EXPRESSION AND ANALYSIS OF ENDO BETA–1,4-MANNANASE OF *ASPERGILLUS FUMIGATUS* IN HETEROLOGOUS HOSTS

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ABSTRACT

EXPRESSION AND ANALYSIS OF ENDO BETA-1,4-MANNANASE OF *ASPERGILLUS FUMIGATUS* IN HETEROLOGOUS HOSTS

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Extracellular endo-1,4-β-mannanase (EC 3.2.1.78) gene of *Aspergillus fumigatus* IMI 385708 (formerly known as *Thermomyces lanuginosus* IMI 158749) was cloned and transformed into *Aspergillus sojae* (ATCC 11906) and *Pichia pastoris* GS115. High level of expression was achieved in both expression systems. Attempts to produce heterologous mannanase in *Arabidopsis thaliana*, suitable for large scale production, were not successful. Comparison of the expression levels of heterologous mannanase reveals that *A. sojae* is a better expression system than *P. pastoris* with respect to extracellular mannanase activity. The production of mannanase in *A. sojae* (AsT1) after 3 days of incubation reached 204 U/ml in YpSs containing 1 % glucose. In *P. pastoris* (PpT1), highest production was observed after 10 hrs of induction with methanol (61 U/ml). Expressed enzymes were purified and analyzed. Both enzymes have specific activity *c.* 349 U/mg protein with pH and temperature optimum of *c.* 4.5 and *c.* 60 °C for mannanases from AsT1 and *c.* 5.2-5.6 and *c.* 45 °C for mannanases from PpT1. A truncated form of mannanase (MAN-S) deleted at amino acids from P_{291} to P_{368}, which still displayed hydrolytic activity was also isolated and characterized. MAN-S has pH and temperature optimum of *c.* 6.5-8.0 and *c.* 60 °C. During incubation of the mannanase on locust bean gum, transglycosylation reactions, in which longer or rare prebiotic oligosaccharides could be produced catalyzed by glycolysis, was detected. The products

of hydrolytic activity of the enzyme on various carbohydrates were analyzed by PACE and MALDI-TOF. Accordingly, hexamannose and smaller oligosaccharides were characterized.

Keywords:

β-Mannanase, Expressional cloning, *Aspergillus, Pichia pastoris,* Transglycosylation, *Arabidopsis*

ÖZ

ASPERGILLUS FUMIGATUS ENDO BETA-1,4-MANNANAZININ HETEROLOG EV SAHİBİ ORGANİZMALARDA EKSPRESYONU VE ANALİZİ

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Aralık 2007, 132 sayfa

Aspergillus fumigatus IMI 385708 (daha önce *Thermomyces lanuginosus* IMI 158749 olarak bilinen) küfünün hücre dışı endo-1,4-β-mannanaz (EC 3.2.1.78) geni klonlanmış ve *Aspergillus sojae* (ATCC 11906) ve *Pichia pastoris* GS115 'e aktarılmıştır. Her iki ekspresyon sisteminde de yüksek oranda üretim sağlanmıştır. *Arabidopsis thaliana* 'da büyük ölçekli heterolog mannanaz üretimi girişimleri başarılı olamamıştır. İfade düzeyleri karşılaştırıldığında hücre dışı mannanaz aktivitelerine göre *A. sojae P. pastoris'*den daha iyi bir sistem olduğu görülmüştür. %1 glikoz içeren YpSs ortamında üç günlük inkübasyon sonunda mannanaz üretimi *A. sojae* (AsT1)'de 204 U/ml'ye ulaşmıştır. *P. Pastoris* (PpT1)'de ise metanol ile uyarıldıktan 10 saat sonra en yüksek mannanaz aktivitesi değerine ulaşmıştır (61 U/ml). Ekspres edilen enzimler saflaştırılmış ve incelenmiştir. Buna göre her iki enzimin spesifik aktivitesi 349 U/mg protein, AsT1' de optimum pH ve sıcaklık yaklaşık 4,5 ve 45 °C ve PpT1 'de ise 5,2-5,6 ve 60 °C olarak bulunmuştur. Hidrolitik aktivite gösteren P_{291} - P_{368} amino asitleri arası içermeyen kırılmış bir mannanaz formu (MAN-S) izole ve karakterize edilmiştir. MAN-S enzimi yaklaşık 6,5-8,0 aralığında pH ile 60 °C 'de sıcaklık optimumuna sahiptir. Keçi boynuzu sakızının mannanaz ile inkübasyonda glikoliz tarafından katalizlenen uzun veya nadir prebiyotik oligosakkarit üretimini olanak veren transglikozilasyon reaksiyonları

gözlemlenmiştir. Değişik karbon kaynakları üzerindeki hidrolitik aktivite ürünleri PACE ve MALDI-TOF ile analizi yapılmıştır. Buna göre hekzamannoz ve daha küçük oligosakkaritler karakterize edilmiştir.

Anahtar Sözcükler:

β-Mannanaz, Ekspresyonel klonlama, *Aspergillus*, *Pichia pastoris,* Transglikozilasyon, *Arabidopsis*

To My Parents,

Aynur Duruksu & Hüseyin Duruksu

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CHAPTER 1

INTRODUCTION

1.1. Endo-β-Mannanase

Endo-β-mannanases (Mannan endo-1,4-β-mannosidases) are extracellular enzymes hydrolysing randomly the 1,4-β-D-mannosidic linkages in mannans, galactomannans, glucomannans and galactoglucomannans. Mannan-containing polysaccharides are the major components of the hemicellulose fraction in both hardwoods and softwoods as well as in the endosperm in many leguminous seeds and in carob beans.

Galactomannan is a water-soluble polysaccharide consisting of a linear backbone of β-1,4-linked mannose units with α-1,6-linked galactose units as side chains (Fig. 1.1). The ratio of mannose to galactose units in guar gum is about 1.6:1 and in locust bean gum, it is 4:1(Tayal *et al*., 1999; Painter *et al*., 1979; McCleary *et al*., 1985).

β-Mannanases (E.C. 3.2.1.78) are categorized under the two titles within the glycosyl hydrolase family according to their amino acid sequences. Most of the fungal origin mannanases are grouped into family 5. The mannanases of *A. fumigatus* and those of other Aspergilli are members of this family. The other group, glycosyl hydrolase family 26, partially consists of mannanase producing microorganisms that are either bacteria or anaerobic fungi. These two groups show considerably different characteristics. The enzymes in family 5 are less stable at alkaline medium than family 26. They have their optimum activities in the acidic range between pH 3 and 5. They are not stable at extreme temperatures and have the optimum temperatures between 50° and 60 °C. (Henrissat, 1991, http://afmb.cnrs-mrs.fr/CAZY/).

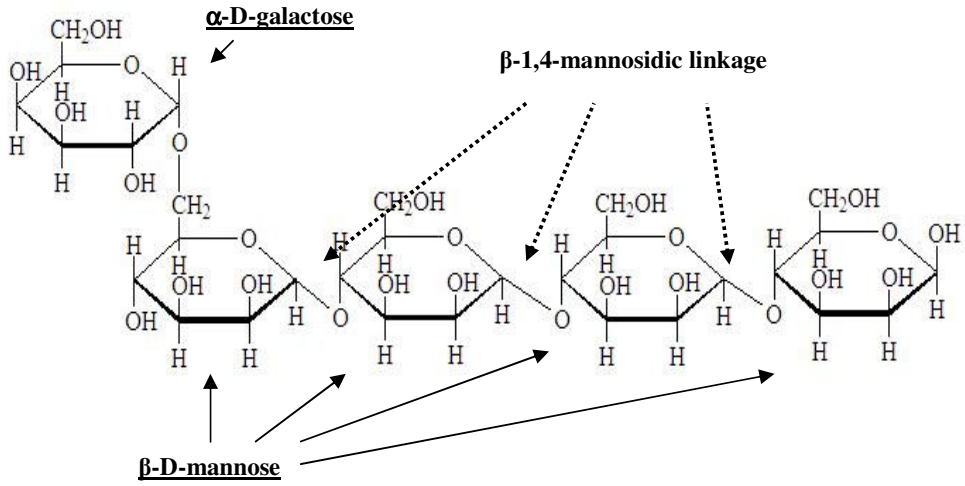


Figure 1.1 Structure of Locust Bean Gum (LBG). Cleavage sites of endo β-mannanase were indicated by arrows.

The three-dimensional crystal structures of *Trichoderma reesei, Thermomonospora fusca* and tomato (*Lycopersicon esculentum*) mannanases were determined (Bourgault *et al*., 2005; Hilge *et al*., 1998; Sabini *et al*., 2000). All three enzyme adopt the $(\alpha/\beta)_8$ fold common to the members of glycohydrolase family GH5.

For the mannanase from tomato, there is a conserved three-stranded β-sheet located near the N terminus that stacks against the central β-barrel at the end opposite the active site. Three noncanonical α-helices surround the active site. Similar helices were found in *T. reesei* but not *T. fusca* mannanase. By analogy with other mannanases, the catalytic acid/base residue is E204 and the nucleophile residue is E318 (Bourgault *et al*., 2005). Subsites of enzyme exist for the binding of multiple sugar groups. These are numbered −4, −3, −2, −1, +1, and +2, from the nonreducing end to the reducing end of the polysaccharide (Davies *et al*. 1997). As it was indicated on the Figure 1.2, cleavage occurs between the mannosyl residues occupying the −1 and +1 subsites (Bourgault *et al*., 2005) The position of the active site was clearly identified in the structural study of the homologous family 5 subgroup A8 bacterial *T. fusca* mannanase. The two catalytic

glutamate residues lie in a groove across the enzyme's surface at one end of the $(\beta/\alpha)_8$ barrel. Four subsites were identified in the complexes of *Therm. fusca* mannanase with mannose oligomers, with sugars being bound in the subsites -4, -3 and -2 and the position of site -1 being deduced from the position of the glutamates (Sabini *et al*., 2000).

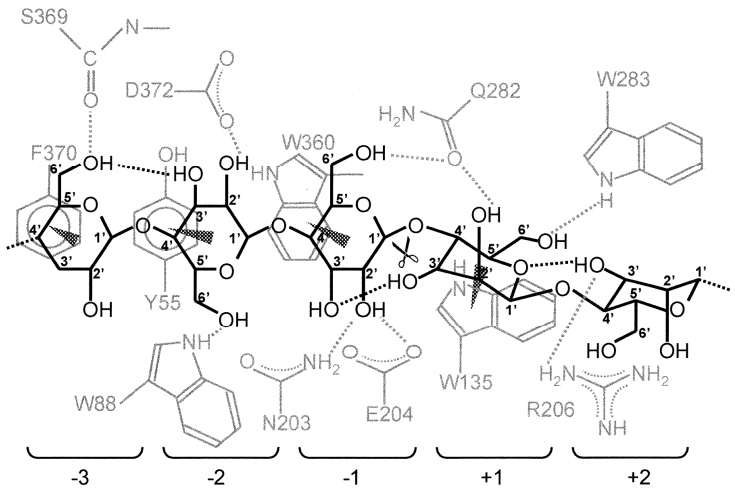


Figure 1.2 Schematic representations of the interactions of mannopentaose with mannanase in the 1-nsec MD simulation of the complex. Hydrogen bonds are indicated as dashed lines; ring-stacking interactions are indicated as semitransparent lines drawn from the protein (thin end) to the sugar ring (thick end). The scissile bond in the substrate is indicated (scissor). The carbon atoms in the substrate (1' to 6') are labeled. The subsites of each sugar molecules bind in (−3 to +2) are indicated (Bourgault *et al*., 2005).

In filamentous fungi production of the hemicellulose-degrading enzymes, hemicellulases are controlled at the transcriptional level by the available carbon source. Carbon catabolite repression in *Aspergillus* is predominantly mediated by the DNA-binding repressor protein CreA (Ruijter and Visser 1997). In the presence of carbon sources which can be metabolised easily (e.g. D-glucose, D-fructose) CreA represses the expression of a large number of genes by binding to specific sites [SYGGRG; Kulmburg *et al*. (1993)] in the promoters of these genes. The expression levels of creA itself are influenced by the presence of repressive carbon sources indicating auto regulation (Strauss *et al*. 1999). CreA plays an important role in the regulation of the expression of

genes from Aspergillus encoding plant cell wall-degrading systems. Repression by CreA has been reported for genes encoding arabinanases, endoxylanases, β-xylosidase, arabinoxylan arabinofuranohydrolase, feruloyl esterases and several pectinases (de Vries, 2003). CreA-mediated repression is not only observed in the presence of D-glucose or D-fructose, but is also triggered by other monomeric carbon sources. A study into the expression of several xylanolytic genes showed that the expression of these genes increases with reducing D-xylose concentration in a wild type *A. niger* strain (de Vries, 2003). Another study showed that the presence of different monomeric compounds results in different levels of CreA-mediated repression of ferulic acid induced expression of genes encoding *A. niger* feruloyl esterases (faeA and faeB) (de Vries, 2003). Although there was no publication related to regulation of mannanase expression and the role of CreA, the induction by mannans and galacto (gluco) mannans could be repressed by CreA gene family (de Vries, 2003)

1.1.1 Plant Cell Wall Polysaccharides

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They make up 90% of the plant cell wall and can be divided into three groups: cellulose, hemicellulose and pectins (Minic and Jouanin, 2006).

Plant cells undergo two type of cell wall deposition: the primary and secondary cell walls. The primary cell wall is synthesized during cell expansion at the first stage of the development and is composed of cellulose, hemicellulose, pectic polysaccharides and many proteins. The secondary cell wall is deposited in fully expanded and specialized cells (xylem and fibers). Compared to primary walls, secondary walls contain more cellulose with a higher degree of polymerization and crystallinity. A minor amount of the hemicellulose is composed of galacto (gluco) mannan.

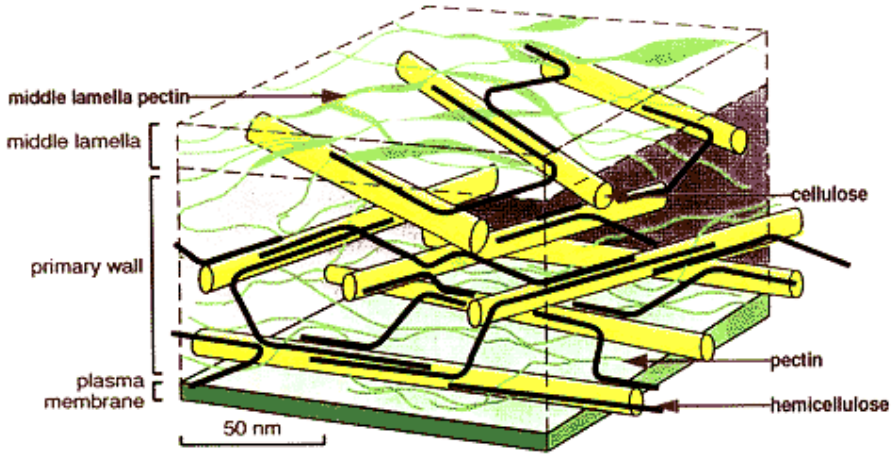


Figure 1.3 Plant cell wall structure (http://www.daviddarling.info/encyclopedia).

Galactomannans and galactoglucomannans consist of a backbone of β-1,4-linked D-mannopyranose residues, which can be substituted with α-1,6-linked D-galactopyranose residues. The amount of galactose linked to the mannose backbone depends on the origin of the galacto(gluco)mannan. In galactoglucomannan, the mannose backbone is interrupted with single β-1,4-linked D-glucopyranose residues (de Vries, 2003). Two types of galactoglucomannan have been identified, a water-soluble galactoglucomannan that contains acetyl residues linked to O2 or O3 of glucose or mannose, and a water-insoluble galactoglucomannan that does not contain acetyl residues and has a lower galactose substitution (Lindberg *et al*. 1973, Timell 1967). The presence of a disaccharide, consisting of two D-galactose residues connected via a β-1,2-linkage, was reported that was attached to the backbone of a galactoglucomannan of *Nicotania plumbaginifolia* via an α-1,6-linkage (Sims *et al*. 1997; de Vries, 2003). Galacto(gluco)mannans are abundant polysaccharides in the thickened secondary walls of gymnosperms and *Arabidopsis* (Minic and Jouanin, 2006). Mannan polysaccharides may have a structural role in cross-linking of cellulose fibrils. However, apart from the established role as a storage polysaccharide, the specific roles of these related polysaccharides are unclear (Handford *et al*., 2003). Since mannose comprises 5-9 mol percent of neutral sugars in *Arabidopsis* leaves, mannan polysaccharides might be a significant component of walls, but become lost in some cell wall fractionation procedures. Alternatively, the

mannose might be derived from the N-linked glycans of glycoproteins (Handford *et al*., 2003).

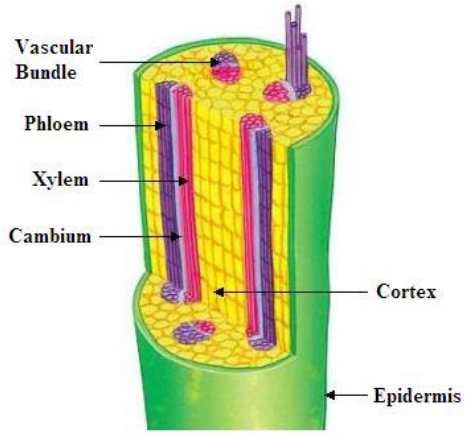


Figure 1.4 Secondary wall structures of stem (http://www.iadeaf.k12.ia.us)

In seeds, relatively large amounts of energy must be stored to guarantee survival of the germinating plant. Beside water immiscible fats, the sugars may be polymerized to thermodynamically stable solid phase outside the aqueous cytoplasm. When energy demand is high, these insoluble reserve polysaccharides can be enzymatically degraded to their components. In some plants, such substances have been adapted to take over the role of reserve polysaccharide. The galactomannans are one such group of polysaccharides. Replacing the storage compound, starch, with the galactomannan increased the ability of adaptation to arid environment. In some species of leguminous plants, galactomannans are stored outside of the cell membrane. Currently, three species are utilized in commercial gum production: locust bean (*Ceratonia siliqua*), guar (*Cyamopsis tetragogonolobus*) and tara (*Caesalpinia spinosa*) gum. Other species which are of interest but are not commercially available include *Cassia* species, mesquite (*Prosopsis* species) and fenugreek (*Trigonella foenum-graecum*) (Fox, 1997).

The locust bean plant is an evergreen plant cultivated mostly in the Mediterranean area. Each pods of the plant contain 10-15 seeds (or carob beans), which are the source of

the polysaccharide. Generally, the seeds weigh approximately 0.25 g each, of which about 38% is galactomannan. About 30% of the seed weight is, however, recovered as food-grade galactomannan (Fox, 1997).

Guar is mostly grown in the arid semi-desert regions of northwest India and Pakistan. The guar seeds weigh approximately 35 mg each, of which about 36% is galactomannan. The seed yield 25-30% of its weight as gum of commercial interest (Fox, 1997).

The tara gum is obtained from the seeds of a shrub indigenous to Ecuador and Peru. The seeds of fruits weigh 0.25 g each, of which approximately 18% is galactomannan (Fox, 1997).

1.1.2 Roles of β-Mannanases in Plant Developement

Endo-β-mannanase is not important only as an extracellular enzyme, but it plays also important roles in plant seed and fruit ripening. Seeds of several tribes of the leguminous plants contain extensive hemicelluloses deposition– in the form of galactomannans - within the walls of their endosperms (Bewley, 1997). Many of these seeds are not dormant, and the galactomannan reserves are mobilized following germination to support the growing seedling. The β-1,4-mannose Iinks in the mannose backbone are hydrolyzed via the action of endo-β-mannanase and the side-chains are released by α-galactosidases. β-Mannoside mannohydrolase (mannosidase; EC 3.2.1.25) further catalyzes the hydrolysis of the oligomannans produced by endo-β-mannanase activity (McCleary, 1979). Mobilization of galactomannans from the endosperms of some legume seeds [e.g. fenugreek (*Trigonella foenumgraecum),* guar *(Gledilsia triachanthos)* and clover (*Trifolium incarnatum* and *T. repens*)] requires the participation of an aleurone layer that surrounds the non-living galactomannan-filled cells. This layer synthesizes and secretes the three cell wall hydrolases (Reid *et al*., 1977). In other endospermic legumes [e.g. carob (*Ceratonia siliqua*)], the cells of the endosperm are not occluded by cell wall galactomannan during development; they remain metabolically active following germination and are the source of the hydrolases (Seiler, 1977; Bewley, 1997).

Ripening related softening of tomato has been intensely studied in this fruit than any other and yet to date the underlying biochemical changes in the fruit cell wall which achieve this remain hard to define (Carrington *et al*., 2002; Brummel *et al*. 2001). Endo-(1,4)-β-mannanase is one cell wall hydrolase identified in ripening tomato (Pressey,

1989). Its presumed substrates, unsubstituted mannans and the heteromannans, galactomannan, glucomannan and galactoglucomannan, become important storage reserves in some seeds, including tomato, and certain monocotyledonous bulbs (Wozniewski *et al*., 1992). It is conceivable that this minor class of cell wall polysaccharides could play a key strengthening role in fruit cell walls and be an important target in ripening related softening (Nonogaki *et al*., 2000).

Tomato fruit shows also physiological disorders when exposed to temperatures at or above 30°C. As the α- and β-galactosidases, as well as the endo-β- mannanase play roles during fruit ripening, the disorder is closely related with the degree of inhibitions of the activity at high temperatures (Sozzi *et al*., 1996). A positive relationship was found between germination ability at low temperature and endo-β-mannanase activity (Leviatov *et al*., 1995).

Interestingly, endo-β-mannanase is also present in many other seeds (including their embryos), and in vegetative tissues. None of which is known to contain appreciable quantities of mannans in their cell walls (Dirk *et al*., 1995). Other possible roles for the activity of endo-β-mannanase includes cell wall extension, defense and the limited mobilization of polymeric hemicellulose, in conjunction with other enzymes, to provide a supply of carbohydrate during specific seed and plant growth phases (Bewley, 1997).

1.2 The Thermotolerant Filamentous Fungus *Aspergillus fumigatus*

Among the eukaryotic organisms, only a few species of fungi have the ability to thrive at temperatures between 45 °C and 55 °C. Such fungi comprise thermophilic and thermo-tolerant forms, which are arbitrarily distinguished on the basis of their minimum and maximum temperature of growth (Cooney and Emerson, 1964).

Thermophilic and thermotolerant fungi are the chief components of the microflora that develops in heaped masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter wherein the warm, humid, and aerobic environment provides the basic conditions for their development (Maheshwari *et al.*, 2000). They constitute a heterogeneous physiology group of various genera in the Phyco-mycetes, Ascomycetes, Fungi Imperfecti, and Mycelia Sterilia (Mouchacca, 1997).

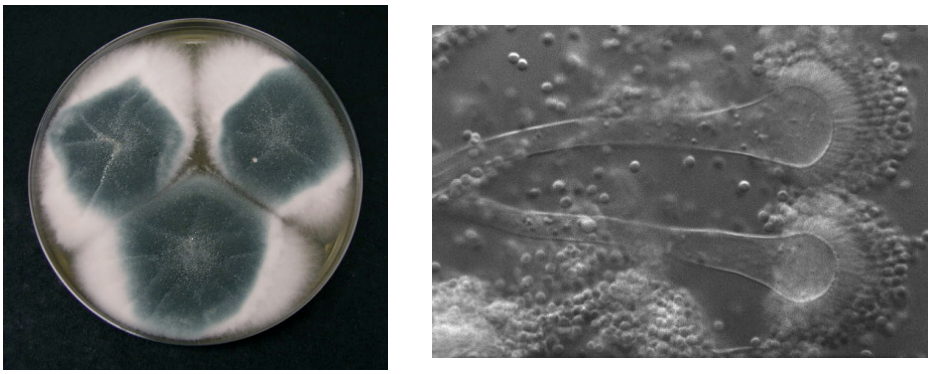


Figure 1.5 *Aspergillus fumigatus* (Photo by Dr. David Midgley).

Enzymes of thermophilic and thermotolerant fungi have been studied primarily to explore their suitability in bioprocesses. Modern studies on thermophilic and thermotolerant fungi were stimulated by the prospect of finding fungi capable of secreting high levels of enzymes and of finding novel enzyme variants with high temperature optima. The extracellular enzymes are appreciably thermostable (Maheshwari *et al.*, 2000).

The possibility of cloning and expressing selected enzymes has facilitated a switch from the production of enzyme mixtures to recombinant single enzyme components (Dalboge and Heldt-Hansen, 1994).

Among the 182 recognized species of Aspergillus (Pitt *et al.*, 2000), *Aspergillus fumigatus* is the most common human and animal pathogen (Denning, 1998, Smith 1989). *A. fumigatus* is a thermotolerant fungus and its natural niche is decaying organic material; as a consequence, it is one of the most common inhabitants of compost (Latgé, 1999). It produces mycotoxins fumigaclavine A, B, and C; fumigatoxin, fumigillin, fumitremorgen A and B; gliotoxin, and helvolic acid. Although human inhale Aspergillus spores at the rate of hundreds per day, they rarely experience complications. Under special circumstances, Aspergillus specis can produce a spectrum of diseases (Immaculata *et al*., 2004). Infection is usually associated with the pulmonary system, although eye, ear, and sinus cavities can also be affected. It is also a principal agent of

aspergillosis and inhalation mycosis, allergic asthma, hypersensitivity pneumonitis, aspergilloma, which were mostly seen in immunocompromised patients suffering from AIDS, hematological malignancy, solid cancer or solid organ transplantation (Denning, 1998; Smith, 1989). Although the thermo-tolerant nature could be very useful in many applications, its industrial use is limited by its pathogenic nature. The genome project of *A. fumigatus* is completed for the strain Af293 in 2003 and the sequence information is publicaly available. *A. fumigatus* has a total genome size of 32 Mb.

1.2.1 β-Mannanases of *Aspergillus fumigatus*

Mannanase of *A. fumigatus* has a special domain at N-terminus called as cellulose binding module (CBM or cellulose binding domain, CBD), which binds to celluloses. The role of the CBM in mannanase activity is not well understood although it has been suggested that adsorption of the CBMs increases the concentration of the catalytic modules near the substrate, thereby increasing hydrolytic activity (Wu *et al*., 2007). This module, however, does not bind to the mannan backbone, as the name indicates the affinity is towards cellulose structures. Due to the nature of the resources, the substrates are found to be in the form of a matrix consisting of celluloses and mannans. Mannanase with CBM (AfMAN1) was studied by Puchart (2004) before. They also purified AfMAN1 and two isoforms (MANI and MANII) were identified. IEF afforded several glycoprotein bands with p*I* values in the range of 4.9–5.2 for MANI and 4.75–4.9 for MANII, each exhibiting enzyme activity. MANI as well as MANII showed highest activity at pH 4.5 and 60 °C and were stable in the pH range 4.5–8.5 and up to 55 °C (Puchart *et al*., 2004).

1.3 Measuring the Activity of endo-β-Mannanase

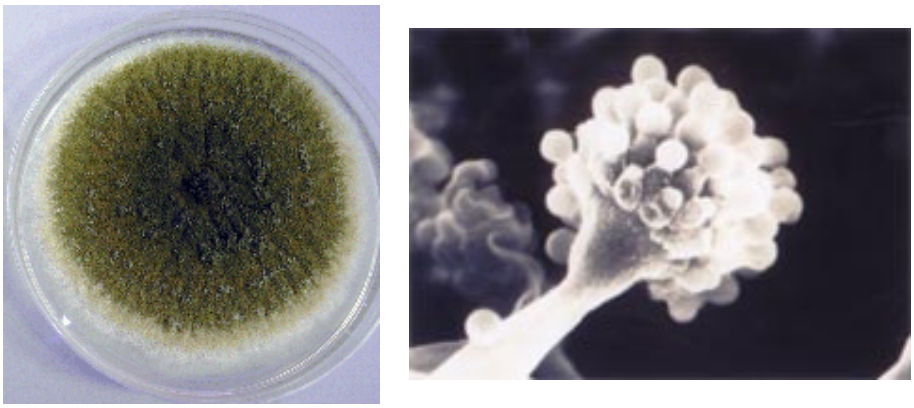
The studies on the activity of this enzyme relied upon viscometric (Halmer *et al*., 1976; McCleary *et al*., 1975; Reid *et al*., 1977) and colorimetric (McCleary, 1978) techniques or the use of a reducing sugar assay of the enzymic products (Villarroya *et al*., 1978). Each of these techniques has its limitations. Although accurate, assaying endo-β-mannanase activity by viscometry is time-consuming and not convenient for large-scale analysis. A more rapid variation was developed to assay the enzyme in fractions following density-gradient centrifugation, using a vertical capillary tube (Halmer, 1989). However, for accuracy, fractions showing activity were reassayed using the standard Cannon–Ubbelhode suspended level viscometer (Halmer *et al*., 1976). The colorimetric

assay, using carob galactomannan dyed with Remazol brilliant blue (RBB) (McCleary, 1978) appears to work well for endo-β-mannanase. Galactose substitution on the mannan backbone also influences the efficacy of RBB–galactomannan as a substrate (McCleary, 1978). The reducing sugar assay for the products of galactomannan hydrolysis does not discriminate between endo- (endo-β-mannanase) and exo- (β-mannoside mannohydrolase) enzymes, or from that of accompanying α-galactosidase activity (Bourgault *et al*., 2002).

A colorimetric assay for endo-β-mannanase was developed using Congo red dye to stain galactomannan incorporated into a Phytagel matrix (Downie *et al*., 1994). This assay is as sensitive as the viscometric one, is linear over five orders of magnitude, and has been used successfully to detect endo-β-mannanase in many seeds, fruits, and vegetative tissues (e.g., leaf abscission zones). Technically, the assay involves making wells in a Phytagel–galactomannan matrix poured into 9-cm diameter Petri dishes and placing the enzyme extract in the wells for up to 24 h. Substrate hydrolysis is detected by staining the galactomannan with Congo red and measuring the diameters of the clearing zones around the wells. The extent of substrate breakdown is recorded in relation to that achieved by known concentrations of a commercial *Aspergillus* endo-β-mannanase. Minor variations in this Petri dish assay include the use of agarose as the matrix and different stain-development protocols (Still *et al*., 1997; Bourgault *et al*., 2002).

1.4 A*spergillus sojae* Expression System

Aspergillus sojae belongs to *Aspergillus* section *Flavi*. Like *A. oryzae*, *A. sojae* is a species of industrial importance. It is widely used in food fermentations, such as soy sauce and bean paste production. *Aspergillus sojae*, which is believed to be a domesticated strain of *Aspergillus parasiticus*, contains all of the aflatoxin biosynthetic genes but is unable to produce aflatoxins and is generally recognized as safe (GRAS) for producing fermented foods (Chang *et al*., 2007).



A **B**

Figure 1.6 *Aspergillus sojae.* Its appearance when grown on minimal medium (A) and the conidia and conidiospores under the microscope (B).

A new expression host from *A. sojae* ATCC strain was developed by Margreet Heerikhuisen, Cees van den Hondel and Peter Punt, TNO Nutrition and Food Research, Dept. of Microbiology (Zeist, The Netherlands). In this study, *A. sojae* ATTCC11906 strain which shows the lowest proteolytic activity was selected for transformation purposes. An auxotrophic marker system was developed for *A. sojae* involving the usage of *pyrG* as a selection marker. Auxotrophic mutant of *Aspergillus sojae* which lacks the ability to synthesize uridine was used. By co-transformation of pAMDSPYRG, with *amd*S and *pyrG* genes encoded by the expressional vector, the true transformants could grow in the minimal medium while untransformed *pyr*G- mutants could not grow in the absence of uracil or uridine from the selective medium as they were blocked in pyrimidine biosynthesis because they lack orotidine-5'-phosphate-decarboxylase. In a relevant study, it has been shown that the efficiency of gene targeting (homologous integration) was unaltered when transcription was induced at different stages in the transformation procedure. The most dramatic factor affecting targeting efficiency was the studied specific locus *amd*S (acetamide utilization) (Koukaki *et al*., 2003).

1.5 *Pichia pastoris* Expression System

Many heterologous proteins have been succesfully expressed in the yeast, *Saccharomyces cerevisiae*. A large amount of knowledge has been accumulated about its genetics and physiology. While this species has been used for the production of some eukaryotic foreign proteins, it has several limitations. Generally, the yields of product are low. Yields reach a maximum of 1-5 percent of the total protein. The presence of foreign gene products creates additional stress on the cells. The production of the protein during the growth phase decreases the growth rate. Even the use of inducible plasmid promoters to achieve a partial separation between the growth and protein production phase, has not been effective due to the instability of plasmid (Buckholz *et al*., 1991). Instability is especially high when the foreign protein product is toxic to the yeast. In addition to the difficulties with scaling up protein production to get higher yields, several reports have noted the hyperglycosylation of secreted glycoproteins which may cause differences in immunogenicity, diminished activity, and decreased stability of the foreign protein. Also, many of the secreted proteins of *S. cerevisiae* are not secreted in the medium, but rather in the periplasmic space. This leads to problems with purification and further decreases product yield (Buckholz *et al*., 1991). Due to these problems, several other species of yeast have been analyzed (Faber *et al*., 1995).

One of the alternative species that has been looked at is *Pichia pastoris*. There are several reasons that this particular species is appealing. The protocols for its growth did not have to be worked out because it can be grown under conditions that are similar to *Saccharomyces cerevisiae*. *Pichia pastoris* has a strong, inducible promoter that can be used for protein production. It is capable of performing post-translational modifications. Isolation of foreign protein is facilitated by the fact that *P. pastoris* does not secrete a lot of its own proteins.

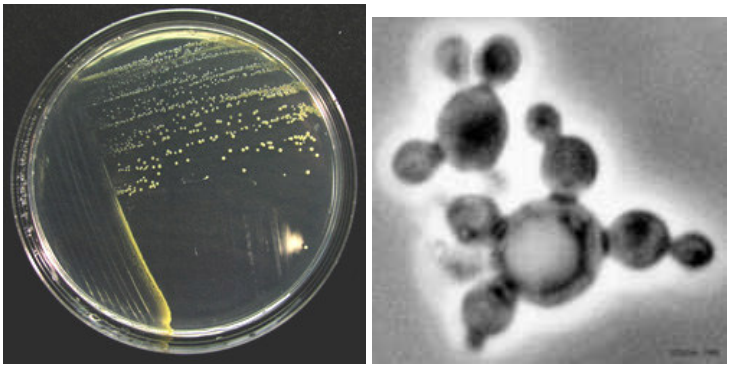


Figure 1.7 *Pichia pastoris* on PDA agar and under the microscope (photo by Ching-Tsan Huang)

The inducible promoter of *Pichia pastoris* is related to its methyltropic nature. The first step in the utilization of methanol is the oxidation of methanol to formaldehyde and hydrogen peroxide (Ledeboer *et al*., 1985). This step is catalyzed by the enzyme alcohol oxidase. The expression of this gene is tightly regulated. When the yeast is grown on glucose or ethanol, alcohol oxidase is not detectable in the cells. However, when the yeast is grown on methanol, alcohol oxidase can make up to thirty-five percent of the total cellular protein. The control of the amount of alcohol oxidase is largely transcriptional (Cregg *et al*., 1985).

There are two alcohol oxidase genes: AOX1 and AOX2. The protein coding regions of the genes are largely homologous, 92 percent and 97 percent at the nucleotide and amino acid sequence levels respectively (Ohi *et al*., 1994; Ellis *et al*., 1985). The promoters share very little homology. No mRNA of the two genes is detectable when the yeast is grown on glycerol. The promoter region for AOX2 has a repressor region that leads to the inhibition of gene expression, and an activation region that leads to the enhancement of gene expression. The AOX1 gene promoter probably has a similar mechanism (Ohi *et al*., 1994; Koutz *et al*., 1989).

The key enzymes for methanol metabolism are compartmentalized in the peroxisomes. The proliferation of peroxisomes is a reflection of environmental conditions. When the cells are grown on glucose very few peroxisomes are present. When grown on methanol, peroxisomes may take up to 80 percent of the total cell volume (Cregg *et al*., 1989).

Previous results clearly show that the alcohol oxidase promoter is both tightly regulated and is a strong promoter. The production of foreign proteins can be repressed until the culture is saturated with colonies, and then the production of the foreign protein can begin with the derepression and induction of the gene. In addition to being able to regulate the production of the protein very tightly, the post- translational modifications made by *Pichia pastoris* are more suitable for use in humans. The structure of carbohydrate added to secreted proteins is known to be very organism specific. Many proteins secreted from *S. cerevisiae* have been demonstrated to be antigenic when introduced into mammals. Thus the use of glycoprotein products synthesized by yeast for therapeutic purposes has been avoided. A comparison of a *S. cerevisiae* protein secreted from *S. cerevisiae* and *P. pastoris* has shown distinct differences between N-linked oligosaccharide structures added to proteins secreted from this yeast. The majority of the N-linked oligosaccharide chains are rich in mannose. However, the length of the carbohydrate chains is much shorter in *P. pastoris*. Even the longest chains of protein produced in *P. pastoris* contained only approximately thirty mannose residues, which is significantly shorter than the 50 to 150 mannose residue chains typically found on *S. cerevisiae* glycoproteins. The second major significant difference between the glycosylation by *S. cerevisiae* and *P. pastoris* is that glycans from *P. pastoris* don't have alpha 1,3-linked mannose residues that are characteristic of *S. cerevisiae* (Cregg *et al*., 1993). The enzyme that makes alpha 1,3 linkages is α-1,3-mannosyl transferase and it is undetectable in *P. pastoris*. This is significant, because the alpha 1,3 linkages on *S. cerevisiae* glycans are primarily responsible for the highly antigenic nature of glycoproteins used for therapeutic products (Cregg *et al*., 1993).

The purpose of mass producing proteins is to purify them and then use them to treat diseases. One of the first steps is the isolation and purification of a foreign protein product. *P. pastoris* grows on a simple mineral media and does not secrete high amounts of endogenous proteins. Therefore the heterologous protein secreted into the culture is relatively pure and purification is easier to accomplish (Faber *et al*., 1995). Secretion of the foreign protein is accomplished by recombining a signal sequence in front of the desired foreign gene when it is inserted into the host DNA (Tschopp *et al*., 1987).

1.6 *Arabidopsis thaliana* Expression System

During the last two decades the use of industrial enzymes expanded dramatically in areas such as bio-pulping, food processing, carbohydrate conversions, chemical

conversions, food and animal feed additives, cleaning, detoxification of environmental toxins. So far several plants have been suggested as candidates for use as bioreactors including tobacco, potato, soybean, alfalfa, corn and rapeseed (Hood *et al*., 1999). They have many advantages including the low cost production, stability of enzyme in the storage tissue such as seeds and tubes ease of the scale-up and probably the most important advantage is that the possibility of the direct addition of plant parts to the industrial processes. The ability to apply the product directly to industrial processes enables to minimize the handling and enzyme manipulation and preparation. The scale-up time for enzyme production from identified transgenic lines is short and inexpensive, mostly involving planting of increased acreage (Hood, 2002).

The production of enzymes in the plants has some advantages over the conventional enzyme production methods such as those in bacteria. For example, the lack of post-transcriptional processes in bacteria makes it unfavorable to use the prokaryotic system. Incorrect folding of enzymes is yet another problem regarding heterologous hosts.

The problem with the yeast mediated production is the hyperglycosylation of enzymes. Production via insect cells or mammalian cells is also possible, but the cost of the product is increased. Generally, the difficulty of scale-up in the conventional fermentation method and the preservation of enzyme activity and its stability of enzymes during the storage are the main drawbacks of such production techniques. Both the storage of enzyme and the packaging are the main factors affecting the price of the product (Souppe *et al*., 2002).

The plant serves as a fermentor in the production of the enzymes. As the scale-up can be achieved easily by growing the plant in larger area and the enzyme is stored in the specific compartments as vacuoles, those problems are eliminated and the marketing cost is decreased. Since the plant sources are used in many industrial applications, plants, producing the desired enzymes, can be directly used as the raw materials in the processing medium without any requirement for purification or concentration (Austin-Phillips *et al*., 2001).

The main problem with this method is the low expression rate of the protein in the plant. By the extended researches on the plant promoters and other expression systems on the plants is expected to solve this problem (Pen *et al*., 1992; Jensen *et al*., 1996; Herbers *et al*., 1995).

Figure 1.8 *Arabidopsis thaliana* Col-0.

Arabidopsis is widely used by plant molecular geneticists to study both basic and applied problems. Most results can be extended to larger field species. It is a small dicotyledonous species, a member of the Brassicaceae or mustard family. The short life cycle and prolific seed production (*c.* 10,000 seeds/plant) of *A. thaliana* makes it an ideal plant for experimental studies, including transformation and regeneration. Moreover, it has a small, completely sequenced genome of 124,000 kbp/haploid copy.

A recent survey of genes in *Arabidopsis thaliana* has indeed indicated that the proportion of plant genes that encode carbohydrate-acting enzymes, such as glycosylhydrolases and glycosyltransferases, is greater than that in humans or other fully sequenced organisms (Coutinho *et al*., 2003).

Many of the recent advances in understanding of wall biosynthesis and function result from the use of *Arabidopsis thaliana*, as a model plant that is amenable to genetic analysis (Reiter, 2002). Forward and reverse genetic screens for Arabidopsis mutants in

cell wall synthesis are being carried out in an increasing number of laboratories. Arabidopsis is also becoming an important model for wood fibre biosynthesis, since the secondary wall formation appears to be similar to that of woody plants. In order to understand the phenotype of these mutants, sensitive tools for polysaccharide analysis, such as mass spectrometry, polysaccharide analysis by carbohydrate gel electrophoresis (PACE) and panels of specific antibodies are being developed (Handford *et al*., 2003).

1.7 Applications of β-Mannanases

Guar is used extensively in food, oil and paper industries due to its excellent viscosifying properties and low cost (Brant *et al*., 1981; Shay *et al*., 1989). Unlike the locust bean gum, guar gum can dissolve in cold water. In many of its applications, however, the chemical architecture or chain size of the guar molecule needs to be modified (Bulpin *et al*., 1990). In the oil and gas industry, guar is used with sand (proppant) to fracture oil- or gas-bearing rock in a process known as hydraulic fracturing. Subsequently, the guar gel needs to be degraded, preferably in a controlled manner, to facilitate oil and gas flow (Prud'homme *et al*., 1989; Kesavan *et al*., 1992). In both these applications, enzymes offer a convenient and efficient way to degrade and hydrolyze the galactomannan (Tayal *et al*., 1999).

Gums are also used to evaluate the effects of hemicelluloses when added to poultry or swine diets. These non-starch polysaccharides have been shown to diminish the growth performance and inhibit nutrient absorption in poultry (Vorha *et al*., 1964; Verma *et al*., 1982) and swine (Blackburn *et al*., 1981; Rainbird *et al*., 1984; Edwards *et al*., 1988). A β-mannanase preparation added to corn-soybean meal-based diets improved feed efficiency in late-nursery pigs and improved daily gain and feed efficiency in growing finishing pigs. Additionally, β-mannanase increased lean gain of pigs. The effect of this enzyme is just equivalent to the fed diets containing 2% soybean oil (energy equivalent of 100 kcal/kg). It was also suggested β-mannanase elicits improvements in growth performance not only through degrading β-mannans, but also via an indirect effect on regulatory hormones (Pettey *et al*., 2002).

Mannanase has also been recently added into laundry washing powders (PuraBrite^{TM}). Gums are widely used in large quantities in the processed food industry. Seed gums, mainly guar and locust bean gums, are used as thickeners and for gel texture in food and

cosmetics products. Consequently, the addition of carbohydrolase enzymes in detergents capable of breaking down these gums is gaining in popularity (Boswell, 2002).

1.8 Prebiotics and Effect of Mannose and Manno-oligosaccharides on Human Health

A prebiotic is defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson *et al*., 1995). Modification by prebiotics of the composition of the colonic microflora leads to the predominance of a few of the potentially health-promoting bacteria, especially, but not exclusively, lactobacilli and bifidobacteria (Roberfroid, 2000).

The only prebiotics for which sufficient data have been generated to allow an evaluation of their possible classification as functional food ingredients are the inulin-type fructans, which include native inulin, enzymatically hydrolyzed inulin or oligofructose, and synthetic fructooligosaccharides. Inulin-type fructans are indigestible oligosaccharides for which a wide range of scientific observations are already available and which demonstrate an array of potential health benefits (Roberfroid *et al*., 1998a; Roberfroid *et al*., 1998b; Mitsuoka, 1990). Other dietary carbohydrates make up also a large family of miscellaneous compounds with different physiological effects and diverse nutritional properties that deserve the attention of nutritionists. In the carbohydrate family, the indigestible oligosaccharides are of particular interest and may, in the next decade, be one of the most fascinating functional food ingredients (Roberfroid, 2000).

The EU-funded project PROTECH (QLK1-CT-2000-30042) dealt with the selection and improvement of unique processing technologies related to the production and improvements of probiotics and prebiotics as well as the interaction of both in the production, distribution and utilization pipeline, in order to better maintain or even improve the health-related performance of these products. Work has been performed on enzymatic modification of prebiotics to allow further development of their improved forms. In particular, better understanding has been gained on the mechanism of galactan-modifying enzymes of bifidobacteria in the degradation of galactan and galacto-oligosaccharide. Furthermore, work on the physical modification of starch has demonstrated the beneficial effect of high hydrostatic pressure treatment in enhancing the content of resistant starch, which is regarded to exert prebiotic effect (Knorr, 2005).

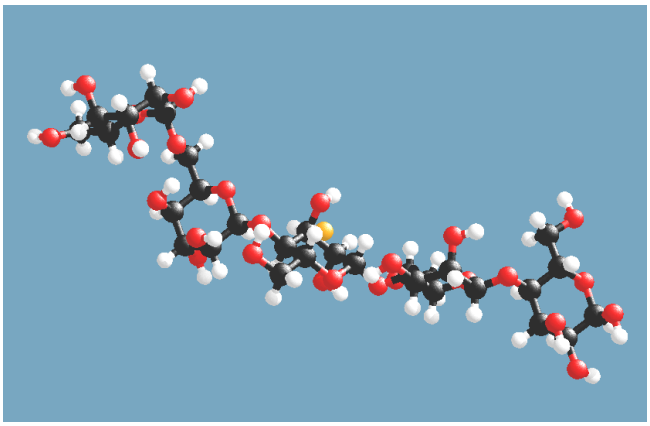


Figure 1.9 Mannotetraose with α-(1→6)-linked galactose side chain

Mannanase is suitable for mannooligosaccharide production, which is used as a functional food additive for the selective growth of human-beneficial intestinal microflora (Kobayashi *et al*., 1987). The ability of mannanases to degrade mannotriose was shown by Takahashi *et al*. (1984). Some fungal mannanases can convert mannotetraose to mannotriose and mannobiose through the transglycosylation reaction (Puchart *et al*., 2004). For most mannanases, a degree of polymerization of at least four is required for a significant hydrolysis rate (Jiang Z. *et al*., 2006).

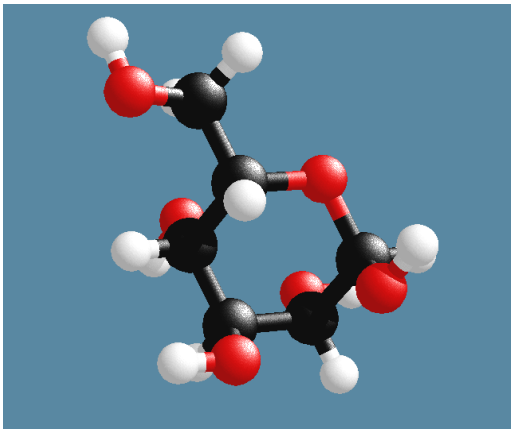


Figure 1.10 D-(+)-mannose

Urinary tract infection (UTI) is a common and distressing disease that is a bacterial infection (caused mostly by the bacteria *E. coli*) that affects the inside lining tissue of the urinary system. The tissues of urinary tract become inflamed, irritated, and swollen so that urinary ducts can partially obstruct normal flow, making it painful and difficult to pass to the urine. *E. coli* that causes most UTIs are among the most common bacteria in the gastrointestinal tract, where they aid digestion and produce a few vitamins. However, when *E. coli* gain entry to the urinary tract via the urethra, they may attach to the internal lining of the bladder, multiply, and spread. *E. coli* adhere to the body tissue while the infection in UTI, and attach to cells lining the bladder and urinary tract using filmy, hair-like projections called fimbria on their cell walls (Fowler *et al*., 1977). At the tip of each fimbrium is a glycoprotein called a lectin that is programmed to bind to the first molecule of the sugar mannose that naturally exists on the surfaces of these cells, acts as receptors, inviting the fimbria of *E. coli* to attach, and allowing them to bind to the tissue in a tight, Velcro- like grip (Ofek *et al*., 1982). In the case of the presence of mannoses not just on the surface of the epithelial cells but also in the urine as well, hinders the attachment of bacteria on cells and prevents the infection (Ofek *et al*., 1978; Bar-Shavit *et al*., 1980).

1.9 Scope of the Study

In this study, it is aimed to clone the mannanase gene of *Aspergillus fumigatus* for expression in the heterologous hosts, *Aspergillus sojae*, *Pichia pastoris* and *Arabidopsis thaliana*. Most of the enzymes of *Aspergillus fumigatus* appear to be thermostable owing to the thermotolerant nature of the fungus. The thermostable nature of enzymes is often an advantage in industrial applications. Taking into account the wide range of useful applications of mannanases, it is aimed to initiate research on the production of the enzyme on an industrial scale. For an economical large-scale process it is essential to produce mannanase at large quantities in a safe organism. However, *A. fumigatus* is not suitable as an industrial organism due to its pathogenic nature. Therefore, the gene coding for mannanase was cloned and transformed into GRAS microorganisms and the catalytic properties of the heterologous mannanases were investigated including their possible use for prebiotic mannooligosaccharide production. Indeed, locust bean gum was digested mainly to mannotetraose, mannobiose and mannotriose when incubated with mannanase, suggesting that this enzyme is suitable for prebiotics mannooligosaccharide production. The observed unique properties of the purified β-mannanase make this enzyme attractive for biotechnological applications.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Organisms and Growth Conditions

Aspergillus fumigatus IMI 385708 (formaerly known as *Thermomyces lanuginosus* IMI 158479) was kindly provided by Dr. Peter Biely from the Slovak Academy of Sciences, Institute of Chemistry. Stock cultures of *A. fumigatus* were grown on YpSs agar slants containing starch as the only carbon source (Appendix B) at 30°C and maintained at 4°C.

The *Aspergillus sojae* (ATCC11906) strain was kindly supplied by Dr. Peter Punt from TNO Nutrition and Food Research, Department of Microbiology Holland. Stock cultures of *A. sojae* were grown on complete medium agar (Appendix B) at 30°C and maintained at 4°C..

For the induction of mannanase, *A. fumigatus* was cultivated in 400 ml YpSs broth containing 0.5% LBG instead of starch (Appendix B) in 1000 ml erlenmeyer flasks at 30°C and 155 rpm. *A. sojae* was cultivated in 400 ml YpSs broth containing 1% glucose (Appendix B) instead of starch in 1000 ml erlenmeyer flasks at 30°C and 155 rpm.

Pichia pastoris strain GS115 was purchased from the company, Invitrogen Life Technologies, as a part of the yeast expression system. Before transformation, the yeast was maintained in YPD medium (Appendix B). After transformation, it was grown in 250 ml YpSs medium supplemented with 1% methanol to induce the protein synthesis. As selective agent for true transformants, Zeocin was added to the medium at a final concentration of 100 µg/ml.

Agrobacterium tumefaciens strain pGV3101 pmp90 was an in-house organism used in the transformation of Arabidopsis. It was cultivated in YEB medium (Appendix B)

supplemented with 50 µg/ml kanamycin, 25 µg/ml gentamycin and 100 µg/ml rifampicin. *Arabidopsis thaliana* Col-0 (ecotypes Columbia 0) was used for transformation purposes.

E.coli strain XL1-Blue MRF (RecA -) was purchased from Stratagene and used in the sub-cloning studies. The strain was grown in LB-medium broth at 37°C and 155 rpm or on LB-medium agar at 37°C.

2.1.2 Chemicals and Enzymes

The list of chemicals and enzymes used and their suppliers are given in Appendix A.

2.1.3 Growth Media, Buffers and Solutions

The preparation of the growth media, buffers and solutions used are given in Appendix B.

2.1.4 Vectors

The list of vectors is given in Appendix C.

2.2 Methods

2.2.1 Nucleic Acid Isolation

2.2.1.1 Genomic DNA Isolation

Mycelia were filtered through Whatman 3MM filter paper and washed with Tris-HCl buffer (pH 7.5) until the flow-through was colourless. Excess water of mycelia was removed with the aid of filter paper. Dried mycelia were immediately frozen in liquid nitrogen and ground into powder by a mortar and pestle. Powdered mycelia were transferred in a sterile 50 ml falcon tube. Then 25 ml TTE buffer (Appendix B) was added. After homogenization, the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and 15 ml of lysis buffer (Appendix B) was added on the pellet and mixed by inversion. Samples were incubated at 37°C for 15 minutes. After the addition of an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), the mixture was centrifuged at 5500 rpm for 8 minutes, upper phase was transferred to a new tube and

phenol:chloroform extraction was repeated until the interphase was clear. The upper phase from the last extraction was transferred to a sterile Sorvall tube of 10 ml. 0.1 volume sodium acetate (3 M, pH 5.2) (Appendix B) and 2 volume cold absolute ethanol was added to the collected upper phase. The mixture was left overnight at -20°C. Then the tubes were centrifuged at 12 000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 10 ml 70% ethanol (Appendix B). The tubes were centrifuged at 6000 rpm for 3 minutes at 4°C and the supernatant was discarded. The tubes were centrifuged at 6000 rpm for 3 minutes at 4°C and the liquid part was pipetted out of the tube. After being allowed to air-dry for 10 minutes, the pellet was dissolved in 100 µl sterile double distilled water at 4°C overnight. To the dissolved DNA solution, 10 µl, 10 mg/ml DNase free RNase (Appendix B) was added and incubated at 37°C for 2 hours.

To estimate the concentration of resulting DNA, agarose gel electrophoresis was performed. The isolates were run on 0.8% agarose gel (Appendix B) along with a marker of known concentration (eg. DNA/EcoRI+HindIII) and their intensities were compared.

The concentration was calculated according to the following formula:

$$\frac{L_{FRAGMENT}}{TL_{MARKER}} \times C_{MARKER} \times V_{MARKER} \times I_{BAND} \times \frac{1}{V_{DNA}}$$

Where $L_{FRAGMENT}$ is the length of the fragment, TL_{MARKER} is the total length of the marker, C_{MARKER} is the concentration of marker; V_{MARKER}, is the volume of marker, I_{BAND} is the intensity of the band and $V_{DNA}$is the volume of loaded DNA

2.2.1.2 Total RNA Isolation

Mycelia that were grown in appropriate media were filtered through Whatman 3MM filter paper and washed with Tris-HCl buffer (pH 7.5) until the flow through was colourless. Filtered and paper-dried mycelia were imidiatelly frozen in liquid nitrogen and ground into powder with a DEPC-treated mortar and pestle for 2 minutes. 100 mg of

mycelia powder was transferred into precooled 1.5 ml eppendorf tube and was shock frozen in liquid nitrogen, and stored until use at –80°C.

Total RNA was isolated by three methods; either by guanidine thiocyanate extraction, by Trizol extraction or by spin column, supplied as a kit. In the RNA isolation, all plastic wares were treated with 0.1% DEPC and autoclaved following baking the drained out plastics at 100 °C for 24 hours.

2.2.1.2.1 Guanidine Thiocyanate (GTC) Extraction

To isolate total RNA by GTC extraction method, "RNagent Total RNA Isolation Kit" (Promega) was used. 100 mg grinded mycelium was transferred to a precooled 1.5 ml eppendorf tube and 600 µl of denaturating solution supplied with the kit, containing GTC and β-mercaptoethanol, was added into each tube and the suspension was homogenized by pipetting several times. Then, 60 µl of 2 M sodium acetate (pH,4) and 600 µl were added and the mixture was shaken vigorously. After incubating the tubes on ice for 15 minutes, the upper phase was transferred into a new 1.5 ml eppendorf tube and centrifuged for 10 minutes at maximum speed. The upper phase was transferred into a new tube and 500 µl denaturating solution was added. After a short spin, 500 µl of supernatant was transferred into another 1.5 ml eppendorf tube. 500 µl of ice-cold isopropanol was added on top of the supernatant and incubated at –20 °C for 60 minutes. The tubes were centrifuged for 20 min at 4°C at max speed to pellet the RNA, the pellet was washed with 1 ml of 75% ethanol and centrifuged for 10 min at 4°C at max speed. The RNA pellet was dissolved in 30 µl nuclease-free-water by incubating the tubes at 0 °C overnight.

2.2.1.2.2 Trizol Extraction

100 mg grinded mycelium was transferred to a precooled 1.5 ml eppendorf tube, 1 ml of TRIzol reagent (Invitrogen) was added and the mixture was vortexed for 15 min at room temperature. The tubes were centrifuged for 5 min at room temperature at maximum speed. Then, 900 µl of the supernatant was transferred into a new tube by avoiding any contact with the pellet. 180 µl chloroform was added into each tube and mixture was shaken vigorously for 15 seconds and incubated at room temperature for 3 min. After centrifugation for 15 min at 4°C at max speed, 450 µl liquid from the upper

phase was carefully pipeted into a new 1.5 ml tube and 200 µl chloroform was added. Tubes were vigorously shaken for 15 seconds and incubated at room temperature for 3 min. They were then centrifuged for 5 min at room temperature at max speed and 400-450 µl from the upper phase was transferred it into a new 1.5 ml eppendorf tube by avoiding any contact with the interphase. Then 1 volume of isopropanol was added and the content was mixed by inverting several times and incubated for 10 min at room temperature. RNA was peletted by centrifugation in an Eppendorf centrifuge for 10 min at room temperature at max speed. The supernatant was decanted and 1.0 ml of 75% ethanol was added. To wash the pellet, the mixture was vortexed and left for 3 min at room temperature. The tubes were spun down for 5 min at room temperature at max speed. Then, supernatant was removed and centrifuged for an additional 15 sec to collect the pellet at the bottom of the tube. Any visible liquid was removed and the tubes were covered with aluminum foil and let air dry for 10 min. Then, 50 µl DEPC-water was added and the tubes were incubated for 15 min at 65 ℃ to dissolve the pellet.

2.2.1.2.3 Total RNA Isolation by Spin Column

As an alternative method to isolate RNA, RNeasy Plant Mini Kit (Qiagen) was used. This kit was designed generally for purification of total RNA from plant cells and tissues and filamentous fungi. This method is the combination of the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane.

Almost 100 mg of frozen dried powdered mycelia were placed in 1.5 ml tube, 450 µl RLC Buffer was added and vortexed vigorously. The lysate was transferred to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuged for 2 min at full speed. The supernatant of the flow-through was carefully transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. 0.5 volume of ethanol (96–100%) was added to the cleared lysate, and mixed immediately by pipetting without centrifugation. The 650 µl sample was transferred in a 2 ml collection tube and centrifuged for 15 s at 10,000 rpm. The flow-through was discarded. 700 µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at 10,000 rpm. 500 µl Buffer RPE was added to the RNeasy spin column. The flow-through was discarded. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 2 min at 10,000 rpm. An additinal centrifugation at top speed for 1 min was performed. The

RNeasy spin column was placed in a new 1.5 ml collection tube and 30 µl RNase-free water was added directly onto the spin column membrane. To elute the RNA, the tube with filter was centrifuged for 1 min at 10,000 rpm. Using the eluate, the last step was repeated with a final centrifugation for 3 min at 10,000 rpm.

2.2.1.2.4 Determination of RNA Quality and Quantity

RNA concentration was determined by measuring absorbance at 260 nm on a UV-spectrophotometer in 10 mM Tris/HCl pH 8.0 (one absorbance unit = 40 µg/mL RNA). The RNA concentration should be > 1.1 µg/µl. The integrity of the RNA was tested by agarose gel electrophoresis. For this purpose, RNase-free 1% agarose gel in TAE buffer was prepared. The samples were diluted to approx. 100-150 ng/µl. RNA samples were loaded on the gel and run for 45 min at 80 V.

2.2.1.3 mRNA Isolation

mRNA was isolated directly from the fresh tissue by using FastTrack 2.0 mRNA isolation kit (Invitrogen). 1-2 g harvested mycelium was quickly frozen in liquid nitrogen. The frozen mass was placed in a mortar precooled with liquid nitrogen and ground with a pestle until a fine powder was obtained. The powder was transferred with the liquid nitrogen to a sterile, 50 ml centrifuge tube. Liquid nitrogen was let to evaporate and 15 ml FastTrack® 2.0 Lysis Buffer was added. After homogenization of the mixture, the cell lysate produced was incubated at 45°C for 60 minutes. The tube was centrifuged at 6000 rpm for 5 minutes at room temperature and the supernatant was transferred into a new tube. The NaCl concentration of the lysate was adjusted to 0.5 M final concentration by adding 950 µl of the 5 M NaCl stock solution to each 15 ml lysate and mixed thoroughly by inversion. The lysate was forced to pass 3 to 4 times through a sterile plastic syringe fitted with an 18-21 gauge needle to shear any remaining DNA. A vial of oligo_(dT) cellulose was added to the lysate. The tube was incubated 2 minutes at room temperature and rocked gently at room temperature for 60 minutes. The oligo_(dT) cellulose was pelletted at 6000 rpm for 5 minutes at room temperature. The supernatant was carefully decanted from the resin bed. The oligo_(dT) cellulose was resuspended in 20 ml of binding buffer and centrifuged at 5000 rpm for 5 minutes at room temperature. The binding buffer was removed from the resin bed, resin was gently resuspended in 10 ml binding buffer and centrifuged as previously. The resin was resuspended in 10 ml low salt wash buffer and centrifuged at 6000 rpm for 5 min. The resuspension and centrifugation

steps with low salt buffer were repeated 3 times. After the last wash, the oligo_(dT) cellulose was resuspended in 800 µl low salt wash. The oligo_(dT) cellulose was transferred into a spin-column and centrifuged at 10.000 rpm for 10 seconds at room temperature. The liquid inside the tube was decanted. The washings with low salt buffer were repeated 3 times until the OD260 of the flow-through was < 0.05. The spin-column was placed into a new (sterile and RNase-free) microcentrifuge tube and 200 µl of elution buffer was added. The buffer was mixed into the cellulose bed with a sterile pipette tip and centrifuged for 30 seconds. A second 200 µl of elution buffer was added to the column, mixed into the cellulose, and centrifuged again for 30 seconds. The column was removed from the tube containing 400 µl elution buffer. mRNA was precipitated with 0.15 volume (~60 µl) of 2 M sodium acetate and 2.5 volume (1 ml) of 100% ethanol. The tube was incubated at –20 °C for 60 minutes and centrifuged in a microcentrifuge at 13.000 speed for 15 minutes at +4°C. Ethanol was decanted and traces of ethanol were removed by brief centrifugation. The RNA pellet was dissolved in 40 µl of Elution Buffer (10 mM Tris, pH 7.5), and concentration was determined by measuring absorption at OD_{260}.

2.2.2 The Polymerase Chain Reaction (PCR)

Standard PCR mixtures contained:

- Sterile double distilled water
- 10X reaction buffer to give a final concentration of 1X
- 10 mM dNTP to give a final concentration of 0.2 mM dNTP
- 2.5 unit Taq DNA polymerase for 50 µl reaction buffer
- 50 pmoles from each primer
- 0.25 µg genomic DNA

20 µl mineral oil was added to prevent evaporation.

Unless otherwise indicated, the thermocycler parameters were adjusted as follows for reaction buffers containing Taq DNA polymerase:

1. Initial denaturation	95°C, 3 min	
	95 °C, 1 min	
2. Annealing	$T_{annealling}$, 1 min	X 35
	72 °C, 1 min / kb	
3. Final Extension	72 °C, 1 min	

,where $T_{annealling}$ is the annealing temperature, ranging from 55°C to 60°C, according to the melting temperature of the primers and their specificities.

2.2.3 Visualization and Documentation of DNA & RNA Gels

For the visualization and analysis of genomic DNA and PCR products, 0.8% (w/v) agarose gel (Appendix B) was used. The gel was melted and cooled to 50-60 °C. After the addition of ethidium bromide to a final concentration of 0.5 µg/ml, the gel was poured into a mould and allowed to solidify for 1 hour. Then it was placed into the horizontal electrophoresis tank and covered with 1X TAE buffer (Appendix B).

The samples to be visualized were mixed with a 6X loading dye at a ratio of 5:1 and gently loaded to the wells. Electrophoresis was carried out at 80 V for 50 minutes for genomic DNA isolates and at 90 V for 45 minutes for PCR results.

Finally, the gel was visualized on a UV transilluminator and photographed by a digital camera (Nikon Coolpix 4500). The photographs were taken black and white with auto focus. The aperture was ranged from 4.2-7.2 with respect to distance of the lens to the object and exposure varying as 1, 2 and 4 seconds according to the brightness. Alternatively, the gels were also documented by the digital camera of the Gel Documentation (BioRad) Instrument.

2.2.4 Nucleic Acid Labeling, Hybridization and Detection Methods

2.2.4.1 Southern Blot Hybridization

2.2.4.1.1 Preparation of Probe DNA

For the preparation of the probe to be used in hybridization, amplification with PCR, isolation and purification of the fragment and labelling were done. To prepare the probe, the genome fragment of the appropriate length was amplified with the specific primers designed according to the information obtained from *Aspergillus fumigatus* genome database (Sanger Institute). To obtain the intended concentration of DNA before labeling series of PCR reactions were performed. The samples were run on the 0.8% agarose gel at 90 V for 45 min. then the bands were visualized under UV-light. Bands of interest were cut out of the gel and slices were washed to remove the salts by incubating in distilled water for 15 min. at room temperature. Then the excess water on the slices was removed by filter paper and gels were put into a *Promega Wizard MiniColumn.* The columns were placed on the 1.5 ml sterile eppendorf tubes and centrifuged at 13000 rpm for 20 min. The liquid content of gel with dissolved DNA was collected at the bottom. The flow-through was later mixed with 2 volume of cold 96% ethanol and 0.1 volume of sodium acetate (3 M, pH = 5.2) (Appendix B). The mixture was left overnight at -20°C. Then the tubes were centrifuged at 13 000 rpm for 20 minutes at 0°C. The supernatant was discarded and the pellet was washed with 1 ml cold 70% ethanol (Appendix B). The tubes were centrifuged at 13 000 rpm for 10 minutes at 0°C and the supernatant was discarded. The tubes were again centrifuged at 3000 rpm for 30 sec and the liquid part was pipetted out of the tube. After being allowed to air-dry for 5 minutes, the pellet was dissolved in an appropriate amount of sterile double distilled water.

The concentration of resulting DNA was determined by running the DNA on 0.8% agarose gel, along with a marker of known concentration, and by comparing the intensity of the bands with the marker band, nearest in size.

2.2.4.1.2 Probe Labeling

15 µl isolated PCR fragment having a concentration between 20 - 100 ng/µl was heat-denatured in boiling water for 10 minutes and immediately chilled on ice for 30 seconds. On ice, 2 µl hexanucleotide mixture (10X), 2 µl dNTP labeling mixture (10X), 1 µl

Klenow enzyme (10U/µl) were added respectively. The reaction was incubated at 37°C overnight, preferably 20 hours. Then the reaction was terminated by adding 2 µl 0.2 M EDTA, pH = 8.0 (Appendix B).

2.2.4.1.3 Southern Blotting

The DNA samples were run on an agarose gel. To determine the efficiency of blotting, a positive control (unlabeled probe DNA usually) was run with the samples. The marker DNA, used to determine the size of the bands run in the gel, plays also an important role as a negative control. After electrophoresis, a photograph of the gel was taken together with a ruler. Then the gel was placed in 10 ml of denaturation solution (Appendix B) and gently shaken for 15 minutes at room temperature. Then the gel was rinsed with distilled water and placed in 10 ml neutralization solution (Appendix B) and again gently shaken for 15 minutes at room temperature.

For the preparation of the neutral southern transfer apparatus, a tray was filled with 20X SSC (Appendix B) as the transfer buffer. The platform was covered with a sheet of Whatman 3MM filter paper saturated with 20X SSC. The gel was rinsed with distilled water and equilibrated with 20X SSC, then the unused parts of the gels were trimmed out and the gel was placed on top of the filter papers avoiding air bubbles remaining between the gel and filter papers. The nylon membrane (Roche) was cut to exact size of the gel and placed on top of the gel, carefully again avoiding air bubbles. The wet areas of the surface were masked with one sheet of saran wrap such that the leakage of the transfer buffer was prevented. On top of the nylon Membrane, sequentially three sheets of Whatman 3MM filter paper wetted with 20X SSC, plenty of paper towels and 500g of weight were placed.

The transfer was allowed to proceed overnight. After blotting, the apparatus was dismantled and the nylon membrane was removed. The nylon membrane was placed on a sheet of Whatman 3MM filter paper and the transferred DNA was fixed under the UV-transluminator for 3 minutes. Along with the Southern blotting, a piece of nylon membrane was spotted with the positive and the negative control DNA.

2.2.4.1.4 Dot Blotting

1 µg RNA or DNA samples were loeded on the nylon membrane. If the dot blotting were performed for the control of Southern hybridization, the amount of the positive control solution, which was the unlabelled PCR product, should not exceed 40 ng on the membrane. As the size of the drops on membrane should be limited, the concentration of samples was adjusted by ethanol precipitation and 2-10 µl samples were loaded. The membrane was dried at room temperature for 5 min. The samples were fixed on membrane under the UV-transluminator for 3 minutes.

2.2.4.1.5 Hybridization

The hybridization was performed in the hybridization oven (Stuart) in a roller bottle with Teflon lid at constant rotation at 50 rpm. The illuminated nylon membrane was placed in a roller bottle and prehybridized at 65°C at least 30 minutes using 20 ml prehybridization solution (DIG Easy Hyb, ready-to-use hybridization solution, Roche). The DIG-labelled probe was heat-denatured by boiling in a water bath for 10 minutes and immediately chilled in –20°C deep-freeze for 30 seconds. The probe was added to 15 ml hybridization solution (DIG Easy Hyb, ready-to-use hybridization solution, Roche) at a concentration of 25 ng/ml. The prehybridization solution was discarded and hybridization solution was added. The membrane was hybridized to the probe at 65°C overnight. The nylon membrane was washed twice with 25 ml low stringency wash buffer (Appendix B) for 5 minutes at room temperature, then twice with 25 ml high stringency wash buffer (Appendix B) for 15 minutes at 65°C as it was indicated in the manual of DIG labelling and detection.

2.2.4.1.6 Detection

The nylon membrane was washed with 25 ml washing buffer (Appendix B) for 5 minutes, and then blocked with 30 ml 1X blocking solution (Appendix B) for 30 minutes. After removing the blocking solution, 15 ml antibody solution (Appendix B) was added. The nylon membrane was washed twice with 30 ml washing buffer (Appendix B) to remove unbound antibody for 15 minutes. In continuation, the nylon membrane was equilibrated with 30 ml detection buffer (Appendix B) for 2 minutes. All incubations were performed at room temperature with shaking and great care was taken not to dry the nylon membrane between the steps. Finally, the nylon membranes were placed on a sheet

of plastic transparent film, DNA side facing up. For each 100 cm^2 of nylon membrane, 500 µl CSPD diluted with detection buffer (Appendix B, 1:100 dilution of 25 mM stock) was added. The damp nylon membrane was covered with a second sheet of transparent film in a sandwich like form. Excess liquid was dripped off from the nylon membrane and the transparency was heat sealed. The sealed bag, containing the nylon membrane was exposed to X-ray film (XBM Blue Sensitive, RETINA) in the Hypercasette™ (Amersham) for at least 30 minutes, at most 24 hours at 37°C.

2.2.5 Enzyme Activity

2.2.5.1 Qualitative Method: Gel Diffusion Assay & Congo Red Staining

The endo-β-mannanase activity was measured by the colorimetric assay. The medium, in which the *A. fumigatus* were grown, was filtered through Whatman 3MM filter paper. The biomass was used in the total RNA isolation. 1 ml of filtrate was taken and stored at 4 °C to be used within 8 days.

Agar locust bean gum plate (0.5% LBG and 1.5% agar) was prepared and approximately 4 mm wells were made. 15 µl of the filtrate medium was injected and incubated at 50 °C or at 55 °C overnight. The next plates were dyed with the 0.1% Congo Red solution overnight at room temperature by gentle shaking. The plates were washed with the 1 M NaCl solution for 2-3 hours at room temperature. The background colour was turned from red to violet-blue colour by 15% acetic acid.

2.2.5.2 Quantitative Method: DNS Assay

The endo-β-mannanase activity was measured by the DNS (3,5-Dinitrosalicylic Acid) assay. The supernatant was filtered through Whatman 3MM filter paper. The filtrate was taken and stored at 4 °C to be used within 8 days. Before the activity measurement the supernatant was diluted 10 times with appropriate buffer. As substrate, locust bean gum (galactomannan) was used. It was dissolved in 0.1 M phosphate-citrate buffer of appropriate pH at a concentration of 0.5% (w/v). 0.9 ml of substrate solution was added to 6 ml screw capped glass tube with Teflon coated cap. LBG was heated to 50 °C before the addition of 100 µl diluted filtrate. After incubation for exactly 300 s (5 min) at 50 °C in a heating block, 1.5 ml DNS solution was mixed to stop the hydrolysis. The tube was heated to 100 °C for 15 min for color formation and afterwards, the tubes were left to

cool on bench. The absorbance was measured at 540 nm by a spectrophotometer (Shimadzu UV-1700).

2.2.6 cDNA Synthesis

The cDNA was synthetised with the Superscript.First-Strand Synthesis System for RT-PCR (Invitrogen). Following mixture was prepared:

- 500 ng mRNA
- 10 mM dNTP mix to give a final concentration of 1mM
- Oligo(dT)12-18 (0.5 µg/µl) to give a final concentration of 0.05 µg/µl

Each sample was incubated at 65°C for 5 min and placed on ice for at least 1 min. The mixture was mixed with a second mixture, whose composition is indicated below:

- 10X RT buffer to give a final concentration of 1X
- 25 mM $MgCl_2$ to give a final concentration of 2.5 mM
- 0.1 M DTT to give a final concentration of 0.01 M
- RNaseOUT. Recombinant RNase Inhibitor (1U)

The tube was incubated at 42°C for 2 min and 50 U of SuperScript II RT was added. The tube was incubated at 42°C for 50 min. The reaction was terminated at 70°C for 15 min. and chilled on ice. Before proceeding with the PCR reaction with specific primers, RNase H was added and incubated for 20 min at 37°C.

2.3 Cloning

2.3.1 Zero Blunt® TOPO® PCR Cloning

Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) was used to clone blunt end PCR fragments. The ligated plasmid was transformed into compotent *E.coli* XL1 Blue.

Table 2.1 Reaction composition for Zero Blunt® TOPO® PCR Cloning

Component	Volume/reaction
Fresh PCR product	0.5 to 4 µl
Salt Solution	1 µl
pCRII-Blunt-TOPO	1 µl
Double distelled water to final volume of 6 µl	
Final Volume	**6 µl**

The PCR fragments were directly used in cloning without the need for further purification. Reagents mentioned on the Table 2.1 were mixed and incubated for 15 min at room temperature (22–23°C). The tubes were then placed on ice to proceed with the transformation of competent cells.

2.3.2 UA PCR Cloning

PCR Cloning Kit (QIAGEN) takes advantage of the single adenine overhang at each end of the PCR fragments generated using *Taq* (non-proofreading) DNA polymerases. The pDrive Cloning Vector, supplied in a linear form with a uridine overhang at each end, hybridizes with high specificity to such PCR products. Therefore any PCR fragment that was amplified with a proofreading DNA polymerase should be processed.

After purification of the PCR fragments, a PCR mixture was prepared except for the primers in mixture. The mixture was incubated at 72°C for 30 min in the presence of 5 U *Taq* DNA polymerase and the product was directly used in the ligation. The reaction mixture (Table 2.2) was mixed and incubated for 30 min at 16°C. The tubes were placed on ice before proceeding with the transformation of competent cells.

Table 2.2 Reaction composition for UA PCR Cloning

Component	Volume/reaction
Fresh PCR product	1 to 4 µl
Ligation Master Mix, 2x	5 µl
pDrive Cloning Vector (50 ng/µl)	1 µl
Double distelled water to final volume of 10 µl	
Final Volume	**10 µl**

2.3.3 Ligation to pAN52-4, pPICZαC and pBinAR

PCR fragments and the plasmids were digested with appropriate restriction enzymes (Table 2.3).

Table 2.3 Plasmids and the appropriate restriction enzymes

Plasmids	Restriction Enzymes
pAN52-4	***BamH*I & *Hind*III**
pPICZαC	***Kpn*I & *Xba*I**
pBinAR	***BamH*I & *Xba*I**

100-500 ng PCR fragments and the 50-100 ng plasmid DNA were mixed in a ratio of 3:1. Ligation mixture was prepared by the addition of 10x ligation buffer to give a final concentration of 1x, 1 U T4 DNA ligase (Roche), sterile double distilled water was added to give a final volume of 10 µl and incubated at 16 °C overnight. Following incubation using ligation mixture, plasmids were transformed into *E. coli* XL1-Blue.

2.4 Transformations

2.4.1 Transformation into *E. coli* (Sub-Cloning)

One tube of competent *E. coli* XL1-Blue was placed on ice. Then, 2-8 µl of the ligation reaction was added and mixed gently. The mixture was incubated on ice for 15 minutes. DNA uptake was achieved by applying heat to the cells for 90 s at 42°C without shaking. The tubes were then transferred on ice immediately. After the addition of 1 ml of LB medium, the tubes were mixed at 155 rpm at 37°C for 1 hr. 50-200 µl of mixture from each transformation was spreaded on a prewarmed selective plate (2xYT medium supplied with approciated antibiotics) and incubated overnight at 37°C.

2.4.2 Transformation into *Aspergillus sojae*

Transformation method to *A. sojae* ATCC11906 was adapted from the transformation studies of Punt & van den Hondel and L.H de Graaff. The method was based on the co-transformation of pAN52-4 fungal expression vector construct and pAMDSPYRG containing the *pyrG* selection marker, in a 1:10 ratio to protoplasts. Transformants were selected for uridine prototrophy.

For the preparation of spore suspension, the fungus was inoculated on the agar medium and allowed to grow until all the agar surface was completely covered with sporylated fungi. 10 ml saline-Tween solution was pipetted on the spore-mat. Spores were scraped off using an inoculation loop. The spore suspension was transferred to a sterile bottle. 20-fold dilution (50 µl + 950 µl saline-Tween) and the concentration of spores were determined by counting the diluted sample in a heamocytometer. The concentration of spores was calculated according to the following equation:

$$\frac{\#\text{ of spores counted x } 1000\text{ mm}}{\#\text{ of squares counted x width}^2\text{ x dept}} \text{ x dilution} = \#\text{ / ml}$$

width^2 : 0.05 mm

dept: 0.02 mm^2

To prepare the protoplasts, 4x 250 ml of minimal medium with uridine/uracil were inoculated with *A. sojae* (1 x 10^6 spores/ml) and cultivated for 10-14 hrs at 30 °C and 155 rpm. Mycelia were harvested on nylon gauze (cheese cloth) using Büchner funnel. The collected mass were placed in Falcon tube (10 ml) and washed once with 0.27 M $CaCl_2$, 0.6 M NaCl buffer (0.5 g mycelia/10 ml). After centrifugation at 2000 rpm for 10 min, the aqueous phase was discarded and the pellets were suspended in 10 ml STC with 100 mg lysing enzymes (lysing enzyms from *Trichoderma harzianum*, Sigma). From this point, protoplasts were formed while incubating at 30 °C with slow agitation (50 rpm). At 30 min intervals protoplast formation is checked on microscope. The enzyme treatment was ceased when more then 1 x10^8 protoplasts were observed. The mycelial debris was removed by filtration over a sterile glass wool plug. The filtrate was collected in a 10 ml falcon tube.

The protoplasts were centrifuged at 2000 rpm for 10 min at 4 °C and carefully resuspended in STC, three times. The protoplasts were resupended, finally in STC buffer at a density of 1 x 10^8 spor / ml.

200 µl of the protoplast suspension was pipetted into an eppendorf tube, 1 µg of pAMDSPYRG (in 2 µl TE buffer) and 10 µg of recombinant enzyme (in 15 µl TE buffer). In general, the total volume of plasmid mixture should not exceed 20 µl. Then, 50 µl of PEG buffer was added, mixed gently by shaking and incubated at RT for 20 minutes. To check the reliability of the transformation, 1 µg / 20 µl of sole pAMDSPYRG was used as a positive control and 20 µl of TE (no DNA) as a negative control. The rest of the protoplast suspension is kept on ice. 2 ml of PEG buffer was added mixed gently, incubated at RT for another 5 minutes and subsequently 4 ml STC was added, and mixed gently. Selective MMS-top agar was added to the transformation mixture so that the tube is almost completely filled. The tube was mixed by inverting several times and the mixture was poured onto two ∅150 mm selective MMS-plates. The plates were incubated for 7 days at 30 °C. Each day, the plates were screened for the hyphal growth. If any growth was observed on the plates, the culture was transferred to another selective MMS-plate. Each isolated strain was serially transferred on selective MMS-plate for 4 times to check the stability of the genetic transformation.

2.4.3 Transformation into *Pichia pastoris*

The vectors were amplified in *E. coli* and isolated by the plasmid purification kit of Machery-Nagel (NucleoSpin Plasmid Isolation Kit). The vector was linerized by *Pme* I restriction enzyme to achieve high recombination efficiency, which was recommended by the kit supplier.

Before transformation into *P. pastoris*, the yeast was grown in 5ml YPD media overnight. The next day, prewarmed 50 ml YPD medium was inoculated with the overnight culture. The culture was grown on rotary shaker at 30°C, 200 rpm to an OD_{600} 0.6. The yeast cells were harvested by centrifugation at 3000g for 5 min. The pellet was chilled twice with ice cold water, as the manual of the yeast expression kit (*P. pastoris* Expression Kit, Invitrogen, USA) indicated. The yeast was transformed by electroporation. The parameter for the electroporator (BioRad, GenePulser II) was adjusted to 1.5 kV in potential, 25 μF in capacitance and 200 Ω in resistance, while using the cuvettes with 2 mm openning. After transformation, the transformants were regenerated on YEB medium supplemented with 1% sorbitol and in the presence of the selective agent, Zeocin (100 μg/ml). After incubation at 28 °C for 2 days, the colonies were picked and the presence of the gene was checked with PCR. The positive colonies were grown in YpSs medium with 1% methanol to induce the protein synthesis. Last, the enzyme activity was checked by DNS-assay and positives were identified.

2.4.4 Transformation into *Arabidopsis thaliana*

Transformation into *Arabidopsis thaliana* was a three stage process. After ligation of mannanase gene onto the plant transformation vector, pBinAR, the construct was transformed first into *E. coli,* to increase the number of plasmids, and next, transferred into *A. tumefaciens* pGV3101 pmp90 by electroporation. The parameter for the electroporator (BioRad) was adjusted to 2.5 kV in potential, 25 μF in capacitance and 200 Ω in resistance, while using the cuvettes with 2 mm openning. The positive colonies were selected under the three antibiotics and proven by PCR and restriction enzyme digestion. Due to the high nuclease content of *A. tumefaciens*, the isolated plasmid from *A. tumefaciens* was retransformed into *E. coli* and the presence of the gene was proven both by restriction digestion and by PCR, later. The positive agrobacteria were used for transformation to plant by floral dipping infiltration. *Agrobacterium tumefaciens* was

cultivated first in 5 ml preculture (YEB medium with 100 mg/L rifampicin, 25 mg/L gentamycin and 25 mg/L kanamycin) for 2 days at 28 °C. 2 ml of preculture was used to inoculate 200 ml LB medium supplemented with 25 mg/L gentamycin and 50 mg/L kanamycin and cultivated. Bacteria were harvested by centrifugation at 4000 rpm for 40 min and resuspended in 400 ml of infiltration media.

The Arabidopsis transformation protocol is based upon the report from Zhang (Zhang *et al*., 2006). For transformation T_0 plants were grown. Seeds were germinated on a tray in a short day (SD) growth chamber (9 hrs light, 22°C). Three weeks after sowing, 12 seedlings were transferred to a 10 cm pot. 10 to 12 pots were prepared per construct to be transformed. Since under SD growth conditions the plants form strong rosettes, it is not necessary to use nylon window screen or similar material to prevent the plants/soil mixture from falling during the infiltration treatment. The plants were further grown for at least 2 more weeks under SD. Afterwards they were transferred to long day (LD) conditions (16 hrs light, 22°C) to promote bolting. Once the primary inflorescence shoots have reached about 10 cm (about 2 to 3 weeks after transfer to LD), they were removed in order to induce the development of rosette inflorescence shoots. The plants were ready for infiltration 6 to 7 days later. A 30 cm diameter glass bell jar was used. The bell jar can fit 2 glass trays (29 cm x 10 cm x 5 cm) filled each with 400 ml of the Agrobacterium suspension. Each tray can hold two inverted pots (only the inflorescence shoots are submerged in the Agrobacterium suspension). No vacuum treatment was applied. The bacterial suspension was reused twice.

After the infiltration treatment the plants looked greyish green. Plants were taken back to a LD growth chamber or to the greenhouse (provided that the temperature in the greenhouse is not higher than 25°C).

To harvest the T_1 seeds, the seeds were collected in 13 cm x 24 cm paper bags. The 12 plants that were originally in each pot were bagged in two pools of six plants each. Before bagging the plants were kept until the basal siliques started open to. These siliques carry seeds that have originated before the infiltration treatment. These seeds usually fall when the plants are bagged. Bagged plants were watered for one more week and then allowed to dry for 1 to 2 more weeks. Before the selection of transformants, disinfection was carried out as follows: 300 to 350 seeds per seed pool were collected and washed 3 times with 70% EtOH, one time with 0.01% hydrogen perchloride for 7 min and 5 times with sterile water. The sterile seeds were distributed on two 9 cm diameter petri dishes of

0.7% agarose/ MS medium without sucrose + 100 mg/l kanamycin. The dishes were kept for one day in 4°C cold-room, 2 days in 37°C incubation-room and then transferred to a 22°C growth room (LD photoperiod). Depending on the selection agent used, it is generally possible to distinguish the transformants, 2 weeks after plating the seed (green, fully expanded cotyledons; first pair of true leaves growing; long root).

2.5 Purification of Mannanase

Purification of mannanase was performed according to the study of Puchart (2004). The method is based on a two step process involving separation by ionic strenght and the hydrophobic nature of the protein.

Cell-free culture fluid was concentrated by increasing salt gradient to 40%, 60% and 80% ammonium sulphate. This step was not applied later, because the enzyme production was so high that even unconcentrated culture fluid had enough mannanase genes to plug the coloumn.

The filtrate was first applied to DEAE Sepharose Fast Flow (IEX-Ion Exchange) column (2.5 × 25 cm) and equilibrated with 50 mM sodium phosphate buffer, pH 5.0, containing 0.02% (w/v) sodiumninhydrin (Sol. A). Elution was performed at the flow rate of 0.55 ml/min and 11 ml fractions were collected. The protein was liberated with a linear gradient of NaCl in Sol. A (Sol. B) from 0 to 0.5 M NaCl concentrations (0-100%).

The fractions were tested for mannanase activity. The ones that show positive activity were further purified on Phenyl Sepharose Fast Flow column (0.9×9 cm) equilibrated with Sol. B. Elution was done at a flow rate of 0.28 ml/min and 1.8-ml fractions were collected. Mannanase was liberated from the column with a linear gradient of 0–50% (v/v) ethylene glycol in Sol. A. β-Mannanase active fractions were pooled and desalted.

2.6 Characterization of Recombinant Enzymes

2.6.1 Determination of pH and temperature optima and stabilities

The optimum pH was determined by mannanase activity assay over a pH range of 3.0 to 7.0 by using sodium-citrate-phosphate buffer at 50 °C. The optimum temperature was determined by DNS-assay (Section 2.2.5.2) at a temperature range of 30 °C to 70 °C. To

measure the thermal stability of the enzyme, samples were incubated at different temperatures ranging between 37 °C and 80 °C in sodium-citrate-phosphate buffer at pH 5.0. The residual activity was determined at 50 °C. The pH stability of the enzyme was determined in the pH range of 2.0 to 10.0 in the following 0.1 M buffers containing 0.5 mg/ml of bovine serum albumin: HCl/KCl (pH 2.0–2.5), glycin/HCl (pH 2.5–4.5), sodium acetate (pH 4.5-5.0), sodium phosphate (pH 5.0–7.5), Tris/HCl (pH 7.5–8.5) and glycine/NaOH (pH 8.5–10.0). After incubation of the enzyme-substate mixture at 50 °C for 5 h, aliquots were taken to assay the residual mannanase activity.

2.6.2 SDS-Polyacrylamide Gel Electrophoresis and Native PAGE

SDS-PAGE was done according to the standard protocol of Laemmli (1970). Electrophoresis system, Serva BlueFlash S, 15 x 28 x 8.5 cm was used. To separate the proteins, 10% (w/v) polyacrylamide gel was used with a thickness of 0.75 cm. Later the gels were either stained with coomasie blue G-250 or silver nitrate.

The activity of mannanase in the gel was checked by adding 0.1% LBG to the separating gel. After electrophoresis, SDS was removed from the gel by multiple washings with distilled water. Later the gel was incubated at 55 °C in 0.1 M Na-citrate buffer for 10 min. Finally the gel was soaked in 0.1% Congo Red dye for 60 min to stain the undigested LBG following three times washing off with 1M NaCl, as mentioned in the qualitative enzyme activity assay.

2.6.3 Substrate Specificity

The substrate specificity of the enzyme was checked by the DNS assay (Section 2.2.5.2) by only altering the carbon sources. The carbon sources are indicated on Table 2.4.

Table 2.4 Galactomannans and other Polysaccharides

Carbon Source	Concentration	Composition
Locust Bean Gum (LBG) -galactomannan-	0.5 %	Man:Gal (4:1)
Guar Gum -galactomannan-	0.5 %	Man:Gal (2:1)
Fenugreek -galactomannan-	0.5 %	Man:Gal (1:1)
Konjac Gum -glucomannan-	0.5 %	Man:Glc (1.6:1)
Xylan, Birchwood -xylan-	1 %	Xyl
Carboxymethyl Cellulose (CMC) -cellulose-	1 %	Glc

Substrate was heated to 50 °C before the addition of 100 µl dil. enzyme solution. After incubation for exactly 300 s (5 min) at 50 °C, 1.5 ml DNS solution was mixed to stop the hydrolysis. The tube was heated to 100 °C for 15 min for color developement and afterwards, the tubes were left to cool at room temperature. The absorbance was measured at 540 nm by spectrometry.

2.6.4 Paper Test Assay

The preparation of test-paper started by placing 1% (w/v) LBG solution in a tray (Fry, 1997). The filter paper was passed quickly over the surface of the solution to wet only one side of the paper. The paper was, then, dried by hanging. After the drying the filter paper was dipped in a solution of 5 µM labelled mannose in 75% (v/v) acetone and the paper was re-dried. Last, the filter-paper was fixed to a support with acetate sheet and adhesive band.

To measure the transferase activity, 3.5-5.0 µl of enzyme sample was spotted on to the marked position on the test-paper without allowing to dry, as mentioned on the Figure 2.3. In the case of pipetting takes place more than 5 min, the stopping step was performed

at 4 °C in the cold room. The test-paper was covered with a second sheet of acetate to maintain the humidity. The sheet was placed between layers of soft-papers (tissue paper) on a flat surface and was loaded evenly. The setup was incubated at 50 °C for 1 hr and later incubated in a fresh mixture of ethanol/90% of formic acid/water (1:1:1) for 2 hrs without agitation. The paper was rinsed off with water for 5 min and dried. The paper was examined under UV-light.

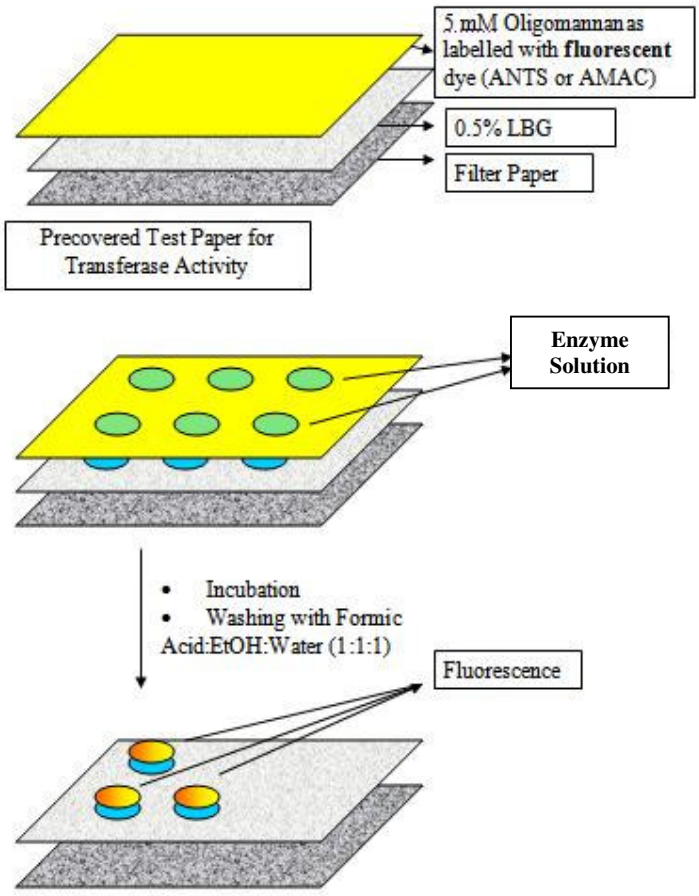


Figure 2.3 Paper Test Assay**.** The fluorescent labelled oligomannans (yellow) , as well as mannose, can only bind to LBG (light gray) by the enzyme, if the enzyme has the transglycosylation activity. LBG cannot be washed-off from the paper at the washing step.

2.6.5 MALDI-TOF

5 µl of filtrate was transferred to a 0.5 ml tube containing 5-10 prewashed Biorex MSZ 501 resin beads (cation exchanging) and was kept for 8-10 min. Then, 1 µl matrix (DHB, 10mg/ml in water) was spotted onto target plate and dried under vacuum. Next 1 µl of treated filtrate was dropped on the dried DHB and the plate was spotted with samples within maximum of 3 min. After 2 min incubation vaccum was applied to the samples. The samples were analysed in MALDI-TOF.

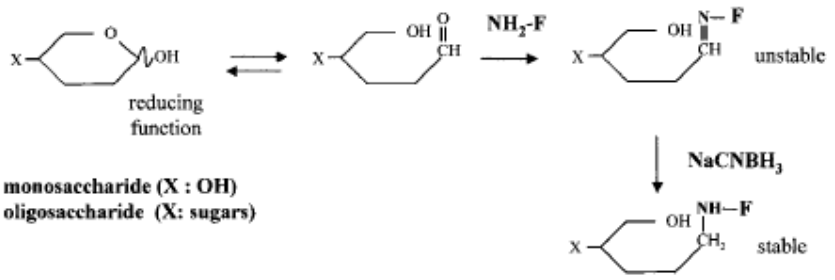
2.6.6 PACE

This method was based on the paper of Goubet (2002). Polysaccharide analysis using carbohydrate gel electrophoresis (PACE) based on derivatization of reducing ends of sugars and oligosaccharides with a fluorophore (AMAC; 2-aminoacridone or ANTS; 8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt), followed by electrophoresis in polyacrylamide gels.

2.6.6.1 Labelling of Saccharides

5 µl of 1 mM sugar was added in a 1.5 ml PP tube. The sample was vacuum-dried. Then 0.1 M AMAC (in acetic acid: DMSO, (3:17; v/v)) or 0.2 M ANTS (in acetic acid: water, (3:17; v/v)) was prepared for labeling and 1 M fresh $NaCNBH_3$ was prepared in water (if AMAC was used) or DMSO (if ANTS was used). On the dry pellet, 5 µl fluorophore (AMAC or ANTS) and 5 µl $NaCNBH_3$ was added and mixed by the help of the pippet tip. The mixture was centrifuged and incubated overnight at 37°C (Figure 2.4). After incubation, the samples were vacuum dried at 40°C for 3 hrs and resuspended in 100 µl of 6 M urea. Samples were stored at -20°C.

A



B

Sugar	Optimal separation conditions	Fluorophore	Excitation	Emission
Uncharged oligosaccharides	Tris-borate pH 8.2	ANTS (Charged)	365 nm	515 nm
Uncharged monosaccharides	Tris-borate pH 8.2	AMAC (Uncharged)	425 nm	520 nm
Charged monosaccharides Charged oligosaccharides	Tris-glycine pH 8.5			

Figure 2.4 Fluorophore derivatization of sugars for PACE. (A) Principle of derivatization of sugars. NH_2-F; Fluorophore. (B) Use of fluorophores for PACE of various sugar categories (Goubet *et al*., 2002).

2.6.6.2 Gel Electrophoresis

To separate the sugars, polyacrylamide gel was prepared. The compoition of the gel is given on Table 2.5. The gel was run in 0.1 M Tris-Borate Buffer (pH 8.2) at constant 200 V for about 60 min at 4°C. The gel was quickly visualized using the transilluminator.

Table 2.5 Polyacrylamide Gel Composition for SDS-PAGE

	Stacking Gel (2 x)	Separating Gel (2 x)
Buffer (1 M Tris-Borate Buffer, pH 8.2)	0.5 ml	1 ml
Acrylamide Sol. (30:1)	0.8 ml	3.4 ml
ddH2O	3.5 ml	5.6 ml
APS (25%)	40 µl	60 µl
TEMED	4 µl	6 µl

2.7 Analysis of Mannanase Expression in Arabidopsis

2.7.1 Liquid Cultivation

Before cultivation of plant, the seeds were sterilized. About 200-300 seeds were collected first in 1.5 ml PP tube. The seeds were washed 3 times with 1 ml EtOH solution (70%). The seeds should not exceed the contact time with ethanol more than one minute at each washing step. Then the seeds were sterilized with 1 ml of 0.005% hydrogen perchloride for 7 min. At every 2 min, the tube was inverted by several times. The sterilant was decanted and 1 ml water was added to wash the seeds. Washing was repeated 5 times and at the end 200 µl water was added to form a seed-water suspension. The mixture was spotted on a steril filter paper and dried under laminar flow.

For liqiud cultivation, 50 ml MS broth with 1% glucose and 50 µg/l Kanamycin (selective agent) was inoculated with about 20-30 seeds. The seeds were cultivated at 100 rpm for 2 weeks at 26 °C and a ligth intensity of 120 µF (no dark period).

2.7.2 Alditol Acetate Assay

To analyze the plant cell wall composition, first the cell wall was prepared and dried. Then the samples were hydrolyzed by triflouracetic acid (TFA) and reduced to alditols by sodium borohydride. Then the sugars were acetylated by acetic anhydride and the samples were dissolved in acetone followed by GC-MS analysis. All the samples were repeated at least 4 times (2 biological and 2 technical replica).

50 mg plant material (~10 hypocotyles) was washed three times with ddH_2O to remove sugars from the medium. The samples were placed in 2 ml PP tube and frozen immediately in liquid nitrogen. Then, 1 ml pure methanol was added onto frozen plant material and homogenized with metal ball at dismembranazer for 1 min at 2500 rpm. Next the tubes were centrifuged at 14 000 rpm for 10 min and the supernatant was poured off. 1 ml chloroform: methanol (v/v; 1:1) was added, centrifuged at 14 000 rpm for 10 min and the supernatant was decanted. This process was repeated usually 5-6 times until no green colour is left in the liquid phase. 1 ml acetone was added, centrifuged at 14 000

rpm for 10 min. The supernatant was removed. The pellet was dried overnight in vacuum centrifuge.

2-4 mg of lypholized cell wall material was weighted into a screw capped glass tube. 50 µg inositol was added as internal standard and liqiuid fraction was evaporated until dryness. 250 µl of 2 M trifluoracidic acid (TFA) was added and the tube was closed with Teflon coated lid. After incubation for 1 h at 121°C, 300 µl isopropanol was added, and the content was evaporated at 40°C; the process was repeated 3 times until all the acid was removed. Next, 400 µl water was added, vortexed and sonicated. The pellet can be used to analyse the crystalline cellulose content of the cell wall by Updegraff method. The liquid phase was removed after centrifugation at top-speed for 10 min. The solution was evaporated and crust remained on the glass.

250 µl reduction reagent [sodium borohydride in 1 M ammonium hydroxide (10mg/ml)] was added and incubated at room temperature for 1 h. Then 20 µl of glacial acetic acid was added to neutralize. After ceasing of bubbling, 250 µl acetic acid/methanol (1:9, v/v) was added and evaporated. This process was repeated 3 times in total. 4 x 250 µl methanol was added and evaporated.

50 µl acetic anhydride and 50 µl pyridine was added and the tube was incubated for 20 min at 121 °C. 200 µl toluene was added and evaporated. This step was repeated twice. Next, 500 µl distilled water and 500 µl methylenchloride were added and vortexed. The lower phase (methylenchlorid) was transferred into a 2ml PP tube. It is important that no water is transferred to the tube. The solvent was rapidly evaporated at room temperature and 500 µl acetone was added and 100 µl solvent was transferred into GC-vial. The vials were capped with Teflon seals. For GC, 2 µl sample was sufficient for analysis. Samples were stored at 4°C.

2.7.3 Amylase treatment

To remove starch from the cell wall preparates, the lypholized cell wall materials to be used for alditol acetate assay were treated with amylase. The pellet was resuspended in 1.5 ml 0.25 M sodium acetate buffer (pH 4.0) and heated for 20 min at 80°C to inactivate the endogenous enzymes of the plant. The suspension was cooled on ice and the pH was adjusted to 5.0 using 1 M NaOH (approximately 13 drops). Then, 1 µl enzyme mixture

(0.01% sodium azide (NaN_3), 1.0 µl amylase (50 µg/1mL H_2O; bacillus species; SIGMA) and 0.5 µl pullulanase (bacillus acidopullulyticus; SIGMA)) was added. The mixture was incubated overnight at 37°C. The enzymes were inactivated by heating the suspension at 100°C for 10 min. The samples were centrifuged at 14.000 rpm for 20 min at 24°C. The solution was discharged and 1.5 ml water was added to the pellet, centrifuged at 14.000 rpm for 20 min at 24°C. The solution was again vacuum-sucked and the pellet was washed until no neutral sugars could be detected. The detection of sugar was performed by the anthrone assay.

2.7.4 Anthrone Assay

This method was based on the report of Dische (1964), which gives positive reactions for hexoses, aldopentoses, 6-deoxyhexoses and hexuronic acids.

The solid sample was first dissolved in 250 µl water. If the effluent of the wash solution was used to analyze, the 250 µl liquid was directyl used. 500 µl anthrone reagent (0.2% (w/w) anthrone in conc. H_2SO_4) was added (twice of sample). After mixing, the tubes were heated for 5 min in boiling waterbath and then cooled at room temperature for 15 min. The absorbance was determined at 620 nm.

CHAPTER 3

RESULTS AND DISCUSSION

Based on the previous studies by Puchart *et al.* (2004), this study was aimed at the cloning the mannanase gene of *Aspergillus fumigatus* for expression in the heterologous hosts, *Aspergillus sojae*, *Pichia pastoris* and *Arabidopsis thaliana*. According to Puchart *et al.* (2004) *A. fumigatus* produces large amount of endo-β-1,4-mannanase, however, the fungus is not suitable for the industrial use due to its opportunistic saprophytic nature. Therefore, the gene was transferred into *A. sojae, P. pastoris* and *A. thaliana.* Accordingly, in this thesis experimental strategies were developed for the comperative analysis of heterologous expression of endo-β-mannanase of *A. fumigatus* in yeast, filamentous fungi and plant expression systems, and for characterization of recombinant enzymes.

3.1 Experimental Strategies

The experimental strategies for expressional cloning of endo mannanase genes of *Aspergillus fumigatus* and their analysis are shown in Figure 3.1. Studies began with the search of possible mannanase genes on *A. fumigatus* genome, which was opened to the public access, but was not annotated at the time. Two mannnase genes were found, where only one contained a putative cellulose binding module (CBM). Primers for the mannanase gene with CBM sequence were designed. The induction level of mannanase by different carbon sources were checked and best substrate was used in the isolation of mannanase cDNA. The isolated fragment was cloned into pAN52-4 under the control of the *gpdA* promoter and the transformed into *A. sojae*. The best transformants were selected. The cDNA of mannanase was isolated from *A. sojae* and transformed into *P. pastoris* and *A. thaliana* after cloning into suitable expression vectors. Transformants were then analysed. Because of the adverse effect of the mannanase on plant, the transformants of *A. thaliana* could not be grown for further analysis. The *P. pastoris* and *A. sojae* transformants were analysed for their enzyme production. The recombinant enzymes were purified, characterized and compared with the native form.

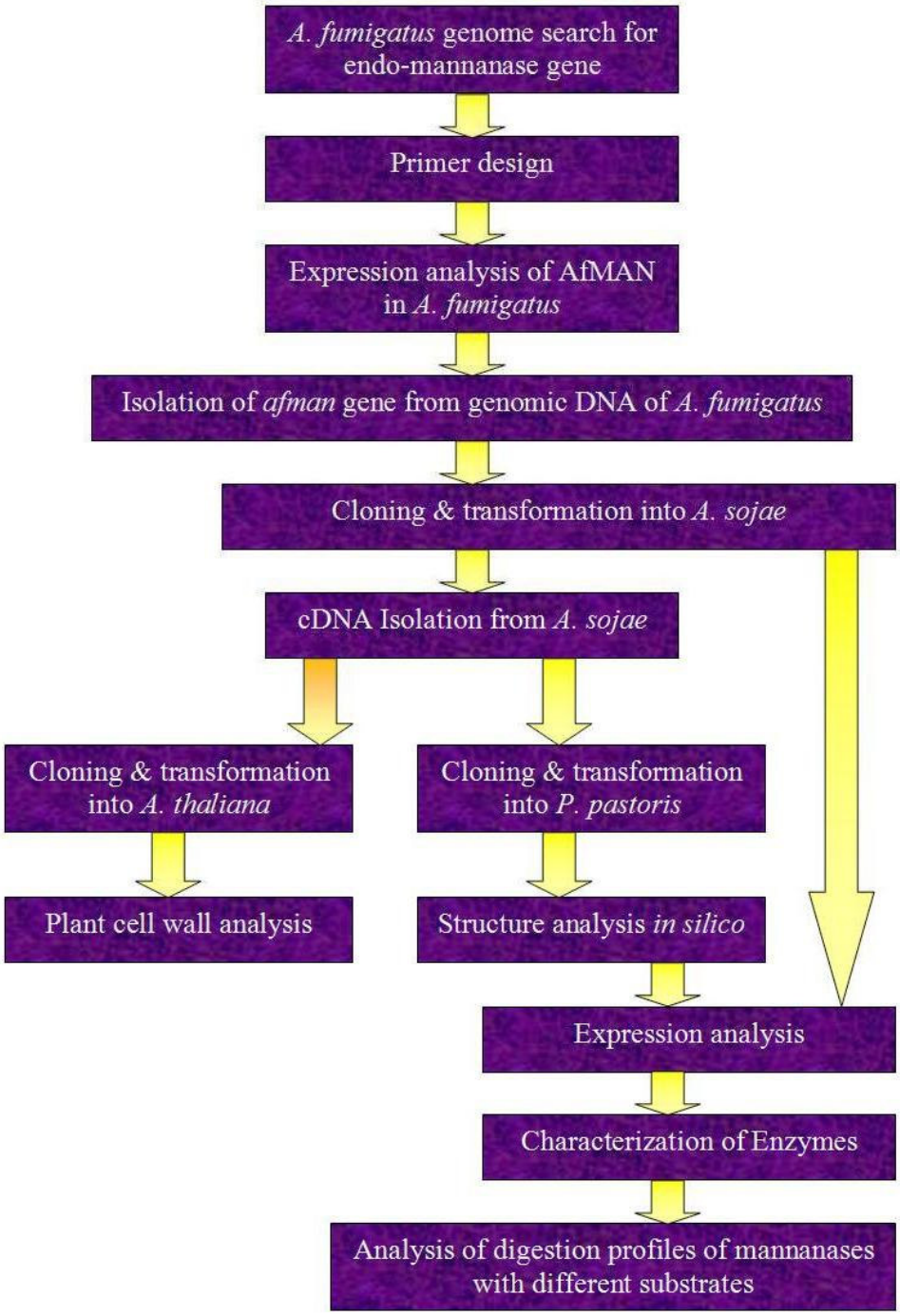


Figure 3.1 Flow chart of the experimental strategies

Analysis of digestion profiles of mannanases with different substrates were performed and screened by MALDI-TOF and gel based techniques.

3.2 Mannanase genes of *Aspergillus fumigatus*

Genome sequence of *Aspergillus fumigatus* Af293 (*A. fumigatus* genome database of the Institute for Genomic Research website at http://www.tigr.org) was analysed for the presence of mannanase genes using BLAST (blastn and tblastn). BLAST analysis gave two sequences of mannanase genes named as *afman1* & *afman2* on the *A. fumigatus* genome. Both of these two genes appeared to belong to glycosylhydroxylase family 5.

afman1 (*Man55*) is located on the 8th chromosome (contig 55) and has a gene size of 1490 bp including 1314 bp long exon with three introns (56 bp, 61 bp and 59 bp). Its nucleotide sequence includes 51 bp signal peptide sequence besides 108 bp putative CBM at its N-terminus.

afman2 (*Man66*) is located on the 7th chromosome (contig 66). The second mannanase is transcribed from a different part of the genome. This enzyme has no cellulose binding domain on its structure and its structure resembles those of bacterial mannanases rather than the fungal enzymes. Its corresponding gene has a size of 951 bp that 888 bp exon region was devided with a single 63 bp long intron.

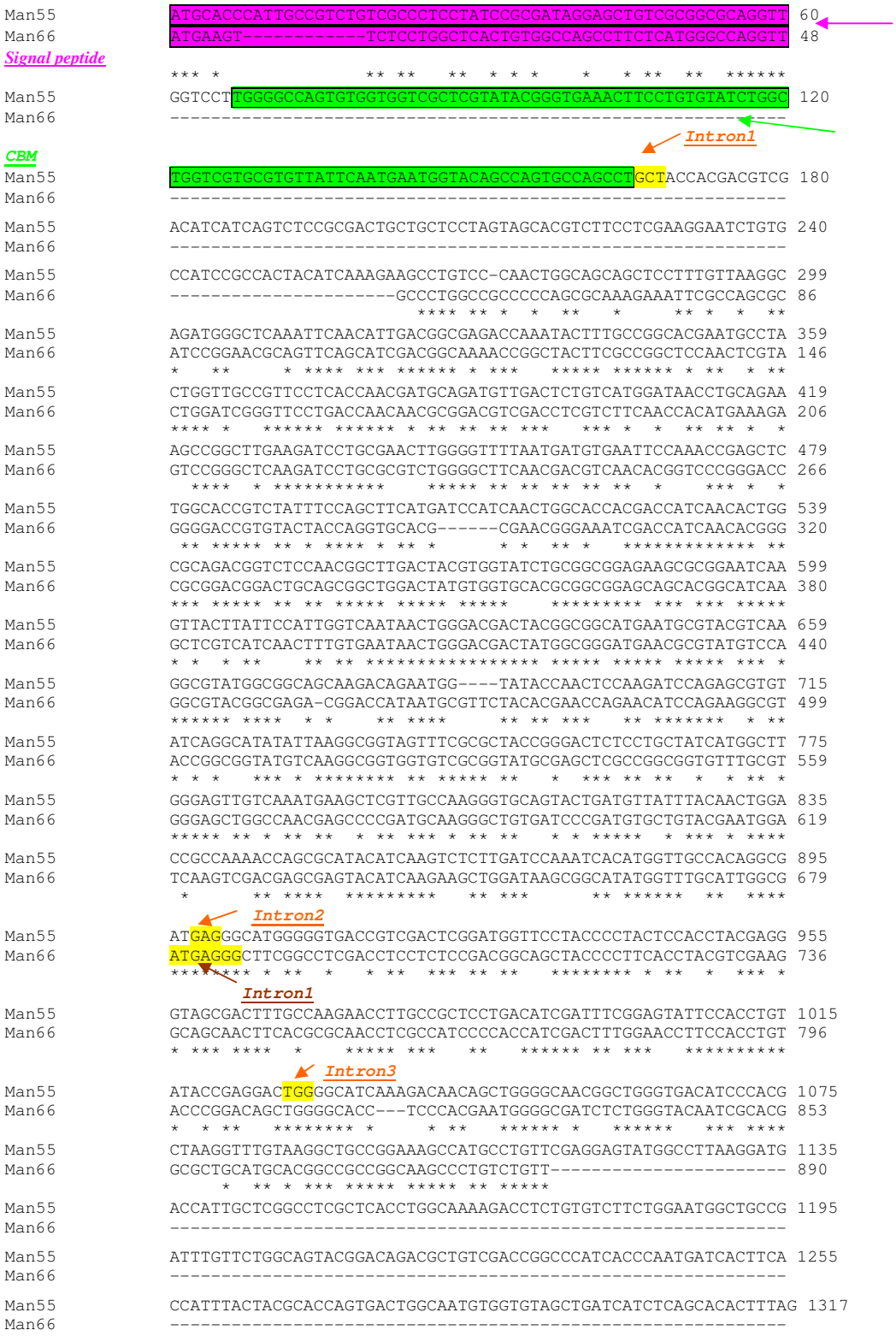


Figure 3.2 Comparison of the *afman1* and *afman2* genes (excluding introns). Signal peptide sequences (pink), CBM (green) and the splice site of introns (yellow) are indicated with arrows.

Figure 3.3 Comparison of AfMAN1 and AfMAN2 amino acid sequences. Signal peptide sequences (pink), CBM (green) and the splice site of introns are indicated with arrows (red).

The two mannanases have a similarity of 58% at the level of nucleotide sequence and 57% at the of level amino acid sequence. The genes are located on different chromosomes without belonging to a known gene cluster. The second intron of afman1 and the intron of afman2 splice sites were preserved, although the intron sequences were not preserved.

3.3 Design of Primers

To be cleared the gene of the enzyme previously isolated and characterized by Puchart *et al.* (2004), primers were designed using the sequence information of putative *A. fumigatus* mannanase gene (a*fman1*). Two groups of primers were designed. F1 and B2 primers were for screening and expressional analysis,while N3 and C3 for directional cloning purposes.

Table 3.1 Sequence and melting temperature information of the primers used in *afman1* cloning.

Primer	Sequence	$T_{melting}$ (°C)
F1	5′-GGTTTTAATGATGTGAAT-3′	60.5
B2	5′-GCTTCATTTGACAACTCCC-3′	61.0
N3	5′-TCCGCGATAATATATGGATCCGCGCAGGTTGGTCCTTGG-3′	62.8
C3	5′-ACGCGTCCTCTGAGCTTCAAGCTTCTAAAGTGTGC-3′	62.2

N3 and C3 were designed for expressional cloning. The sequence information and the melting temperatures of primers in PCR were indicated in Table 3.1 and the annealing sites of primers on *afman1* are given in Figure 3.4. Possibility of primer dimer formations was also checked by using the software "OligoAnalyzer 3.0".

The amplification product of F1 & B2 is 360 bp and for the primer pairs N3 & C3 1324 bp (without introns) and 1511 bp (with 3 introns). N3 & C3 primers amplifies the region excluding the native signal sequence.

ATGCACCCATTGCCGTCTGTCGCCCTCCTATCCGCGATAGGAGCTGTCGCGGCGCAGGTTGGTCC

N3

TTGGGGCCAGTGTGGTGGTCGCTCGTATACGGGTGAAACTTCCTGTGTATCTGGCTGGTCGTGCGT
GTTATTCAATGAATGGTACAGCCAGTGCCAGCCTGGTGAGCAATACACTGCTTCTCTGTGCGGCCG
AGATGATAGTCCTAACATATTCAAGCTACCACGACGTCGACATCATCAGTCTCCGCGACTGCTGCT
CCTAGTAGCACGTCTTCCTCGAAGGAATCTGTGCCATCCGCCACTACATCAAAGAAGCCTGTCCCA
ACTGGCAGCAGCTCCTTTGTTAAGGCAGATGGGCTCAAATTCAACATTGACGGCGAGACCAAATAC
TTTGCCGGCACGAATGCCTACTGGTTGCCGTTCCTCACCAACGATGCAGATGTTGACTCTGTCATG
GATAACCTGCAGAAAGCCGGCTTGAAGATCCTGCGAACTTGGGGTTTTAATGATGTGAA

F1

TTCCAAACCGAGCTCTGGCACCGTCTATTTCCAGCTTCATGATCCATCAACTGGCACCACGACCAT
CAACACTGGCGCAGACGGTCTCCAACGGCTTGACTACGTGGTATCTGCGGCGGAGAAGCGCGGAAT
CAAGTTACTTATTCCATTGGTCAATAACTGGGACGACTACGGCGGCATGAATGCGTACGTCAAGGC
GTATGGCGGCAGCAAGACAGAATGGTATACCAACTCCAAGATCCAGAGCGTGTATCAGGCATATAT
TAAGGCGGTAGTTTCGCGCTACCGGGACTCTCCTGCTATCATGGCTTGGGAGTTGTCAAAT

B2

GAAGCTCGTTGCCAAGGGTGCAGTACTGATGTTATTTACAACTGGACCGCCAAAACCAGCGCATAC
ATCAAGTCTCTTGATCCAAATCACATGGTTGCCACAGGCGATGGTACGCTAAGCACTCCCCTCTCA
CATACCTGATGTCACGCAGGTGACTAACTCCAGTATAGAGGGCATGGGGGTGACCGTCGACTCGGA
TGGTTCCTACCCCTACTCCACCTACGAGGGTAGCGACTTTGCCAAGAACCTTGCCGCTCCTGACAT
CGATTTCGGAGTATTCCACCTGTATACCGAGGACTGTGAGTATTGTTCCACCCTCGTCGTCACTGG
ACTGTCAATTGACGCATCGTACCAGGGGGCATCAAAGACAACAGCTGGGGCAACGGCTGGGTGACA
TCCCACGCTAAGGTTTGTAAGGCTGCCGGAAAGCCATGCCTGTTCGAGGAGTATGGCCTTAAGGAT
GACCATTGCTCGGCCTCGCTCACCTGGCAAAAGACCTCTGTGTCTTCTGGAATGGCTGCCGATTTG
TTCTGGCAGTACGGACAGACGCTGTCGACCGGCCCATCACCCAATGATCACTTCACCATTTACTAC
GGCACCAGTGACTGGCAATGTGGTGTAGCTGATCATCTCAGCACACTTTAGAAGCTTGAAGCTCA
GAGGACGCGT

C3

Figure 3.4 Annealing sites of primers used in the amplification of *afman*1 gene. The attachment sites of primers are marked on the sequence with black. Signal peptide sequence (pink), introns (orange) and the sequence after stop codon (yellow; TAG) are indicated.

3.4 Analysis of Mannanase Expression by *A. fumigatus*

Because of the gel matrix formation of galactomannan with salts during the RNA isolation, the extraction method became less efficient. To get rid of this problem, other carbon sources other than the locust bean gum (LBG) were tested for their induction capabilities. The substrates (Table 3.2) were added into the growth medium and gel diffusion assay (Section 2.2.5.1) was performed.

To determine the day of highest mannanase expression, two alternative methods were used: First was RNA dot blot analysis and the other was based on measuring the enzyme activity by colorimetric enzyme assay.

3.4.1 Preparation of Probe DNA for *afman1* and RNA Dot-Blot Analysis

Probe was prepared using F1 & B2 primer pair, which yields a 360 bp long product (Figure 3.5). This region was selected because of its highly conserved sequence in both *afman1* and *afman2*. This would allow analysis of the expression of both mannanases. This amplicon was sequenced and it was found that its sequence was identical to *afman1* based on the sequence data in the database. The probe was labeled with DIG DNA labeling kit (Roche, Mannheim, Germany).

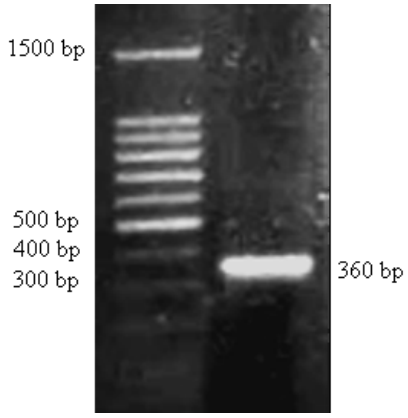


Figure 3.5 Amplification product of F1/B2 on *A. fumigatus* gDNA described in Section 2.2.2.

The expression of endo-β-mannanase gene was proven by total RNA isolation following a dot blot analysis; as described in Sections 2.2.1.2.3 & 2.2.4.1.3. With respect to the result of hybridization with the probe amplified with primers N3 & C3, the maximum level of mannanase gene expression was determined at the 4th day of growth (Figure 3.6).

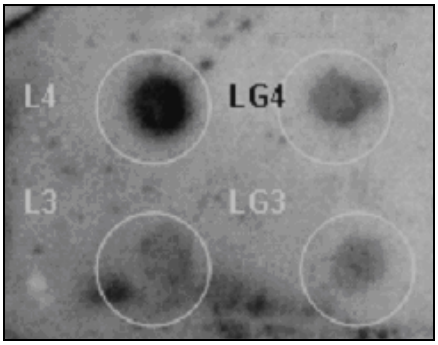


Figure 3.6: RNA Dot-Blot Analysis. Lx: total RNA isolated from *A. fumigatus* grown on 0.5% LBG isolated on xth day. LGx: total RNA isolated from *A. fumigatus* grown on 0.5% LBG and 0.5% glucose isolated on xth day.

The repression of endo mannanase expression by glucose can be clearly seen on Figure 3.6. Accordingly, expression levels are influenced by the presence of repressive carbon sources suggesting regulation by *creA*, which plays an important role in the regulation of genes from Aspergillus encoding plant cell wall-degrading systems (Strauss *et al*. 1999). Repression by CreA has been reported for genes encoding arabinanases, endoxylanases, β-xylosidase, arabinoxylan arabinofuranohydrolase, feruloyl esterases and several pectinases (de Vries, 2003). Although there was no publication related to regulation of mannanase expression and the role of CreA, the induction by mannans and galacto(gluco)mannans could be repressed by CreA gene family (de Vries, 2003).

3.4.2 Analysis of Mannanase Expression by Plate Assay

Accordingly, locust bean gum was the best inducer of endo-β-mannanase production among the other carbon sources shown in Table 3.2. It was also observed that birch wood xylan, carboxymethyl and avicel microcrystalline celluloses can also induce the mannanase expression, but not as much as LBG. In the presence of xylan, the induction of mannanase by LBG was decreased by *c.* 38% (Figure 3.7). This result shows that the expression of endo mannanase in complex media containing xylan would be not as high as in the galactomannan rich media.

Table 3.2 Substrates used in the induction analysis of endo mannanase

Carbon Source	Composition of Medium (%)
LBG (locust bean gum)	0.5
LBG+glucose	0.5 + 0.5
LBG+galactose	0.5 + 0.5
LBG+mannose	0.5 + 0.5
LBG+xylan	0.5 + 0.5
Carboxymethyl cellulose	2
Avicel microcrystalline cellulose	2
D-(+)-mannose	1.5
D-(+)-trehalose	1.5
D-(+)-galactose	2
Xylan	1
D-(+)-glucose monohydrate	2
Sorbitol	2

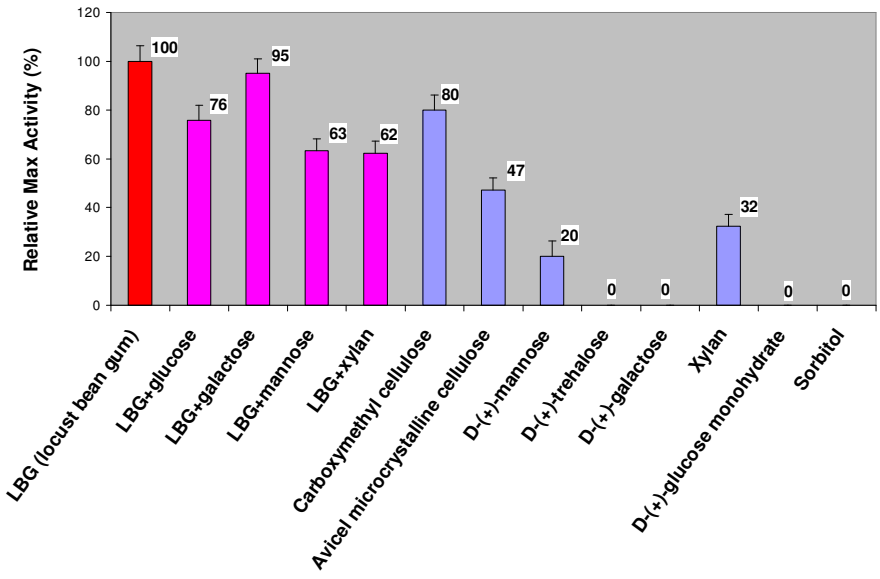


Figure 3.7 Comparison of inductions of endo mannanase with different carbon sources.

In filamentous fungi expression of the cellulose- and hemicellulose-degrading enzymes is under strict control at the transcriptional level by the available carbon source. In fungi, the production of cellulolytic enzymes is subject to transcriptional regulation by the available carbon source. So far, the known cellulase regulators include the CREI/CreA carbon catabolite repressors from *T. reesei* and aspergilli and the activators ACEII from *T. reesei* and XlnR from *A. niger*. Most of the hemicellulase genes in aspergilli are controlled by CREI, the wide-domain carbon catabolite repressor, and induced in the presence of the cellulase inducers. In general, gene expression of celluloses and hemicelluloses is determined by the balance of both positively and negatively acting regulators, including ACEII, ACEI, and CREI (Aro *et al*., 2003). The regulatory factors are identical. Induction of mannanase by different complex polymeric substrates is, therefore, not surprising.

Considering mixtures of the LBG and monosaccharides, galactose did not have any effect on the repression of expression, while mannose and glucose repressed expression of the endo mannanase. CreA-mediated repression is not only observed in the presence of D-glucose or D-fructose, but is also triggered by other monomeric carbon sources. A study into the expression of several xylanolytic genes showed that the expression of these

genes increases with reducing D-xylose concentration in a wild type *A. niger* strain (de Vries, 2003). Another study showed that the presence of different monomeric compounds results in different levels of CreA-mediated repression of ferulic acid induced expression of genes encoding *A. niger* feruloyl esterases (faeA and faeB) (de Vries, 2003).

The comparison of substrates with respect to the induction level of *afman1* showed that LBG was the best inducer among others. Some other complex carbon sources caused also induction, but both the low induction level and the adverse effect of the monomers on the isolation procedures the *afman1* was isolated from genomic DNA for the A. sojae transformation at the beginning.

3.5 Cloning and Heterologous Expression of *afman1*

3.5.1 Amplification of *afman1* by Genomic PCR

N3 & C3 primers were designed with respect to *afman1* gene for cloning. These primers include unique restriction sites for directional cloning onto the vector pAN52-4. N3 primer has restriction enzyme recognition site for *B*am*H*I and C3 primer has restriction enzyme site for *H*indIII. Amplification product of N3 & C3 primers from the genomic DNA had a length of 1511 bp. The band was verified to be *afman1* by Southern blotting and hybridization (Figure 3.8) using the 360 bp fragment, which was previously sequenced (Section 3.4.1).

The band was isolated from the agarose gel and cloned to pDrive (Qiagen) vector for sequence analysis. The insert was amplified via PCR with N3 & C3 primer pair, isolated from the agarose gel (0.8%) and finally dissolved in the ddH_2O. The insert was poly(A) tailled by PCR with Taq DNA polymerase and ligated in the cloning vector, pDrive (named as pMan1).

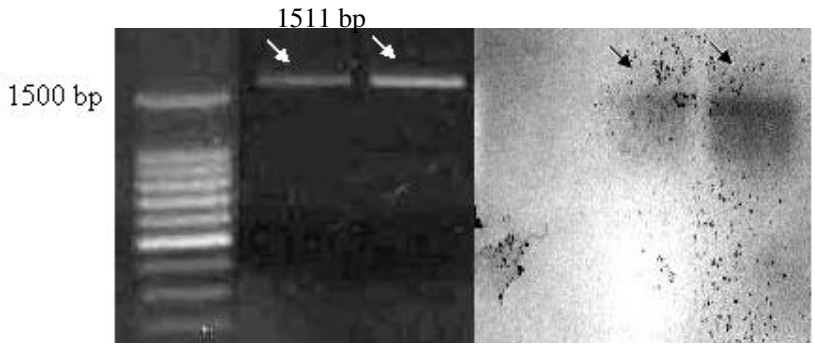


Figure 3.8 Amplification of *afman1* with primers N3 & C3 using *A. fumigatus* genomic DNA as template (left: white arrows) and the result of Southern blotting and hybridization (right: black arrows).

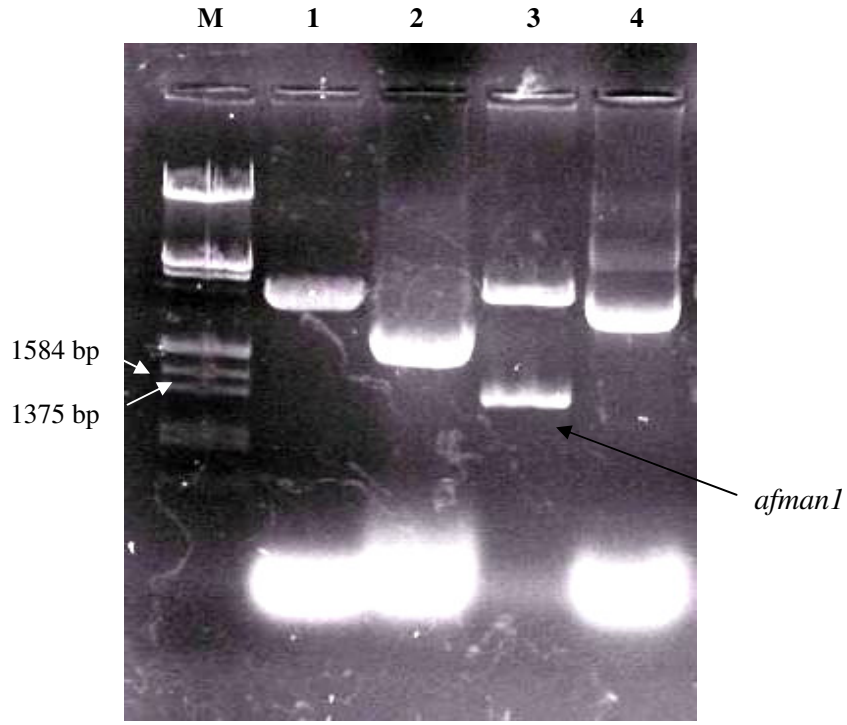


Figure 3.9 Restriction digestions of pMan1 and pDrive (empty vector) with *BamH*I and *Hind*III. M: Marker, Lambda DNA/ *EcoR*I + *Hind*III. 1: pDrive digested (*BamH*I + *Hind*III). 2: undigested pDrive. 3: pMan1 digested (*BamH*I + *Hind*III). 4: pMan1 undigested

The transformed *E. coli* were selected on the 2xYT agar (+ X-gal+IPTG, + Amp_{100}). After two generations, 2 transformants were selected being stably transformed and containing insert. (Figure 3.9).

3.5.2 Transformation into *Aspergillus sojae*

The *A. sojae* expression system employs the *gpdA* promoter from *Aspergillus nidulans,* which allows expression on simple carbon sources such as glucose (Dienes *et al.,* 2006). In fungal expression systems, strong *cbh1* promoter has also been used by the construction of an overexpression strain. Since most of the cellulases are co-expressed, using this promoter the deletion or deactivation of disturbing cellulase genes is required in order to avoid the need for enzyme purification (Ilmén *et al*., 1997). An alternative approach for enrichment of MAN, not requiring tedious gene deletion, is the use of the *gpdA* promoter of *Aspergillus nidulans* (Collén *et al*., 2001). In this case, although the enzyme production level is much less compared to the use of the *cbh*1 promoter, cultivations can be performed on glucose medium and thus the production of the major cellulases is repressed (Ilmén *et al*., 1997).

The insert on vector pMan1 was selected for transformation into *A. sojae*. The insert was cloned into the *BamH*I/*Hind*III restriction sites of the fungal expression vector pAN52-4. The gene was inserted downstream of the *gpd*A promoter region of pAN52-4 to attain high level constitutive expression in *A. sojae*. *gpd*A promoter originated from *Aspergillus nidulans* and is the promoter of the gene glyceraldehyde-3-phosphate dehydrogenase, an enzyme in glycolysis . Another adventage of this promoter was the capacity of the expression without the requirement of mannan containing carbon sources and the lack of a transcriptional repression control system. Furthermore, the signal sequence of *afman1* was replaced by the *A. niger* glucoamylase signal peptide sequence (accession no. X00712) and the pro-peptide region with a dibasic processing site (LYS-ARG). Such a fusionis expected to enhance secretion of the heterologous protein. The newly constructed vector was named as p3.2 and was used in further transformation studies after the conformation of the sequence by DNA sequence analysis.

The vector construct was co-transformed into *A. sojae* ATCC 11906 protoplasts (Figure 3.11) with the selection vector, pAMDSPYG. Among 39 transformants, after serial cultivations, only 8 were stable on the minimal medium. Transformants were then

tested for activity in shake flask containing 1% glucose, as the sole carbon source (Figure 3.12).

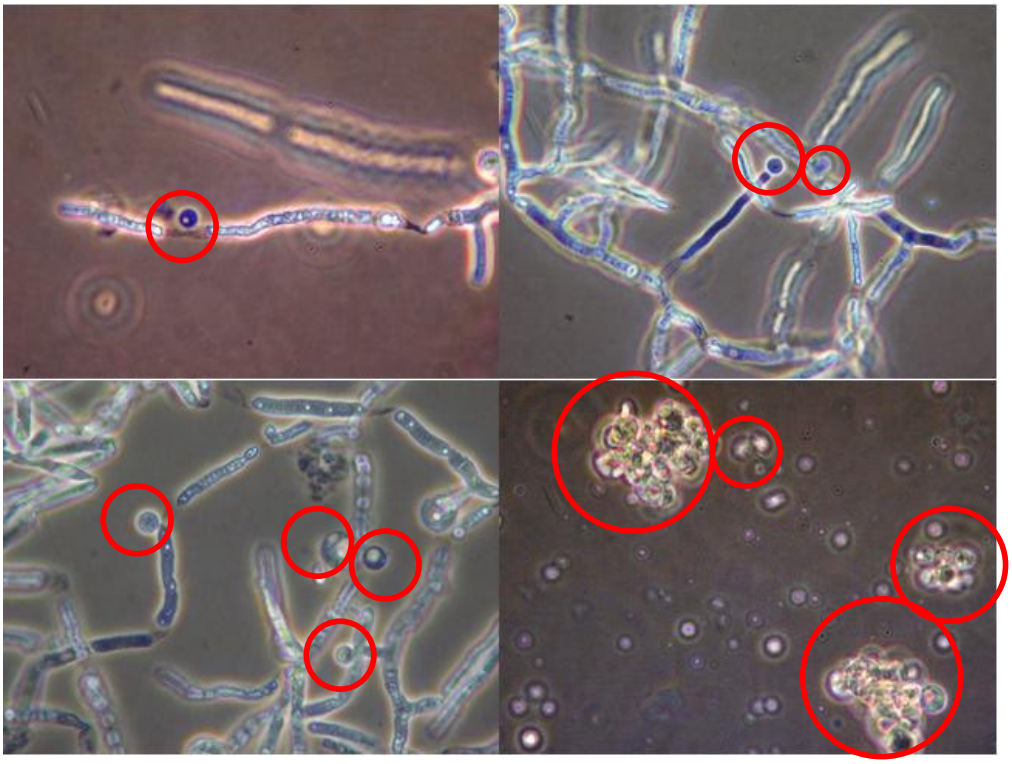


Figure 3.11 Protoplast formations in lysis buffer.

Mannanase production by *A. sojae* transformants in YpSs containing 1% glucose instead of starch was determined for 72 hrs (3 days). Two of the transformants showed highest activity (195 U/ml for p3.2.1 and 204 U/ml for p3.2.2). Comparison of the recombinant strains with the wild type endo mannanase production of *A. fumigatus*, shown to produce highest mannanase on the 3rd day as 30 U/ml (Puchart *et al.*, 1999), indicated an increase of 6 fold in the production of mannanase. The strain p3.2.4 did not show any activity for 3 days, pointing out that only the insertion of pAMDSPYG but not pAN52-4-*afman1* took place into the genome. The control organism was the commercial Bakers yeast (*Saccharomyces cerevisiae*), which was known to be non-mannanolytic.

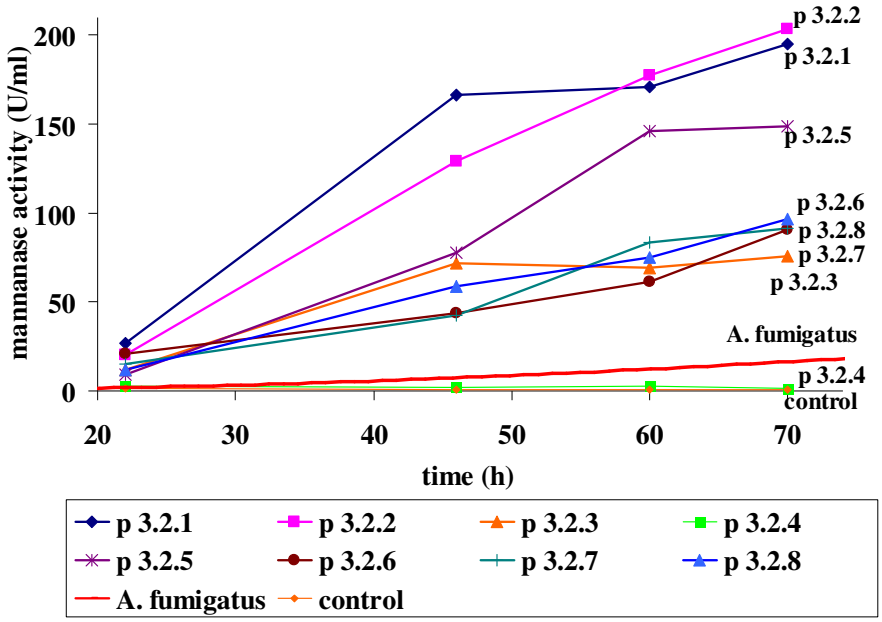


Figure 3.12 Mannanase Activity Measurements of Transformants (p3.2.1-p3.2.8) grown on 1% glucose containing medium by the DNS Method.

The best mannanase producers, *A. sojae* p3.2.1 and p3.2.2 were named as AsT1 and AsT2, respectively. Mannanase expressed by AsT1 is equal to *c.* 1 g mannanase per liter cultivation medium, which corresponds to *c.* 10% of extracellular total proteins.

3.5.3 cDNA Synthesis and Transformation into *P. pastoris*

Due to failure in attempts to isolate the cDNA of *afman1* from *A. fumigatus* RNA isolates, it was decided to use AsT1 as the source of *afman1* cDNA for further cloning into *P. pastoris*. Therefore, total RNA was isolated from AsT1 and cDNA was amplified using the primers N3 & C3. After cDNA synthesis and amplification 2 bands, about 1000 bp (small: *s*) and about 1300 bp (long*: l*) long were detected. Both of these bands were isolated and sub-cloned on the pCR2.1 vector.

DNA sequence analysis has revealed that the large fragment (*man-l*) is 1266 bp long and is completely processed cDNA of *afman1*. The smaller fragment (*man-s*) turned out to be a truncated form of the full-length cDNA. These two fragments were amplified with

the same primers N3 & C3, which were designed according to the N- and C-termini of the *afman*1 gene (Figure 3.13).

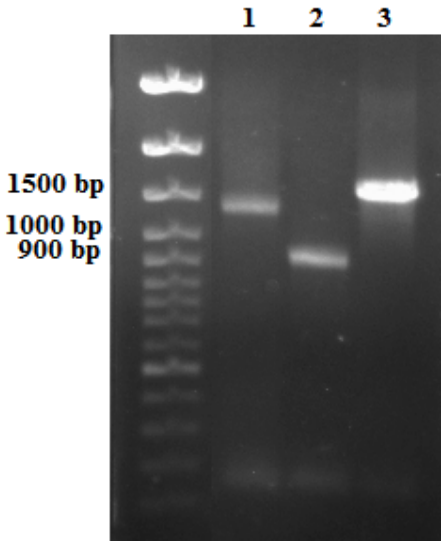


Figure 3.13 Amplification of isolated cDNAs from *Aspergillus sojae.* (1), large band (*man-l*); (2) and small band (*man-s*); and gene from *Aspergillus fumigatus* (3), *afman1*.

```
MAN-L           MHPLPSVALLSAIGAVAAQVGPWGQCGGRSYTGETSCVSGWSCVLFNEWYSQCQPATTTS 60
MAN-S           MHPLPSVALLSAIGAVAAQVGPWGQCGGRSYTGETSCVSGWSCVLFNEWYSQCQPATTTS 60
                ************************************************************

MAN-L           TSSVSATAAPSSTSSSKESVPSATTSKKPVPTGSSSFVKADGLKFNIDGETKYFAGTNAY 120
MAN-S           TSSVSATAAPSSTSSSKESVPSATTSKKPVPTGSSSFVKADGLKFNIDGETKYFAGTNAY 120
                ************************************************************

MAN-L           WLPFLTNDADVDSVMDNLQKAGLKILRTWGFNDVNSKPSSGTVYFQLHDPSTGTTTINTG 180
MAN-S           WLPFLTNDADVDSVMDNLQKAGLKILRTWGFNDVNSKPSSGTVYFQLHDPSTGTTTINTG 180
                ************************************************************

MAN-L           ADGLQRLDYVVSAAEKRGIKLLIPLVNNWDDYGGMNAYVKAYGGSKTEWYTNSKIQSVYQ 240
MAN-S           ADGLQRLDYVVSAAEKRGIKLLIPLVNNWDDYGGMNAYVKAYGGSKTEWYTNSKIQSVYQ 240
                ************************************************************

MAN-L           AYIKAVVSRYRDSPAIMAWELSNEARCQGCSTDVIYNWTAKTSAYIKSLDPNHMVATGDE 300
MAN-S           AYIKAVVSRYRDSPAIMAWELSNEARCQGCSTDVIYNWTAKTSAYIKSLD---------- 290
                ***********************************************

MAN-L           GMGVTVDSDGSYPYSTYEGSDFAKNLAAPDIDFGVFHLYTEDWGIKDNSWGNGWVTSHAK 360
MAN-S           ------------------------------------------------------------


MAN-L           VCKAAGKPCLFEEYGLKDDHCSASLTWQKTSVSSGMAADLFWQYGQTLSTGPSPNDHFTI 420
MAN-S           --------CLFEEYGLKDDHCSASLTWQKTSVSSGMAADLFWQYGQTLSTGPSPNDHFTI 342
                        ***********************************************

MAN-L           YYGTSDWQCGVADHLSTL 438
MAN-S           YYGTSDWQCGVADHLSTL 360
                ******************
```

Figure 3.14 Alignment of amino acid sequences of MAN-L and MAN-S. The deletion covers a region of 78 a.a., start with P_{291} to P_{368} with respect to the MAN-L sequence.

Compared with the large fragment, the smaller fragment had a segment of 234 bp deleted on it sequence, which includes the sites of the two introns. The deleted site correspond a 78 amino acid long sequence from P_{291} to P_{368} of MAN-L (Figure 3.14). As the lacking fragment did not disturb the open reading frame, the rest of the gene is expected to be translated correctly. This fragment is 1032 bp long (Appendix F).

The large and small fragment (*man-l* and *man-s*) were subcloned onto pPICZαC vector (Invitrogen) taking into account the open reading frame. It was aimed to secrete the proteins under the strong promoter, P_{AOX1} (alcohol oxidase gene, which is tightly regulated with methanol). Therefore, the genes were ligated downstream of the *AOX1* promoter and the α-factor secretion signal peptide (*Saccharomyces cerevisiae* α factor prepro peptide) sequence. *AOX1* promoter is a 942 bp long fragment that allows methanol-inducible, high-level expression of the gene of interest in *Pichia pastoris*. Additionally, this region also targets plasmid integration into the *AOX1* locus. α-factor secretion signal peptide sequence was obtained from *Saccharomyces cerevisiae* and allows for efficient secretion of most proteins from *Pichia.* The vector construct would

make possible to detect and purify the secreted protein by its C-terminal tags, but due to the stop codon on the fragments, His-tag region was not translated. The primary structure of the expressed protein of *manl* would be similar to the mannanase of AsT1 or AsT2. The ligated vectors with mannanase cDNAs (*man-l* and *man-s*) were named as plαC and psαC, with respect to *manl* and *mans*.

After transformation by electroporation, 42 colonies transformed with *man-l* and 40 colonies transformed with *man-s* were screened by PCR for the presence of mannanase cDNA. As the *P. pastoris* is not mannanolytic, it was not expected to have a mannanase gene on its genome. The recombinant strains obtained after transformation with *man-l* and *man-s* were named as PpT1 and PpT2, respectively (Figure 3.15).

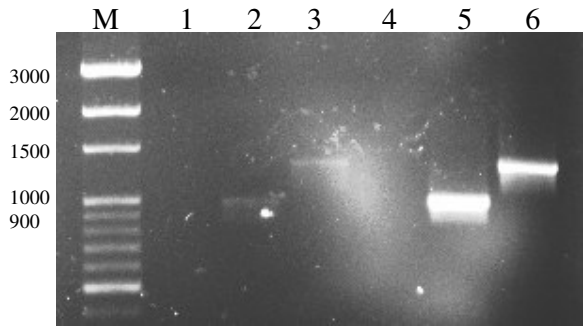


Figure 3.15 Amplification of mannanase cDNA by PCR with the primers N3 & C3 using the following templates genomic DNA of untransformed *P. pastoris* GS115 (1); gDNA of PpT2 (2); gDNA of PpT1 (3); *man-s* (5) and *man-l* (6).

PpT1 and PpT2 were cultivated on YpSs based medium containing 1% glucose instead of starch supplemented with 1% methanol for induction. Because the expression of the recombinant mannanase was possible only in the presence of volatile methanol, the medium was supplemented with methanol (0.5%, v/v) at the end of every 24 hrs of cultivation. The biomass and the mannanase production were screened for 60 hrs and 84 hrs for PpT1 and PpT2, respectively (Figure 3.16 and 3.17).

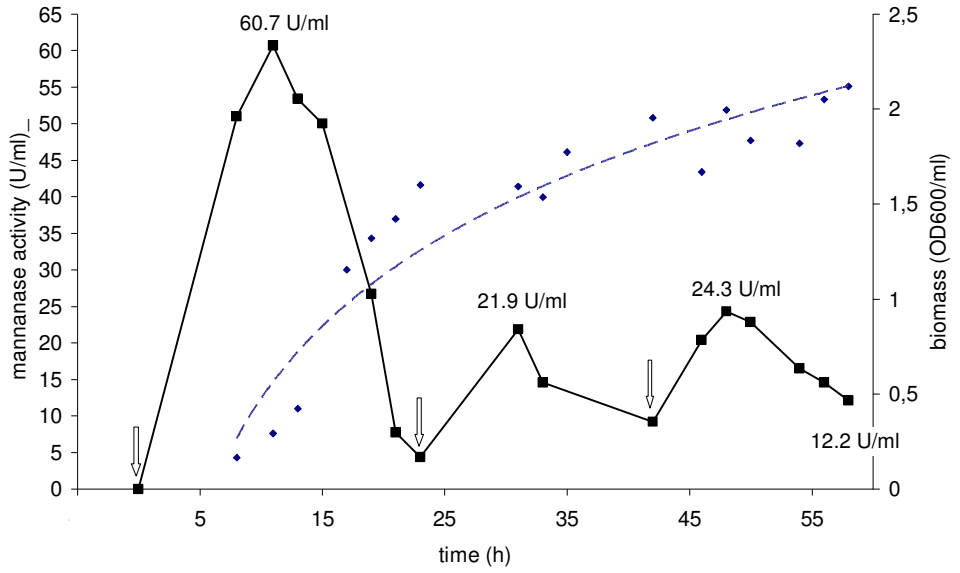


Figure 3.16 Time course of PpT1's mannanase production (continuous line) and its biomass production (dashed line). Arrows indicate the time points of methanol induction to a final concentration of 0.5% (v/v).

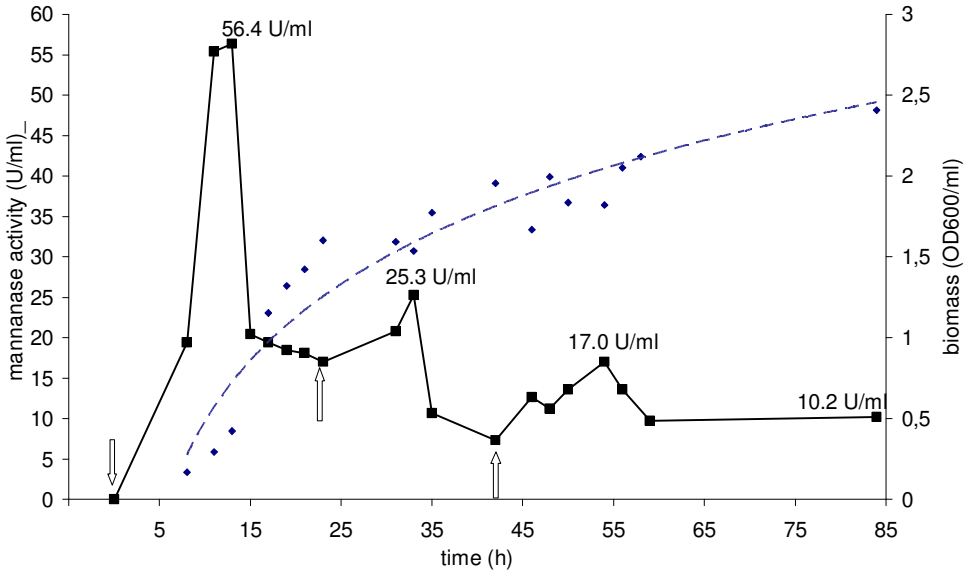


Figure 3.17 Time course of PpT2's mannanase production (continuous line) and its biomass production (dashed line). Arrows indicate the time points of methanol induction to a final concentration of 0.5% (v/v).

After each induction, the organisms showed response by increasing the mannanase production. However, the response was higher during the initial phases of growth, when the growth rate was high. Later, eventhough the number of the cells was higher than before, the response remained low. These results suggest a continuous cultivation system may be more suitable in endo mannanase production by the recombinant yeasts (PpT1 and PpT2) than the batch fermentation, which was more appropriate for AsT1 and AsT2 cultivation. Mannanase activity was not detected in the culture medium of wild-type *P. pastoris* GS115 strain after methanol induction even after 60 h. The highest expression was observed at 10^{th} h of initial inoculation with methanol in PpT1at the exponential growth phase. Mannanase was produced at the level of 0.17 mg/ml cultivation medium, which was equivalent to *c.* 3.7% of extracellular total proteins. However, when the yeast is grown on methanol, alcohol oxidase can make up to thirty-five percent of the total cellular protein (Cregg *et al*., 1985).

In terms of the mannanase production efficiency with respect to the time of the operation, the *P. pastoris* transformant (6.1 U $ml^{-1}h^{-1}$) was better than the *A. sojae* construct (max. 2.83 U $ml^{-1}h^{-1}$). This result suggests that the system of methylotrophic yeast, *P. pastoris* may be more suitable for the production of mannanase by continuous operation. Mannanase production by PpT1 and PpT2 reached their highest level at *c.* 61 U/ml and *c.* 56 U/ml at 10 and 12 hrs of cultivation, respectively. Comparison with the maximum mannanase production level (30 U/ml) of *A. fumigatus* after 72 hrs, these levels correspond to approximately 2 fold increase in the production by the *Pichia* systems.

3.6 Transformation into *Arabidopsis thaliana*

The enzyme was aimed to be expressed constitutively in the apoplastic region of plant under the control of the CaMV35S promoter. First, cDNA of mannanase gene (*manl*) was cloned to pBinAR vector. This vector is a derivative of Bin19 binary vector containing expression cassette for constitutive expression of chimeric genes in plants. It is an in-house developed vector (Max Planck Institute, Molecular Plant Physiology, Potsdam, Germany), meaning that it was not commercially available. The gene was inserted within the region of the CaMV35S promoter (constitutive) and the OCS3' terminator.

Agrobacterium-mediated transformation of *A. thaliana* was performed by using the floral dip method. After first transformation, seeds of 4 lines were collected, sterilized

and germinated on the MS agar supplemented with 100 µg/ml kanamycin. Almost all the seeds were germinated. Hypocotyls that were resistant to antibiotic had the phenotype of dark green and the ones that were sensitive to selection agent had a pale green to white coloured leaves. However, all the hypocotyls that were resistant to kanamycin could not develop further to true plant and died after 3 weeks. However, the plants transformed with empty vector developed and grew similar to the wild type cultivars. The seedlings were observed only on the plate and were not transferred on the soil.

The transformation was repeated and again 4 lines were obtained germinating on 50 µg/ml kanamycin containing plates. The seedlings were grown for 2 weeks and green healthy seedlings were transferred on soil, however true plant parts and higher plant structures did not develop. They remained as seedlings on soil and died. On the contrary, the plants transformed with the empty vector grew on soil and did not show any significant difference compared to wild-type cultivars.

To observe the potential effect of the enzyme on wild-type plant, the seeds were germinated in the MS liquid medium with the supplementation of *Bacilli* endo mannanase (Megazyme) (~0.6 U/ml), but the seedlings grew without having any effect. This suggests that either the enzyme had no effect on the plant or the enzyme (47 kDa) was too large to diffuse into the plant tissues.

The seeds from the second transformation were also germinated in liquid MS medium with kanamycin (50 µg/ml). In that case, the medium was not supplemented with any enzyme. Normally the seeds in liquid medium germinated and grew within 2 weeks. In the following days, the growth was so extensive that the wild type plant leaves, shoots and roots mixed to each other forming one single block structure, which did not allow separating a single plant without damaging its structure. This was what was observed in the flasks of the control plant, which was transformed with empty pBinAR structure. The plants transformed with mannanase gene, however, showed delayed development in cultivation. After 5 weeks, the seedlings were still alive and did not grow as much as the wild-type seedlings in their 2 weeks of cultivation (Figure 3.18).

Figure 3.18 Liquid culture of *A. thaliana* transformed with pBinAR, empy vector (left) and with pBinAR_*Manl* (right).

5 weeks of growth in liquid culture was unusually long cultivation, which had to be ceased at the time, when the contaminating yeasts started to grow (white precipitate on the background; Figure 3.19).

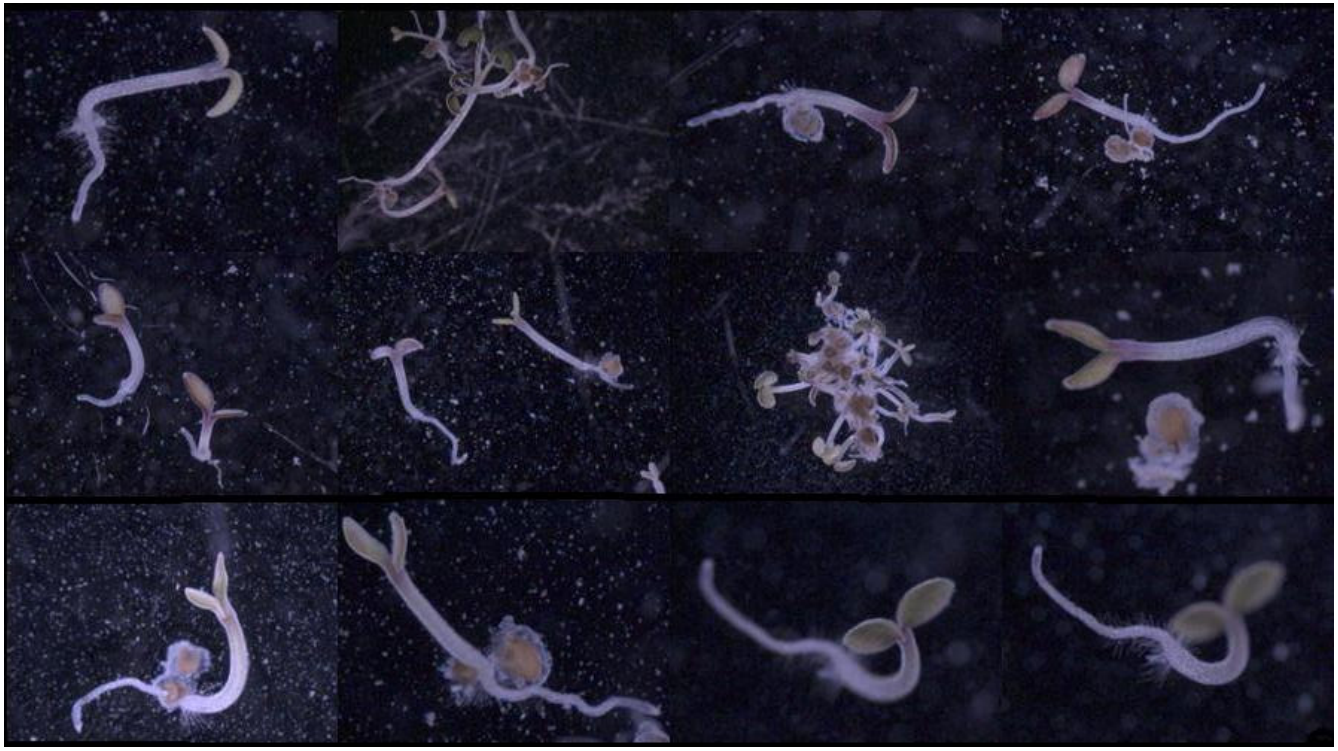


Figure 3.19 *Arabidopsis thaliana* Col-0 transformed with pBinAR_*Manl*. The seeds were incubated for 2 days in dark room at 37 °C for imbibition in 50 ml MS-brot supplemented with kanamycin (50 µg/ml). The seedlings were incubated for 5 weeks by continuous shaking at 100 rpm.

The mannose content of plant cell wall is quite low (a molar ratio of *c.* 2.3%) compared to other sugars such as galactose, xylose, arabinose revealing that the mannan and heteromannan content is so low that such any change in mannan amount on the wall composition is not expected to have any effect on the plant physiology. The cultivation of transformed plants showed that mannanase had an effect on the growth directly or indirectly. To explain the effect of the enzyme to the plant development, some other experiments should be carried out, but this was out of the scope of this thesis.

After transformation, mannanase is expected to have its activity towards the plant cell wall. In the lab of Dr. Markus Pauly (Molecular Plant Physiology, Max Planck Institute, Potsdam, Germany), it was shown that plants modify their cell wall structure as a defence response to such an enzyme, such that the sugar compostion is changed as a result. The sugar composition of hypocotyls was analyzed by alditol acetate assay following by GC-MS. Although the leaves were harvested in the dark, glucose content was high (Figure 3.20).

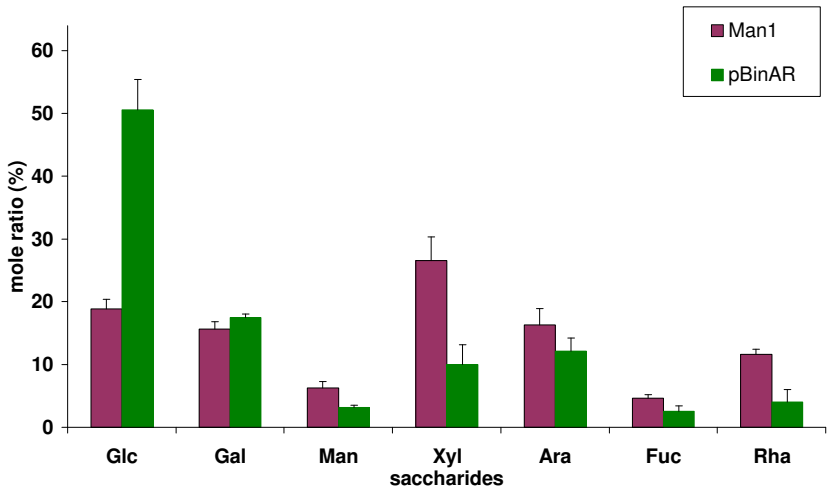


Figure 3.20 Mole percentages of monosaccharides in plant cell wall composition without amylase treatment. The graph shows the mole ratio of indicated 7 monosaccharides (total 100%, of each plant line) in plant cell wall.

Harvesting in the dark was decided, because in the presence of light, the plant begins photosynthesis and the glucose content increases enourmously. In this case, the decrease of the glucose ratio could be explained by the decreased cellulose content. However, the requirement of the cell wall material for this assay was so high, it could not be performed. The calculations for cell wall sugar composition were repeated for the same assay, in this case without calculating the glucose values. Figure 3.21 shows that the ratio of galactose was decreased in the cell wall. As the mannose chain in galactomannan is substituted with galactose units, the decrease of mannose possibly resulted in the elimination of galactose binding site. On the contrary, the mannose ratio was increased slightly. It means that the transgenic plant was increased in its glucomannan, which is a substrate less hydrolyzed by mannanase. The xylose content was also increased in the recombinant plants. As one type of hemicellulose is decreased, other types are likely to increase, such as xylans and pectins.

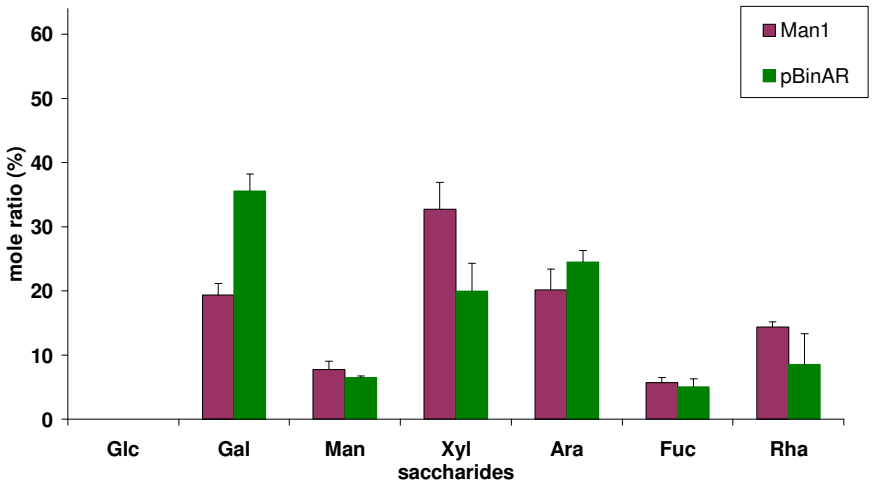


Figure 3.21 Mole percentage of monosaccharides in plant cell wall composition. The monosaccharide composition was calculated excluding the glucose ratio in the cell wall.

The assay was also repeated for the amylase treated pBinAR cell wall samples (Figure 3.21). The effect of the enzyme on the cell wall composition was analysed by excluding the glucose value from calculations such that the mole ratio of plant cell wall

monosaccharide was calculated only within 6 sugars. In general significant increase in the xylose, mannose and rhamnose content was observed. The mol ratio of galactose in the cell wall was decreased**.**

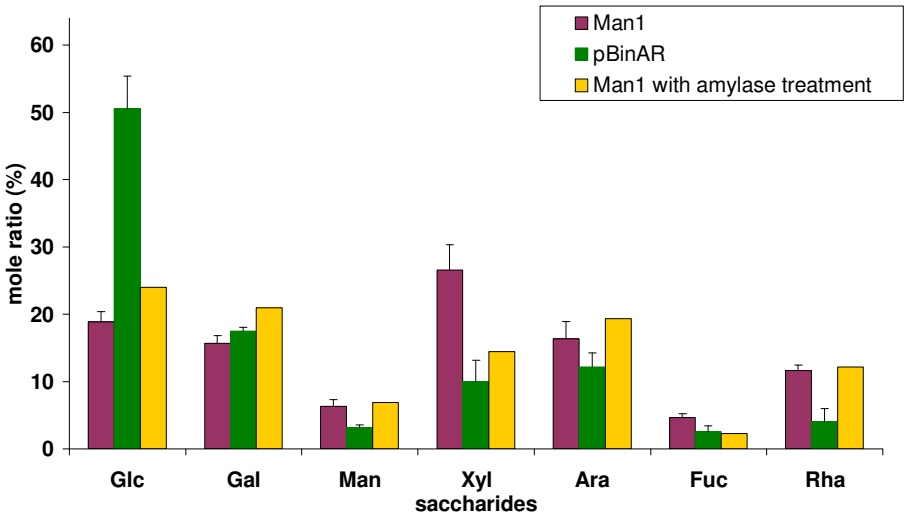


Figure 3.22 Mole percentage of monosaccharides in plant cell wall with and without amylase treated pBinAR sample and without amylase treated pBinAR_*afmanl*.

The results of transformation into Arabidopsis showed that the expression of mannanase gene in the early stages of plant development has a lethal effect. This showed that the plant system was not a good system for the constitutive enzyme expression, which is required for a feasible mannanase production system.

3.7 Characterization of Recombinant Mannanases

AsT1 was grown in 500 ml of YpSs medium containing 2% glucose. Not to induce any native mannanase genes, no supplementation of locust been gum or any complex mannan polymers were used in the cultivation medium. The sole carbon source in the medium was glucose, which was also shown for the *Aspergillus fumigatus* as the repressor for mannanase activity in Section 3.4.2. PpT1 and PpT2 were inoculated in 200 ml YpSs medium with 1% glucose and 1% (v/v) methanol. In general, mannanases of AsT1 and PpT1 retained 70% of their activities after 4 week and 10% of their activities

after 9 weeks storage at 4 °C in crude form. PpT2, however, lost its activity after two weeks storage in the refrigerator 4 °C.

3.7.1 Purification of Mannanases

Purification of heterologous mannanases from *A. sojae* and *P. pastoris* were performed as described in Section 2.5. First, proteins of the sample were separated by HiTrap DEAE FF column, which is a weak anion exchange coloumn composed of 6% highly cross-linked agarose with a total ionic capacity of 0.11-0.16 mmol Cl^-/ml medium and a dynamic binding capacity of 110 mg HSA/ml medium. The binding of charged sample molecules to oppositely charged groups attached to the insoluble matrix is electrostatic and reversible.

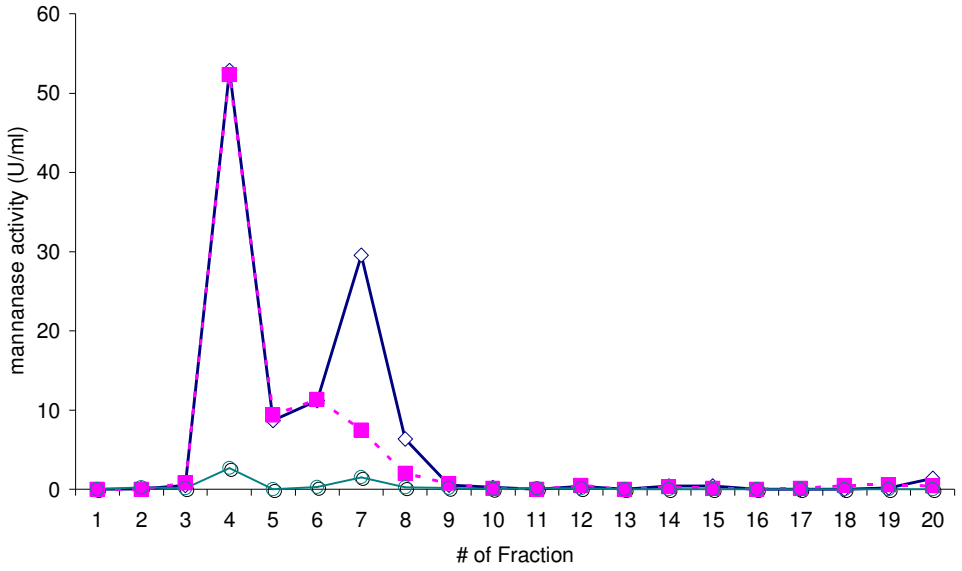


Figure 3.23 IEX column seperation of MAN of AsT1(◊), PpT1 (■) and PpT2 (O).

Cell-free culture fluid was concentrated by increasing salt gradient to 40%, 60% and 80% ammonium sulphate. It was observed that MAN precipitate at salt concentration of 80%. This step was not applied later, because the enzyme production was so high that even unconcentrated culture fluid was enough to plug the column. Elution profile with

respect to enzyme activities of this purification step is shown in Figure 3.23. Majority of the proteins including mannanases was not trapped on the column and eluted in early fractions. The separation of AsT1 proteins gave two mannanase activity peaks; one in the 4th and the other in the 7th fractions. After ion exchange column purification, the fractions, in which the major mannanase activities were measured, were colleted and used for separation in the hydrophobic interaction column (HIC).

The hydrophobic column is packed with a phenyl agarose matrix. In the presence of high salt concentrations the phenyl groups on this matrix bind hydrophobic portions of proteins and both by reducing the NaCl concentration and adding ethylene glycerol different column-bound proteins are eluted. Results of HIC column separation of the partially purified extracts are shown in Figures 3.24 and 3.25.

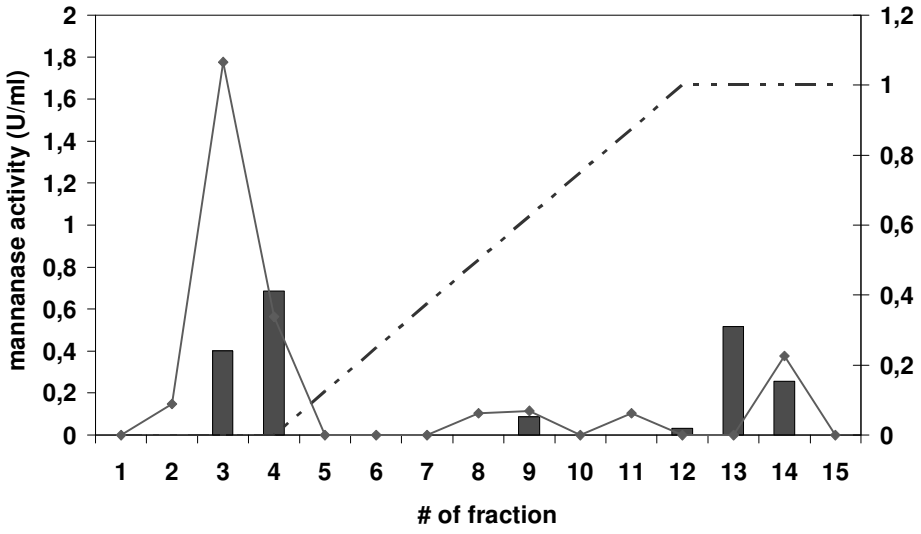


Figure 3.24 HIC column separation of MAN of AsT1. Mannanase activity (continuous line), total protein concentration (solid bar), percentage of ethylene glycol (dashed line).

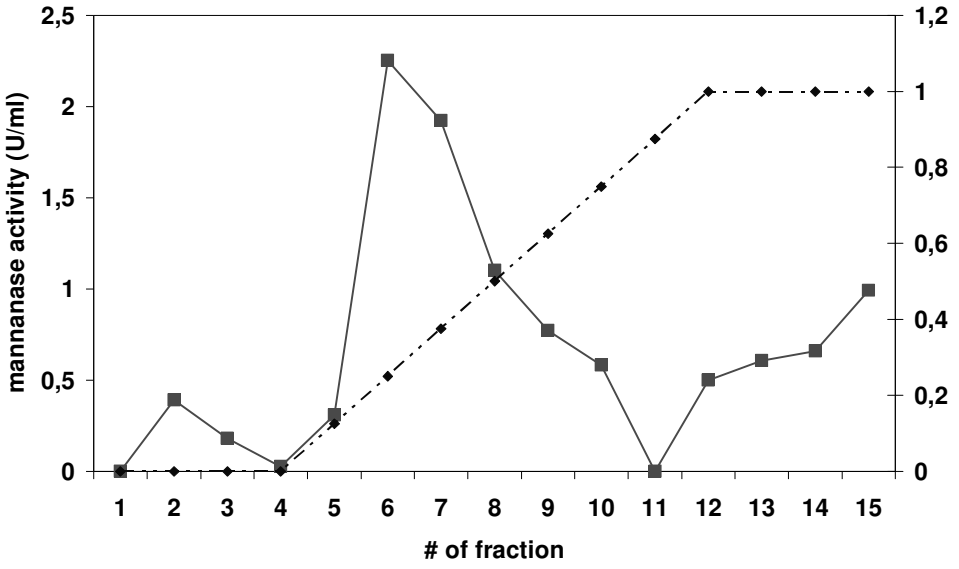


Figure 3.25 HIC column separation of MAN of PpT1. Mannanase activity (continuous line), percentage of ethylene glycol (dashed line).

Both mannanases from *A. sojae* and *P. pastoris* had specific activities of 349 U mg^{-1} protein. The molecular mass of the mannanases from both organisms was estimated as 60 kDa (Figure 3.26) and the molecular size of the truncated mannanase, PpT2 as 40 kDa (Figure 3.27) by SDS-PAGE.

The mannanase was analysed with activity staining by mixing 10% of polyacrylamide gel with LBG (Figure 3.28-3.31). After removal of SDS from the gel and incubation, the carbohydrate polymer, LBG, was stained with Congo red as described in Section 2.2.5.1 .

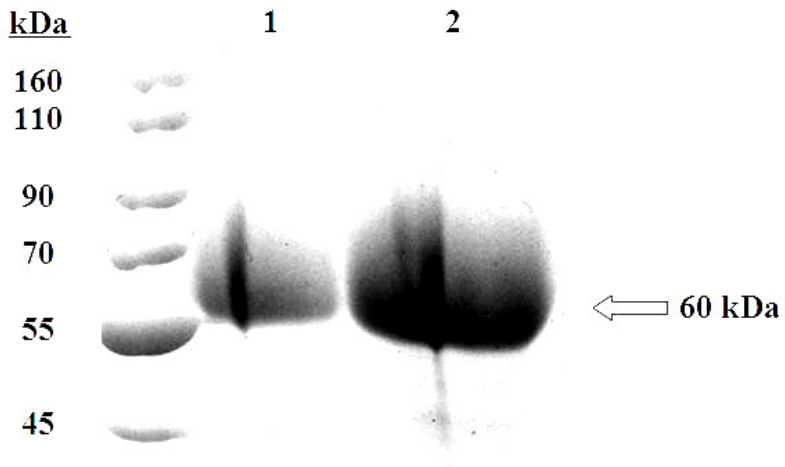


Figure 3.26 SDS-PAGE of purified PpT1 (1) and AsT1 (2) mannanases after staining with Coomassie brilliant blue G-250.

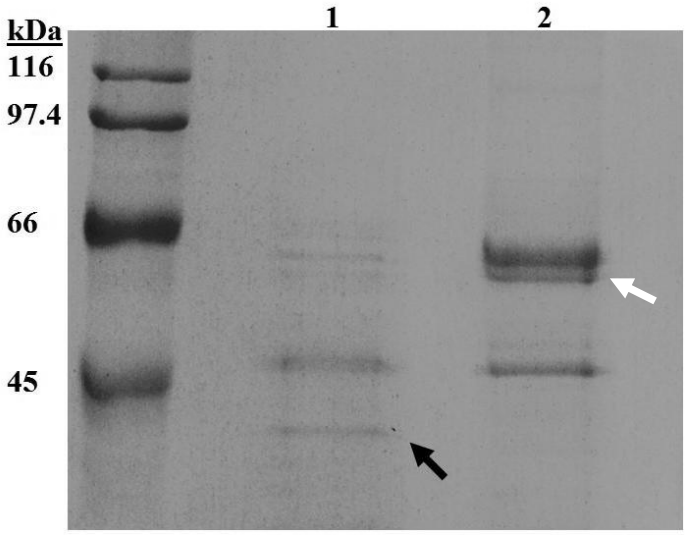


Figure 3.27 SDS-PAGE of PpT1 and PpT2 after staining with Coomassie brilliant blue G-250. Lane 1: PpT2 and Lane 2: PpT1. Mannanases were shown with arrows.

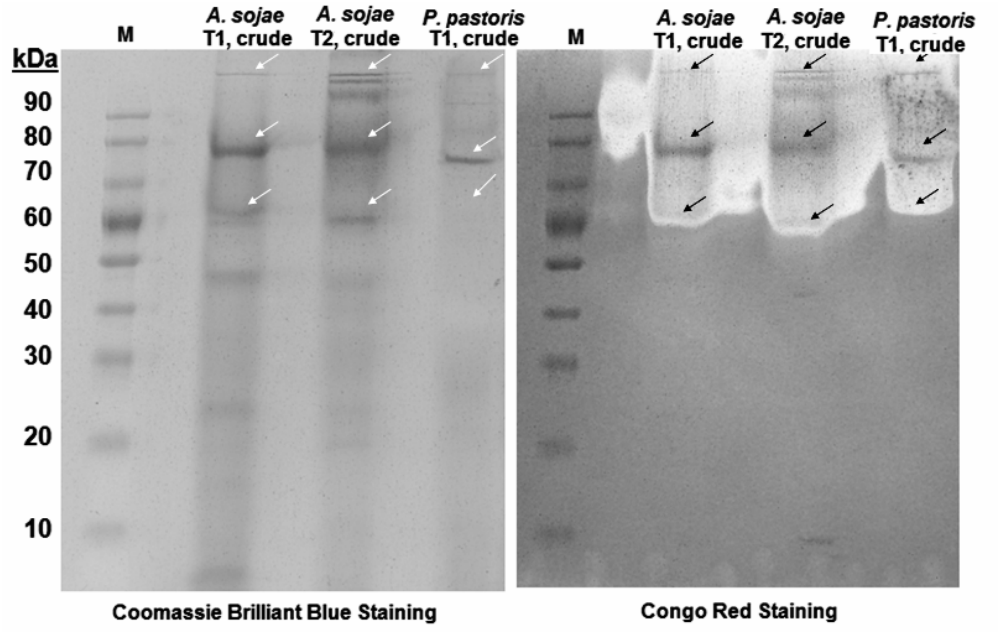


Figure 3.28 Native PAGE and Activity Staining. PAGE was supplemented with 0.1% LBG before gellification. After staining of proteins with Coomasie brilliant blue G-250, unhydrolized LBG was stained with 0.1% Congo Red.

In Figure 3.28, multiple forms of MAN were observed. This may indicate the existence of conformational differences in the native form such as the degree of glycosylation. At least 3 forms could be noticed.

The molecular size and activity of the enzymes were also analyzed in crude culture filtrates by separating both boiled and unboiled samples on the same gel (Figures 3.29 and 3.30).

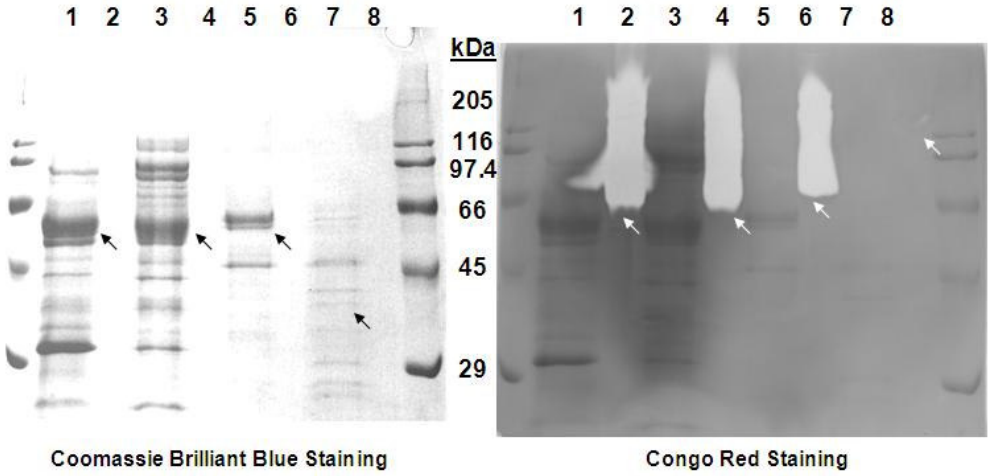


Figure 3.29 Activity staining. Odd numbered lanes were denatured culture filtrates and even numbers were untreated (enzymatically active) culture filtrates of AsT1, AsT2, PpT1 and PpT2, respectly.

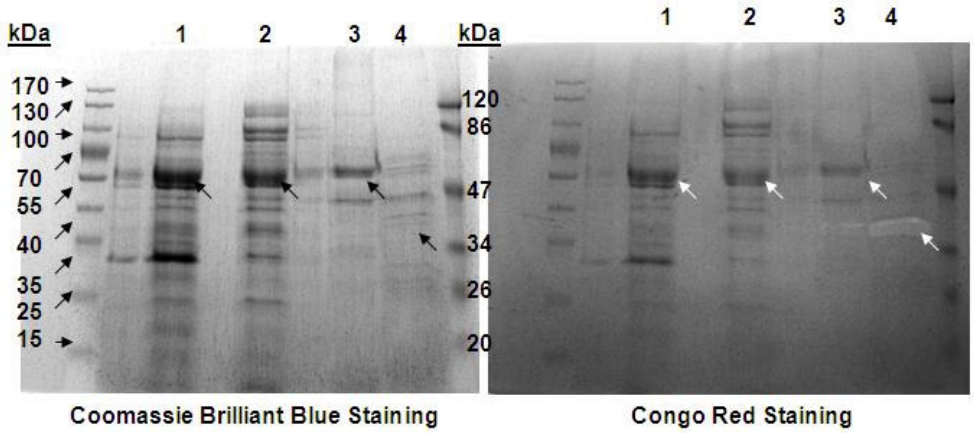


Figure 3.30 Activity Staining. Heat denatured culture filtrates of 1, AsT1; 2, AsT2; 3, PpT1; 4, PpT2.

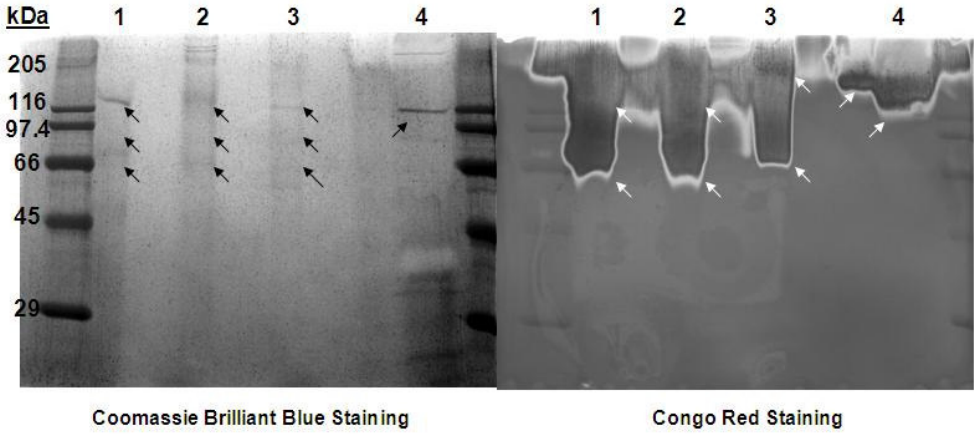


Figure 3.31 Activity Staining. Culture filtrates of 1, AsT1; 2, AsT2; 3, PpT1; 4, PpT2.

In activity staining experiments mannanases from PpT2 showed activity bands in unexpectedly large fragments (Figure 3.30). Only when the boiling time was decreased from 3 min to 1 min, activity was observed in the 40 kDa region (Fig. 3.31). These observations may suggest that mannanases of PpT2 exists in multimeric forms in its native state. The multimers possibly separate upon boiling, and the enzyme remains in its active form after 1 min, but not after 3 min of boiling. This is interesting, because it indicates that the accidentally generated form of MAN, namely MAN-S, is more stable to boiling, which may or may not be linked to the possible formation of multimers.

3.8 Characterization of Mannanases Expressed in *Aspergillus sojae* and *Pichia pastoris*

In the determination of the optimum pH for activity of MAN in the crude filtrate of AsT1, two peaks at pH 4.6, the minor, and at 5.6, the major peak were observed. These experiments were repeated several times. After purification of MAN of AsT1, the optimum was found only at pH 4.5. For MAN of PpT1, two peaks of activity were observed even in the pure form; namely at 4.0 and at a range of 5.2-5.6 (Figure 3.32). Analysis of the native mannanase by Puchart *et al*. (2004) showed two enzymes with different degrees of glycosylation and highest activities at pH 4–5.

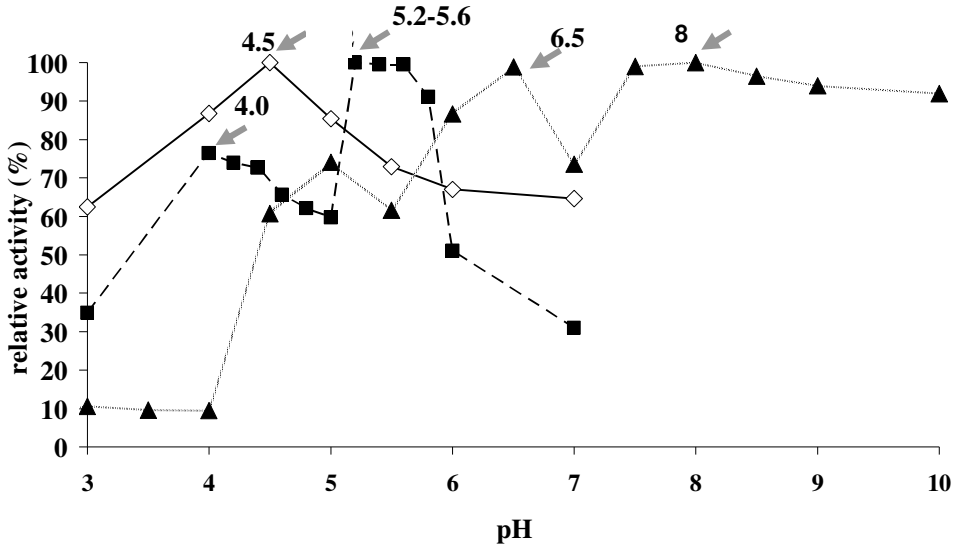


Figure 3.32 Effects of pH on the activity of purified MAN from AsT1 (◊), PpT1 (■) and PpT2 (▲).

In the measurements of the optimum pH, it should be noticed that the acidic hydrolysis of the locust been gum below pH 3.0 there was so much reduced sugar in the enzyme substrate, without the need of the spectral analysis, the difference in the color could be identified by naked eye. Therefore any pH optimum measurements with LBG at pH lower than 3.0 were not reliable. The truncated form PpT2, however, represents a quit different result. It has a pH optimum at around pH 8.0. This mannanase form showed higher activities at neutral and basic ranges as like mannanases from bacterial strains.

The temperature optima for the enzymes were also different from each other, namely 60 °C and 45 °C for MAN of AsT1 and PpT1, respectively (Figure 3.33). The optimum temperature value for PpT2 is 60 °C. The difference between MAN of AsT1 and PpT1 might be explained by the rate of the glycosylation of the protein by the yeast system. Analysis of the native mannanases from *A. fumigatus* by Puchart *et al*. (2004) showed two enzymes with different degrees of glycosylation and highest activities at 60–65 °C. As the primary structure of the PpT2 mannanase are different than the recombinant mannanases of AsT1 and PpT1, different effects of pH and temperature are expected.

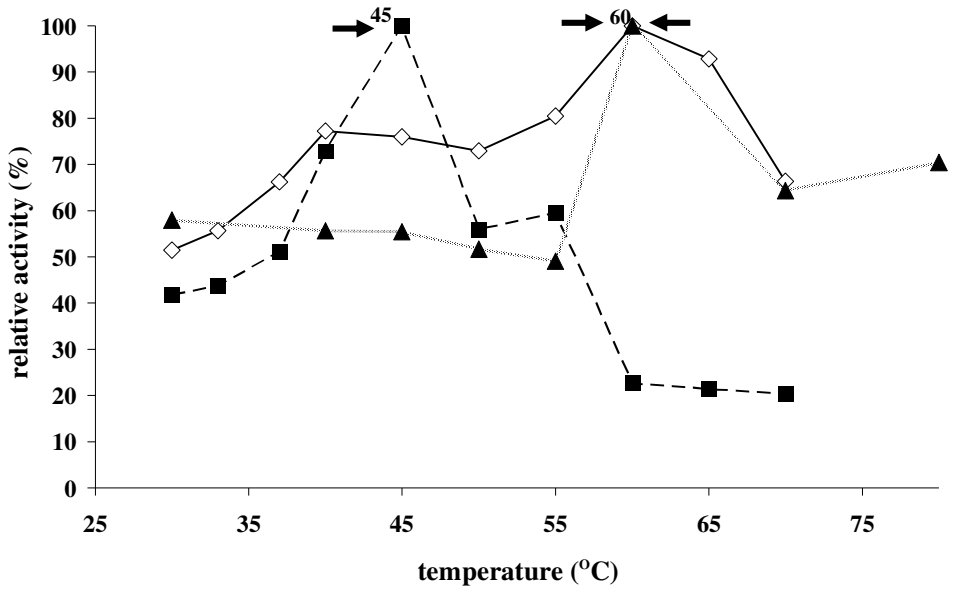


Figure 3.33 Effect of temperature on the activity of purified MAN from AsT1 (◊), PpT1 (■) and PpT2 (▲).

According to the literature, native fungal mannanases of GH 5 generally represent similar optimum temperatures (50-70 °C) and pH (pH 3-5) (Ademark *et al.*, 1998; Christgau *et al.*, 1994; Ferreira *et al.*, 2004; Puchart *et al.*, 2004; Stalbrand *et al.*, 1993). Further fungal mannanases cover a wide pH stability range (from pH 3.5 to pH 8.5). This was also observed in the case of MAN produced by AsT1 & PpT1 (Fig. 3.34). However, temperature stabilities were lower (Figure 3.35-3.37). MAN from AsT1 lost most of its activity after 10 h of incubation at 60 °C. the enzyme form PpT1 was more stable and retained most its activity even after 24 h incubation at 50 °C.

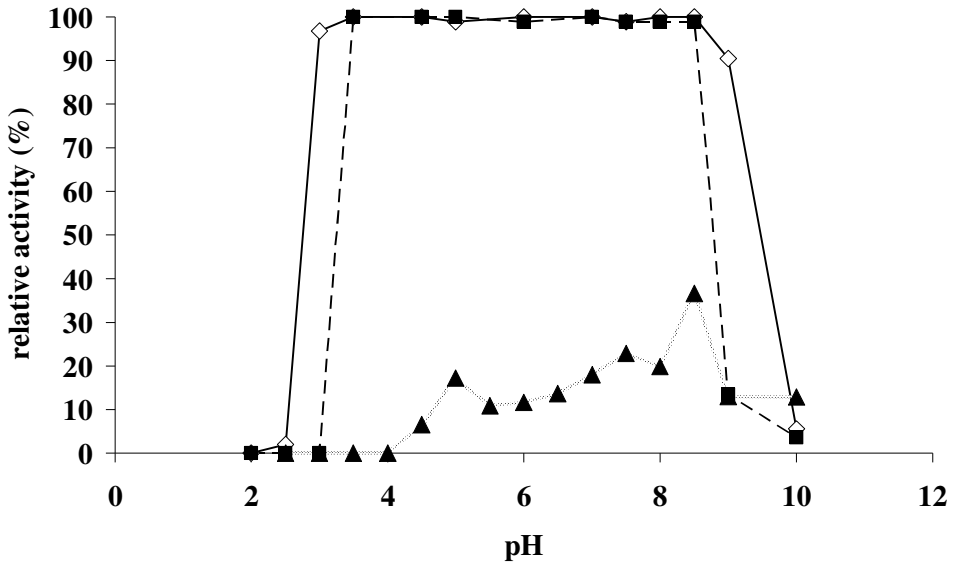


Figure 3.34 Effect of pH on the enzyme stability of of purified MAN from AsT1 (◊), from PpT1 (■) and from PpT2 (▲).

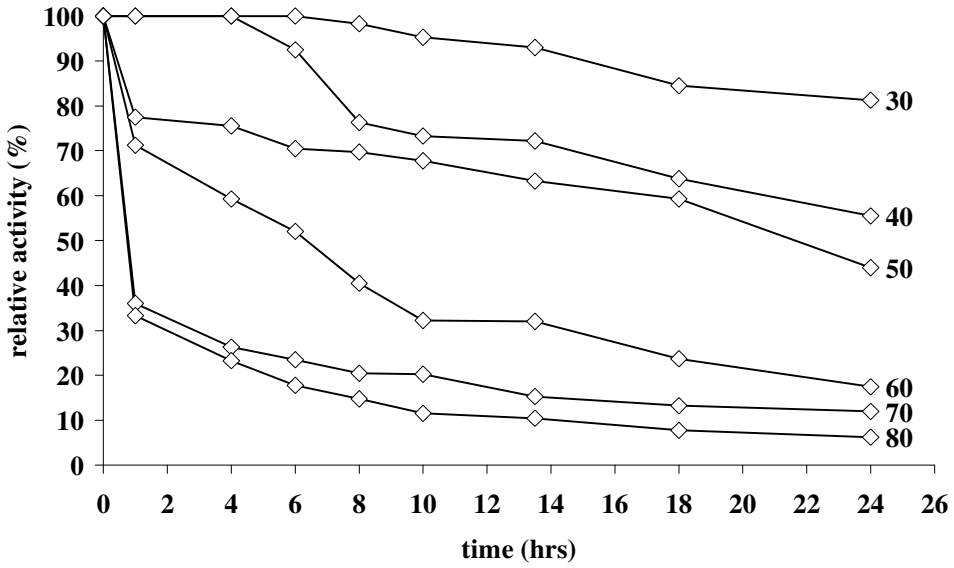


Figure 3.35 Effects of temperature on the stability of purified MAN from AsT1 (◊).

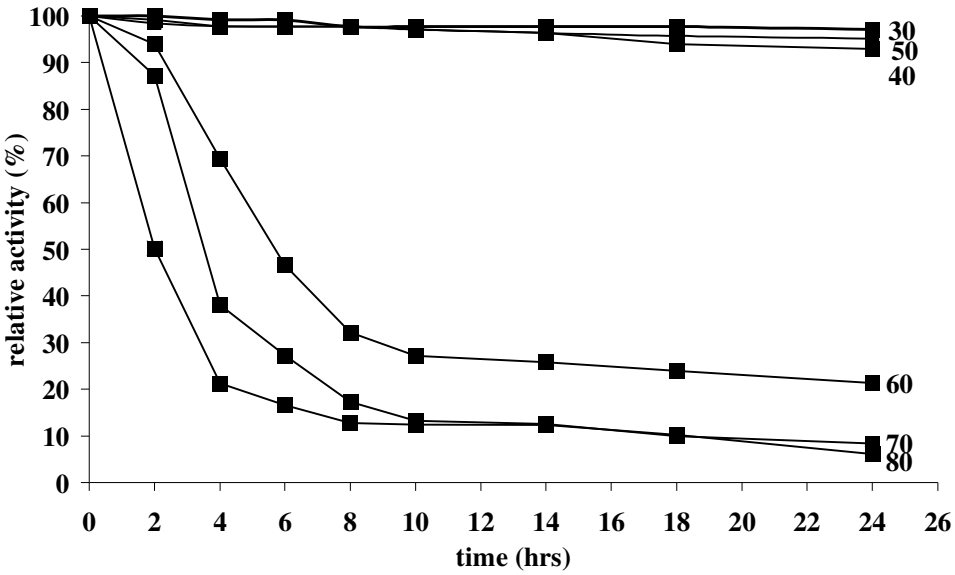


Figure 3.36 Effect of temperature on the stability of purified MAN from PpT1 (■).

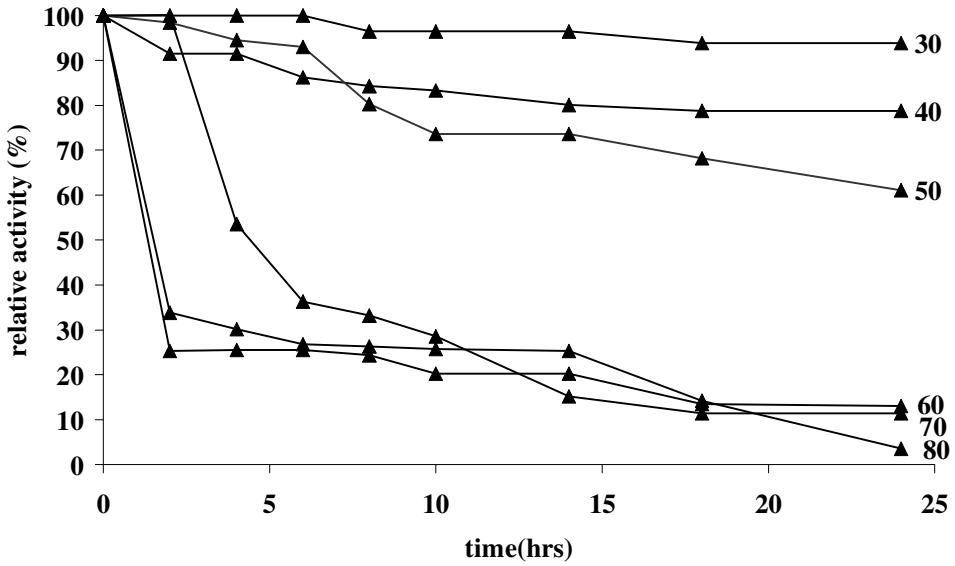


Figure 3.37 Effect of temperature on the stability of purified MAN from PpT2 (▲).

PpT2, however, is less stable than two other enzymes. It is not stable in acidic range. After incubation at 50 °C for 5 hrs in acidic medium below pH 4.0 caused total inactivation. It shows the moderate stability at slightly basic range, but it can preserve only 37% of its activity at pH 8.5.

Table 3.2. Comparison of biochemical properties of fungal mannanases and recombinant strains

Species	pH_{opt}	$Temp_{opt}$ (°C)	Temp stability[a] (°C)	pH stability[b]	MW (kDa)	Reference
AsT1	4.5	60	40	3.5-8.5	60	in this study
PpT1	5.2-5.6	45	50	3.5-8.5	60	in this study
PpT2	6.5-8.0	60	40		40	in this study
A. fumigatus	4.5	60	55	4.5–8.5	60	Puchart et al., 2004
A. aculeatus	5.0	60-70	<70	2.5-10	45	Christgau et al., 1994
A. niger	3.5	-	50	3.5–7	40	Ademark et al., 1998
A. sulfureus	2.4	50	40	2.2–8.0	48	Chen et al., 2007
T. reesei	3.5-4.0	70	60	3.4-6.0	53	Stalbrand et al., 1993
T. harzianum	3.0	55	60	-	36.5	Ferreira et al., 2004
P. purpurogenum	5	70	65	4.5-8	57	Park et al., 1987

[a] The highest temperature that the enzymes remained stable for 2 h or longer.
[b] The pH range that the enzymes remained stable for 24 h or longer.
[c] One unit of enzyme is defined as 1 µmol of sugar released per min using either locust bean gum or galactomannan as substrate.

The observed differences in enzyme properties may be caused by a difference in protein modification patterns of the two host systems. *P. pastoris* is capable of adding both *O*- and *N*-linked carbohydrate moieties (composed of mannose residues) to secreted proteins (Goochee *et al.*, 1991), but different hosts may add *O*-linked sugars on different threonine and serine residues in the same protein. Consequently, *P. pastoris* might

glycosylate a heterologous protein even though the protein is not glycosylated by its native host (Cereghino and Cregg, 2000). Glycosylation can alter the pH optima, temperature optima, or thermostabilities of enzymes (Fatima and Husain, 2007; Görlach *et al.*, 1998). Difference in glycosylation and other protein modification patterns in PpT1 MAN may have also influenced the observed differences in temperature stabilities.

These characterized fungal mannanases represent a variety of modular structures. The cellulose-binding domain of the mannanase from *A. fumigatus* is near the N-terminus, that of *T. reesei* is near the C-terminus, and the mannanases from *A. aculeatus* and *A. sulfureus* do not contain a cellulose-binding domain. The biochemical properties of mannanases were compared in Table 3.2. Despite their differences in modular structure and sequence diversities, all these GH 5 mannanases have similar optimum temperature and temperature stability. The fungal enzymes function optimally at acidic pH. The presence of carbohydrate binding modules (CBM1) in the structure of mannanases from *A. bisporus*, *A. fumigatus* and *T. reesei* implies their potential to bind cellulose. Cellulose-binding activity had been demonstrated for the *A. bisporus*, *A. fumigatus*, and *T. reesei* mannanases (Puchart et al., 2004). Similarly, over 80% of the *P. chrysosporium* Man activity bound specifically to Avicel cellulose and the activity could be eluted with ethylene glycol (Benech et al., 2007). Binding to Avicel cellulose may therefore provide a convenient batch method to purify recombinant mannanase in the future (Benech et al., 2007). The presence of the carbohydrate binding module is thought to enhance enzyme activity towards cellulose-conjugated mannan. Removal of CBM1 from the *T. reesei* mannanase reduced its activity on mannan/cellulose complexes but had no effect on its activity on locus bean gum or mannopentaose (Hagglund et al., 2003). These results support the idea that the binding of CBM1 to the cellulose present in the mannan/cellulose complex facilitates the action of the mannanase by increasing substrate proximity and local enzyme concentration on the substrate surface. However, deletion of CBM1 from the *A. bisporus* enzyme significantly reduced the specific activity in the culture filtrates for locust bean gum (Tang et al., 2001). Thus, in addition to cellulose binding, CBM1 in fungal mannanases may also play a role in stabilizing the enzyme activity and/or promoting secretion (Benech et al., 2007).

As the mannanase of PpT2 show low pH stability, it makes less favorable enzyme in various industrial processes. For that reason, the enzyme was not analysed further.

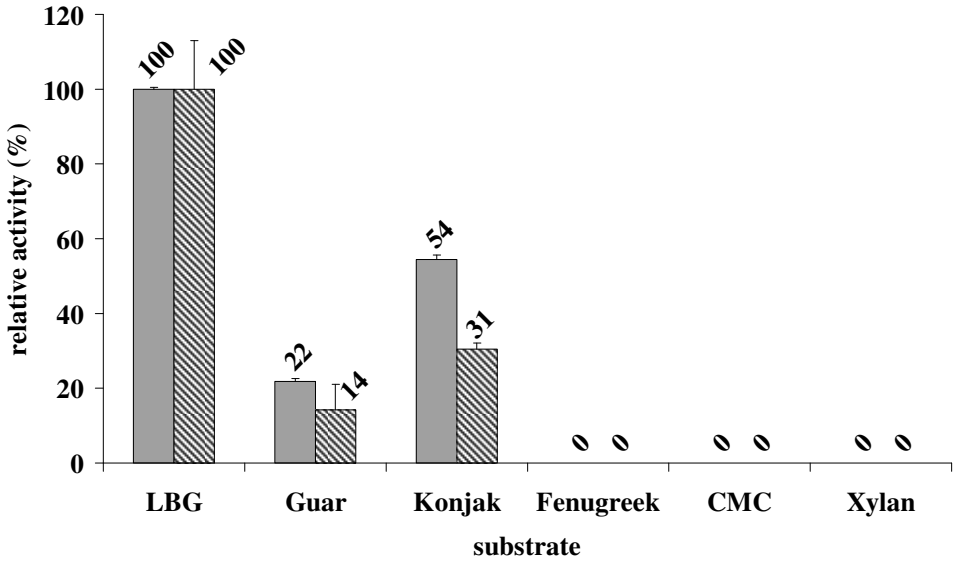


Figure 3.38 Enzyme substrate specificities of MAN from AsT1 (solid bar) and PpT1 (dashed bar). Purified MAN were added to 0.5% substrate at pH 5.0 and incubated at 50°C.

Substrate specificities of enzymes of AsT1 and PpT1 were compared in Figure 3.38. Accordingly, similar patterns but different catalytic degree were observed. The activities were determined and relative values were calculated by taking the degredation value of LBG as 100% in each enzyme. The highest activities were determined for LBG, in which a galactose subunit was bound at every 4-5 mannoses on the mannan backbone. As the galactosylation on the backbone increases (in guar gum; mannose/galactose ratio is 2:1 and in fenugreek gum; 1:1), the activity decreases and even no activity was observed in the highly galactosylated fenugreek galactomannan. In Konjac glucomannan, there are no galactose side chains attached, but glucose is present on the backbone (mannose:glucose (1.6:1)), in addition to acetyl groups, which may lower the degree of hydrolysis. Nevertheless, the activity of recombinant mannanases on Konjac gum was still higher than the activity toward guar gum. The hydrolysis of carboxymethylcellulose (CMC) and xylan (birchwood) by the recombinant MANs were analysed, but no activity was determined.

3.9 Transglycosylation

Transglycosylation activity is very common in fungal mannanases, however its determination requirs high molecular weight isotopes and NMR. Therefore, an alterntive method was developed for tracing transferase activity, called “paper test assay” (Steele et al., 2000).

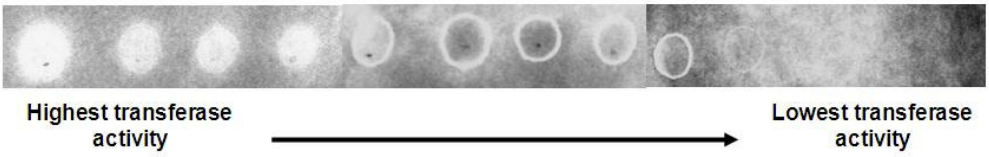


Figure 3.39 An example of the observation of scale produced by using xyloglucan endotransferase transglycosylation activity with the substrates xyloglucan and xylogluco-oligosaccharides on test paper.

Quantification is possible by using the fluoro spectrophotometer, but in this thesis, the activity was analysed only qualitatively. From the transglycosylation scale (Figure 3.39), it can be noticed that as the activity decreases, the dot turns into the shape of a ring and then disappears.

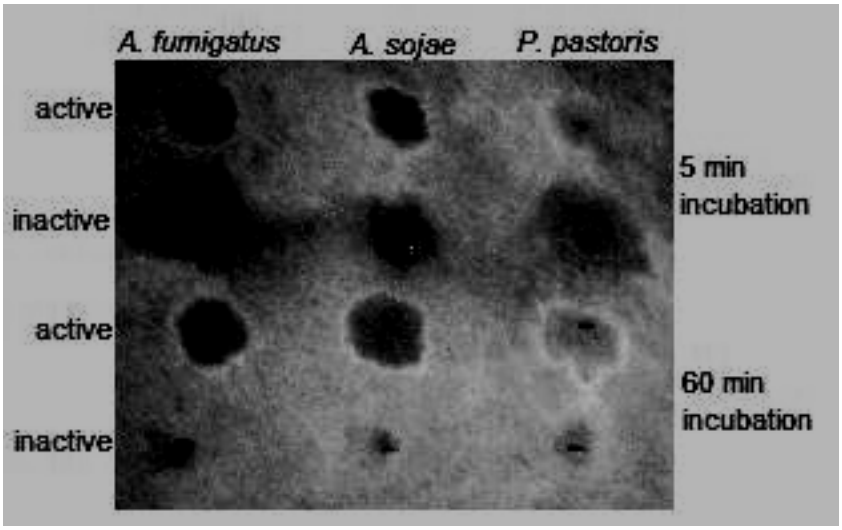


Figure 3.40 Paper test assay for *A. fumigatus*, *A. sojae* and *P. pastoris*.

The assay was applied both for active (~10 U enzyme for each dot) and heat inactivated (10 min, in boiling water) enzymes. The spots were incubated at 50 °C covered with acetate. Accordingly, the incubation time increases, the intensity of fluorescence increased. This was not observed in the heat inactivated samples. The incubation time could not be further extended due to drying.

3.10 Digestion Profile Determination by PACE and MALDI-TOF

3.10.1 Digestion of Locust Bean Gum by Recombinant Mannanases

Polysaccharide analysis using carbohydrate gel electrophoresis (PACE), also called as fluorophore assisted carbohydrate gel electrophoresis (FACE) was used to analyse the digestion pattern of mannanases, against different polymeric substrates. The samples were first digested and then labelled with a fluorophore, ANTS (8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt; Figure 3.41).

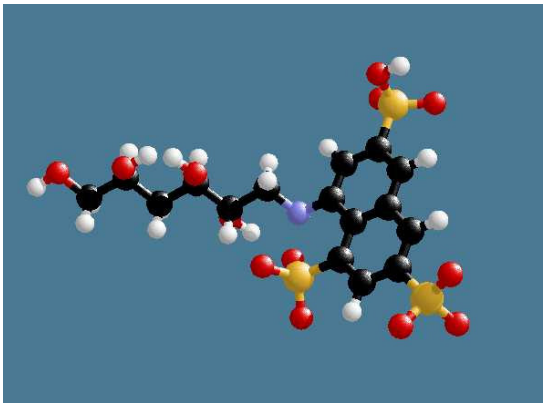


Figure 3.41. D-(+)-mannose labelled with ANTS

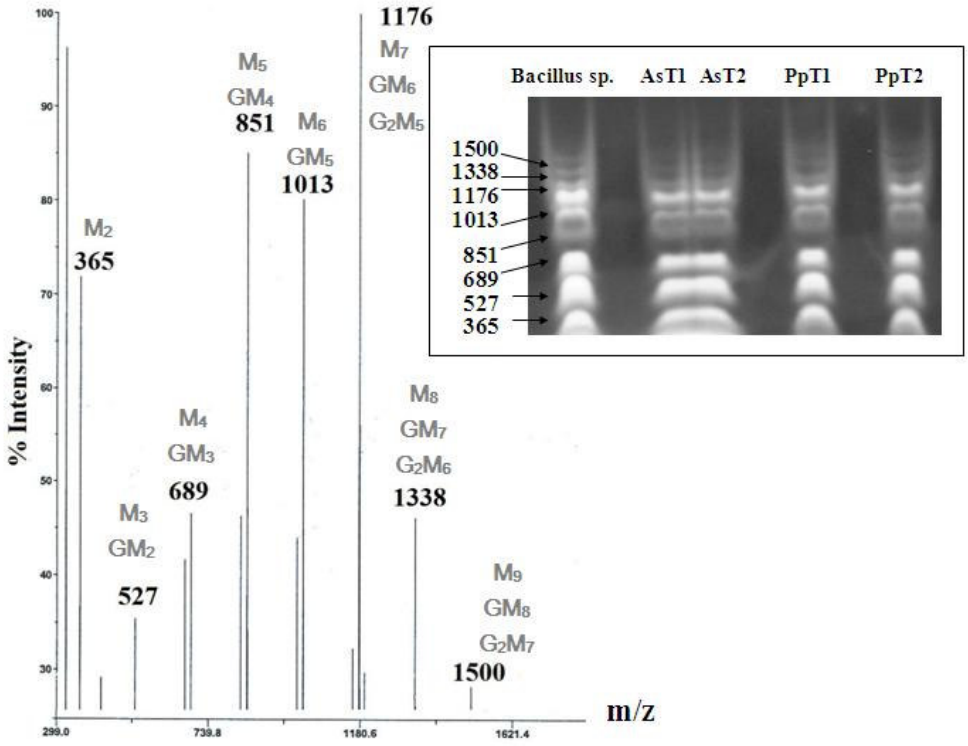


Figure 3.42 Characterization of the band sizes of LBG digestion product on gel by MALDI-TOF. The molecular weight of each band appeared as peaks on the MALDI-TOF data sheet. Above the defined peaks, the expected structure of digestion product was indicated.

The size of the bands on PACE gel was determined by MALDI-TOF analysis without the requirement of a standard. MALDI-TOF also allows rapid detection including acetylation of the polymer. The drawback of this method, however, is that it is not a quantitative method. If quantification is desired, HPLC analysis should be performed in parallel.

The digestion pattern of recombinant mannanases are shown in Figure 3.42 & Figure 3.43 and compared with the commercially available mannanase from Bacillus sp. (Megazyme, Ireland). The strain of Bacillus, whose mannanase was used as a control in reactions, was not mentioned by the producer. After hydrolysis of 0.5% LBG with *c.* 40 U of enzyme for 24 hrs, a major difference in the digestion pattern of bacterial and fungal mannanases was not observed. Similar digestion patterns were obtained for mannanases of AsT1, AsT2, PpT1, PpT2 and Bacillus species.

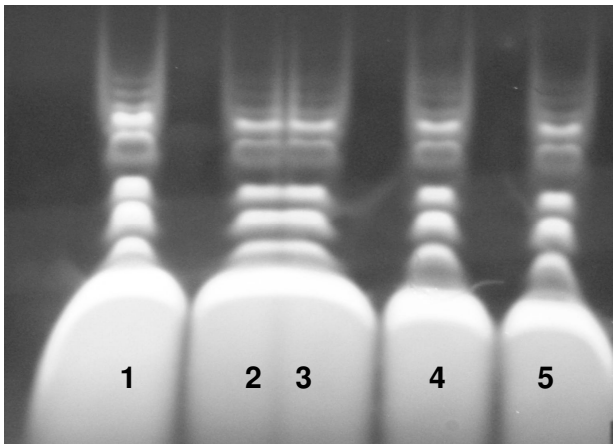


Figure 3.43 PACE of LBG digestion pattern (fingerprint) of mannanases from; Bacillus sp. (1), AsT1 (2), AsT2 (3), PpT1 (4) and PpT2 (5). Incubation time: 24 hrs.

After 48 hrs incubation, the digestion of LBG by mannanases from AsT1 and PpT1 displayed a difference such that tetramanno-oligosaccharide (M_4) disappeared in AsT1 mannanase reaction (Fig. 3.43). This could be explained by the transglycosylation of the products, which could diminish M_3 through hydrolysis and transglycosylation.

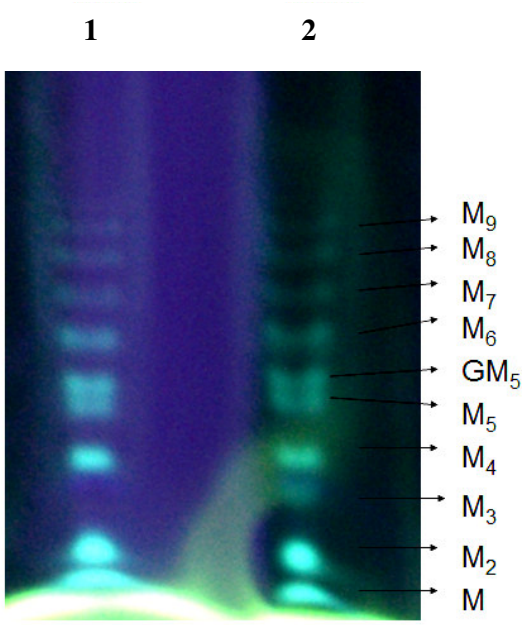


Figure 3.44 PACE of LBG digestion pattern of AsT1 (1) and PpT1 (2). Incubation time: 48 hrs.

Digestions of different substrates with mannanases are shown in Figure 3.45. In general LBG was digested efficiently by mannanases. In (A), (B) and (C), fenugreek galactomannan, which is highly galactosylated mannan, was not digested. However, in (D), the fenugreek was digested by MAN-S from PpT2. The small structure of the enzyme allows the protein to gain of the property of having access to the backbone of the fenugreek polymers. In (E), α-galactosidase was used in the hydrolysis besides MAN of AsT1. It is seen that α-galactosidase treatment has increased access of AsT1 MAN into the backbobe of the galactomannan polymer. Thereby, the formation of M_1, M_2, M_3, M_4, M_5 and M_6 bands were increased, as expected. The digestion of guar gum with the enzyme mixure of AsT1 MAN and α-galactosidase (E) was better than the digestion of guar gum with only of AsT1 MAN (A), because guar gum is composed of highly galactosylated mannan backbone. After elimination of the galactose branching units by α-galactosidase from the backbone, MAN easily attacked to mannan backbone and hydrolized the β-1,4-mannosidic linkage. The hydrolysis of fenugreek was remarkably increased with α-galactosidase.

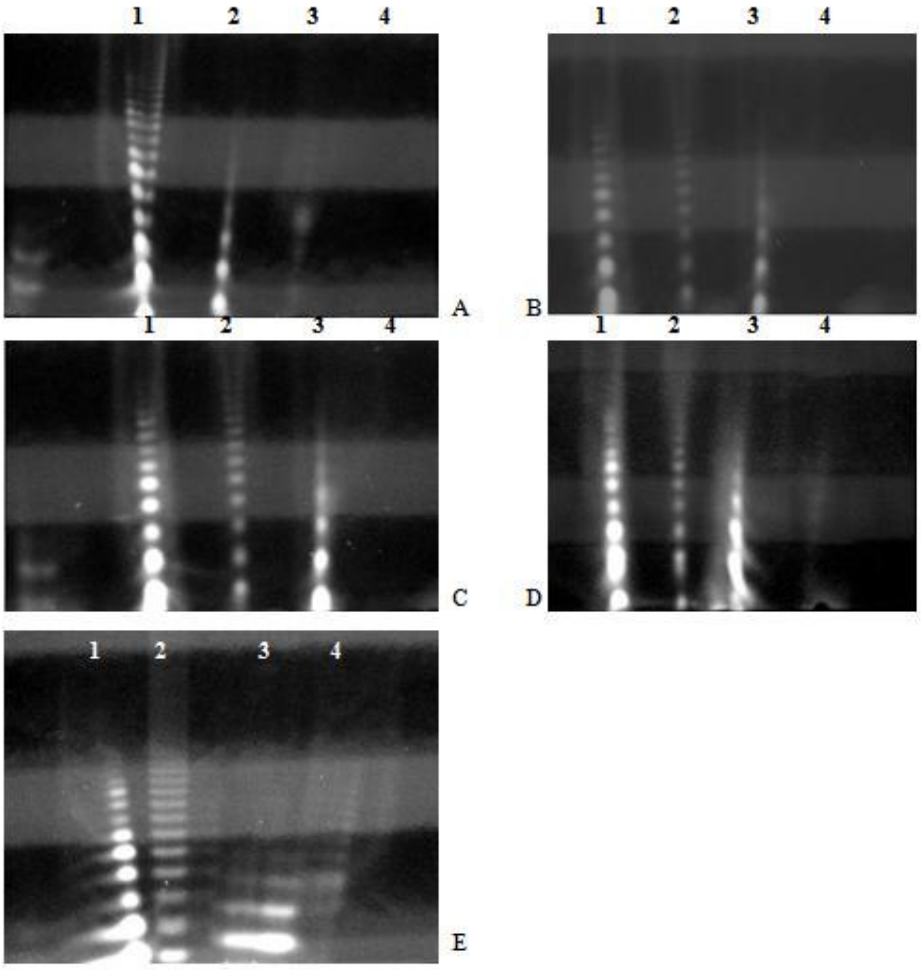


Figure 3.45 PACE of digestion of different substrates with mannanases. Four substrates were selected: (1): LBG (2): Guar Gum (3): Konjac Gum (4): Fenugreek Gum. Each gel represents digestion with a different enzyme; (A): AsT1, (B): AsT2, (C): PpT1, (D): PpT2, (E): [AsT1 + α-galactosidase].

3.11. Predicted 3D-Structures of Endo Mannanase of *Aspergillus fumigatus* and The Isolated Small Form

The 3D structure of both mannanases of AsT1 and PpT1 is predicted to be $(\beta/\alpha)_8$ TIM barrel. The 3D- structure of the mannnase were developed using the software "DeepView /Swiss-Pdbviewer" by the SWISS-MODEL; the catalytic domain (Figure 3.46) and the cellulose binding module, CBM (Figure 3.47).

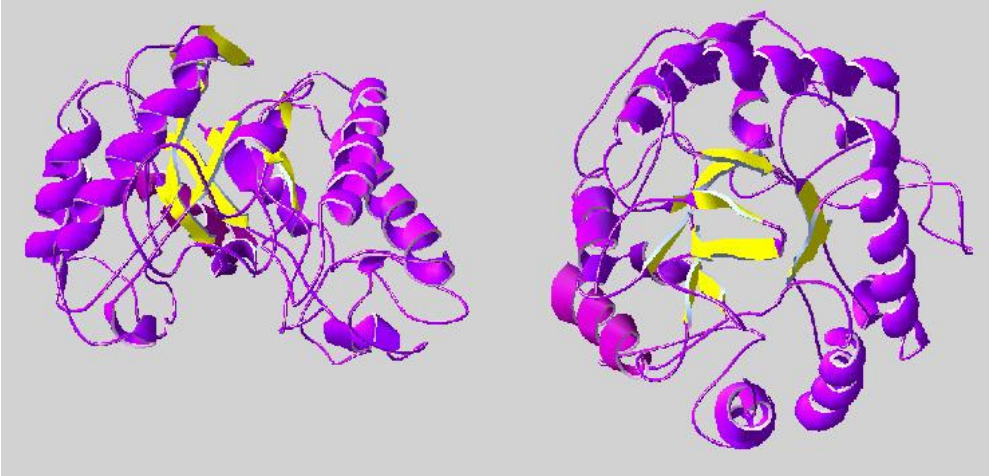


Figure 3.46 Predicted 3-D Structure of AfMAN1 without CBM. β-sheets are shown with yellow.

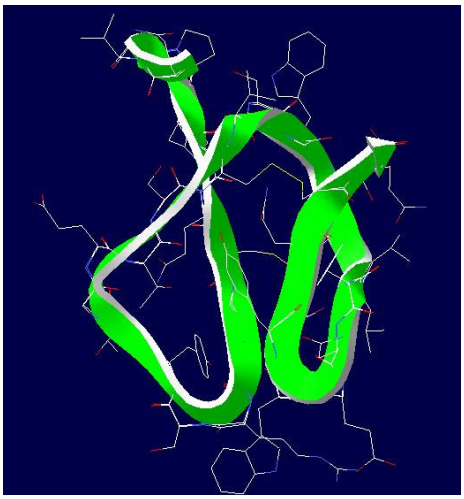


Figure 3.47 Predicted 3-D Structure of CBM of AfMAN1, located at the N-terminus of the enzyme.

3D crystal structure of *Trichoderma reesei* endo mannanase and predicted 3D structure of AfMAN1 show the classical $(\beta/\alpha)_8$-barrel architecture typical of the family 5 glycosyl hydrolases, which belong to the GH-A clan, display a $(\beta/\alpha)_8$-barrel motif. The

overall fold of the *T. reesei* mannanase is very similar to that of the *Thermomonospora fusca*. However, visual comparison of the fungal and the bacterial structures revealed several interesting differences. Both structures contain two short β -strands at the N-terminus which cover the bottom of the barrel, but the *T. reesei* endo mannanase contains two additional β –sheets that extend over the putative -3 substrate subsite and could be involved in the interaction with the substrate: this implies the existence of more sites in the fungal than in the bacterial enzyme (Sabini et al., 2000).

The active site of the *T. reesei* endo-mannanase is in a similar position compared to that of AFMAN1, namely, at one end of the $(\beta/\alpha)_8$-barrel. Sequence alignment reveals that only eight residues are strictly conserved in all family 5 mannanases and cellulases (Hilge *et al.*, 1998). In *T. reesei* endo mannanase, they are Arg54, His102, Asn168, Glu169, His241, Tyr243, Glu276 and Trp306.

Table 3.5 Common amino acids at the actives site of mannanases from *T. reesei, A. fumigatus* and *L. Esculentum*

T. reesei mannanase (Sabini et al., 2000)	AfMAN	*L. esculentum* mannanase (Bourgault *et al*. (2005))
Arg 54		
His 102		
Asn 168	Asn 168	Asn 203
Glu 169	Glu 171	Glu 204
His 241		
Tyr 243		
Glu 276	Glu 280	Glu 318
Trp 306	Trp 334	Trp 360

The significance of seven of these residues is clear, as they all lie in and around the active site. Glu169 and Glu276 have the roles of catalytic acid/base and nucleophile, respectively. Arg54 is hydrogen bonded to Asn168, which is in turn hydrogen bonded to Glu169. His241 is also hydrogen bonded to Glu169. Tyr243 is hydrogen bonded to

Glu276. Trp306 forms the hydrophobic sugar-binding platform in subsite -1. Only His102 lies on the opposite side of the molecule, with no obvious functional role in catalysis or substrate binding (Sabini et al., 2000). There is a non-prolyl *cis* peptide bond between residues Trp306 and Gln307. It is believed to be essential for the enzyme function, since it constrains the position of Trp306, which is involved in the interactions with the -1 subsite (Sabini et al., 2000).

Common amino acids are listed in Table 3.5. The listed amino acids for AfMAN1 were determined according to the position on the mannanase structure and the distance between the interacting units. The amino acids of *T. reesei* and *L. esculentum* mannanases were determined with respect to their functions defined by Sabinini *et al.* (2000) and by Bourgault *et al*. (2005).

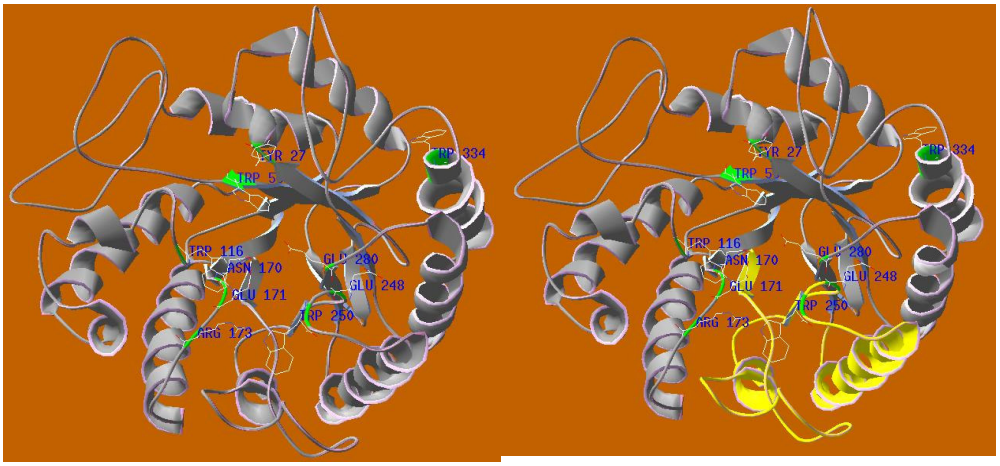


Figure 3.48 Predicted active site of AfMAN1. On the left the truncated part of the PpT2 mannanase were shown with yellow.

Although a large part of the protein, corresponding to about 25 % of its original size, was truncated, mannanase was still active. The deletion of two α-helices and one β-sheet did not affect the amino acids in the predicted active site, but the stability was significantly decreased.

CHAPTER 4

CONCLUSIONS

- Two possible mannanase genes, *afman1* and *afman2*, were identified on the *A. fumigatus* Af293 genome according to the similarity analysis of Blast program. The *afman1* gene has a total length of 1490 bp including 51 bp long signal peptide sequence, 108 bp long CBM and 3 introns in total length of 176 bp. The *afman2* gene was characterized as 951 bp long sequence with a 63 bp intron. The two mannanases have a similarity of 58% at the level of nucleotide sequence and 57% at the of level amino acid sequence.

- According to the mannanase expression analysis in *A. fumigatus* IMI 385708, LBG galactomannan was found to be the best inducer of mannanase genes. The induction effect of Avicel cellulose, CMC and xylan was observed beside the repression effect of glucose and mannose.

- The *afman1* gene was inserted into the fungal expression vector pAN52-4, downstream of the *gpd*A promotor, which is a constitutive promoter isolated from *A. nidulans*. Transformation into *A. sojae* ATCC 11906 yielded 7 stable transformants with *afman1* gene. The production of mannanase in *A. sojae* (AsT1) was achieved to *c.* 204 U/ml (about 7 fold: max. 2.83 U $ml^{-1}h^{-1}$) on the 3rd day, which corresponds to *c.* 10% of the extracellular proteins.

- Two cDNA of *afman1* gene were isolated and amplified with the primers N3 & C3. The sequence analysis of these fragments demonstrated a full processed cDNA of *afman*1 in length of 1266 bp (*man-l*) and a truncated form of the *afman*1 gene in length of 1032 bp (*man-s*). The comparison of two amino acid sequences showed that the deleted region corresponds to 78 amino acid long fragment from P_{291} to P_{368} of MAN-L. Both genes were cloned into pPICZαC vector under the control of AOX1 promoter, a methanol inducible promoter of *S. cerevisiae*. Transformation of vector constructs into *P. pastoris* GS115 yielded

two transformants, one with *afman1* cDNA (PpT1) and other with truncated cDNA of *afman1* (PpT2).

- In PpT1, *c.* 61 U/ml production level (*c.* 2 fold) was reached after 10 hrs of cultivation with a production rate of 6.1 U $ml^{-1}h^{-1}$ and at a level of 0.17 mg/ml cultivation medium, which was equivalent to *c.* 3.7% of the extracellular proteins.

- In PpT2, *c.* 56 U/ml production level (*c.* 2 fold) was reached after 12 hrs of cultivation with a production rate of 4.7 U $ml^{-1}h^{-1}$.

- The full cDNA of *afman1* was ligated into pBinAR vector and transformed into *A. thaliana*. The expression of mannanase gene in the early stages of plant development had a growth retarding effect, which makes the plant an unfavorable expression system for endo-mannanase. The expression of enzyme in plant caused increase in the galactose and xylose content of the plant cell wall.

- Recombinant enzymes were purified and molecular weight of the proteins were determined as *c.* 60 kDa for AsT1 and PpT1 and *c.* 40 kDa for PpT2.

- The characterization of AsT1 mannanase showed a pH and temperature optimum at pH 4.5 and 60°C. The enzyme was stable between pH 3.5-8.5 and up to 40°C.

- The PpT1 mannanase was characterized as having a pH and temperature optimum at pH 5.2 and 45 °C and pH and heat stablity between pH 3.5-8.5 and up to 50 °C. PpT2 mannanase has a pH and temperature optimum at pH 8 and 60 °C. The enzyme was stable between pH 4.5-9 and up to 40 °C. In general PpT2 was not a highly stable enzyme and highly affected by the pH of the medium.

- By the paper test assay, it was shown that the AsT1 and PpT1 mannanases have transglycosylation activity. Activity increase was observed with the time course of incubation.

- PACE method was used to visualize the digestion products, which involves labeling of the oligosaccharides with the fluorophore, ANTS, and separating on

polyacrylamide gel. The bands of hexamannose and smaller oligosaccharides on the gel were later characterized with MALDI-TOF.

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APPENDIX A

CHEMICALS, ENZYMES AND THEIR SUPPLIERS

3,5-dinitrosalicylic acid (DNS)	Sigma
Agar	Invitrogen
Agarose	Sigma
ANTS	Sigma
Chloroform	Merck
Disodium Phosphate	Merck
dNTP mix	MBI Fermentas
EDTA	Merck
EtBr	Sigma
Ethanol	Merck
Glucose Monohydrate	Merck
Isopropanol	Merck
K2HPO4	Merck
MgSO4.7H2O	Merck
NaCl	Merck
NaOH	Merck
Phenol	Applichem
Pfx DNA Polymerase	Invitrogen
Sodium Acetate	Merck
Sodium Citrate	Merck
Sodium Chloride	Merck
Sodium Diphosphate	Merck
Sorbitol	Merck
Sucrose	Merck
T4 DNA Ligase	Roche
Taq DNA Polymerase	Invitrogen
Tris	Merck
Triton X-100	Sigma

TRIzol	Invitrogen
Tryptone	Difco
Tween 20	Sigma
Tween 80	Sigma
Yeast Extract	Merck
YEB-medium	Difco

APPENDIX B

PREPARATIONS OF GROWTH MEDIA, BUFFERS AND SOLUTIONS

1. Agarose Gel 0.8% (w/v)

0.8 g agarose and 2 ml 50 X TBE are added and dissolved in 100 ml distilled water by microwave.

2. DNase free RNase

RNase A is dissolved in 0.01 M Sodium acetate (pH 5.2) to give a final concentration of 10 mg/ml. The solution is heated to 100ºC for 15 min in a boiling water bath for the inactivation of DNase. It is cooled slowly to room temperature. 0.1 volume of 1 M Tris-HCl (pH 7.4) is added until the pH of the solution is 7.0. The solution is dispensed into aliquots and stored at -20ºC.

3. EDTA (0.5M, pH 8.0)

186.1 g of ethylenedinitrilotetraacetic acid disodium salt dihydrate added to 800 ml of distilled water. It is stirred vigorously on a magnetic stirrer while the pH is adjusted to 8.0 with NaOH pellets. The solution is dispensed into aliquots and sterilized by autoclaving.

4. Ethanol (70%, 100 ml)

70 ml absolute ethanol mixed with 30 ml distilled sterile water.

5. Glucose (25% w/v, 10 ml)

2.5 g of glucose is dissolved in 8 ml H_2O. The volume is adjusted to 10 ml and sterilized by filtration.

6. LB Broth (per Liter)

10 g tryptone

5 g yeast extract

10 g NaCl

Final volume is adjusted to 1 liter with distilled water and pH is adjusted to 7.0 with NaOH and autoclaved. 150 µl / 100 ml ampicilin is added whenever it is used. The medium is stored at 4 °C.

7. LB Agar (per Liter with ampicilin)

10 g tryptone

5 g yeast extract

10 g NaCl

20 g agar

Final volume is adjusted to 1 liter with distilled water and pH is adjusted to 7.0 with NaOH and autoclaved. When it cools to 55 °C 150 µl / 100 ml ampicilin is added and poured to petri dishes. The plates stored at 4 °C.

8. NaAC (3 M, per Liter)

408.3 g of $NaAC.3H_2O$ is dissolved in 800 ml of H_2O. The pH is adjusted to the desired value with glacial acetic acid. The volume of the solution is then adjusted to 1 liter with distilled water, dispensed into aliquots and sterilized by autoclaving.

9. NaOH (10 N, 100 ml)

40g of NaOH pellets is added slowly to 80 ml of H_2O. When the pellets have dissolved completely, the volume is adjusted to 1 liter with H_2O. The solution is stored at room temperature. Sterilization is not necessary.

10. Saline Tween (ST)

0.8% NaCl

0.005% Tween-80 (1:100 dilution from 0.5% (v/v) Tween-80 stock.

11. YEB

5 g/l Gibco beef extract

1 g/l Bacto yeast extract

1 g/l Bacto peptone

5 g/l sucrose

pH 7.4

added 2 ml 1M $MgSO_4$ after autoclave

12. Infiltration medium (IM)

1/2 MS salts

1x B5 vitamins

5% sucrose

0.044 uM Benzylaminopurine (10ul/l of a 1mg/ml stock)

pH 5.7 (KOH)

13. Germination medium (GM)

1x MS medium (salts and vitamins)

no sucrose

8 g/l Agar

pH 5.7 (KOH)

after autoclaving cool to 45 ^{o}C, add antibiotics

14. YpSs Broth Media

Yeast extract 4.0 g/L

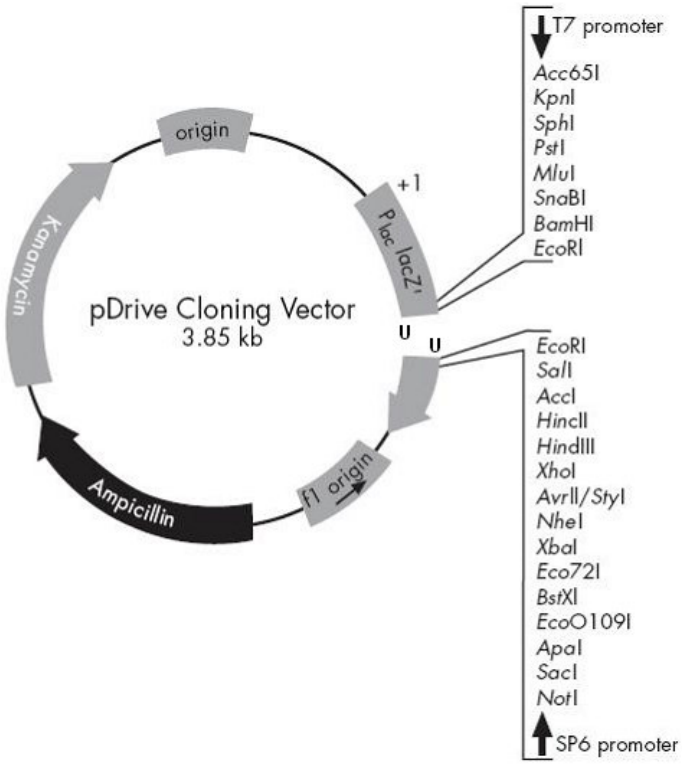
K_2HPO_4 1.0 g/L

$MgSO_4.7\ H_2O$. 5.0 g/L

Glucose monohydrate 44.0 g/L

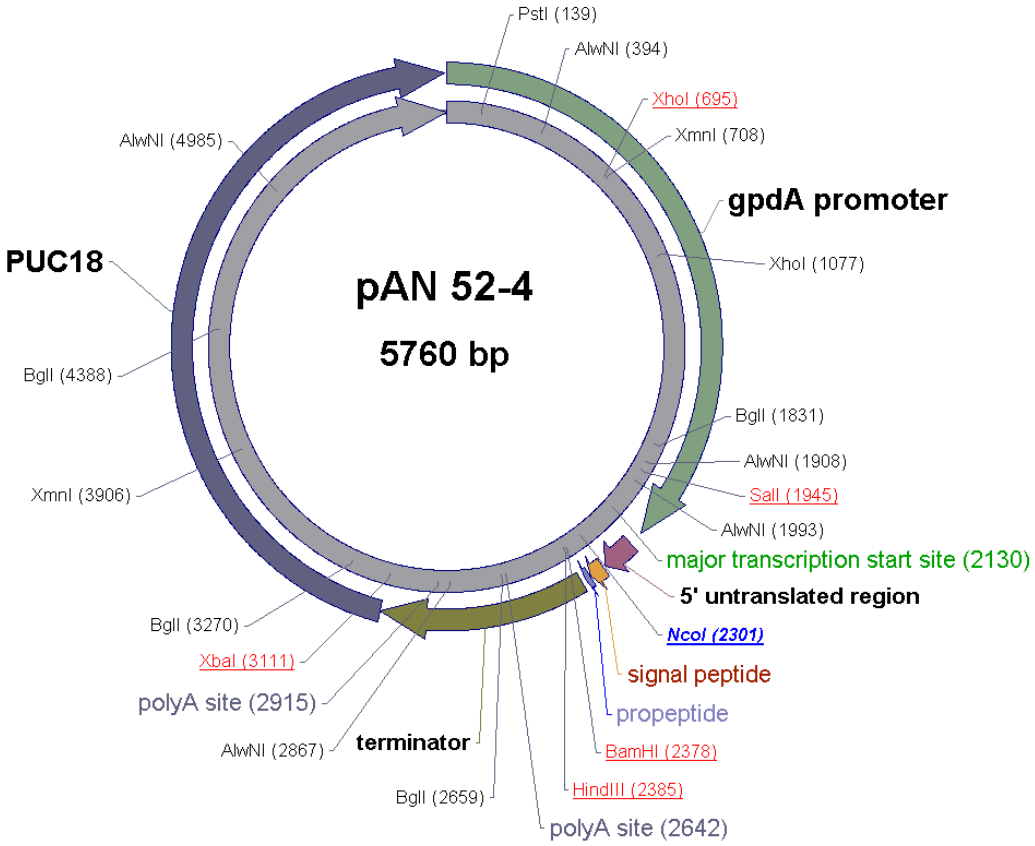
APPENDIX C

VECTOR MAPS

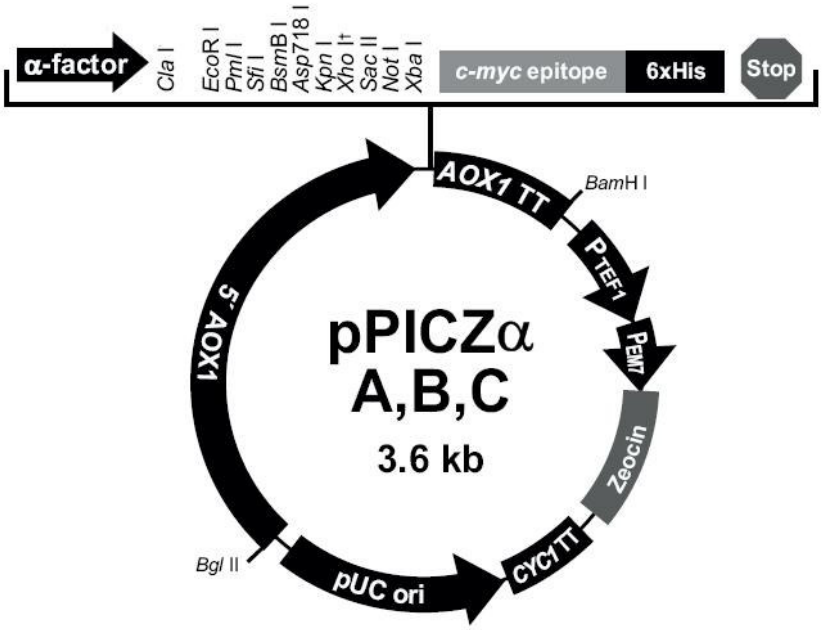


C.1. Map of sub-cloning vector, pDrive (Qiagen)

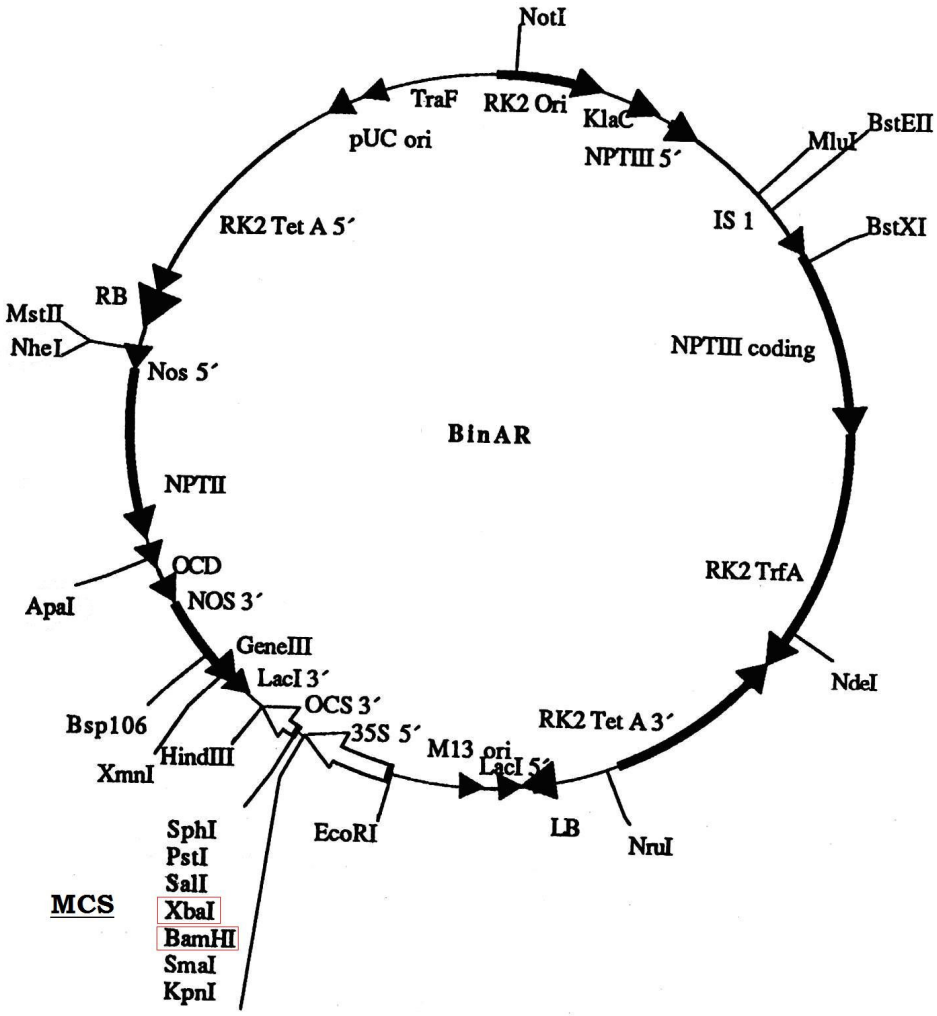
C.2. Map of sub-cloning vector, pBlunt II TOPO (Invitrogen)



C.3. Map of *Aspergillus sojae* Expression vector, pAN52-4.



C.4. Map of *Pichia pastoris* Expression vector, pPICZαC.



C.5. Map of *Arabidopsis thaliana* Expression vector, pBinAR.

APPENDIX D

ALIGNMENT OF MANNANASES

of

A. fumigatus, A. clavatus & N. fischeri

```
Afman1          ATGCACCCATTGCCGTCTGTCGCCCTCCTATCCGCGATAGGAGCTGTCGCGGCGCAGGTT 60
Afman1+int      ATGCACCCATTGCCGTCTGTCGCCCTCCTATCCGCGATAGGAGCTGTCGCGGCGCAGGTT 60
Nfman1          ATGCGCCCATTGTCGTCTGCCGCTCTCCTATCCGCGATAGGAGCTGTCGCGGCGCAGGTT 60
Acman1          ATGCGTTCCTTGTCGTCTGTTGCTCTCCTGTCTGCGATAGGAGCTGCCTCCGCCCAGGCT 60
                ****   * *** ******  ** ***** ** ************ * * ** **** *

Afman1          GGTCCTTGGGGCCAGTGTGGTGGTCGCTCGTATACGGGTGAAACTTCCTGTGTATCTGGC 120
Afman1+int      GGTCCTTGGGGCCAGTGTGGTGGTCGCTCGTATACGGGTGAAACTTCCTGTGTATCTGGC 120
Nfman1          GGTCCTTGGGGCCAGTGTGGTGGGCAGTCATATACGGGTGGAACCTCCTGTGTATCTGGA 120
Acman1          GGCCCTTGGGGACAATGCGCAGGCATCTCCCATACTGGACCTACCACTTGCGAGTCGGGA 120
                ** ******** ** ** *  **    **  **** **    **  * ** *  ** **

Afman1          TGGTCGTGCGTGTTATTCAATGAATGGTACAGCCAGTGCCAGCCTG-------------- 166
Afman1+int      TGGTCGTGCGTGTTATTCAATGAATGGTACAGCCAGTGCCAGCCTGGTGAGCA-ATACAC 179
Nfman1          TGGGCATGCGTGTTTTTGAATGATTGGTATAGCCAGTGCCAGCCTGGTGCGGA-ATATAC 179
Acman1          TGGTCTTGCGTCTATCTGAATGACTGGTACAGCCAGTGCCAACCTGGTAAGAGCATGCAC 180
                *** * ***** *   * ***** ***** ********** ****

Afman1          ---------------------------------------------CTACCACGACGTCGAC 182
Afman1+int      TGCTTCTCTGTGCGGCCGAGATGATAGTCCTAACATATT-CAAGCTACCACGACGTCGAC 238
Nfman1          TCCCCCTCTGTGCGGTAGAAATGATAATCCTAACATATTTCAAGCTACCACGACGTCAAC 239
Acman1          TGGGTCCCTTGCCCATTTA----------CCAGGAGCTGACACCCCATAGGAGCTGCAAC 230
                                                        * *      *  * **

Afman1          ATCATCAGTCTCCGCGACTGCTGCTCCTAGTAGCACGTCTTCCTCGAAGGAATCTGTGCC 242
Afman1+int      ATCATCAGTCTCCGCGACTGCTGCTCCTAGTAGCACGTCTTCCTCGAAGGAATCTGTGCC 298
Nfman1          AT---CAGTCTCAGCGACTGCACCTCCTAGTAGCACGTCTTCCTCGACAGCATCCGTGTC 296
Acman1          ATCTTCA---TCGACCACCGTCTCTTCGACCAAGCAGCCTTCGTCCACGGTTGCTGCTCC 287
                **   **   **  * ** *   ** * *  *    * **** ** *  *   * *   *

Afman1          ATCCGCCACTACATCAAAGAAGCCTGTCCCAACTGGCAGCAGCTCCTTTGTTAAGGCAGA 302
Afman1+int      ATCCGCCACTACATCAAAGAAGCCTGTCCCAACTGGCAGCAGCTCCTTTGTTAAGGCAGA 358
Nfman1          ATCCTCCACTTCATC---GACACCTATCCCAACTAGCAGTGGCTCTTTTGTCAAGGCAGA 353
Acman1          GTCTTCGACTACATCCGCTCATACGCTGCCCACTGGTAGCGGCTCTTTTGCCAAGACGGA 347
                 **  * *** ****        *  * ** *** * **  **** ****  *** * **

Afman1          TGGGCTCAAATTCAACATTGACGGCGAGACCAAATACTTTGCCGGCACGAATGCCTACTG 362
Afman1+int      TGGGCTCAAATTCAACATTGACGGCGAGACCAAATACTTTGCCGGCACGAATGCCTACTG 418
Nfman1          AGGGCTCAAATTTAACATTGACGGCGAGACAAAGTACTTTGCCGGCACGAATGCCTACTG 413
Acman1          TGGACTCAAGTTCAACATCGACGGCAAGACTAAATACTTTGCTGGCACCAACGCCTACTG 407
                 ** ***** ** ***** ****** **** ** ******** ***** ** ********

Afman1          GTTGCCGTTCCTCACCAACGATGCAGATGTTGACTCTGTCATGGATAACCTGCAGAAAGC 422
Afman1+int      GTTGCCGTTCCTCACCAACGATGCAGATGTTGACTCTGTCATGGATAACCTGCAGAAAGC 478
Nfman1          GCTGCCGTTCCTCACCAACAATGCAGATGTTGACTCTGTTTTTGATCACCTGCAGCAGAC 473
Acman1          GCTGCCGTTCCTGACCAACAATGCCGATGTTGACGCTGTCTTCGACCATCTGCAGCAGAC 467
                * ********** ****** **** ********* ****  * **  * ****** *  *

Afman1          CGGCTTGAAGATCCTGCGAACTTGGGGTTTTAATGATGTGAATTCCAAACCGAGCTCTGG 482
Afman1+int      CGGCTTGAAGATCCTGCGAACTTGGGGTTTTAATGATGTGAATTCCAAACCGAGCTCTGG 538
Nfman1          AGGCTTAAAGATCCTGCGGACCTGGGGTTTTAATGATGTGAATTCCGTTCCGAACCCCGG 533
Acman1          CGGCTTGAAGATCCTCCGCACTTGGGGTTTCAATGATGTGAACACCATCCCCGGCTCTGG 527
                 ***** ******** ** ** ******** ***********  **   **   * * **
```

```
Afman1          CACCGTCTATTTCCAGCTTCATGATCCATCAACTGGCACCACGACCATCAACACTGGCGC 542
Afman1+int      CACCGTCTATTTCCAGCTTCATGATCCATCAACTGGCACCACGACCATCAACACTGGCGC 598
Nfman1          AACCGTATATTTCCAGCTTCATGACCCATCAACCAGCACCACGACCATCAACACTGGCGC 593
Acman1          AACCGTCTACTTCCAGCTCCACGACAAGGCAACCGGTACCAGCACCATCAACACCGGAGC 587
                 ***** ** ******** ** **     ****  * ****  *********** ** **

Afman1          AGACGGTCTCCAACGGCTTGACTACGTGGTATCTGCGGCGGAGAAGCGCGGAATCAAGTT 602
Afman1+int      AGACGGTCTCCAACGGCTTGACTACGTGGTATCTGCGGCGGAGAAGCGCGGAATCAAGTT 658
Nfman1          AGACGGTCTCCAAAGGCTCGACTACGTGGTATCTGCGGCGGAGAAGCACGGAATAAAGTT 653
Acman1          CAACGGTCTGCAGCGCCTGGACTACGTTATCTCCGCTGCTGAAAAGCACGGCATCAAGCT 647
                  ******* **  * ** ********  * ** ** ** ** **** *** ** *** *

Afman1          ACTTATTCCATTGGTCAATAACTGGGACGACTACGGCGGCATGAATGCGTACGTCAAGGC 662
Afman1+int      ACTTATTCCATTGGTCAATAACTGGGACGACTACGGCGGCATGAATGCGTACGTCAAGGC 718
Nfman1          ACTTATCCCATTAGTCAATAATTGGGACGACTACGGCGGCATGAATGCGTACATCAAAGC 713
Acman1          GATCATTCCTTTTGTCAACAACTGGGATGACTATGGTGGCATGAACGCCTACATCAATGC 707
                  * ** ** ** ***** ** ***** ***** ** ******** ** *** **** **

Afman1          GTATGGCGGCAGCAAGACAGAATGGTATACCAACTCCAAGATCCAGAGCGTGTATCAGGC 722
Afman1+int      GTATGGCGGCAGCAAGACAGAATGGTATACCAACTCCAAGATCCAGAGCGTGTATCAGGC 778
Nfman1          GTATGGCGGCAGCAAGACAGAATGGTACACCAACTCCAAGATCCAGAGCGTGTATCAGGC 773
Acman1          CTACGGTGGCAGCAAGACCGAATGGTACACCAACGAGAAGATCCAGAGCGTCTATCAGGC 767
                 ** ** *********** ******** ******   ************** ********

Afman1          ATATATTAAGGCGGTAGTTTCGCGCTACCGGGACTCTCCTGCTATCATGGCTTGGGAGTT 782
Afman1+int      ATATATTAAGGCGGTAGTTTCGCGCTACCGGGACTCTCCTGCTATCATGGCTTGGGAGTT 838
Nfman1          ATATATAAAGGCGGTAGTTTCCCGCTACCGGGACTCTCCTGCTATTATGGCTTGGGAGTT 833
Acman1          GTACATCAAGGCGATTGTCTCTCGCTACAGGGACTCTCCTGCCATCTTTGCGTGGGAATT 827
                 ** ** ****** * ** ** ****** ************* **  * ** ***** **

Afman1          GTCAAATGAAGCTCGTTGCCAAGGGTGCAGTACTGATGTTATTTACAACTGGACCGCCAA 842
Afman1+int      GTCAAATGAAGCTCGTTGCCAAGGGTGCAGTACTGATGTTATTTACAACTGGACCGCCAA 898
Nfman1          ATCAAATGAAGCTCGTTGCCAAGGGTGCAGTACTGATGTTATTTACAACTGGGCCACCAA 893
Acman1          GGGCAATGAGCCCCGCTGCAAGGGCTGCAGCACCGATGTCATCTACAACTGGGTGGCTAA 887
                    *****  * ** *** * ** ***** ** ***** ** *********    * **

Afman1          AACCAGCGCATACATCAAGTCTCTTGATCCAAATCACATGGTTGCCACAGGCGATG---- 898
Afman1+int      AACCAGCGCATACATCAAGTCTCTTGATCCAAATCACATGGTTGCCACAGGCGATGGTAC 958
Nfman1          AACCAGTGCATACATCAAGTCTCTTGATCCAAATCACATGGTTGCCACAGGTGAAGGTAC 953
Acman1          GACCAGTGCTTACATCAAGTCTCTGGATCCCAACCACATGGTGACAACGGGTGAAGGTTT 947
                 ***** ** ************** ***** ** ********  * ** ** ** * *
Afman1          ------------------------------------------------------------AGG 901
Afman1+int      GCTAAGCACTCCCCTCTCACATACCTGATGTCACGCAGGTGACTAACTCCAGTATAGAGG 1018
Nfman1          GCTA-GTACTCCCCTCTCACGTACCTGATGCCACGCAGGCGACTAACTCCGGTATAGAGG 1012
Acman1          GTTT----TCCTAAAACCACAATTGTTGTGTGTCATGATTTCTAACACACCGTGCAGAGG 1003
                                                                        ***

Afman1          GCATGGGGGTGACCGTCGACTCGGATGGTTCCTACCCCTACTCCACCTACGAGGGTAGCG 961
Afman1+int      GCATGGGGGTGACCGTCGACTCGGATGGTTCCTACCCCTACTCCACCTACGAGGGTAGCG 1078
Nfman1          GCATGGGGTTGACCGTCGACTCGGATGGTTCCTACCCCTACTCCACCTACGAGGGTAGCG 1072
Acman1          GCATGGGTCTGACTGTCGACTCCGATGGCTCCTATCCCTACTCCAAGGACGAGGGCAGCG 1063
                *******  **** ******** ***** ***** **********   ******* ****

Afman1          ACTTTGCCAAGAACCTTGCCGCTCCTGACATCGATTTCGGAGTATTCCACCTGTATACCG 1021
Afman1+int      ACTTTGCCAAGAACCTTGCCGCTCCTGACATCGATTTCGGAGTATTCCACCTGTATACCG 1138
Nfman1          ACTTTGAAAAGAACCTTGCCATTCCTCACATAGATTTTGGAGTATTCCACCTGTATACCG 1132
Acman1          ACTTCGCCAGGAACCTTGCCGCCCCCGATATCGACTTTGGAGTCTACCACCTCTACGTTG 1123
                **** *  * **********   **  * ** ** ** ***** * ****** **    *

Afman1          AGGAC---------------------------------------------------------- 1026
Afman1+int      AGGACTGTGAGTATTGTTCCACCCTCGTCGTCACTGGACT----GTCAATTGACGCATCG 1194
Nfman1          CTGATTGTGAGTACTGTTCCACTCTCGTCTTCACTGTACTCTGTGTCAATTGACGCATTG 1192
Acman1          CCGACTGTAAGTATAGT-----CCTCGTCTATCGAGAAGTTACTCCGAACTAACCCACTG 1178
                  **
Afman1          --------GGCATCAAAGACAACAGCTGGGGCAACGGCTGGGTGACATCCCACGCTAAGG 1078
Afman1+int      TACCAGGGGGCATCAAAGACAACAGCTGGGGCAACGGCTGGGTGACATCCCACGCTAAGG 1254
Nfman1          TAATAGGGGGCATCACAGACAACAGCTGGGGCAACAGATGGGTGACATCCCACGCAAAGC 1252
Acman1          TA---GGGGGAGTTTCGGACAACGCCTGGGGCAACCGCTGGATCAAGTCCCACGCCAAGG 1235
                        **  *    ******  ********** * *** * *  ******** ***

Afman1          TTTGTAAGGCTGCCGGAAAGCCATGCCTGTTCGAGGAGTATGGCCTTAAGGATGACCATT 1138
Afman1+int      TTTGTAAGGCTGCCGGAAAGCCATGCCTGTTCGAGGAGTATGGCCTTAAGGATGACCATT 1314
Nfman1          TTTGTGAGGCTGCCGGAAAGCCATGCCTGTTCGAGGAATATGGCCTTAAAGATGACCATT 1312
Acman1          TCTGCGAGGCTGCTGGAAAGCCTTGCCTGTTCGAGGAATACGGTATCAAGGATGACCACT 1295
                * **  ******* ******** ************** ** **  * ** ******** *
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Afman1          GCTCGGCCTCGCTCACCTGGCAAAAGACCTCTGTGTCTTCTG---GAATGGCTGCCGATT 1195
Afman1+int      GCTCGGCCTCGCTCACCTGGCAAAAGACCTCTGTGTCTTCTG---GAATGGCTGCCGATT 1371
Nfman1          GCTCGGCCGCGGTCGTCTGGCAAAAGACCTCTTTGACTACTGCCGGAATGGCTGCCGACT 1372
Acman1          GCGGAGACTCGCTCAAGTGGCAGAAGACCTCTCTGACTACCACCGCCAACTCTGCGGATC 1355
                **   * * ** **   ***** ********* ** ** *       *   **** **

Afman1          TGTTCTGGCAGTACGGACAGACGCTGTCGACCGGCCCATCACCCAATGATCACTTCACCA 1255
Afman1+int      TGTTCTGGCAGTACGGACAGACGCTGTCGACCGGCCCATCACCCAATGATCACTTCACCA 1431
Nfman1          TGTTCTGGCAGTACGGGCAGACACTGTCGACCGGTCAATCACCCAATGATCGCTACACCA 1432
Acman1          TGTTCTGGCAGTATGGCCAGCAGCTGTCCACCGGTGCCTCGCCAAATGATCACTACACCA 1415
                ************ ** ***   ***** *****    ** ** ******* ** *****

Afman1          TTTACTACGGCACCAGTGACTGGCAATGTGGTGTAGCTGATCATCTCAGCACACTTTAG 1314
Afman1+int      TTTACTACGGCACCAGTGACTGGCAATGTGGTGTAGCTGATCATCTCAGCACACTTTAG 1490
Nfman1          TTTACTACGGCACCAGTGACTGGCAATGTGCTGTAATTGATCATGTCAGCCGAATCTAG 1491
Acman1          TCTACTACGGCACTGATGACTGGAAGTGCGCTGTCATCGACCACATCAGCCAGATCTAA 1474
                * ***********   ******* * ** * ***    ** **  *****    * **
```
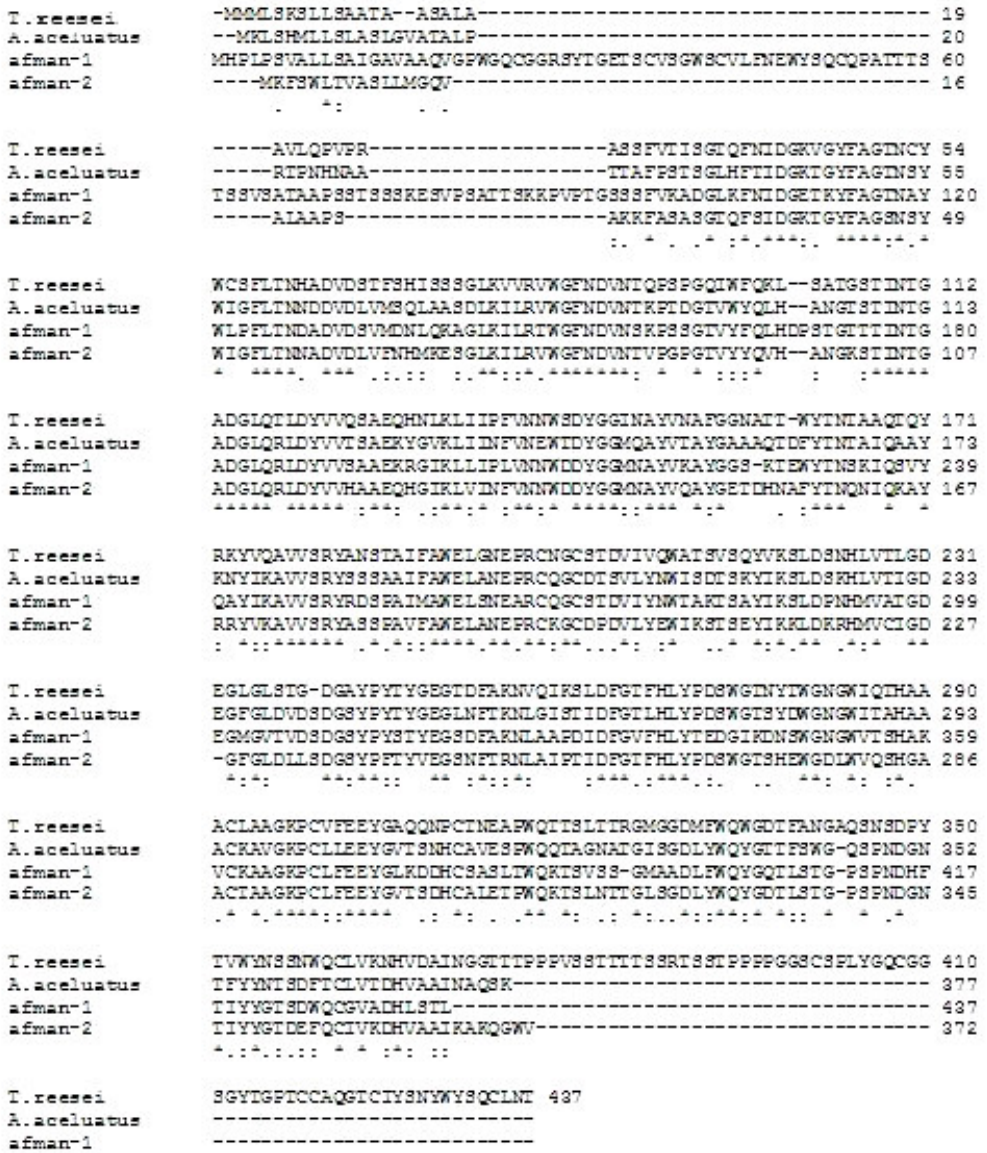

APPENDIX E

ALIGNMENT OF MANNANASES

of

A. fumigatus, A. aceluatus & T. reesei



APPENDIX F

ALIGNMENT OF
FULLY PROCESSED (AfMAN1)
AND
TRUNCATED AfMAN1 (MAN-S) CDNA

```
mans            ATCATGGCTTGGGAGTTGTCAAATGAAGCTCGTTGCCAAGGGTGCAGTACTGATGTTATT 780
manl            ATCATGGCTTGGGAGTTGTCAAATGAAGCTCGTTGCCAAGGGTGCAGTACTGATGTTATT 780
                ************************************************************

mans            TACAACTGGACCGCCAAAACCAGCGCATACATCAAGTCTCTTGAT---------------- 825
manl            TACAACTGGACCGCCAAAACCAGCGCATACATCAAGTCTCTTGATCCAAATCACATGGTT 840
                ******************************************

mans            ------------------------------------------------------------
manl            GCCACAGGCGATGAGGGCATGGGGGTGACCGTCGACTCGGATGGTTCCTACCCCTACTCC 900
                                  Intron 2
mans            ------------------------------------------------------------
manl            ACCTACGAGGGTAGCGACTTTGCCAAGAACCTTGCCGCTCCTGACATCGATTTCGGAGTA 960

mans            ------------------------------------------------------------
manl            TTCCACCTGTATACCGAGGACTGGGCATCAAAGACAACAGCTGGGGCAACGGCTGGGTGA 1020
                                        Intron 3
mans            ----------------------------------------TGCCTGTTCGAGGAGTATGGCC 847
manl            CATCCCACGCTAAGGTTTGTAAGGCTGCCGGAAAGCCATGCCTGTTCGAGGAGTATGGCC 1080
                                                       **********************

mans            TTAAGGATGACCATTGCTCGGCCTCGCTCACCTGGCAAAAGACCTCTGTGTCTTCTGGAA 907
manl            TTAAGGATGACCATTGCTCGGCCTCGCTCACCTGGCAAAAGACCTCTGTGTCTTCTGGAA 1140
                ************************************************************
```

F1. Alignment of *man-s* and *man-l*. The fragment missing in *mans* is highlighted. Intron splicing sites were indicated with arrows.

CURRICULUM VITAE

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2002 - Present	METU Dept. of Biotechnology	Research Assistant
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FOREIGN LANGUAGES

Advanced English, Advanced German

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Swimming, Dance