sequenced, and the nucleotide sequence showed 99.7% identity with the sequence of the *A. aculeatus* KSM510 as reported by Christgau *et al.* (8). Three base-pair descrepancies were detected on the DNA level and one resulted in an amino acid sequence difference (Ser → Thr) at position 225. This region is variable according to sequence alignment by Hilge *et. al* (18) and is not crucial for its structure and function. The neutral Ser→Thr substitution is thus not likely to affect the enzyme activity.
Expression and production of active Man5A in *S. cerevisiae*

The *manl* gene amplified by PCR was also ligated to *URA3*-based yeast multicopy shuttle vectors under the transcriptional control of the *ADH2* and the *PGK*, generating plasmid pMES1 and pMES2, respectively. These plasmids were introduced into *S. cerevisiae* strain Y294, and selected on SC-URA plates. In addition, the chromosomal *FUR1* gene of the recombinant yeast strains was replaced with the *fur1::LEU2* allele. The gene replacement was confirmed by Southern blot analysis performed on the genomic DNA isolated from the parental strain *S. cerevisiae* Y294 and from the two recombinant strains: *S. cerevisiae* Man5ADH2 and *S. cerevisiae* Man5PGK1. The non-disrupted wild type *FUR1* gene was highlighted as a 1.3 kb fragment and the disrupted gene in the recombinant strains as 3.27 kb fragments (data not shown). This disruption resulted in auto-selective Man5A producing *S. cerevisiae* strains, which could be grown and maintained on any medium of choice.

Northern blot analysis was used to determine the transcription of the β-mannanase encoding gene *manl* in the recombinant yeast strains. The *manl* gene is efficiently transcribed in the yeast under the regulation of both the *ADH2* and *PGK1* (Fig. 1). The yeast strains secreted active Man5A into the medium, reaching levels as high as 521 nkat/ml and 379 nkat/ml under the *ADH2* and *PGK1*, respectively (Fig.2).
Fig. 1. A Northern blot analysis of total RNA isolated from *S. cerevisiae* Y294 (1), *S. cerevisiae* Man5ADH2 (2), and *S. cerevisiae* Man5PGK1 (3), shows the transcripts encoded by the *man1* gene in the two transformants after 24 hours of growth in a complex YPD medium. The *man1* fragment isolated by PCR from first strand cDNA was used as an [α-32P]-labelled probe.
Fig. 2. Growth and production of an active Man 5A by the recombinant yeast strains: *S. cerevisiae* Man5ADH2 (A) and *S. cerevisiae* Man5PGK1 (B). Growth ($\nu$) was measured as cell density at 620 nm, and activity ($\lambda$) as the release of reducing sugars from locust bean gum galactomannan.

**Biochemical properties of the native and recombinant Man5A**

Purification of the native and recombinant Man5A was achieved in a three-step process which involved either ammonium sulphate or acetone precipitation, anion exchange and gel-filtration chromatography from the culture filtrates of *A. aculeatus* Iizuka and *S. cerevisiae* Man5ADH2. Table 2 shows the purification profile of the two enzymes, Fig. 3 (lanes 1 and 2) show the purified fractions after gel filtration. The recombinant enzyme was purified 20 fold to specific activity of 1077 nkat/mg and the native protein was purified 60 fold to electrophoretic homogeneity with the specific activity of 4402 nkat/mg. The enzymes behaved very similar showing optimal activity around pH 3, at 50°C, Fig. 4A. When incubated at the same pH values without the substrate both enzymes retained 90% activity at pH 4 – 6 after 24 hrs (Fig. 4B). The temperature optima and stability profiles showed that the enzymes have optimal
Table 2. Purification table of the recombinant and native Man5A

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total activity (nkat)</th>
<th>Total protein (mg)</th>
<th>Specific activity (nkat/mg)</th>
<th>Purification fold</th>
<th>Activity yield (%)</th>
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<tbody>
<tr>
<td>Purification from <em>S. cerevisiae</em> Man5ADH2</td>
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<td>Culture filtrate</td>
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<td>(100)</td>
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<td>Acetone precipitation</td>
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<td>2949</td>
<td>8.7</td>
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<td>0.8</td>
<td>4402</td>
<td>64</td>
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</table>
Molecular mass and glycosylation

Analysis with SDS-PAGE gave the apparent molecular mass of 50 kDa and 45 kDa for the purified recombinant and native Man5A, respectively (Fig. 3 lanes 1 and 2). A faint smear of higher molecular mass material ranging from 51 – 95 kDa was visible in the recombinant protein preparation. The molecular mass as highlighted by Western blot analysis corresponded with the purified proteins (Fig. 3, lanes 3 and 5). The high molecular mass material in the recombinant Man5A was immuno-reactive, indicating Man5A with heavy outer-chain glycosylation. Treatment of the recombinant and native Man5A with PNGase F resulted in a mobility shift in both
proteins yielding a product with the same molecular mass of 41 kDa (Fig. 3. lane 4 and 6).

Fig. 4. The effect of pH on the activity (A) and stability (B) of the purified native Man5A (○) and the recombinant Man5A (λ) as determined at 50°C. The stability was measured after a 24-h incubation of the enzymes at different pH values in the absence of the substrate.

Fig. 5. The effect of change in temperature on the activity (A) and stability (B) of the purified native Man5A (○) and recombinant Man5A (λ) at pH 5.3. All stability studies were carried out in the presence of 100 µg/ml of BSA.
Substrate hydrolysis and kinetic properties

Some of the hydrolytic and kinetic properties were determined for the purified recombinant and native Man5A. Ivory nut mannan, an insoluble polymer of β-1,4-linked mannose residues was hydrolysed with native and recombinant Man5A at 40°C for 48 h. The products detected at the end of the hydrolysis were mainly mannose, mannobiose and mannotriose (Fig. 6). The molar ratio of mannobiose/mannotriose released by the recombinant Man5A was calculated to be 1.8 whereas that of the native Man5A was 1.5.

The native enzyme showed slightly higher affinity towards galactomannan with a $K_m$ of 0.15 mg/ml ± 0.02 whereas that of the recombinant mannanase was 0.3 mg/ml ± 0.06. The $V_{\text{max}}$ was calculated as 180 μmol/min/mg for the native and 82 μmol/min/mg for the recombinant enzyme.

Fig. 6. A chromatographic representation of products detected after hydrolysis of ivory nut mannan with purified Man5A. (A), the chromatogram obtained from non-hydrolysed mannan. (B), represents the sugars released after hydrolysis with purified native Man5A, and (C), with recombinant Man5A. M1 (mannose), M2 (mannobiose), M3 (mannotriose).
DISCUSSION

This paper presents high expression levels of the Man5A coded for by the man1 gene from A. aculeatus MRC11624 in S. cerevisiae. The man1 gene was cloned and expressed in S. cerevisiae under the ura3 fur1 auto-selection system that ensures maintenance of the plasmid carrying the gene of interest irrespective of the cultivation conditions and promotes increased biomass and product formation (26,40). The man1 gene was expressed under the regulation of two yeast promoters, PGK1p and ADH2p. The PGK1p promoter, is a glycolytic promoter which can be expressed to levels greater than 1% of total RNA or 4 -10% of the soluble protein on fermentable carbon sources (20,40,44). The ADH2p promoter is powerful and tightly regulated by glucose repression, however cultures can be grown to high cell density and derepression of the promoter results in high expression levels (37,40,44). In the present study, 379 nkat/ml of active extracellular recombinant Man5A were produced under the transcriptional control of the PGK1p, and 521 nkat/ml under the ADH2p, after 60 hrs of growth in YPD medium. As estimated from the specific activity of the native enzyme, the activity levels that were measured are equivalent to 86 mg/L and 118 mg/L of Man5A produced, respectively. β-Mannanase encoding genes of various origins have been cloned and expressed either in yeast or bacterial host strains which do not secrete cellulases or other hemicellulases, eventually reducing unnecessary purification processes. Table 3 summarises β-mannanase activities and production levels reported in different hosts. The production levels attained in the current study are relatively higher than those reported for other heterologously expressed β-mannanases. The Man5A level produced by S. cerevisiae Man5PGK1 is about 570 fold higher than that reported for the expression of the Man5A of T. reesei in S. cerevisiae under the regulation of the same promoter (45). When man1 is expressed in S. cerevisiae Man5ADH2 the Man5A production level increased by 37%. This high production of the recombinant Man5A can be attributed to the ability to grow cultures in complex medium without the risk of losing the episomal plasmid.

The purified recombinant Man5A had a higher molecular mass than the native Man5A, but after enzymatic deglycosylation they both migrated with the apparent
Table 3. β-Mannanase activity levels measured from expression in different hosts.

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Gene</th>
<th>Expression Host</th>
<th>Gene Product Name</th>
<th>Enzyme Activity (nkat/ml)</th>
<th>Production Levels (mg/l)</th>
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<td>E. coli RR28</td>
<td>ManA</td>
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<td>10.2</td>
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<tr>
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<td>man1</td>
<td>S. cerevisiae DBY746</td>
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<td>0.15</td>
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<td>man1</td>
<td>A. oryzae</td>
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<td>nd</td>
<td>nd</td>
<td>8</td>
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<td>S. cerevisiae Y294</td>
<td>Man5A</td>
<td>521 (ADH2_p)</td>
<td>118</td>
<td>This study</td>
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<tr>
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<td>S. cerevisiae Y294</td>
<td>Man5A</td>
<td>379 (PGK1_p)</td>
<td>86</td>
<td>This study</td>
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nd: not determined
molecular mass of 41 kDa. This correlates with the calculated molecular weight of the *A. aculeatus* Man5A (8) and is also comparable to the value reported for the *A. niger* β-mannanase (1). The recombinant Man5A had an additional 32 kDa immuno-reactive product, which could possibly be due to proteolysis. Mannose, mannobiose and mannotriose were the main products detected after hydrolysis of ivory nut mannan with either the native or recombinant enzyme. This indicates that Man5A is able to hydrolyse substrates with the DP$_4$ up to 4. Interestingly, the *A. aculeatus* enzyme in the present investigation released significantly higher proportions of mannose, which was not detectable for the *T. reesei* and *A. niger* enzymes. Torto et al. (50,51) and Ademark et al. (1) showed that the major products are mannotriose and mannobiose, and mannose may be formed due to secondary hydrolysis of mannotriose.

The kinetic parameters of the native and recombinant man5A were slightly different, with the $K_m$ of 0.15 mg/ml and 0.3 mg/ml on LBG, respectively. The difference could potentially be the effect of the higher degree of glycosylation of the recombinant enzyme. The $K_m$ of the *A. aculeatus* Man5A produced in *A. oryzae* has been reported as 0.7 mg/ml on the same substrate (8).

In conclusion, the recombinant Man5A showed similar properties to the native enzyme, that correlate with those reported by Christgau et al. (8), and also to other fungal β-mannanases (1,8,29,46). Therefore, the recombinant yeast expression system is valuable for industrial applications, as *S. cerevisiae* does not produce other enzyme activities, which are likely to interfere during processing. Expression of the *A. aculeatus* MRC11624 mannanase in *S. cerevisiae* under auto-selective conditions, presents an option for producing increased amounts of Man5A free from other hemicellulases. In this study we achieved high β-mannanase production levels in *S. cerevisiae*. These levels are comparable with those achieved for other hydrolases in shake flask cultivation using *Pichia pastoris* as the expression host (21,23,35,39). Higher levels can be attained by designing fermentation and downstream processing strategies that promote better expression, secretion and recovery of Man5A in *S. cerevisiae*. 

72
Acknowledgements
The National Research Foundation (NRF) in South Africa and the Swedish Foundation for International Co-operation in Research and Higher Education (STINT) supported this study. The Swedish Research Council for Engineering Sciences (TFR) is gratefully acknowledged for a grant to Henrik Stålbrand.

Abbreviations used:
PCR, polymerase chain reaction; \(PGK_{PT}\), phosphoglycerate kinase promoter and terminator; \(ADH2_{PT}\), alcohol dehydrogenase promoter and terminator; \(K_m\), Michaelis constant; \(V_{max}\), maximum reaction velocity; CBD, cellulose-binding domain; SC, synthetic complete medium, minimal medium containing 2% glucose, 0.67% yeast nitrogen base (without amino acids), supplemented with necessary amino acids required for growth and plasmid maintenance; YPD, 1% yeast extract/ 2% peptone/ 2% glucose; UPRTase, uracil phosphoribosyl transferase; UMP, uridine 5'-monophosphate; LBG, locust bean gum a galactomannan polysaccharide from seeds of Ceratonia siliqua; DNS, dinitro-salicylic acid; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide-gel electrophoresis; DTT, dithiotritol; PNGase F, peptide N-Glycosidase F; BCIP, 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt; NBT, p-nitro blue tetrazolium chloride; BSA, bovine serum albumin; Da, Dalton; DP, degree of polymerisation.

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CHAPTER 3

The effect of signal peptide replacement and cultivation temperature on the production and secretion of fungal endo-1,4-β-mannanases in Saccharomyces cerevisiae

Submitted to

FEMS Yeast Research
THE EFFECT OF SIGNAL PEPTIDE REPLACEMENT AND CULTIVATION TEMPERATURE ON THE PRODUCTION AND SECRETION OF FUNGAL ENDO-1,4-β-MANNANASES IN SACCHAROMYCES CEREVISIAE

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Abstract
Heterologous expression and production of the family 5 endo-1,4-β-mannanases (Man5A) from the ascomycetous fungi Aspergillus aculeatus (Aa-Man5A) and Trichoderma reesei (Tr-Man5A) in Saccharomyces cerevisiae were evaluated. The Man5A encoding genes (man1) were expressed in episomal multicopy shuttle vectors using the constitutive phosphoglycerate kinase promoter and terminator (PGK1PT) sequences to regulate expression. Recombinant autoselective fur1::LEU2 S. cerevisiae strains expressing Man5A were constructed. S. cerevisiae AMan5-1 and S. cerevisiae AMan5-2 produced Aa-Man5A using either the native β-mannanase secretion signal or the yeast MFα1S secretion signal, and secreted 99.4 mg l⁻¹ and 86 mg l⁻¹ of active enzyme, respectively. S. cerevisiae TMan5-1 secreted 13 mg l⁻¹ of Tr-Man5A directed by the native secretion signal. Removal of the cellulose-binding domain (CBD) from Tr-Man5A (yielding Tr-Man5AΔCBD) in S. cerevisiae TMan5-2 yielded 10 mg l⁻¹ active enzyme, and did not affect secretion from its native secretion signal significantly. S. cerevisiae TMan5-3 secreted Tr-Man5AΔCBD with the yeast MFα1S secretion signal at a level of 16 mg l⁻¹ active enzyme. Replacement of the native secretion signals of Aa-Man5A and Tr-Man5AΔCBD with the yeast MFα1S secretion signal had marginal effects on their secretion efficiencies. However, S. cerevisiae producing Aa-Man5A with its
native secretion signal exhibited limited cell growth, whereas *S. cerevisiae* producing Aa-Man5A with the MFα1s secretion signal displayed normal cell growth. Interestingly, Man5A production in *S. cerevisiae* AMan5-1 increased with an increase in cultivation temperature (30°C>25°C>20°C). In contrast, *S. cerevisiae* TMan5-1 and TMan5-2 produced more enzyme at lower cultivation temperature (20°C>25°C>30°C).

INTRODUCTION

Endo-1,4-β-mannanases are plant cell wall degrading enzymes that are widely distributed in nature and have been isolated, sequenced and characterized from various sources [1-7]. β-Mannanases are classified into two families of glycosyl hydrolases, GH-5 and GH-6, and are designated Man5 and Man26, respectively [8]. Both Man5 and Man26 are retaining endohydrolases and display a prominent cleft in which the active site is situated [9-11]. Typically this architecture allows cleavage of the substrate in the middle of the chain [12]. β-Mannanases randomly hydrolyze mannan polysaccharides into oligosaccharides of various lengths, which together with β-mannosidase and α-galactosidase are degraded into monosaccharides [13].

β-Mannanases naturally occur as single catalytic domains or as part of modular proteins. These modular proteins sometimes include other catalytic domains such as endoglucanase. Putative carbohydrate binding modules (CBMs) such as cellulose binding domains (CBDs) and mannan binding domains (MBDs) are also included [1,4,6,14]. Different domains are joined by recognizable Pro-Ser-Thr rich spacers often referred to as PT-boxes that act as flexible hinges between functionally distinct portions of the modular enzymes [15]. CBMs are thought to enhance activity by increasing local enzyme concentration on the substrate surface or by disrupting non-covalent interactions and thereby increasing substrate accessibility [16]. *Trichoderma reesei* (teleomorph: *Hypocrea jecorina*) produces a two-domain β-mannanase (Tr-Man5A), comprising a GH-5 catalytic core and a CBD connected by a PT-box, which is presumably O-glycosylated in the native structure [6]. Recently the three-dimensional structure of the Tr-Man5A catalytic domain was resolved [11]. Another ascomycetous fungus,
Aspergillus aculeatus, secretes a single domain enzyme (Aa-Man5A) with Man5 catalytic activity [2]. The man1 genes encoding the Man5A from both fungi have previously been cloned and the encoded proteins share 60% identity. Seven amino acids found in and around the active site of Tr-Man5A are also conserved in Aa-Man5A. Both these β-mannanases have been heterologously expressed in S. cerevisiae [6,13]. Aa-Man5A was efficiently produced in S. cerevisiae at levels that were comparable to enzymes produced in Pichia pastoris [13]. Conversely, production of Tr-Man5A in S. cerevisiae was reported to be relatively poor [6].

β-Mannanases have potential applications in the modification of poultry feeds based on legume seeds such as soybean, guar bean and alfalfa [17]. The seeds contain high concentrations of galactomannan, which if fed in large quantity results in reduced nitrogen retention, fat absorption and amino acid uptake, adversely affecting chicken growth [17-18]. Addition of β-mannanase to the feed has been shown to alleviate growth depression in chickens [17-18]. β-Mannanase producing S. cerevisiae strains could serve as an important probiotic in this type of application. However, efficient secretion of recombinant β-mannanases is important for these biotechnological applications.

The prepro-leader of the yeast mating pheromone α-factor (MFα1) is regarded as the classical yeast secretion signal, and has frequently been used to direct secretion of heterologous proteins from S. cerevisiae [19-20]. However, signal peptides are recognized with low specificity in eukaryotes and in prokaryotes [21-22]. Consequently, heterologous proteins have been successfully secreted using either native secretion signals, or as hybrids with foreign secretion signals [22-26]. Synthetic prepro-leaders lacking consensus N-linked glycosylation sites and the dibasic KEX2 endoprotease processing site have also been used to facilitate secretion [20]. Recently, the involvement of monobasic-specific aspartyl proteases (yapsin 1 and yapsin 2) of S. cerevisiae in secretion was identified. These proteases act as “secretases” that cleave secretion signals adjacent to basic amino acids, with a preference for monobasic sites [27,28].

Environmental factors such as temperature and pH can influence the secretion capacity of heterologous proteins [29-32]. Temperature in particular has often been exploited to improve the secretion of heterologous proteins in yeast [30-32]. Nagashima et al. [30] showed that high production levels can be achieved by shifting cultivation
temperature from optimal growth temperature to lower temperatures. This concept was shown to work favorably for secretion of several other proteins [31-32].

This paper reports the influence of the nature of the secretion signal and cultivation conditions on the production and secretion of Aa-Man5A and Tr-man5A in S. cerevisiae. Our objective was to evaluate the secretion efficiency of autoselective Man5A producing S. cerevisiae strains using either their native or the yeast MFα1S secretion signal, and to study the influence of cultivation temperature on secretion. In addition, the effect of the removal of the CBD of Tr-Man5A on secretion was also evaluated.

**Materials and methods**

**Microbial strains and cultivation conditions**

Microbial strains and plasmids used in the current study are summarized in Table 1. Recombinant *Escherichia coli* and *S. cerevisiae* strains were cultivated and maintained as previously described [13]. Yeast was cultivated in 100 ml yeast/peptone/glucose (YPD) medium in 500 ml conical flask at 30°C and 120 rpm on a rotary shaker.

**Plasmid constructions**

The Man5A encoding gene (*manl*) of *Trichoderma reesei* was isolated from plasmid pHSM1 [6] as an EcoRI/XhoI fragment and ligated to plasmid pJC1 [33] digested with the same enzymes to generate plasmid pMES3. Plasmid pPH003 [34] was first digested with *SacII* and the overhanging ends filled in with Klenow. The plasmid was subsequently digested with *BamHI* to release the truncated *manl* fragment, *manlΔcbd*. This fragment was ligated to plasmid pJC1 that had been digested with *EcoRI* overhanging ends filled in, followed by digestion with *BglII* resulting in plasmid pMES6.
Table 1. A list of plasmid vectors and microbial strains used in this study

<table>
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<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Reference</th>
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<td><em>E. coli</em> XLI-Blue</td>
<td>MRF’ endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F’proAB lacIq ZΔM15 Tn10(tet)]</td>
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<td><em>S. cerevisiae</em> Y294</td>
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<td><em>S. cerevisiae</em> Man5PGK1</td>
<td>bla MAN1</td>
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<td><strong>2.3 Plasmids</strong></td>
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<td>bla man1</td>
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<tr>
<td>pPH003</td>
<td>bla man1Δcbd</td>
<td>[34]</td>
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<td>bla URA3 PGK1&lt;sub&gt;PT&lt;/sub&gt;</td>
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<tr>
<td>pPE</td>
<td>bla TRP1 FUR1</td>
<td>[37]</td>
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<td>pDF1</td>
<td>bla fur1::LEU2</td>
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<td>pMES3</td>
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<td>pMES13</td>
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<td>This study</td>
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The yeast MFα₁<sub>S</sub> secretion signal was isolated from plasmid pRLR1 (Table 1) as an EcoRI/XhoI fragment and ligated into pBluescript SK<sup>+</sup> pre-cut with the same enzymes, to generate plasmid pMF01. Primers (Table 2) were designed to amplify sequences coding for Aa-Man5A from pMES2 [13] and Tr-Man5A from pMES6, respectively, by polymerase chain reaction (PCR) on a GeneAmp<sup>®</sup>PCR system 2400 (Perkin-Elmer, Norwalk, CT USA).
Table 2. A list of primers used to isolate sequences coding for mature β-mannanase from A. aculeatus and T. reesei and to construct MFα1-man1 fusion proteins.

<table>
<thead>
<tr>
<th>Primer sequences (5’ to 3’)</th>
<th></th>
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<tbody>
<tr>
<td><strong>A. aculeatus</strong> NFOR (ClaI restriction site; 24-mer):</td>
<td>GCTTATACGATACACCACCGCCTTC</td>
</tr>
<tr>
<td><strong>A. aculeatus</strong> MANR (XhoI restriction site; 28-mer):</td>
<td>CTAATCGCGGCGCAGCAGTCTACTTCG</td>
</tr>
<tr>
<td><strong>T. reesei</strong> FHIND (HindIII restriction site; 30-mer):</td>
<td>GCTGAAGCTTATGCTTCGAGCTTTGTAACC</td>
</tr>
<tr>
<td><strong>T. reesei</strong> CREV (BamHI restriction site; 24-mer):</td>
<td>TGGTGAGGAGGAGGAGATCCCTAGG</td>
</tr>
</tbody>
</table>

The PCR fragments were fused to the MFα1S secretion signal in pMF01. The man1 gene of A. aculeatus was fused as a ClaI/XhoI fragment, whereas the man1 gene of T. reesei was fused into the HindIII/BglII sites as a HindIII/BamHI fragment. The MFα1 Glu-Ala dipeptides were retained in both constructs. MFα1S-Man5A fusion series were isolated with EcoRI/XhoI and ligated into pJC1 to generate plasmids pMES13 and pMES14 containing the fusion genes for MFα1S-AaMan5A and MFα1S-Tr-Man5A, respectively. Fig. 1 summarizes these different constructs used for expression of man1. Plasmids were propagated in E. coli XL-1 Blue and routine DNA manipulations were carried out following standard procedures by Sambrook et al. [35].

Plasmids pMES3, pMES6, pMES13 and pMES14 are shuttle vectors carrying different Man5A encoding constructs under the transcriptional regulation of the phosphoglycerate kinase (PGK1pr) promoter and terminator. The plasmids were introduced into S. cerevisiae Y294 using the lithium acetate method [36]. Subsequently, the FUR1 gene of the recombinant strains was replaced with a fur1::LEU2 disruptive
allele to construct autoselective strains [23]. The FUR1 replacement was confirmed by Southern blot analysis using an α-32P-labeled FUR1 fragment isolated from pPE [37].

![Man5A constructs schematic](image)

**Fig.1.** A schematic representation of man1 constructs used for expression in *S. cerevisiae*. The Man5A catalytic domains are indicated with open boxes, the native secretion signals with cross-hatched boxes, the CBM moiety with a hatched box and the MFα1s secretion signal with as solid box. The plasmids encoding the different Man5A constructs, as well as the corresponding *S. cerevisiae* strains are also indicated.

**RNA isolation and Northern blot analysis**

*S. cerevisiae* strains were cultivated in 100 ml YPD medium at 30°C on a rotary shaker. Ten millilitre samples were collected at 24 h, 36 h, and 48 h intervals, and total RNA was isolated from *S. cerevisiae* Y294, *S. cerevisiae* AMan5-1, *S. cerevisiae* TMan5-1, and *S. cerevisiae* TMan5-2 using the phenol-freeze method [38]. Five micrograms of RNA were separated on formaldehyde denaturing gel, and transferred to Magnacharge nylon membrane (Osmonics Inc. Westborough, MA-USA). man1 transcription was monitored over 48 hours using the relevant [α-32P]-labeled man1 gene of *A. aculeatus* and *T. reesei* as probes. A 470 bp ACT1 PCR amplified fragment was used to probe for actin as an internal standard.
Protein extraction and Western blot analysis

Yeast strains were cultivated in 100 ml YPD for 48 hrs at 20°C and 30°C. Twenty-milliliter samples were collected and centrifuged at 4000 × g. Supernatants were precipitated with 60% ammonium sulphate and re-dissolved in 4 ml of 50 mM citrate buffer pH 5. Periplasmic protein fractions were extracted by making spheroplasts with Zymolyase T100 (ICN Pharmaceuticals, Inc., Costa Mesa, CA-USA) as described by la Grange et al. [23]. Subsequently, the spheroplasts were collected by centrifugation and lysed to release intracellular proteins. Extracellular, periplasmic and intracellular protein fractions were dialyzed against 50 mM citrate buffer pH 5 using a 10 kDa molecular weight cutoff SPECTRA/POR® membrane (Spectrum medical industries, INC., California). Four microgram protein samples made up to a final volume of 20 µl were loaded onto polyvinylidene difluoride (PVDF) membrane using the Bio-Dot apparatus (Bio-Rad Laboratories, California). Membranes were exposed to β-mannanase antiserum (Antibody AB, Lund, Sweden) raised against the Aspergillus niger or T. reesei β-mannanase, followed with a goat anti-rabbit IgG [H+L] alkaline phosphatase conjugate (Bio-Rad laboratories, California). 5-Bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP)/ p-nitroblue tetrazolium chloride (NBT) colour detection reagents (Bio-Rad laboratories, California) were used to develop the membranes.

Enzyme assays

Standard β-mannanase activity assays were performed on 0.5% locust bean gum as previously described [39]. Biomass concentrations (g l⁻¹) were determined from linear correlation established for optical density at 600 nm versus dry weight measurements.
Results

Production of different β-mannanase constructs by recombinant *S. cerevisiae*

Complementary DNA fragments of *T. reesei* encoding the full-length Tr-Man5A and truncated Tr-Man5AΔCBD (lacking its carbohydrate binding module) were cloned and expressed in autoselective strains of *S. cerevisiae* (TMan5-1 and TMan5-2, respectively) under the regulation of the *PGK1* promoter and terminator sequences (Fig. 1). Northern blot analysis performed on total RNA isolated from *S. cerevisiae* AMan5-1, TMan5-1 and TMan5-2 showed that the Man5A encoding cDNA fragments were efficiently transcribed in all the strains (Fig. 2), however, strain AMan5-1 exhibited higher transcript levels. The native secretion signals of Aa-Man5A from *A. aculeatus* and Tr-Man5AΔCBD from *T. reesei* were replaced with the yeast Mfa1S secretion signal (Fig. 1). These constructs were expressed in autoselective strains of *S. cerevisiae* (AMan5-2 and TMan5-3, respectively), producing the mature Mfa1S-Man5A-fusion proteins.

![Northern blot analysis](http://scholar.sun.ac.za)

**Fig. 2.** Northern blot analysis was performed on total RNA isolated from *S. cerevisiae* strains Y294, AMan5-1, TMan5-1 and TMan5-2. Man5A transcripts were detected with the α-32P-labeled *manl* gene of *A. aculeatus* and *T. reesei*. The yeast *ACT1* gene was used as an internal standard.

The recombinant *S. cerevisiae* strains were evaluated for production of active Man5A when cultivated over a period of 96 hrs in rich medium (Fig. 3). A continuous increase in extracellular active enzyme was observed for all the recombinant strains. *S. cerevisiae* AMan5-2, TMan5-1, TMan5-2, and TMan5-3 exhibited similar biomass formation
despite the production of different Man5A constructs. In contrast, *S. cerevisiae* AMan5-1 (previously designated *S. cerevisiae* Man5PGK1 [13] displayed stunted biomass formation but produced more active enzyme than *S. cerevisiae* AMan5-2.

Table 3 summarizes the total Man5A protein and Man5A protein per cell mass produced after 96 hrs for the five recombinant *S. cerevisiae* strains. Replacement of the native *A. aculeatus* secretion signal with the MFα1S secretion signal resulted in two-fold reduced production of Aa-Man5A. *S. cerevisiae* AMan5-1 secreted up to 24.2 mg g<sub>biomass</sub>−1 with the native secretion signal, whereas *S. cerevisiae* AMan5-2 secreted 13.3 mg g<sub>biomass</sub>−1 with the MFα1S secretion signal. *S. cerevisiae* TMan5-1 and TMan5-2 secreted approximately the same amount of enzyme, 1.5 mg g<sub>biomass</sub>−1 and 1.8 mg g<sub>biomass</sub>−1, respectively. This implies that the presence of the CBD and the PT-box had no effect on production and secretion of Tr-Man5A. In contrast, substitution of the native secretion signal with the
MFα1S secretion signal resulted in a slight increase in Man5A production by S. cerevisiae TMan5-3. This strain produced 2.4 mg g\textsubscript{biomass}\textsuperscript{-1} which is approximately 1.5 fold higher than the levels produced by TMan5-1 and TMan5-2.

**Table 3.** Auto-selective S. cerevisiae strains and β-mannanase production levels detected after 96 hours of growth in rich YPD medium. Standard deviations were calculated from three parallel experiments.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Mature protein\textsuperscript{a}</th>
<th>Secreted Man5A\textsuperscript{b} (mg l\textsuperscript{-1})</th>
<th>Specific production levels (mg\textsubscript{Man5A} g\textsubscript{biomass} 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMan5-1</td>
<td>Aa Man5A</td>
<td>99.4 ± 17.2</td>
<td>24.2 ± 1.6</td>
</tr>
<tr>
<td>AMan5-2</td>
<td>Aa MFα1S-Man5A</td>
<td>86.0 ± 10.4</td>
<td>13.3 ± 0.7</td>
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<tr>
<td>TMan5-1</td>
<td>Tr Man5A</td>
<td>13.0 ± 0.04</td>
<td>1.5 ± 0.02</td>
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<td>TMan5-2</td>
<td>Tr Man5AΔCBD</td>
<td>10.3 ± 1.1</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>TMan5-3</td>
<td>Tr MFα1S-Man5AΔCBD</td>
<td>16.4 ± 0.9</td>
<td>2.4 ± 0.04</td>
</tr>
</tbody>
</table>

\textsuperscript{a} designated according to the standardized scheme by Henrissat et al. [8].

\textsuperscript{b} based on the specific activities of the purified enzymes which are 4402 nkat mg\textsuperscript{-1} for Aa-Man5A [13], 6157 nkat mg\textsuperscript{-1} for Tr-Man5A, and 7907 nkat mg\textsuperscript{-1} for Tr-Man5AΔCBD [34].

The effect of temperature on β-mannanase production by S. cerevisiae

S. cerevisiae AMan5-1, TMan5-1 and TMan5-2 were cultivated at 20°C, 25°C and 30°C, and Man5A production was monitored for 5 days. The effect of temperature on the production efficiency of the three strains differed. In S. cerevisiae AMan5-1 producing Aa-Man5A, there was an increase in enzyme production with an increase in cultivation temperature (Fig. 4A). The Aa-Man5A produced per cell mass was 8.42 mg g\textsuperscript{-1}, 15.88 mg g\textsuperscript{-1} and 24.73 mg g\textsuperscript{-1} at 20°C, 25°C and 30°C, respectively, thus a 3-fold increase in enzyme produced from 20°C to 30°C. In contrast, S. cerevisiae TMan5-1 produced 5.60 mg g\textsuperscript{-1}, 3.26 mg g\textsuperscript{-1} and 1.36 mg g\textsuperscript{-1}, and TMan5-2 produced 5.41 mg g\textsuperscript{-1}, 2.99 mg g\textsuperscript{-1} and 1.11 mg g\textsuperscript{-1} Tr-Man5A per cell mass at 20°C, 25°C and 30°C, respectively, thus a 5-fold decline in enzyme produced with an increase in cultivation temperature from 20°C to
30°C (Fig. 4B and C). Removal of the CBD moiety did not affect the production of Tr-Man5A significantly.

Fig. 4. The effect of temperature on production of Man5A by _S. cerevisiae_ AMan5-1 (A), _S. cerevisiae_ TMan5-1 (B), and _S. cerevisiae_ TMan5-2 (C). Cultivations were carried out at 20°C (●), 25°C (■), and 30°C (▲) in YPD medium. The cell mass (CM) yields of the different recombinant _S. cerevisiae_ strains after incubation at the three different temperatures are noted in brackets. The results are representative of the average of three independent experiments.

Western blot analysis was performed on extracellular, periplasmic, and intracellular protein fractions extracted from the three strains after 48 hrs of growth at 20°C and 30°C. Man5A was predominantly secreted in all the _S. cerevisiae_ strains (Fig. 5). In addition
Western blot analysis of the extracellular fractions after separation on an SDS-PAGE gel showed that the proteins were hyperglycosylated at both temperatures (Fig. 6).

![Western blot analysis](image)

**Fig. 5.** A. dot blot performed on extracellular (E), periplasmic (P), and intracellular (I) protein fractions isolated from the yeast strains after 48 hours of growth in YPD medium. Man5A was detected using antiserum raised against purified enzyme from *T. reesei* and *A. niger*.

![Dot blot](image)

**Fig. 6.** Western blot analysis performed on extracellular protein fractions of *S. cerevisiae* AMan5-1 cultivated at 20°C (lane 2) and 30°C (lane 3), *S. cerevisiae* Y294 20°C (lane 4) and 30°C (lane 5) was detected with antiserum raised against *A. niger* Man5A. The fractions from *S. cerevisiae* TMan5-1 20°C (lane 7) and 30°C (lane 8), *S. cerevisiae* TMan5-2 20°C (lane 9) and 30°C (lane 10) were detected with antiserum raised against *T. reesei* Man5A.
Tr-Man5AΔCBD and Aa-Man5A migrated as bands of approximately 50 kDa followed by a heavy smear, probably reflecting heterogeneous glycoforms. Tr-Man5A had a molecular mass of 65 kDa also followed by a smear. At 20°C a smaller immuno-reactive band of 50 kDa was also detected in Tr-Man5A.

Discussion

Endo-1,4-β-mannanase from *A. aculeatus* (Aa-Man5A) and *T. reesei* (Tr-Man5A) were produced and secreted in *S. cerevisiae* using the native fungal secretion signals and as fusion proteins with the yeast MFα1S secretion signal. Processing of the prepeptides of the two fungal Man5A and the yeast MFα1S secretion signal by a signal peptidase between two Ala residues occurs in all three native hosts [2,6,22]. However, processing of the propeptides differs. The MFα1S propeptide is cleaved by a KEX2 endoprotease at the carboxyl terminal side of the dibasic sequence Lys-Arg [22]. The propeptide of Tr-Man5A is possibly cleaved by a monobasic-specific protease at the carboxyl terminus of the arginine in the Pro-Arg sequence [6,27,28]. Cleavage of the presumed propeptide of Aa-Man5A is believed to occur at the carboxyl terminus of Ala-28 in *A. aculeatus*, however, similar processing has not been confirmed in *S. cerevisiae* [2]. This is not a typical monobasic or dibasic cleavage site, and no protease with such activity has to our knowledge been reported for *S. cerevisiae* yet. Interestingly though, *S. cerevisiae* AMan5-1 produced and secreted significantly higher quantities of Aa-Man5A enzyme per gram biomass with the native secretion signal without displaying any detectable intracellular accumulation, however, biomass formation was compromised in this strain. On the contrary, secretion with the MFα1S secretion signal in *S. cerevisiae* AMan5-2 seemed to relieve the adverse effect on biomass formation. This strain secreted comparable amounts of active Man5A reaching a final concentration of 86 mg l⁻¹ compared to 99 mg l⁻¹ of *S. cerevisiae* AMan5-1 (Table 3). It is possible that lack of appropriate proteases to cleave the native Aa-Man5A propeptide may result in slight perturbations in the secretory pathway leading to poor biomass formation. Other researchers have shown that perturbations in the secretory machinery could have an
impact on growth in a similar way as the class A sec mutants [41], and that protein synthesis and transport can continue despite this defect [29,40]. *S. cerevisiae* TMan5-1, TMan5-2 and TMan5-3 produced and secreted low amounts of Man5A in comparison with *S. cerevisiae* AMan5-1 and AMan5-2. This may be due to differences in transcript levels (Fig. 2). *S. cerevisiae* TMan5-3 produced 1.3 and 1.6 fold more active Man5A in contrast to TMan5-1 and TMan5-2, respectively. This slight improvement can be attributed to better secretion with MFα1s secretion signal since this is the only difference between *S. cerevisiae* TMan5-2 and TMan5-3.

Temperature is one of the environmental parameters that has been shown to have an effect on protein production and secretion [30-32]. The production and secretion of Man5A by *S. cerevisiae* AMan5-1 increased about 3-fold with an increase in temperature in the sequence (30°C>25°C>20°C). It is possible that a decrease in cultivation temperature results in lower rates of macromolecule synthesis (transcription and translation), resulting in lower secretory levels of Aa-Man5A protein. In contrast, *S. cerevisiae* TMan5-1 and TMan5-2 secreted more Man5A at 20°C than at 30°C. Western blot analysis showed that *S. cerevisiae* TMan5-1 secreted two species of Tr-Man5A at 20°C (Fig. 6). The smaller protein of 50 kDa is indicative of core-glycosylated protein, whereas the heterogeneous species at 65 kDa followed by a heavy smear indicates outerchain glycosylated protein. Both the native Aa-Man5A and Tr-Man5A catalytic domains have calculated molecular weight of 41 kDa. These phenomena have been shown for other enzymes such as laccase [31], and α-amylase and human lysozyme [30] produced in *S. cerevisiae*. Growth at low temperature is thought to not only affect macromolecule synthesis, but also to allow proper passage through the secretory pathway, thus resulting in restoration of secretion defects brought about by overglycosylation [22,42]. Riederer *et al.* [43] explained that at low temperature there is less thermodynamic energy present, such that the environment allows persistence of folding precursors thus resulting in production of more correctly folded material. Consequently, secretion of glycosylated as well as unglycosylated protein species can be achieved under such conditions [22]. The improved production /secretion of Man5A at 20°C by *S. cerevisiae* TMan5-1 and TMan5-2 could, apart from macromolecule
synthesis, be due to a combination of the above-mentioned factors. However, the role played by low cultivation temperature seems to be more complex and protein dependent. For instance, secretion of carboxypeptidase Y [44], glucoamylase II and α-galactosidase [32] in *S. cerevisiae* was not affected by low cultivation temperature. In contrast, lowering temperature increased secretion of glycosylated human secreted alkaline phosphatase in the baculovirus system [42].

Family 5 and 26 β-mannanases and other β-glycanases are thought to have evolved by reshuffling of catalytic domains and several binding domains. Consequently, some enzymes acquired CBMs that mediate binding of the enzymes to insoluble substrates [45-46]. The CBD of Tr-Man5A was reported to enhance activity on mannan/cellulose complexes, however, it has no affinity towards substituted mannan polysaccharides [34]. In the current study, the truncated Tr-Man5AΔCBD was produced and secreted in *S. cerevisiae* TMan5-2. The PT-box and the CBD seemed to only contribute to the molecular mass of Tr-Man5A, and had no effect on production and secretion of the enzyme or its activity on locust bean gum. This observation indicates that the CBD of Tr-Man5A is not necessary for folding, as has been reported for Tp-Man5A of *Thermoanaerobacterium polysaccharolyticum* [1]. It is perhaps so that Tr-Man5A acquired the CBD as an evolutionary adaptation that allowed the enzyme better access to mannan substrates that are intricately associated with cellulose.

**Acknowledgements**

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References


CHAPTER 4

The effect of endo-1,4-β-mannanase on the properties of coffee extracts: a preliminary investigation

Manuscript

in preparation
THE EFFECT OF ENDO-1,4-β-MANNANASE ON THE PROPERTIES OF COFFEE EXTRACTS

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ABSTRACT

Technically produced coffee extracts from roasted Arabica and Robusta beans contain 20-36% carbohydrates, predominantly in the form of β-1,4-mannan and β-1,3-galactan (9). These carbohydrates contribute significantly to the high viscosity of coffee extracts. The flow dynamics of Arabica coffee extracts were studied at 20%, 40% and 60% w/v extract concentrations. The extracts exhibited pseudoplastic non-Newtonian flow behaviour. Recombinant endo-1,4-β-mannanase produced from S. cerevisiae AMan5-1 was used to hydrolyze 20% and 40% w/v coffee extracts, resulting in significant reduction in viscosity. Coffee extracts were prepared either by an enzyme-aided extraction procedure at 50°C, or by high temperature extraction at 121°C. The extracts were dried and analyzed for volatile compounds. Enzyme-aided extraction resulted in reduced losses of volatile compounds compared to heat extraction.

INTRODUCTION

Polysaccharides such as arabinogalactan, mannan, and cellulose constitute nearly 50% of the green coffee bean weight (3,5). The major polysaccharide of this fraction is a water-insoluble, crystalline mannan that forms approximately 20-30% of the dry weight of Arabica and Robusta beans (5). These carbohydrates play an important role in the retention of volatile substances due to their capacity to bind aromatic compounds at the adsorptive sites (3,10).

Modern instant coffee production entails, cleaning, roasting, and grinding of coffee beans, followed by a split extraction and concentration to achieve a high solids concentrate for a low-energy spray-drying operation (8). During the roasting procedure of
the coffee beans the physical and chemical properties of carbohydrates change drastically (5). For instance, green coffees contain 62% arabinogalactan, 24% galactomannan, and glucans in the high-molecular weight material extracted with water, whereas roasted coffees contain 69% galactomannan and 28% arabinogalactan (3). After roasting and grinding, extraction is the key operation in the large-scale manufacture of instant coffee in which both soluble solids and volatile aroma/flavor compounds are extracted (1). Technically produced extracts from roasted *Arabica* and *Robusta* coffee contain 20-36% carbohydrates depending on the degree of extraction. They are predominantly composed of mannan and galactan in about the same proportions, with glucan and araban making up only 1-3% of the extracts (9).

Coffee extraction techniques lead to extracts of around 25% w/w soluble solids concentration (1). Spray-drying on these concentrations can provide coffee of the required physical form, however, there will be substantial loss of volatile compounds. Consequently, pre-concentration methods for coffee extracts prior to drying have been introduced to circumvent extensive losses (1). The extracts can be concentrated to 45% w/w soluble solids concentration in vacuum evaporators, resulting in lower costs of removing water during drying (8). However, concentrations above 42% w/w solids are difficult to reach, due to the high viscosity of coffee extracts, also, highly viscous extracts will require longer pre-concentration times (1). The viscosity of coffee extracts can be reduced by hydrolyzing the mannan portion to short oligosaccharides (5,11). This would make it possible to concentrate the extracts to concentrations higher than 42% w/w soluble solids.

Endo-1,4-β-mannanases hydrolyze linear mannan polysaccharides and complex substituted mannan polysaccharides such as glucomannan, galactomannan and galactoglucomannan into oligosaccharides of various chain lengths (4,6). Hydrolysis of substituted mannans is greatly affected by the degree and pattern of substitution so that as the galactose content increases, the rate of hydrolysis ($V_{\text{max}}$) by β-mannanase decreases and the $K_m$ increases (2). Endo-1,4-β-mannanases can therefore be used to perform hydrolysis experiments in which the intention is only to modify the properties of polysaccharides without complete degradation. The viscosity of polysaccharides is
proportional to chain length, branching, and entanglement, and reduction of viscosity can be effected through partial hydrolysis. The aim of the current study was (i) to evaluate the effect of a recombinant endo-1,4-β-mannanase produced by *Saccharomyces cerevisiae* AMan5-1 on the viscosity of coffee extracts and (ii) to investigate the influence of enzyme-aided extraction on the retention of volatile compounds and the rate of drying.

**Materials and methods**

**Enzyme production**

The recombinant endo-1,4-β-mannanase producing yeast strain *S. cerevisiae* AMan5-1 (6) was cultivated in minimal synthetic complete medium supplemented with 4% glucose, 0.17% yeast nitrogen base without ammonium sulphate, and an amino acid drop-out mix without methionine. The cultures were incubated at 30°C on a rotary shaker for 4 days, after which the cells were removed by centrifugation. The supernatant (enzyme source) was concentrated through the Minitan cross-flow ultrafiltration devise (Millipore Corporation, Bedford, Massachusetts, USA). The filtrate was freeze-dried and used for hydrolysis experiments.

**Hydrolysis of locust bean gum (galactomannan)**

A 1% (w/v) locust bean gum solution was prepared in 50 mM citrate buffer pH 5 and used as a substrate for viscosity analysis. The viscosity analyses were performed using a Brookfield viscometer model (Brookfield Engineering Laboratories, Inc., Stoughton, Mass. USA). The initial viscosity was determined at 40°C, after which the β-mannanase enzyme was added to the final concentration of 2 nkat/mg substrate. The viscosity of the reaction mixture was measured at different time points and sample were collected concurrently and analyzed for reducing sugars. The reaction was terminated by boiling for 5 min. The reducing sugars were determined using the modified DNS method as previously described (7).
The flow dynamics of coffee extracts

Ground Arabica coffee (Boveldt pure South African) was supplied by SAPEKOE (Pty) Ltd. (Tzaneen, SA). Coffee extracts were prepared by pre-wetting 400 g of coffee in 50 mM citrate buffer at pH 5 for 5 hrs. Extraction was carried out overnight at 80°C with constant stirring. The soluble extract was collected by filtration through a miracloth (CALBIOCHEM), and freeze-dried. The dried extract was used to prepare different extract concentrations (20%, 40% and 60% w/v) for viscosity analysis. The flow dynamics of the extracts were studied at 30°C on a HAAKE RV12 Viscometer (HAAKE Mess-Technik GmbH Co., Germany). The effect of endo-1,4-β-mannanase on the viscosity, and flow dynamics of the coffee extract was evaluated. The enzyme was added to a final concentration of 2 nkat/mg extract and 100 μg/ml BSA was added to stabilize the enzyme. The reaction was carried out at pH 5, and 50°C for 3 h followed enzyme inactivation at 100°C for 5 min.

Extraction processes

Thirty grams of ground Arabica coffee was pre-wet to 50°C in 100 ml of citrate buffer pH 5 for 5 h, followed by hydrolysis with endo-1,4-β-mannanase for 5 h with constant stirring. The enzyme was inactivated, and the extraction was continued for another 2 h. In a parallel experiment, 30 g of coffee was pre-wet under the same conditions, followed by two autoclave cycles, at 121°C for 20 min per cycle. As control, the same amount of coffee was pre-wet with buffer for 5 h, then freeze-dried. In all the treatments, 0.02% sodium azide was added to prevent microbial growth. The soluble extracts were freeze-dried and volatile compounds were analyzed by gas chromatography.

Analytical methods

Gas chromatography analysis was performed on 3.57 g of coffee samples. SPME fiber (100 μm Polydimethylsiloxane) was used for sampling. Extraction was carried out
for 120 min at 65°C (head space), and desorption at 230°C for 5 min. Compounds were separated on a PS089 (0.25 µM film), 40 m x 0.25 mm GC column using 38 cm/s He as a carrier gas. The GC program was 40°C (0 min) to 230°C at 4°C/min.

RESULTS AND DISCUSSION

The effect of endo-1,4-β-mannanase on the viscosity of locust bean gum

Locust bean gum was hydrolyzed with endo-1,4-β-mannanase for a period of 120 min. The change in viscosity of the substrate and the release of reducing sugars were monitored continuously. A rapid loss of viscosity was observed in the first 20 minutes of the reaction (Fig. 1). This was accompanied by a gradual increase in the concentration of reducing sugars. These results indicated that there is a rapid decrease in the average molecular weight of galactomannan, which is followed by a steady state. The viscosity remained constant whereas the concentration of reducing sugars increased continuously.

Fig. 1. Hydrolysis of locust bean gum galactomannan with the recombinant endo-1,4-β-mannanase from S. cerevisiae AMaIn-1. Viscosity (●) was measured with a Brookfield viscometer at 40°C and the reducing sugars (■) were measured using the DNS assay.
Flow dynamics of coffee extracts

Ground *Arabica* coffee was extracted with water at 80°C and the extract was freeze-dried. The fluid properties of 20%, 40% and 60% w/v coffee extracts were studied. Fig. 2 shows the flow curves and viscosity curves of the different extract concentrations. The extracts showed similar rheological properties, and exhibited non-Newtonian flow characteristics. The viscosity of the extracts decreased with increase in shear rate resulting in pseudoplastic behaviour. These results compare with those reported by Sachslehner *et al.* (5), and indicate that galactomannan found in coffee extracts impart on-Newtonian flow properties to the extracts.

![Graph showing flow properties of coffee extracts](image)

**Fig. 2.** The flow properties of coffee extracts determined with 20% (σ), 40% (ν), and 60% (λ) extracts. Closed symbols represent the flow curves, and open symbols represent the viscosity curves.

Treatment of the 20% and 40% extracts with endo-1,4-β-mannanase resulted in significant drop of viscosity (Fig. 3) indicating that the enzyme is capable of hydrolysing coffee galactomannan. The extracts still retained their pseudoplastic properties after
hydrolysis (data not shown), this implies that other polysaccharides such as arabinogalactan could have similar flow properties as galactomannan and that they are not hydrolysed by the β-mannanase. Sachslehner et al. (5) reported that enzymatic treatment of coffee extract with the β-mannanase from Sclerotium rolfsii resulted not only in the cleavage of mannan but also with possible degradation of galactan, however, we did not investigate galactan degradation in the current study. An enzyme-aided treatment of coffee extracts could play a pivotal role in instant coffee production since the hydrolysis of mannan will reduce the viscosity of the extracts, thus improving the pre-concentration process. In addition, the remaining polysaccharides will impart enough viscosity to give coffee its characteristic body.

![Graph](image)

**Fig. 3.** The effect of endo-1,4-β-mannanase on the viscosity of coffee extracts and their fluid dynamics was investigated. Change in viscosity of 20% (σ) and 40% (v) extracts was monitored before treatment (closed symbols) and after treatment (open symbols).
The effect of enzyme-aided extraction on volatile compounds

It has previously been shown that hydrolysis of coffee galactomannan with endo-1,4-β-mannanase results in reduction of viscosity, and that the viscosity remained constant after a few hours of hydrolysis (5). Therefore, it was expected in the current study that hydrolysis of coffee extracts at 50°C for 5 h would generate high concentrations of oligosaccharides which together with high molecular weight arabinogalactan have the capacity to bind volatile compounds (3,10). Due to the mildness of the treatment, retention of higher concentrations of volatile compounds would be achieved. Fig. 4, shows the chromatograms obtained from coffee extract samples that were extracted by enzyme-aided treatment (E-1), and through autoclaving at 121°C (A-2). The profiles of volatile compounds were compared to those detected in ground coffee. The three samples displayed similar profiles which, indicated that they contained the same types of volatile compounds. However, the differences in peak sizes indicate that the autoclaved sample had a significant loss of volatile compounds, relative to the control. Enzyme treatment does also seem to affect the amount of compounds retained, resulting in improved retention of some compounds and slight losses of others. Quantitative analyses have not been performed to allow comparison on the basis of concentration therefore it is not possible to draw conclusions on these results.

The preliminary results indicate that endo-1,4-β-mannanase is capable of hydrolyzing coffee galactomannan, leading to substantial reduction in viscosity. In addition the results seem to indicate that enzyme-aided extraction might be a valuable techniques for instant coffee production since this could allow extractions at lower temperatures, and therefore, better retention of volatile compounds. In addition, lower extract viscosity would allow recovery of concentrates higher than 42% after pre-concentration, and also improve the drying process due to the low water content of the extracts.
Fig. 4. GC profiles of coffee samples. C-3 is the control sample, A-2 is the autoclaved sample, and E-1 is the enzyme treated sample.
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REFERENCES


CHAPTER 5

General Discussion and Conclusion

Thus, the task is, not so much to see what no one has yet seen; but to think what nobody has yet thought, about that which everybody sees.

- Erwin Schrödinger 1887-1961
GENERAL DISCUSSION

Genes encoding endo-1,4-β-mannanase enzymes have been isolated and sequenced from bacteria, fungi, plants and animals (Ch 1, Table 1). So far, fungal β-mannanases have only been isolated from three species, *Piromyces equi* (1), *Trichoderma reesei* (11), and *Aspergillus aculeatus* (3). The β-mannanases from *P. equi* have been classified under glycosyl hydrolase family 26 (GH-26), whereas the *T. reesei* and *A. aculeatus* β-mannanases belong to family 5 (GH-5). Genes encoding the two GH-5 enzymes were cloned and expressed in *Saccharomyces cerevisiae* (10,11). The production yields of the two enzymes showed significant disparity, despite the 60% identity shared by the two enzymes. Some of the observations made in the current study brought up interesting questions pertaining to factors that influence heterologous protein production in *S. cerevisiae*.

The following questions and hypotheses were drawn:

(i) Should *S. cerevisiae* still be considered an efficient host for enzyme production?
(ii) Does post-translational modification of recombinant proteins in *S. cerevisiae* affect their function and biochemical properties?
(iii) Poor recognition of the native Aa-Man5A secretion signal possibly hampers Golgi-trafficking which in turn might affect growth;
(iv) Sub-optimal growth temperature enhances production and secretion of Tr-Man5A and Tr-Man5AΔCBD;
(v) The differences in the yields of Aa-Man5A, Tr-Man5A, and Tr-Man5AΔCBD in *S. cerevisiae* maybe a result of the differences in transcript levels and inherent features of the proteins.

This discussion seeks to elaborate on these hypotheses in the context of protein production, folding and secretion.

*Saccharomyces cerevisiae* as a host for heterologous protein production

When *S. cerevisiae* emerged into recombinant DNA technology as a host for heterologous gene expression, it was considered to be an efficient host compared to
E. coli. However, as other non-Saccharomyces yeast strains such as P. pastoris and H. polymorpha, with higher secretion capacities were discovered, S. cerevisiae became less frequently used for over-expression of heterologous genes. Nevertheless, S. cerevisiae is non-pyrogenic, has GRAS status (Generally Regarded As Safe), and is widely used in the food and beverage industry. Consequently, an abundance of vector systems and fermentation processes have been devised and optimized to improve the yield of heterologous proteins in S. cerevisiae. For instance, in chapter 2, high production levels of recombinant A. aculeatus β-mannanase were achieved by combining multi-copy expression, promoter strength and autoselection. Production levels up to 118 mg/L were achieved under the regulation of the ADH2p promoter. These levels were comparable to production levels of other fungal hydrolases produced in P. pastoris in shake flask cultivations (18). Table 1 summarizes the levels of a variety of microbial hydrolases produced in S. cerevisiae. It is rather difficult to compare data from the literature and various factors should be considered, such as expression systems used, copy number of heterologous genes, medium composition, growth conditions as well as S. cerevisiae strain background. It is notable that recombinant enzyme yields in S. cerevisiae can fluctuate up to 1000 fold. However, certain general comments can be made on recombinant fungal hydrolases expressed in S. cerevisiae:

(i) The recombinant protein appears to be more heterogeneous in size and exhibit higher molecular weight compared to the native enzymes mainly due hyperglycosylation.
(ii) In some cases retention of bulky proteins in the periplasmic space was reported (17).
(iii) The heterologous products mostly retained similar biochemical properties as the native enzymes.

Similar observations were made for the recombinant β-mannanases expressed in S. cerevisiae in chapter 2 and 3. In the current study we were able to show that autoselection offers an added advantage to heterologous protein production as it allows flexibility in the choice of cultivation media. Similar studies have shown that the use of minimal media result in lower product yields (5). Production of fungal proteins by S. cerevisiae resulted in many cases with low yields. Factors such as promoter strength,
plasmid copy number, mRNA stability, folding efficiency and post-transcriptional processing have been shown to affect secretion efficiency (16,29). These factors are however protein dependent.

Over the years knowledge about fungal genetics has been accumulated and opened new ways to use filamentous fungi for the production of homologous and heterologous proteins. Species of the genera Aspergillus and Trichoderma are currently used extensively for large-scale production of proteins. Heterologous proteins are produced at g/l quantities in different strains of Aspergillus e.g. A. niger, A. awamori and A. oryzae and with less overglycosylation. However, despite the naturally high secretion capacity in these strains, the yields of heterologous proteins can be as low as 50 μg/L (27,28). Factors affecting heterologous protein production in fungi, and strategies to improve yields have been reviewed elsewhere (28).

**Post-translational processing**

The efficiency with which the secretion signal directs transport of nascent proteins through the secretion pathway is dependent upon the nature of the secretion signal and the presence of a recognizable cleavage junction (20). Most of the hydrolases listed in Table 1, have been successfully secreted in S. cerevisiae using their native secretion signals. Presumably due to the degeneracy/simplicity of the secretion signals recognized by S. cerevisiae, it can tolerate a high variability fungal secretion signals. In some of the cases the native secretion signals were shown to be more efficient than homologous yeast secretion signals such as the MFα1S and the invertase secretion signals (10,11). Replacement of the native secretion signals of the Aa-Man5A and Tr-Man5A with the MFα1S in chapter 3 did not lead to significant improvement in the secretion of the recombinant β-mannanases.
Table 1. Comparison of hydrolase production in *Saccharomyces cerevisiae* and *Pichia pastoris*.

<table>
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<th>Donor of organism</th>
<th>Enzyme</th>
<th>Promoter</th>
<th>Activity (U/ml)</th>
<th>Product levels (mg/l)</th>
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<th>Reference</th>
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<td>Activity (U/ml)</td>
<td>Product levels (mg/l)</td>
<td>Medium</td>
<td>Reference</td>
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**Expression in Pichia pastoris**

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In the current study, it was observed that *S. cerevisiae* AMan5-1, which secreted Aa-Man5A containing the native secretion signal, exhibited aberrant growth. Poor recognition of the cleavage site of the prepeptide may result in retention in the ER, with concomitant degradation of the protein and subsequent lower secretory levels. Poor recognition of the cleavage site of the propeptide is however not often discussed. Replacement of the native secretion signal with the yeast MFα1s secretion signal alleviated the growth defect, however, the amount of secreted enzyme did not increase. Since both secretion signals possessed common prepeptide cleavage sites, ER retention due to poor signal peptide processing was ruled out. The differences in the lengths of the propeptides and the cleavage site thereof, remained a concerning factor. The native propeptide is only ten amino acids long and is cleaved at the C-terminus of Ala-28, which is not a favorable site for serine, aspartyl, or proline-directed arginyl proteases. Taken together these observations led us to speculate that the cleavage site of the native secretion signal was poorly recognized, thus resulting in extended residency in the Golgi where most endoproteases are localized (7,16). Understandably, this could culminate in limitations in Golgi-trafficking, which represents a major obstacle in secretion. *How does this impact on growth?* There are two possible explanations that can be considered; (i) blockage in the Golgi compartments not only affects secretion of Aa-Man5A, but can also prohibit processing and trafficking of other host glycoproteins that might be essential for growth, (ii) increased demand of endoproteases in the Golgi, could probably cause titration of endoproteases such as *KEX2, MKC7*, and *YAP3* that have been shown to play a role in both secretion and growth (7).

**Cultivation temperature**

Temperature has occasionally been exploited to improve the yield of secreted heterologous proteins in *S. cerevisiae*. Several researchers have shown that reduction of growth temperature to sub-optimal ranges enhances the secretion of heterologous proteins, which are poorly secreted under optimal growth conditions. In chapter 3, the effect of temperature on the secretion efficiency of Aa-Man5A, Tr-Man5A, and Tr-
Man5AΔCBD in recombinant *S. cerevisiae* strains was evaluated. Reduction of cultivation temperature from 30°C to 20°C resulted in a 5-fold increase of secreted Tr-Man5A and Tr-Man5AΔCBD. In contrast, secretion of Aa-Man5A increased proportionally with cultivation temperature. It is conceivable that reduction of temperature slows down the rate of transcription, translation, protein synthesis, and transport of Aa-Man5A to the extracellular medium. However, the manner in which low cultivation temperature improves the secretion of Tr-Man5A and Tr-Man5AΔCBD is not that obvious.

In *E. coli*, low cultivation temperature improves protein solubility, resulting in increased proportion of soluble versus insoluble forms of recombinant protein (2). It is currently generally recognized that aggregation *in vivo* is not a function of the solubility and stability of the native state of the protein, but of its folding intermediates in relation to the environment in which they are folding (6). In addition, low cultivation temperature is thought to provide an environment with low thermodynamic energy, which allows persistence of folding precursors, resulting in production of more correctly folded material (15). Therefore, the influence of low temperature is presumably on the level of folding. *Are the folding intermediates of Tr-Man5A less stable at high temperatures?* It is difficult to give an answer that is plausible, however, we can allude to the fact that protein folding occurs by a progressive stabilization of intermediates, which interact to form secondary structures, which then give rise to the final tertiary structure (15). Perhaps there are inherent characteristics in the intermediates of Tr-Man5A that render them more susceptible to denaturation in the yeast cytosol at elevated temperatures. For this to be validated, one would have to investigate the folding process of Aa-Man5A and Tr-Man5A *in vitro*.

In summary, our results show that there are a number of parameters that need to be considered to improve the production yields of recombinant proteins in *S. cerevisiae*. cultivation temperature, promoter strength, copy number, cultivation media and replacement of leader peptides all affected β-mannanase production in *S. cerevisiae* and can be exploited to improve poor yields of other heterologous proteins in *S. cerevisiae*. The current study is focused on comparing heterologous protein production of fungal
hydrolases of the same family (family 5) that share high homology (60 % identity). In this case factors such as strain background, promoter systems, and secretion signals have been normalized, such that only a few parameters can be considered. Our results highlight the complexity of heterologous protein production, showing that slight differences in mRNA levels and other inherent properties of proteins can contribute considerably towards heterologous protein production.

CONCLUSIONS

Based on the data presented in this study, the following conclusions can be drawn

- Aa-Man5A was properly transcribed and secreted in *S. cerevisiae* under the regulation of the *PGK1* or the *ADH2* promoter and terminator sequences. The *ADH2* promote higher expression of the *man1* gene.
- The recombinant Aa-Man5A was hyperglycosylated in *S. cerevisiae*, resulting in a core-glycosylated protein with 10 kDa higher molecular mass compared to the calculated molecular weight, and additional heterogenous glycoforms with an apparent molecular mass ranging from 51 kDa to 95 kDa.
- Expression and production of Aa-Man5A in *S. cerevisiae* did not affect the biochemical properties of the enzyme, however, the kinetic properties were slightly affected.
- The cellulose-binding domain (CBD) of Tr-Man5A does not affect secretion of the enzyme in *S. cerevisiae* or its activity on locust bean gum.
- Substitution of the native secretion signal with the yeast MFiα-1S secretion signal did not lead to significant improvement of secretion efficiencies, however, alleviated stunt growth in *S. cerevisiae* AM5-2.
- Low cultivation temperature gave rise to more secreted activity of Tr-Man5A in *S. cerevisiae*.
- Replacement of the secretion signal, or changing the cultivation temperature could promote heterologous protein production, but the intrinsic structural features of the recombinant protein still plays a significant role in heterologous production.
FUTURE RESEARCH

Preliminary studies showed that the recombinant *A. aculeatus* endo-1,4-β-mannanase (Aa-Man5A) secreted by *S. cerevisiae* AMan5-1 is capable of hydrolysis coffee galactomannan and reduce the viscosity of coffee extracts. In addition, the enzyme showed potential in aiding the extraction process and retention of volatile compounds. Consequently, future work in this project will be directed towards establishing a liaison with the instant coffee production industry, so that further investigation can be performed on pilot scale, under conditions, which mimic an industrial process. More research will also be conducted to design a fermentation medium in which sulfur-containing compounds can be excluded and downstream processing strategies that will allow high product yield and promote better recovery of Aa-Man5A.

REFERENCES


APPENDIX
Immuno-localization of endo-1,4-β-mannanases in the recombinant
Saccharomyces cerevisiae strains AMan5-1 and TMan5-1

Abstract

In chapter 3 Western blot analysis was used to determine the localization of Aa-Man5A and Tr-Man5A in the recombinant Saccharomyces cerevisiae strains. The results indicated that both enzymes were mainly secreted to the extracellular medium with minimum intracellular accumulation. These observations were further supported by additional results obtained through electron microscopy. Immuno-electron microscopy showed that the enzymes were predominantly cell wall-associated and secreted.

INTRODUCTION

Transmission electron microscopy (TEM) has developed as a useful tool for investigating the distribution of native and heterologous proteins in yeast (1,4,5,6). Sample preparation methods have been optimized so that preparation of well-fixed and infiltrated samples of yeast cells can be easily achieved (7). Immuno-electron microscopy makes it possible to trace proteins of interest and to identify the specific suborganellar location in the cells (2). In addition, this technique can be used to investigate morphological changes of cell organelles that can sometimes occur due to overexpression of heterologous proteins or accumulation of misfolded proteins (6). For instance, Umebayashi et al. (6) observed that accumulation of misfolded delta-pro aggregates in Saccharomyces cerevisiae caused morphological alterations of the endoplasmic reticulum (ER) and the nucleus. Such observations cannot be made by techniques such as Western blot analysis. Therefore, immuno-electron microscopy gives a more complete and informative picture of protein transport, intracellular distribution and the effect of foreign protein expression on the ultrastructure of different organelles.

The results obtained in chapter 3 using Western blot analysis suggested that although the β-mannanases from Aspergillus aculeatus and Trichoderma reesei were efficiently
secreted in *S. cerevisiae*, there was some accumulation in the periplasm. Consequently, immuno-electron microscopy was used to ascertain the location of these enzymes in *S. cerevisiae*.

**Materials and Methods**

1. **Fixation**

The recombinant *S. cerevisiae* strains AMan5-1 and TMan5-1 were cultivated in YPD medium for 36 h at 30°C and prepared for transmission electron microscopy. Yeast cells were harvested and washed with ice cold distilled water, followed by primary fixation in a mixture of 4% formaldehyde and 0.5% glutaraldehyde (EM grade) prepared in 200 mM sodium phosphate pH 7.0. The cells were subsequently washed and secondary fixation was performed using a fixative containing 0.5% OsO₄ and 0.8% K₃Fe(CN)₆ in 100 mM sodium phosphate pH 7.0. The cells were then washed and urched with 1% NaIO₄, followed by treatment with 50 mM NH₄Cl. Following fixation the cells were washed and re-suspended in low melting point agarose. Small blocks (1–2 mm) were cut and washed with distilled water. The fixation protocol was modified from Wright (7) and Byers and Goetsch (2).

2. **Dehydration, infiltration and embedding**

Agarose blocks were treated with 2,2-dimethoxypropane and washed with acetone. Infiltration was initiated with acetone:SPURR resin (3:1), followed by a 1:1 mixture and completed with a 1:3 mixture. Each step was performed for 30 min. The blocks were embedded in pure SPURR resin for 1 h and polymerized for 16 h at 70°C.

3. **Sectioning and stains**

Thin sections were prepared and collected on nickel grids. The sections were labeled with antibodies raised against the *A. niger* and the *T. reesei* endo-1,4-β-mannanase. Immunogold detection was performed using Aurion ImmunoGold reagents GAR EM-10 nm (Aurion, Costerweg 5, 6702 AA Wageningen, The Netherlands) containing Goat-anti-Rabbit IgG (H & L) EM-grade 10 nm. Immuno-staining and detection were done
following the manufacturer’s protocol. The sections were viewed on a Bio-cryo-transmission electron microscope (Phillips CM120 BioTWIN Cryo), equipped with energy filter imaging (Gatan GIF 100).

Results and Discussion

Immuno-electron microscopy was used to determine the localization of the recombinant β-mannanase in the *S. cerevisiae*. Both the β-mannases from *A. aculeauts* and *T. reesei* were concentrated at the cell wall of *S. cerevisiae* (Fig. 1). These results correspond with the observations made in chapter 3 using Western blot analysis, and they indicate that although there is some amount of enzyme intracellularly and in the periplasm, the enzymes are predominantly cell wall-associated and secreted. The presence of the β-mannases in the periplasm could perhaps be directly related to the rate of progression of the enzymes through the cell wall. This is dependent on the average pore size of the yeast cell wall, which has been shown to decrease markedly when the cells go into stationary phase (3). Since both β-mannases have molecular masses higher than 50 kDa and are hyperglycosylated, it is tempting to speculate that their passage through the cell wall is slightly hindered by the porosity of the cell wall as the cells approach stationary phase. Therefore, association of these enzymes with the cell wall does not necessarily imply that the enzymes are retained intracellularly but that the rate at which they traverse the cell wall is hampered.

Acknowledgements

We like to acknowledge Gunnel Karlsson (Biomicroscopy unit, Lund University) for her technical assistance.
Fig. 1. (A) A cross section of the host *S. cerevisiae* Y294 strain, (B) A higher magnification of the cell wall (CW) of *S. cerevisiae* Y294 parental strain and (C and D) show the cell wall of the recombinant yeast strains *S. cerevisiae* AMan5-1, and TMan5-1 after immuno-labeling, respectively. The arrow heads indicate the β-mannanase-bound gold particles.
References


