OPTIMIZATION OF MANNANASE PRODUCTION FROM RECOMBINANT *ASPERGILLUS SOJAE* AND ANALYSIS OF GALACTOMANNAN HYDROLYSIS

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OPTIMIZATION OF MANNANASE PRODUCTION FROM RECOMBINANT *ASPERGILLUS SOJAE* AND ANALYSIS OF GALACTOMANNAN HYDROLYSIS

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

OPTIMIZATION OF MANNANASE PRODUCTION FROM RECOMBINANT *ASPERGILLUS SOJAE* AND ANALYSIS OF GALACTOMANNAN HYDROLYSIS

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Aspergillus fumigatus produces enzymes required for the hydrolysis of galactomannans like locust bean gum. Among these enzymes endo-beta-1,4 mannanase is also produced at high levels. However, the fungus is not safe for use in the food industry. Therefore, the gene encoding endo-beta-1,4-mannanase of *A. fumigatus* IMI 385708 was previously cloned in our laboratory into *Aspergillus sojae* ATCC11906 which is a safe microorganism for use in food applications. Altogether eight transformants were obtained. It was shown that some of these transformants overproduce the enzyme because of expression under the control of glyceraldehyde-3-phosphate dehydrogenase promoter and fusion to the glucoamylase signal and pro-peptide coding region of *Aspergillus niger*. In this study, mannanase production of these transformants was compared with *A. fumigatus* and *A. sojae* transformant AsT1 showed *c.* 12 fold increase with the maximum activity of 352 U/ml. The effects of initial medium pH and

number of spores on activity were investigated and maximum activity was achieved at pH 7.0 and the number of spores was found as 3.6×10^6. Optimization of the growth conditions for maximum mannanase production in shake flasks by using the best mannanase producing transformant AsT1 was carried out by using Box-Behnken design under Response Surface Methodology. The highest beta-mannanase activity on the fourth day of cultivation at 30 ºC was obtained as 363 U/ml in the optimized medium containing 7% sugar beet molasses, 0.43% NH_4NO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4$ as the weight/volume percentage at 207 rpm. On sixth day of cultivation under the optimized conditions, the highest mannanase activity was achieved as 482 U/ml which is 1.4 fold of 352 U/ml activity found on glucose medium previously. After 48 h of LBG hydrolysis by 40 U of mannanase, mannotriose, 6^1-galactosyl-beta-D-mannotriose and 6^3,6^4-di-alpha-galactosyl-beta-1,4-mannopentaose were found as the main products via HPLC analysis.

Keywords: Recombinant *Aspergillus sojae*, glyceraldehyde-3-phosphate dehydrogenase promoter, endo-beta-mannanase, response surface methodology, locust bean gum, mannooligosaccharides.

ÖZ

REKOMBİNANT *ASPERGİLLUS SOJAE* DE MANNANAZ ÜRETİMİNİN OPTİMİZASYONU VE GALAKTOMANNAN HİDROLİZ ANALİZİ

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Nisan 2008, 105 sayfa

Aspergillus fumigatus, keçiboynuzunda da bulunan galaktomannan polimerlerini hidroliz etmek için gerekli olan enzimleri üretmektedir. Bu enzimlerin arasından endo-beta-1,4 mannanaz enzimi yüksek miktarda üretilmektedir. Ancak bu mantar güvenli olmaması nedeni ile gıda endüstrisinde kullanılmamaktadır. Bu nedenle *A. fumigatus* IMI385708'un endo-beta-1,4-mannanaz enzimini kodlayan geni, gıda uygulamalarında kullanımı güvenli olan *Aspergillus sojae* ATCC11906 mantarına (mikroorganizmasına) önceden laboratuvarımızda klonlanmıştır. Sonuç olarak sekiz transformant elde edilmiştir. Bu transformantların bazılarının, gliseraldehit-3-fosfat dehidrogenaz promotör kontrolü altında ifade edilmesinden ve genin *Aspergillus niger*' ın glikoamilaz sinyal ve pro-peptit bölgesine füzyonundan dolayı, enzimi daha fazla ürettikleri görülmüştür. Bu çalışmada, transformantların mannanaz üretimi *A. fumigatus* ile karşılaştırılmış ve *A. sojae* transformantı AsT1, 352 U/ml'lik maksimum aktivite ile 12 katlık bir artış göstermiştir. Baslangıç besiyeri pH'sının ve ekilen spor

sayısının aktivite üzerine etkisi araştırıldığında, maksimum aktivite başlangıç besiyeri pH'sı 7.0 ve ekilen spor sayısı 3.6×10^6 iken bulunmuştur. Çalkalamalı erlenlerde maksimum mannanaz üretimi için büyüme koşullarının, en iyi mannanaz üreticisi olarak bulunan *Aspergillus sojae* transformantı AsT1 kullanılarak optimizasyonu, Yanıt Yüzey Metodu altında Box-Behnken istatistiksel deney tasarımı yapılarak yürütülmüştür. En yüksek mannanaz üretimi 30 ºC'lik inkübasyonun dördüncü gününde, optimum koşullarda, ağırlık/hacim yüzdesi %7 melas, %0.43 NH_4NO_3, %0.1 K_2HPO_4, %0.05 $MgSO_4$ olan besiyerinde 207 rpm'de 363 U/ml olarak bulunmuştur. Optimum koşullarda en yüksek aktivite, altıncı günde 482 U/ml olarak bulunmuş ve bu değerin daha önce glikozlu ortamda elde edilen 352 U/ml'lik aktiviteden 1.4 kat daha fazla olduğu saptanmıştır. 40 Ünite mannanaz enzimi ile 48 saat boyunca hidroliz edilen keçiboynuzu gamından, başlıca mannotrioz, 6^1-galaktozil-beta-D-mannotrioz ve 6^3,6^4-di-alfa-galaktozil-beta-1,4-mannopentaoz oligosakkaritleri elde edilmiştir.

Anahtar Kelimeler: Rekombinant *Aspergillus sojae*, gliseraldehit-3-fosfat dehidrogenaz promotörü, endo-beta-mannanaz, yanıt yüzey metodu, keçiboynuzu gamı, mannooligosakkaritler.

To My Parents
ADİLE and HALİL İBRAHİM ÖZTÜRK
and To My Dear Sister
ÖVGÜ ÖZTÜRK

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CHAPTER 1

INTRODUCTION

1.1 β-Mannanases (EC 3.2.1.78)

β-mannanases (EC 3.2.1.78) are extracellular enzymes hydrolysing the 1,4-beta-D-mannosidic linkages in mannans, galactomannans, glucomannans and galactoglucomannans.

Mannans and heteromannans are widely distributed in nature as part of the hemicellulose fraction in hardwoods and softwoods, seeds of leguminous plants and in beans. Hemicelluloses are copolymers of both hexose and pentose sugars. The branched structure allows hemicellulose to exist in an amorphous form that is more susceptible to hydrolysis. Mannan is the predominant hemicellulosic polysaccharide in softwoods from gymnosperms (e.g. pines (*Pinus*), spruces (*Picea*), larches (*Larix*), have well-established systems of horizontal and vertical ducts filled with resin) but is the minor hemicellulose in hardwood from angiosperms (*Eucalyptus,* beech, oak, maple, and magnolia).

In leguminous seeds, water soluble galactomannan is the main storage carbohydrate, comprising up to 20% of the total dry weight (McCleary, 1983). It

has a α-galactose linked at the O-6 position of some mannose residues and may also have some β-D-glucose residues incorporated in the backbone.

The galactomannans from different leguminous taxonomic groupings differ in their degrees of galactose substitution and M:G ratios between 1.1:1 (high galactose) and 3.5:1 (low galactose) are encountered. For example, locust bean gum is a galactomannan which has 4:1 ratio of mannose to galactose, and tara gum has 3:1 ratio of mannose to galactose, and guar gum is a galactomannan which has 2:1 ratio of mannose to galactose , whereas fenugreek gum is a galactomannan with a M:G ratio of 1:1 and konjac gum can be given as an example of glucomannans.

The structures of mannan, galactomannan, glucomannan and galactoglucomannan are shown in Figure 1.1.

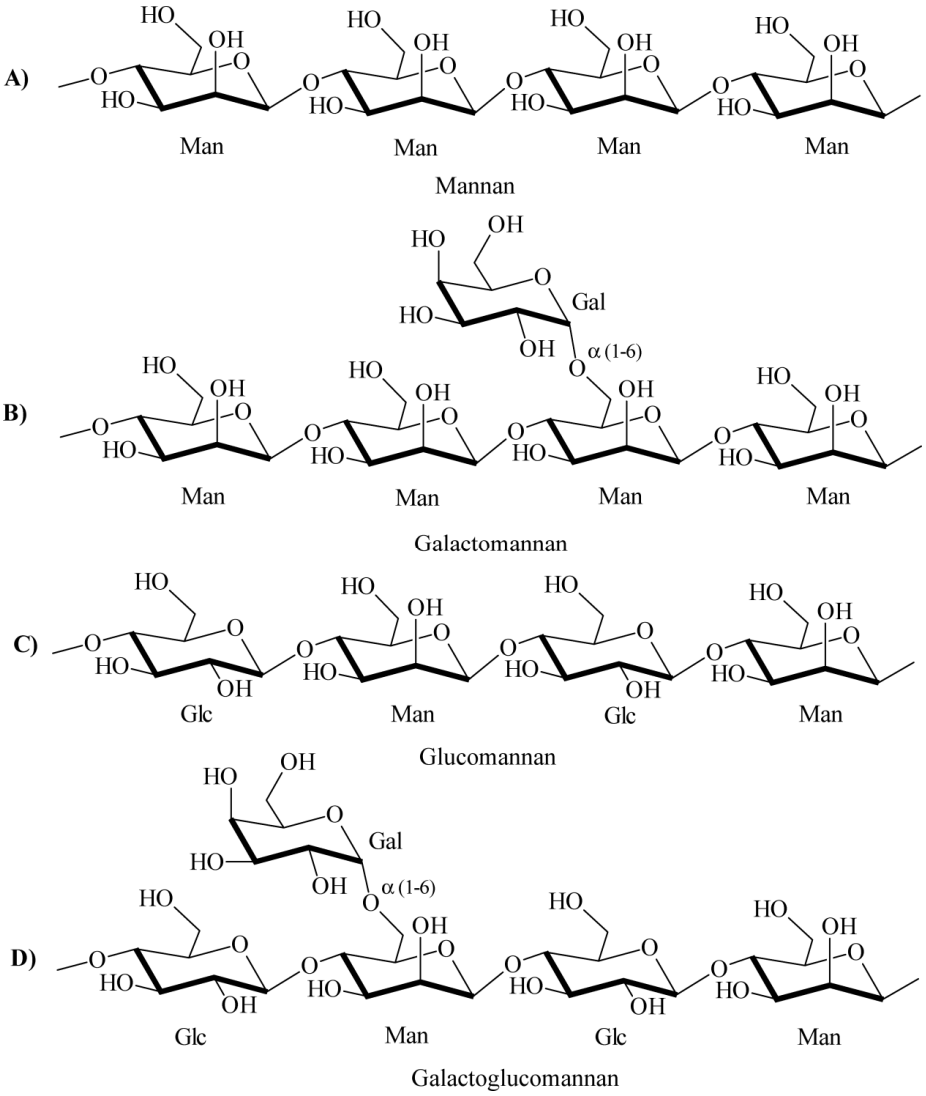


Figure 1.1 General structure of mannan and heteromannans. A) A typical mannan structure, a main chain of $\beta-1,4$ linked mannose (Man) residues; B) A typical galactomannan structure; a main chain of $\beta-1,4$ linked mannose residues with $\alpha-1,6$ linked galactose (Gal) residues attached to some (Man) residues; C) A typical glucomannan structure; a main chain of $\beta-1,4$ linked mannose (Man) and glucose (Glc) residues; D) A typical galactoglucomannan structure; a main chain of $\beta-1,4$ linked mannose (Man) and glucose (Glc) residues, with $\alpha-1,6$ linked galactose (Gal) residues attached to some (Man) residues. (Dhawan and Kaur, 2007).

1.1.1 Enzymatic Hydrolysis of Mannans

The main component of mannan is D-mannose, a six carbon sugar, but due to the heterogeneity and complex chemical nature of plant mannans, its complete breakdown into simple sugars that can be readily used as energy sources by particular microorganisms, the synergistic action of endo-1,4-*β*-mannanases (E.C 3.2.1.78, mannan endo-1,4-*β*-mannosidase) and exoacting *β*-mannosidases (E.C 3.2.1.25) is required (Table 1.1). Additional enzymes, such as *β*-glucosidases (EC 3.2.1.21), *α*-galactosidases (EC 3.2.1.22) and acetyl mannan esterases (Tenkanen *et al*., 1998) are required to remove side chain sugars that are attached at various points on mannans.

Table 1.1 The major mannanases and their classification

Enzymes	***Substrates***	***EC number***	***Family***
Endo-*β*-1,4-mannanase	*β*-1,4-Mannan	3.2.1.78	GH 5, 26
Exo-*β*-1,4-mannosidase	*β*-1,4-Mannooligomers, mannobiose	3.2.1.25	GH 1, 2, 5

1.2 Microorganisms Producing β-Mannanases

Mannanases occur ubiquitously in animals, plants, and microbes. However, microbes are the most potent producers of mannanases and represent the preferred source of enzymes in view of their rapid growth, limited space required

for cultivation, and ready accessibility to genetic manipulation. Microbial mannanases are shown in Table 1.2 (Dhawan and Kaur, 2007).

Table 1.2 Sources of mannanases

Organism	**Reference**
Fungal mannanases	
Agaricus bisporus	Tang *et al*., 2001
Aspergillus tamarii	Civas *et al*., 1984
Aspergillus aculeatus	Christgau *et al*., 1994; Setati *et al*., 2001
Aspergillus awamori	Kurakake *et al.*, 2001
Aspergillus fumigatus	Puchart *et al*., 2004
Aspergillus niger	Ademark *et al*., 1998
Aspergillus oryzae NRRL	Regalado *et al*., 2000
Aspergillus sulphureus	Chen *et al*., 2007
Aspergillus terrus	Huang *et al*., 2007
Phanerochaete chrysosporium	Wymelenberg *et al.*, 2005
Piromyces sp.	Fanutti *et al*.,1995
Sclerotium rolfsii	Sachslehner *et al*., 2000
Polysaccharolyticum *Thermomonospora fusca*	Hilge *et al*., 1998
Trichoderma harzanium strain T4	Franco *et al*., 2004
Trichoderma reesei	Stalbrand *et al*., 1993,1995
Bacterial mannanases	
Bacillus agaradhaerens	Bettiol and Showell, 2002
Bacillus AM001	Akino *et al*., 1989

Table 1.2 (continued)

Bacillus brevis	Araujo and Ward, 1990
Bacillus circulans K-1	Yosida *et al*.,1998
Bacillus polymyxa	Araujo and Ward, 1990
Bacillus sp JAMB-750	Hatada *et al*., 2005
Bacillus sp. 1633	Kauppinen *et al*., 2003
Bacillus sp. M50	Chen *et al*., 2000
Bacillus sp. N 16-5	Yanhe *et al*., 2004
Bacillus stearothermophilus	Talbot and Sygusch, 1990
Bacillus subtilis	Mendoza *et al*., 1994
Bacillus subtilis B36	Li *et al*., 2006
Bacillus subtilis BM9602	Cui *et al*., 1999
Bacillus subtilis SA–22	Sun *et al*., 2003
Bacillus subtilis SA–22	Sun *et al*., 2003
Bacillus subtilis168	Helow and Khattab,1996
Bacteroides ovatus	Gherardini and Salyers, 1987
Bacteroides ruminicola	Matsushita *et al*.,1991
Caldibacillus cellulovorans	Sunna *et al*., 2000
Caldocellulosiruptor saccharolyticus	Morris *et al*., 1995
Caldocellum saccharolyticum	Bicho *et al*., 1991
Cellulomonas fimi	Stoll *et al*., 1999, 2000
Clostridium butyricum/ beijerinckii	Nakajima and Matsuura, 1997
Clostridium cellulolyticum	Perret *et al*., 2004
Clostridium tertium	Kataoka and Tokiwa,1998
Clostridium thermocellum	Halstead *et al*.,1999
Dictyoglomus thermophilum	Gibbs *et al*.,1999
Flavobacterium sp	Zakaria *et al*., 1998
Paenibacillus curdlanolyticus	Pason and Ratanakhanokchai, 2006

Table 1.2 (continued)

Paenibacillus polymyxa	Han *et al*., 2006
Pseudomonas fluorescens subsp. cellulosa	Braithwaite *et al*., 1995
Rhodothermus marinus	Politz *et al*., 2000
Streptomyces galbus	Kansoh and Nagieb, 2004
Streptomyces lividans	Arcand *et al*., 1993
Thermoanaerobacterium	Cann *et al*., 1999
Thermotoga maritima	Parker *et al*., 2001
Thermotoga neapolitana	Duffaud *et al*., 1997
Vibrio sp.	Tamaru *et al*.,1997
Mannanases of other organisms	
Lycopersicon esculentum (plant)	Filichkin *et al*., 2000
Gastropoda pulmonata (snail)	Charrier and Rouland, 2001
Pomacea insulars (snail)	Yamamura *et al*.,1993
Littorina brevicula (mollusk)	Yamamura *et al*., 1996

1.3 Fungal Beta-Mannanases

Glycoside hydrolases (EC 3.2.1.-) from various sources were classified into different families based on their amino acid sequence similarities (Henrissat and Bairoch, 1993; Bolam *et al.,* 1996).

Beta-mannanases (EC 3.2.1.78) are grouped under either glycoside hydrolase family 5 or 26. Most of the fungal origin mannanases including the mannanases of *Aspergillus spp.* are in family 5. The family 26 group mannanases are produced either by bacteria or by anaerobic fungi.

The sequence identity among the members of family 5 is low and an additional classification into eight subfamilies, A1-A8, has been introduced (Beguin, 1990; Hilge *et al.*, 1998). The identity between members of the same subfamily is usually 25% or more and between subfamilies is rarely greater than 20%. Subfamily A7 contains eukaryotic mannanases, while subfamily A8 is composed of bacterial mannanases. Some fungal beta-mannanases are shown in Section 1.2 (Table 1.2).

Three dimensional structure of the *Trichoderma reesei* mannanase can be given as an example for the fungal mannanases. The figure was generated by Sabini *et al.* (2000) using the program BOBSCRIPT.

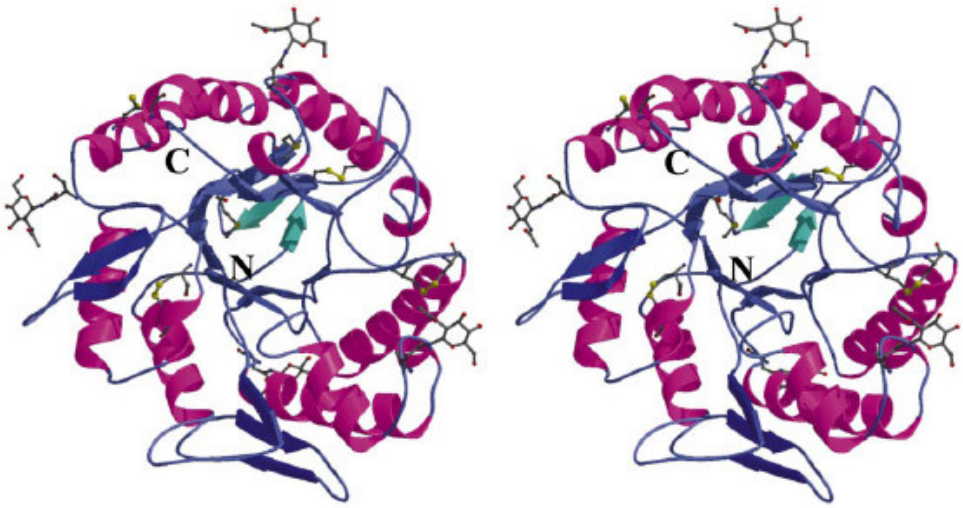


Figure 1.2 Structure of the *T. reesei* β-mannanase. Two short β-strands (sky blue) at the N-terminus form the bottom of the barrel. In addition to these, the fungal enzyme also contains a three-stranded and a two-stranded β-sheet (both in blue) that lie close to the C terminus. The four N-glycosylation sites and the four disulphide bridges are also shown (Sabini *et al.*, 2000).

1.3.1 Mannanases of *Aspergillus spp*.

The genus *Aspergillus* is among the filamentous fungi with 180 officially recognized species. Most of the members are useful microorganisms in nature for the degradation of plant polysaccharides and they are important industrial microorganisms for the large-scale production of both homologous and heterologous enzymes.

Among them *Aspergillus oryzae* and *Aspergillus niger* are on the Generally Recognized as Safe (GRAS) list of the Food and Drug Administration (FDA) in the United States. *A. fumigatus* is the most common infectious cause of human mortality and a major allergen. *A. oryzae*, and *A. sojae*, and *A. tamarii* are used in the food fermentation industry and are considered to be safe because they produce no aflatoxins (Ward *et al.*, 2006). *A. oryzae*, *A. sojae* are used in the production of traditional oriental fermented beverages or foods including soy sauce, miso, and sake (Wicklow *et al.*, 2002).

The wide range of enzymes produced by *Aspergillus* spp. for the degradation of plant cell wall polysaccharides are of major importance to the food and feed industry. Recently, several *Aspergillus* spp. have received increased interest as hosts for heterologous protein production (de Vries and Visser, 2001).

Filamentous fungal fermentation is widely used to commercially produce useful products such as organic acids, enzymes, antibiotics, cholesterol lowering statins, etc. Fungi can be grown in submerged cultures in several different morphological forms: suspended mycelia, clumps, or pellets (Liao *et al.*, 2007).

Several species of *Aspergillus* are used to produce mannanase enzyme. Some of the examples are given below.

Aspergillus tamarii was found to produce beta-mannanase when it was cultivated in a medium containing galactomannan at 29 ºC. The activity of the enzyme in crude extract was found 0.02 U/ml by using Nelson and Somogy method. Specific activity of the mannanase was found as 2.9 U/mg protein (Civas *et al.*, 1984).

The beta-mannanase gene of *Aspergillus aculeatus* was cloned and transformed into *Aspergillus oryzae* by Christgau *et al.* (1994) for overexpression and purification of the enzyme. The recombinant enzyme had a molecular weight of 45 kDa, an isoelectric point of pH 4.5, an optimum pH of 5.0 and a temperature optimum of 60-70 ºC.

The endo-beta-1,4-mannanase encoding gene *man1* of *Aspergillus aculeatus* MRC11624 was cloned and expressed in *Saccharomyces cerevisiae* under the gene regulation of the alcohol dehydrogenase ($ADH2_{PT}$) and phosphoglycerate kinase ($PGK1_{PT}$) promoters and terminators, respectively. The *man1* gene product was designated Man5A. The strains *S. Cerevisiae* Man5*ADH2* and *S. cerevisiae* Man5*PGK1* secreted 521 nkat/ml and 379 nkat/ml of active Man5A after 96 h of growth in a complex medium (Setati *et al.*, 2001).

Two extracellular endo-beta-1,4-mannanases, MAN I (major form) and MAN II (minor form), were purified to electrophoretic homogeneity from a locust bean gum-spent culture fluid of *Aspergillus fumigatus* IMI 385708 (formerly *Thermomyces lanuginosus* IMI 158749). Molecular weights of MAN I and MAN II estimated by SDS-PAGE were 60 and 63 kDa, respectively. MAN I as well as MAN II showed highest activity at pH 4.5 and 60 ºC and were stable in the pH range 4.5–8.5 and up to 55 ºC. Both beta-mannanases contained identical internal amino acid sequence corresponding to glycoside hydrolase family 5 and also a cellulose-binding module. These data suggested that both MAN I and MAN II are products of the same gene differing in posttranslational modification.

Maximum level of beta-mannanase activity (about 30 U/ml) was reached after 72–75 h (Puchart *et al.*, 1999, 2004).

The filamentous fungus *Aspergillus niger* produced beta-mannanase having a molecular mass of 40 kDa. *A. niger* ATCC-46890 was grown at 28°C in shake-flasks containing one of the following carbon sources: 0.5–2% (w:v) locust bean gum galactomannan, 0.5% Solka Floc cellulose, or 1% glucose and showed a mannanase activity of 90 nkat/ml (Ademark *et al.*, 1998).

Beta-mannanase and beta-mannosidase from *Aspergillus awamori* K4 was produced by solid culture with coffee waste and wheat bran. The optimum composition for enzyme production was 40% coffee waste–60% wheat bran. Production of beta-mannanase was 50 U per 1 g of solid medium (coffee waste and wheat bran). Locust bean gum (10%) was hydrolyzed by 1.7 U/ml K4 beta-mannanase yielding di-, tri-, tetra- and pentasaccharides (Kurakake *et al.*, 2001).

Co-expression of phytase and mannanase genes of *Aspergillus terreus* in *Pichia pastoris* was studied and the stable double functional engineered yeast simultaneously expressing extracellular phytase and mannanase was obtained. Enzyme activity of mannanase in supernatant was found as 39.7 U/ml. Mannanase was active under pH 5.5-10.5, and the activity was up to the highest under pH 7.5. Optimum temperature for mannanase was 55 ºC (Huang *et al.*, 2007).

1.4 Applications of β-Mannanases

Microbial mannanases have been used recently in the food, feed and detergent industries. Advances in genetic manipulation of microorganisms result in the production of tailor-made mannanases with novel and desirable properties. The development of recombinant mannanases and their commercialization by P&G,

ChemGen and Genencor is an excellent example of the successful application of modern biology to biotechnology (Dhawan and Kaur, 2007).

A few commercial products have been launched successfully worldwide as shown in Table 1.3.

Table 1.3 List of commercial mannanases, their suppliers and application areas (Dhawan and Kaur, 2007)

Product Trade Name	Supplier	Source Microorganism	Recommended pH/Temperature	Action Pattern	Applications
Hemicell	ChemGen, USA	*Trichoderma longibrachiatum* and *B. lentus*	—	endo-mannanase	Animal feed supplement
Gamanase	Novo Nordisk, Denmark	*Aspergillus niger*	3–6/20–80°C	endo-mannanase	Coconut oil extraction
Mannaway	P&G (Novozyme)	*Bacillus* sp.	—	endo-mannanase	Detergent
Purabrite, Mannastar	Genencor, USA	Fungal mannanase	—	endo-mannanase	Detergent
Pyrolase 160, Pyrolase 200	Diversa (NASDAQ-DVSA)	Organism from hydrothermal vents	5–10/37–93°C	endo-mannanase and exo-mannanase	Oil recovery and well drilling

There are many applications for mannanases in the industrial processes. They are used mainly for improving the quality of food and feed and aiding in enzymatic bleaching of softwood pulps in the paper and pulp industries, in decreasing the viscosity of coffee extracts, in the detergent industry and in oil drilling, in oil extraction of coconut meats, for degradation of thickening agents, in producing non nutritional food additives and in the textile and cellulosic fiber processing industries.

Pulp and paper industry:

The most potent application of mannanase is consistent with its potential use in enzymatic bleaching of softwood pulps. The extraction of lignin from wood fibers is an essential step in bleaching of dissolving pulps. Pulp pretreatment under alkaline conditions hydrolyzes hemicelluloses covalently bound to lignin and thus facilitates subsequent removal of lignin. There is a drawback to alkaline treatment of wood pulps, however, in that it creates an environmental pollution problem. The alternate use of mannanases equally facilitates lignin removal in pulp bleaching and yields results comparable to alkaline pretreatment (Gubitz *et al.*, 1997).

Instant Coffee Industry:

Mannan is the main polysaccharide component of these extracts and is responsible for their high viscosity, which negatively affects the technological processing of instant coffee. Different mannanase preparations obtained from the filamentous fungus *Sclerotium rolfsii* were also used for the hydrolysis of coffee mannan, thus reducing significantly the viscosity of coffee extracts (Sachslehner *et al.*, 2000).

Detergent industry:

Alkaline mannanases stable to constituents of detergents have found application in certain laundry segments as stain removal boosters. Mannanases hydrolyze mannan containing materials like gums (galactomannans, glucomannans and guar gum). These gums are used worldwide as a thickener or stabiliser in many types of household products and foods including ice-creams, BBQ sauces, hair styling gels, shampoos, conditioners and toothpaste. Stains containing mannan are generally difficult to remove because mannans have a tendency to adsorb to cellulose fibers and therefore bind to cotton textiles. Treatment with cleaning or detergent compositions comprising the mannanase can improve whiteness as well as prevent binding of certain soils to the cellulosic material. Accordingly,

mannanase are used in cleaning compositions, including laundry, personal cleansing and oral/dental compositions (Dhawan and Kaur, 2007).

Oil and Gas Drilling:

Mannanases can also be used to enhance the flow of oil or gas in drilling operations. Product flow to the well bore is stimulated by forcing out open crevices in the surrounding bedrock, which is done by flooding the well with a natural polymer (guar gum) solution and sand particles, capping the well and then pressurizing the bedrock until it fractures. The viscous polymer solution carries the sand through the fractures, propping open cracks for oil and gas flow. To facilitate product flow, the polymer solution is thinned. As mannanases are able to hydrolyze guar gum at elevated temperatures, so implementation of oil production by these enzymes could be feasible (McCutchen *et al.*, 1996).

Poultry feed industry:

β-Mannans have been found to be highly deleterious to animal performance, severely compromising weight gain and feed conversion as well as glucose and water absorption. Incorporation of *β*- mannanase into these diets results in decreased intestinal viscosity, thus improving both the weight gain of chicks and their feed conversion efficiency. Manno-oligosaccharides, mannotriose and mannobiose as well as a small amount of mannose are generated when beta-mannanase is included in the diet. Since only mannose can be absorbed in the intestine, mannobiose and mannooligosaccharides may not be absorbed and are therefore unable to supply energy to the host. However, in the sense of the health of the poultry, the production of mannooligosaccharides can improve a chicken's health, either by increasing the population of specific bacteria such as *Bifidiobacteria* because the supply of these types of carbohydrates are a source of feed for bacteria in the caeca and thus suppressing the pathogenic ones, or by flushing out the pathogenic bacteria which attach to the mannooligosaccharides. In fact, mannooligosaccharides are added to the poultry diets for this effect. The

use of mannanase in poultry research has been widespread and proven to be successful (Sundu *et al.*, 2006).

Oil extraction of coconut meals:

Mannanases can be used in enzymatic oil extraction of coconut meat. The main components of the structural cell wall of coconut meat are mannan and galactomannan. Enzymatic process has many advantages over the traditional method of oil extraction by expeller (Chen *et al.*, 2003).

Degradation of Thickening Agents:

Galactomannans such as guar gum and locust bean gum are widely used as thickening agents, e.g. in food and print paste for textile printing such as prints on T-shirts. Mannanase can be used for reducing the viscosity of residual food in processing equipment and thereby facilitate cleaning after processing. The enzyme or enzyme preparation is useful for reducing viscosity of print paste, thereby facilitating wash out of surplus print paste after textile printings (Ademark *et al*., 1998).

Non-nutritional food additive production:

Mannanases also contribute to the human health as they degrade mannans, which are otherwise resistant to mammalian digestive enzymes in the small intestines, but are readily fermented in the large intestine, particularly by probiotic bacteria belonging to the genera *Bifidobacteria* and *Lactobacillus*. Prebiotic oligosaccharides including mannooligosaccharides, are believed to promote the selective growth and proliferation of human beneficial intestinal microflora (Dhawan and Kaur, 2007).

1.4.1 Utilization of Mannooligosaccharides as Prebiotics

Mannanases can be used for the production of mannooligosaccharides from cheap agricultural by-products such as copra or konjac mannan. These oligosaccharides were reported to be excellent prebiotics stimulating growth of beneficial intestinal microorganisms and hence could be used in pharmaceuticals or in food stuffs (Großwindhager *et al.,* 1999).

1.4.1.1 Prebiotics

Prebiotics are non digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health. Prebiotics are commonly oligosaccharides. They resist digestion in the upper GI tract but are metabolised by certain beneficial bacteria, resulting in a healthier gut flora (Roberfroid, 2001).

- To be an effective prebiotic, the oligosaccharides should;
  - neither be hydrolysed nor absorbed in the upper part of the GI tract.
  - have a selective fermentation such that the composition of the large intestinal microbiota is altered towards a healthier composition.
  - referred as bifidogenic factors and are known as anti-adhesive agents

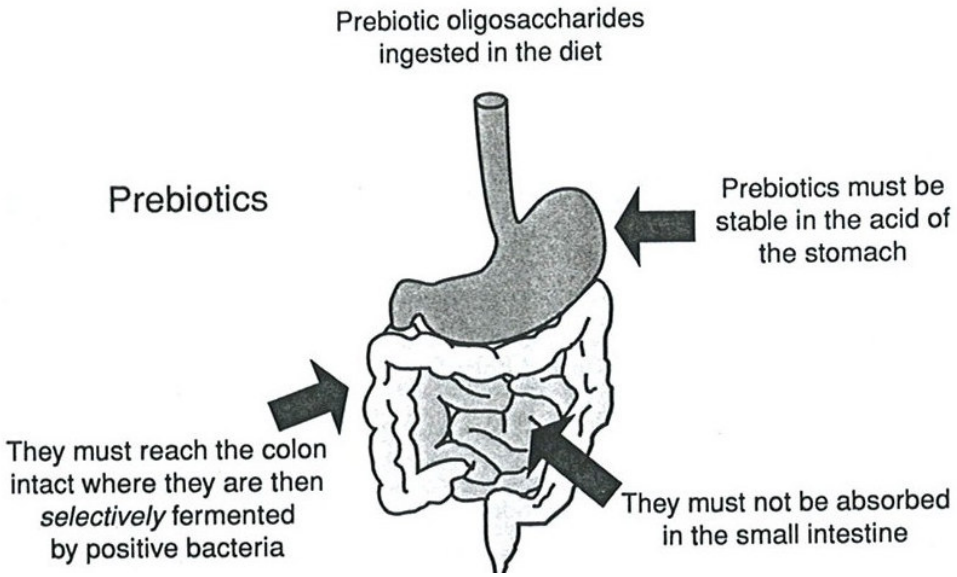


Figure 1.4 The mechanism of prebiotic action (Gibson, 2000).

The mechanism of the prebiotics is shown in Figure 1.4. Most prebiotics, including fructans, galactooligosaccharides, lactulose and raffinose have been shown to have bifidogenic properties (Bouhnik *et al.*, 2004).

- Beneficial effects of prebiotics are:
  - prevention of intestinal disorders; diarrhea and constipation.
  - modulation of immune response
  - prevention of colon carcinogenesis.
  - reduction in serum cholesterol and blood lipids.
  - improved bioavailability of minerals (Ca, Mg..)

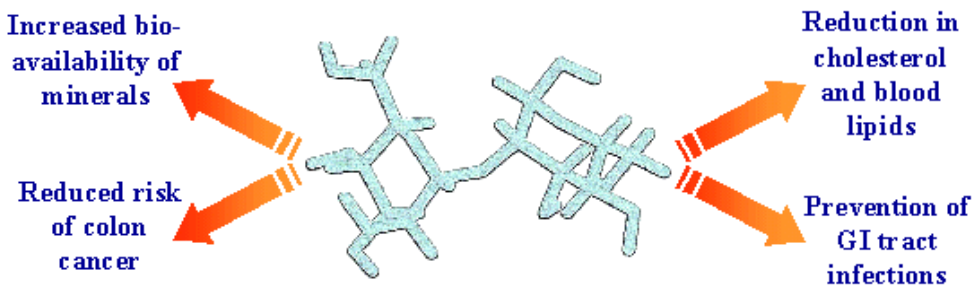


Figure 1.5 The beneficial effects of prebiotic oligosaccharides

Fermentation of food derived indigestible carbohydrates by the bacteria in large intestine causes increased production of short-chain fatty acids, which decreases circulatory cholesterol concentrations either by inhibiting hepatic cholesterol synthesis or by redistributing cholesterol from plasma to the liver. Furthermore, increased bacterial activity in the large intestine results in enhanced bile acid deconjugation. Deconjugated bile acids are not well absorbed by the mucosa of the gut and are excreted. Consequently, cholesterol, being a precursor of bile acids, is used to a greater extent for *de novo* bile acid synthesis. It has been suggested that these actions combined are contributing mechanisms to the association of fermented milk consumption with decreased circulating cholesterol concentrations (Jones, 2002).

Short-chain fatty acids, such as acetate, propionate and butyrate, produced during gut fermentation may also have promising uses in the treatment of colon cancer. Increased production of short-chain fatty acids has been shown to decrease the pH of the contents of the colon and enhance cell proliferation in normal cells, while suppressing proliferation in transformed cells, which in turn has been associated with a reduced incidence of colon cancer in various populations (Jones, 2002).

1.4.1.2 Mannooligosaccharides (MOS)

Mannooligosaccharides are commonly found attached to mammalian glycoconjugates, typically as N-linked oligomannose or high-mannose type glycans in glycoproteins. They form a wide range of structures built from common sub-sequences. Mannooligosaccharides are known to be the cellular receptor site for the mannose-specific FimH adhesion present at the tip of type-1 fimbriae carried by most *Escherichia coli* strains and other enteric micro-organisms.

Prebiotic effect of mannooligosaccharides:

Beneficial micro-organisms, such as lactobacilli and bifidobacteria, use MOS as an energy source, where pathogenic micro-organisms cannot. Therefore it stimulates growth of the beneficial gut flora.

Most pathogenic bacteria need to attach to the intestinal wall in order to exert their pathogenic effect (multiplication, toxin secretion and longer residence time). This attachment is a specific interaction between oligosaccharide receptors on the epithelial cells of the intestine of the animal and fimbria of the pathogenic microorganisms (lectins).

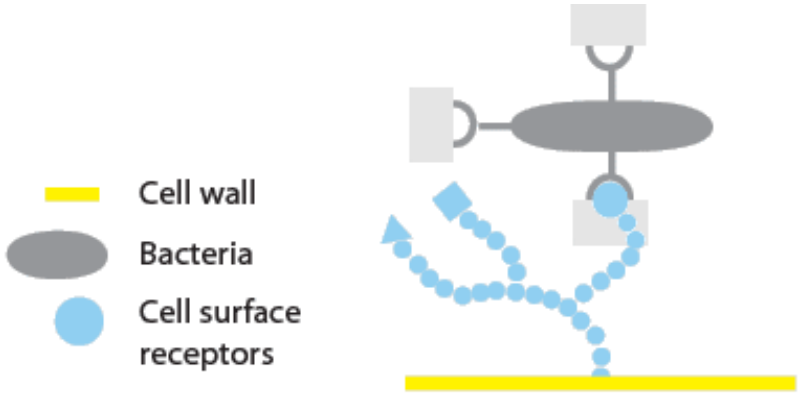


Figure 1.5.a Interaction between oligosaccharide receptors on the epithelial cells of the intestine of the animal and fimbria of the pathogens (http://www.nutrex.be/products/feed/yeast_preparations/yeast_ymos.html).

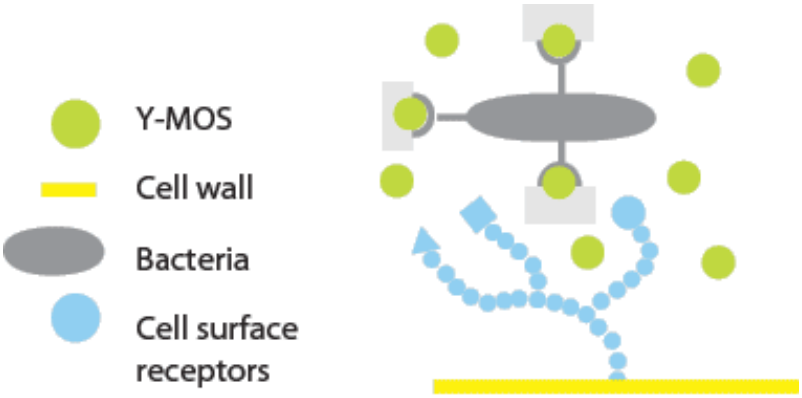


Figure 1.5.b MOS can compete with the epithelial receptors. They can interact with the fimbria, resulting in a prevention of the attachment of pathogens (http://www.nutrex.be/products/feed/yeast_preparations/yeast_ymos.html).

MOS stimulate the development of a stable intestinal flora consisting of favourable bacteria. Such candidate anti-adhesive oligosaccharides can be synthesised chemically at high cost and by enzymatic methods involving the use of sugar nucleotide-dependent glycosyl transferases and glycosidases (Maitin *et al.*, 2004).

Chemical synthesis of oligosaccharides is both highly sophisticated and very complex. For this reason, no large-scale chemical synthesis operations have been established. The most likely developments in the large-scale synthesis of oligosaccharides is the area of enzymatic synthesis (Gibson *et al.*, 2000).

Enzymatic production of MOS by reverse hydrolysis by using 1,6-α-D mannosidase enzyme of *Aspergillus phoenicis* ATCC 14332 (Athanasopoulos *et al.*,2004). Mannooligosaccharides can also be released after the hydrolysis of mannan structures like coffee mannan (Sachslehner *et al.*, 2000).

The effects of Mannan oligosaccharides:

It is revealed that mannan oligosaccharides improve the intestinal mucosa and increase the number of villus and some enzymes (maltase, aminopeptidase and alkaline phosphatase) in jejunum. Mannan oligosaccharides also prevent the colonization of intestinal pathogens. The pathogens bind to mannan oligosaccharides and thus the number of the pathogens which colonize by binding to the intestinal epithelium were reduced and when MOS were added to the feed of the broiler poultry, it was seen that there was a big decrease in the number of pathogenic *Salmonella* and *E.coli* found in the intestines of the turkeys (Yıldız and Akan, 2004).

Mannan oligosaccharides can be supplemented into the feed for broilers in order to provide an alternative to the anticoccidials to improve the growth performance and avoid the coccidiosis in broiler poultries (Elmusharaf *et al.*, 2007).

It has been shown that α-D-mannopyranosyl-(1,2)-D-mannose, α-D-mannopyranosyl-(1,3)-D-mannose and, to a lesser extent, α-D-mannopyranosyl-(1,6)-D-mannose disaccharides and trisaccharides with the same linkages can inhibit both adhesion of *E. coli* to intestinal mucin and haemagglutination (Sajjan and Forstner, 1990).

According to the studies carried out in Japan, it was found that the intake of coffee drink containing MOS (mannan oligosaccharides) 3.0 g per day increased the excreted fat from the body and cause a decrease in fat utilization (Kumao *et al.*, 2006).

1.5 Optimization of Mannanase Production

Beta-Mannanases have numerous applications in the food, feed, as well as pulp and paper industries. Mannanases can be produced by a number of fungi, yeasts, bacteria, and marine algae, as well as from germinating seeds of terrestrial plants, and various invertebrates. Among these options, production of beta-mannanases by microorganisms is more promising due to its low cost, high production rate, and readily controlled conditions (Feng *et al.*, 2003).

Conventional methods for optimization of medium and fermentation conditions involves varying one parameter at a time and keeping the others constant, is time consuming and expensive, when a large number of variables are to be evaluated. To overcome this difficulty and to evaluate and understand the interactions between different physiological and nutritional parameters, response surface methodology (RSM) has been widely used. This methodology brings about the effect of interaction of various parameters, generally resulting in higher production yields and simultaneously limits the number of experiments. It is currently used for optimization studies in several biotechnological and industrial processes (Kaushik *et al.*, 2006).

Response surface methodology may be summarized as a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables (Gangadharan *et al.*, 2007).

To meet the growing demands in the industry it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production. The growth and enzyme production of the organism are strongly influenced by medium composition thus optimization of media components and cultural parameters is the primary task in a biological process (Djekrif Dakhmouche *et al.*, 2006).

It is well documented, that several organisms are able to produce beta-mannanases (Dhawan and Kaur, 2007), but in the literature there are few studies in which statistical methods have been applied for optimization of beta-mannanase (El-Helow *et al.*, 1997; Heck *et al.*, 2005; Regalado *et al.*, 1999).

El-Helow *et al.* (1997) studied formulation of an economic *B. subtilis* beta-mannanase production medium utilizing inexpensive agro-industrial wastes or byproducts that are available in large quantities such as wheat bran and palm seed powder. 2^n factorial design was used to optimize the medium composition. They achieved 102 U/ml mannanase activity in the medium containing palm seed powder (10 g/l) instead of locust bean gum (galactomannan) 4 g/l; NH4NO3, 0.3 g/l; MgSO4.7H2O, 0.2 g/l; FeSO4.7H2O, 0.01 g/l; CaCl2.2H2O, 0.05 g/l; K2HPO4, 7.54 g/l and KH2PO4, 2.32 g/l. This enzyme activity obtained at the optimized growth condition was equivalent to about 319% of the beta-mannanase activity level reached by a galactomannan-based culture.

Heck *et al.* (2005) studied A 2^3 central composite design (CCD) was applied to determine the optimal conditions of cultivation time, aeration and temperature to

xylanase and mannanase production by *Bacillus circulans* BL53. They performed solid state cultivation in the bioreactor by soaking industrial fibrous soy residue into the basic liquid medium which had the composition (g l−1): 0.2 $MgSO_4$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.02 $CaCl_2$ and 0.05 $FeCl_2$. Mannanase production was increased at higher cultivation time and aeration rate and lower temperature. They have found that the highest enzymatic activity obtained was 0.540Umg−1 in 7 days under the optimum conditions (800 ml/min, of aeration and at 30 ◦C) which is higher than than the activity obtained under the conditions they have previously employed (0.150Umg−1 of protein at 5 days, 500 ml min−1 of air flow and 37 ◦C).

Regalado *et al.* (1999) used response surface methodology to maximize the production of β-mannanase from solid state fermentation of soluble coffee wastes using *A. oryzae*. Coffee waste was supplemented with salts (1x, 2.5x and 5x), where 1x concentration was 0.01 g L^{-1} $FeSO_4.7H_2O$; 0.05 g L^{-1} $CaCl_2$; 7.54 g L^{-} K_2HPO_4; 2.32 g L^{-1} KH_2PO_4; organic nitrogen (yeast extract: 5, 10 and 15 g L^{-1}), and inorganic nitrogen (ammonium sulphate: 2, 4 and 6 g L^{-1}); fermentation time was varied (2, 4 and 6 days). A 2^4 fractional factorial design was used to derive a second-order polynomial fitted model, and the response surface of the effect of the four factors was studied. Best conditions were obtained supplementing with 4 g L^{-1} ammonium sulfate, 11.5 g L^{-1} yeast extract, 1x minerals concentration, and 4 days fermentation time. Additional experiments conducted under these conditions gave activities close to the maximum predicted value of 108 mg mannose min^{-1} L^{-1} (0.6 U/ml).

Since beta-mannanase is a very promising enzyme used in many fields of industry, enhancement of mannanase production should be carried out in order to get higher yields from the processes and to reduce the time of processes, so that an economical approach to optimize the enzyme production could be provided.

In this study, the use of a statistical approach called Box-Behnken Design (BBD) of response surface methodology to optimize the physiological and nutritional parameters, which were found optimum by classical 'one-at-a-time' method was reported. The aim was to study the effect of interactions among carbon, nitrogen source and agitation speed on mannanase yield of *A. sojae* Tr1.

1.6 Previous Studies

In previous studies, beta-1,4-mannanase gene of *A. fumigatus* IMI 385708 was cloned in *A. sojae* in our laboratory by using the vector pAN 52-4. On the expression vector the nucleotide sequence of *afman* signal sequence was replaced by the signal and prosequences of *Aspergillus niger gla*A encoding glucoamylase and the gene was put under the control of the glyceraldehyde triphosphate dehydrogenase (*gpd*A) promoter of *Aspergillus nidulans* using the unique *Bam*HI/*Hind*III sites of the vector. The final construct was named as pAN52-4*afman* (Appendix E). The host strain *A. sojae* ATCC11906 was co-transformed with the plasmid pAMDSPYG carrying the *pyr*G gene of *A. niger* encoding orotidine-5'-phosphate-decarboxylase and pAN52-4*afman* for selection of the transformants and as a result eight transformants having mannanase activity were obtained (Duruksu G, 2007).

1.7 Scope of This Study

In this study, it was aimed to compare the production of mannanase by *Aspergillus fumigatus* IMI 385708 and three transformants of *Aspergillus sojae* ATCC11906 producing higher levels of mannanase and to optimize the growth conditions for maximum beta-mannanase production.

Furthermore, the mannooligosaccharides produced by the enzymatic hydrolysis of locust bean gum by the recombinant enzyme were analysed by High Pressure Liquid Chromatography and Thin Layer Chromatography.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Organisms and Growth Conditions

Aspergillus fumigatus IMI 385708 (formerly 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 potato dextrose agar plates (Appendix B) at 30 °C and maintained at 4 °C.

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

2.1.2 Chemicals and Their Suppliers

The list of chemicals 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.

2.2 Methods

2.2.1 Maintenance and Cultivation of the Strains

Stock cultures of *Aspergillus fumigatus* IMI 385708 and recombinant strains of *Aspergillus sojae* (ATCC11906) were grown on PDA plates at 30 °C for 4-5 days and followed by storage at 4 ºC for a maximum of 1 month. Every 1 month, stock cultures were periodically renewed.

Spore suspensions were prepared by pouring sterile saline-tween solution (Appendix B) on top of stock cultures on PDA agar plates and by collecting the mixture containing spores of *A. fumigatus* and *A. sojae* in 15 ml sterile falcon tubes. The number of spores were counted by using "Thoma Haemocytometer" (Chang Bioscience Inc.) under the microscope.

For the analysis of recombinant mannanase production, equal number of spores of *A. fumigatus* and *A. sojae* transformants were first inoculated seperately into 30 ml modified YpSs preculture medium containing yeast extract (0.4% w/v), K_2HPO_4 (%0.1 w/v), $MgSO_4 .7H_2O$ (0.05% w/v) and glucose (1% w/v) instead of starch (Appendix B) for germination. Then, preculture media containing germinated spores were added into 1250 ml main culture containing the same medium as the preculture medium except for the use of 2% glucose instead of 1%.

For the induction of mannanase production, *A.fumigatus* was cultivated in 1250 ml modified YpSs medium containing LBG (2% w/v) instead of starch (Appendix B) in 2L erlenmeyer flask and incubated at 30 ºC, 155 rpm for 7 days and enzyme production was analyzed in culture supernatants.

For the optimization studies, 100 ml modified YpSs media (Appendix B) containing different concentrations of sugar beet molasses instead of glucose and different concentrations of ammonium nitrate (NH_4NO_3) instead of yeast extract, were used for fungal growth.

2.2.2 Mannanase Activity Assay

Endo-beta-1,4-mannanase activities were determined by using the DNS (Dinitrosalicylic acid) method based on the determination of reducing sugar content.

- A solution of locust bean gum substrate (0.05% w/v) in Na-citrate buffer (pH=6.0) was prepared and 1.8 ml substrate was mixed with 0.2 ml of appropriately diluted culture supernatant in a test tube. After 5 min incubation at 50 ºC in water bath, the reaction was stopped with 3 ml of DNS solution (Appendix B) (Miller *et al.*, 1959). The tubes were then boiled at 90 ºC in a water bath for 15 minutes to allow the color to develop and then cooled in an ice bath.

- Absorbances were measured at 540 nm using a UV-Visible Spectrophotometer (PharmaSpec UV-1700, SHIMADZU) (Bailey *et al.*, 1992) and they were used to determine the activities by the help of mannose standard curve (Appendix C). The activity values were calculated in terms of U/ml (Unit/ml supernatant) according to the definition of mannanase activity. One unit of enzyme activity (1 U=16.7 nkat) was determined as the amount

of enzyme liberating reducing sugars equivalent to 1 µmol D-mannose in 1 min (Puchart *et al.*, 2004).

The data to draw the mannose standard curve was obtained by preparing different concentrations of D-mannose (0-10 µmol/ml) and measuring their absorbances (OD) at 540 nm after the DNS method was applied. The equation of the standard curve was found experimentally to be:

$$OD_{540} = a \times C_{mannose} + b \qquad \textbf{(Equation 2.1)}$$

where, a = 0.2651 (slope of the standard curve)

b = -0.1377 (intercept of the standard curve)

$C_{mannose}$ = concentration of mannose (µmole/ml)

- The reducing sugar content of the samples corresponding to the [$OD_{Sample} - (OD_{Substrate\ blank} + OD_{Enzyme\ blank})$] were determined from Equation 2.1. Mannanase activities were calculated from Equation 2.2 by using the reducing sugar content (equivalent to mannose concentration) of each sample.

$$\text{Mannanase Activity (U/ml)} = C_{mannose} \times R_V \times DF / t \qquad \textbf{(Equation 2.2)}$$

where, DF = Dilution Factor

R_V = Volume of total solution in test tube / Volume of enzyme solution

t = duration of the reaction in minutes

2.2.3 Biomass Determination

Biomass was determined by taking 40 ml samples of culture from 1250 ml of the medium every 2 days of cultivation, filtering through a preweighed Whatman No:1 filter paper, and drying at 60 ºC for 24 hours.

Biomass (g/l) was determined by substracting the tare of Whatman No:1 paper from the weight of the paper containing dried mycelia and then dividing by the volume of the culture medium taken as the sample.

2.2.4 Optimization of *A. sojae* Mannanase Production

The optimization of medium components and cultural conditions for mannanase production by the fungus *A. sojae* was carried out in the following steps. First step is the selection of the factors (carbon and nitrogen concentration, agitation speed) and their levels by performing the preliminary experiments on one-factor-at-a-time basis (Frey *et al.*, 2003) and the second step is the estimation of the optimum values by using Box-Behnken design under Response Surface Methodology to obtain maximum mannanase activity (Myers, 1971).

2.2.4.1 Response Surface Methodology

In order to determine the optimum concentrations of carbon and nitrogen in the medium and the rate of agitation that maximize the mannanase activity, the statistical approach, Response Surface Methodology (RSM) was used (Gilmour, 2006; Myers, 1971). For the design of experiments performed by RSM, statistical software MINITAB 13 was employed.

The steps followed during the creation of a response surface design are given below. There are 4 commands used when carrying out response surface designs. Each command in MINITAB 13 is given at the beginning of the steps and explained in details (Response surface designs, MINITAB User's Guide).

1. Stat > DOE > Response Surface > Create Response Surface Design: This command is used to create the response surface design of the experiment.

- The appropriate response surface design is chosen by concerning the experimental region of interest, number of factors and runs that can be performed and other considerations such as cost and time. The Minitab provides two response surface designs namely, Central Composite and Box-Behnken designs.
- After Box-Behnken design was chosen, the number of the factors are specified.
- The names, the lowest and highest values of factors are identified.
- And the number of blocks can be specified. When the number of runs is too large to be completed, it is necessary to be concerned with introducing blocks to the experiment. Running an experiment in blocks allows you to work separately in which block effects (different experimental conditions) are concerned by the program. When the experimental runs can be carried out at the same time, there is no need to introduce blocking to the experiment.
- After carrying out the steps above one by one, a response surface design with definite combinations of runs is created. A table in a Minitab worksheet displaying the combinations of experimental runs is generated by Minitab 13.
- After performing the experiment, the experimental results of the response variable for each run are typed into the column of response in the worksheet.

2. Stat > DOE > Response Surface > Analyze Response Surface Design:

- After typing the responses, the results were analyzed by the command given above.

- The data can be analyzed by using both coded and uncoded units. The coded values −1, +1, 0, show the low level, high level, center points, respectively. Minitab displays the output based in this coded form. However, uncoding performs the analysis using the actual levels of the factors.
- The terms full quadratic or linear can be chosen to be included in the model. Full quadratic terms include the terms which show the linear, squares and the interactions of the factors.
- After the analysis of the design, the display of the results can be obtained in terms of estimated regression coefficients and ANOVA tables presenting the lack fit of the model (p), R^2 values in a separate session window in the same Minitab project.
- Lack of fit (p value) and R^2 values are important for checking the adequacy of the fitted model. If lack of fit (p-value) is smaller than 0.05 (confidence interval was adjusted as 95 %), the model is not appropriate for the design. And R^2 value between 0-1 should be close to 1.
- The estimated regression coefficients are used to determine the second order polynomial equation defined by the relation between variables and the response.

3. Stat > DOE > Response Surface > Analyze Response Surface Design > Contour/surface plots: This command is used to create graphs that define the relation between the response and the factors.

- A contour plot provides a two-dimensional view where all points that have the same response are connected to produce contour lines of constant responses.
- A surface plot provides a three-dimensional view that may provide a clearer picture of the response surface.

4. Stat > DOE > Response Surface > Analyze Response Surface Design > Response Optimizer: This command is used to find the optimum levels of the

factors to give a maximum or target response with a desirability value. According the desirability value and considering the economy of the process. The most appropriate combination of the factors to obtain the maximum activity was found out.

After these steps, the validation of the optimum conditions were done by performing parallel experiments under the optimum conditions found by the program.

2.2.5 HPLC and TLC Analysis of Hydrolysis Products of Locust Bean Gum

The sample for HPLC analysis was prepared by incubating 900 µl of 0.5% (w/v) locust-bean gum solution with 100 µl crude mannanase enzyme (40 Units). Thin-layer chromatography (TLC) was used to identify products. Enzymatic hydrolysis of 10 ml locust bean gum in 50 mM Na-citrate buffer (pH 6.0) was carried out at 50 ºC by using 600 U mannanase. At appropriate times, aliquots (5 µl each) were removed and analysed by TLC (silica gel 60 plates, 10 × 20 cm, Merck) with acetonitrile-H_20 (85:15, v/v) as a solvent system (Jiang *et al.,* 2004). The sugar spots were detected by spraying a solution of 10% (v/v) H_2SO_4, and heating the silica plates at 100 ºC for 15 min.

The mannooligosaccharides (MOS); M2 (mannobiose), M3 (mannotriose), GM2 (6^1-α-galactosyl-β-1,4mannobiose)- the galactopyranosyl group was linked α-1,6 to the reducing mannose residue of 1,4-β-D-mannobiose- and G2M5 (6^3,6^4-di-α-galactosyl-β-1,4-mannopentaose) -two galactopyranosyl groups were linked α-1,6 to the third and fourth mannose residues from the reducing end of 1,4-β-D-mannopentaose- were used as authentic standards.

Hydrolysis products of locust-bean gum were analysed by using columns based on size exclusion principle in HPLC (High Pressure Liquid Chromatography) in Gaziosmanpasa University according to the procedure outlined below:

MOS were chromatographed on a (Perkin Elmer) HPLC system equipped with a refractive index detector (Perkin Elmer Series 200), and column oven (Perkin Elmer Series 200). Before injection, samples were filtered through 0.2 μ filter. Aliquots of filtered sample (20 μL) were injected to the HPLC system. MOS were eluted using distilled-deionized water as the mobile phase from an ion-mediated stationary phase in the silver form (Biorad Aminex HPX 42A). The Aminex HPX 42A column (300 x 7.8 mm), which was preceded by its complimentary de-ashing cartridge (Biorad), was used at 55 ^{o}C and a flow rate of 0.5 mL/min. A complete analysis of MOS was carried out in 30 min. Computing integrators determined the start, retention time and end of the peak, and integrated the area under each peak as a function of height and width of the peak. Concentration of an oligosaccharide was quantified using average peak areas compared with peak areas of standard oligosaccharides and expressed as mg/mL oligosaccharide.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 An Overview of the Study

This thesis aimed to;

- compare the production of mannanase from *A. fumigatus* IMI 385708 and in transformants of *A. sojae* ATCC11906,
- investigate and optimize bioprocess parameters for enhanced mannanase production,
- analyze the mannooligosaccharides resulting from the hydrolysis of locust-bean galactomannan.

For this purpose the following were performed:

1. Mannanase production from different *A. sojae* transformants and the donor strain of *A. fumigatus* were compared and mannanase production was investigated by changing the growth medium composition.

2. The growth parameters (e.g. carbon and nitrogen sources and agitation speed) were optimized by Response Surface Methodology to find the best mannanase production conditions.

3. Locust-bean galactomannan was hydrolysed by the produced beta-mannanase and the hydrolysis products were identified by Thin Layer Chromatography and High Pressure Liquid Chromatography using mannooligosaccharide standards.

3.2 Comparison of the Production of Mannanase from *A. fumigatus* IMI 385708 and Transformants of *A. sojae* (ATCC 11906)

To investigate the mannanase production of *A.sojae* transformants and the gene donor strain of *A. fumigatus,* first these fungi were sporulated on PDA plates and after 3-4 days of incubation at 30 ºC, the number of the spores for each fungus were counted by using Thoma Haemocytometer.

An equal number of spores were taken from four transformants Tr 1, Tr 2, Tr 5, Tr 4 previously obtained by Duruksu (2007) and *A. fumigatus*, respectively and the endo-beta-mannanase activity of the fungal strains were compared by growing cultures under conditions described in Section 2.2.1.

β-(1,4)-endomannanase activity in the culture supernatants were compared by using both locust-bean gum and guar gum as substrates in enzyme assays as described in Section 2.2.2.

3.2.1 Comparison of Mannanase Production by using Locust-bean Gum as the Substrate

Mannanase production by *A. sojae* transformants in YpSs containing 2% glucose instead of starch was determined for 7 days.

The activity of transformants can differ from each other, since the mannanase gene may have integrated on different regions on the genome. According to the results the highest mannnanase producing transformant was found as AsTr1 which yielded an activity of 352 U/ml on 6th day of cultivation. The strain AsT4 did not show any activity for 7 days, pointing out that only the insertion of pAMDSPYG but not pAN52-4-*afman1* (see Section 1.7) took place into the genome. The wild type endo-mannanase producer *A. fumigatus* showed the highest activity on the 6th day as 88.3 U/ml (Figure 3.1).

Because of the fact that the DNS method was used to follow mannanase activity, which determines the reducing sugar content, the enyzme activity detected during the cultivation of *A. fumigatus*, indicated not only endo-beta-mannanase production, but also alpha-galactosidase and β-mannosidase productions inducted by the locust-bean gum used as the carbon source. So, the activity in *A. fumigatus* supernatants is a result of these three enzymes. Therefore, the actual mannanase production by *A. fumigatus* is lower than 88.3 U/ml.

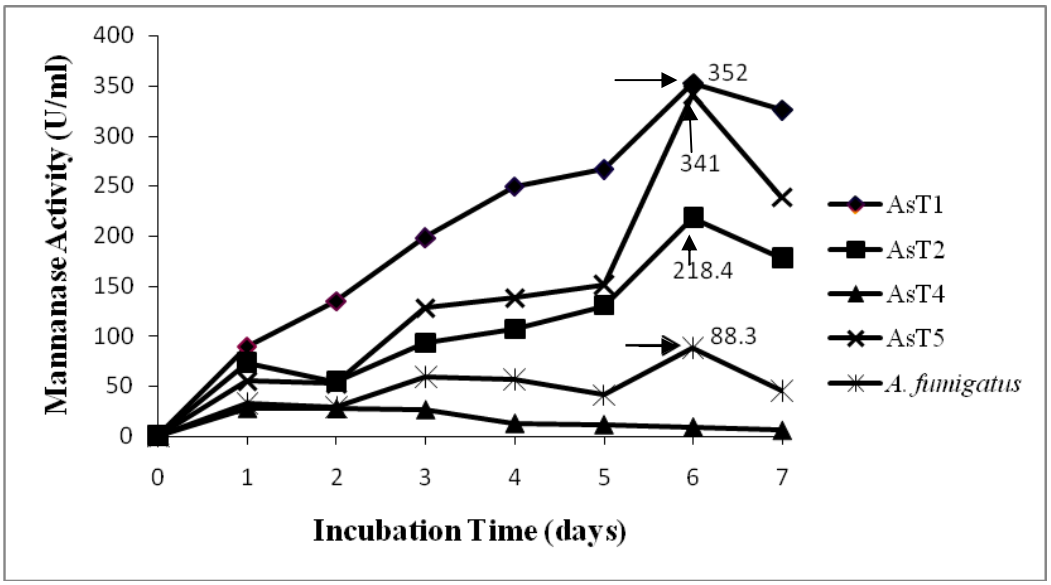


Figure 3.1 Comparison of mannanase production of *A. sojae* transformants and *A. fumigatus* IMI 385708 in modified YpSs medium with 2% glucose (*A. sojae*) and 2% LBG (*A. fumigatus*), at 30 ºC, 155 rpm.

According to the definition of mannanase activity; one unit of enzyme activity (1 U=16.7 nkat) is defined as the amount of enzyme liberating reducing sugar equivalent to 1 µmol of D-mannose in 1 min (Puchart *et al.*, 2004). Mannanase activity level of recombinant AsT1 is found as 352 U/ml = 5876 nkat/ml and the highest activities of both AsT1 and AsT5 were very close. Thus, AsT5 was also a good producer of endo-beta- mannanase (Figure 3.1).

A. sojae transformants (see Section 1.6) carrying the *afman1* gene of *A. fumigatus* IMI 385708 produced higher amounts of mannanase than *A. fumigatus* IMI 385708 produced. Since *A. fumigatus* had a total activity of three enzymes (endo-beta-mannanase production, but also alpha-galactosidase and β-mannosidase) as 88.3 U/ml and mannanase could be assumed to have one-third

of the total activity, AsT1 showed approximately 12 fold increase in mannanase activity when compared with *A. fumigatus* IMI 385708 (Table 3.1).

Table 3.1 A comparison of endo-beta-mannanase production in *A. sojae* transformants and *A. fumigatus* IMI 385708

Organism	Mannanase levels	
	nkat/ml	U/ml
AsT1	5876	352
AsT2	3647.3	218.4
AsT5	5695	341
A. fumigatus IMI 385708	1475	88.3

* The strains were grown in the medium containing 2% LBG (*A. fumigatus* IMI 385708) and 2% glucose (AsT1, AsT2, AsT5), at 30 ºC.

- All activity assays were carried out by using DNS method.

When the mannanase production of AsT1 is compared with those of other fungi in the literature, it is seen that recombinant mannanase of *A. sojae* T1 has the highest activity with at least 1.8 fold increase (Table 3.2).

Table 3.2 A comparison of endo-beta-mannanase production in some fungi and bacteria

Organism	Mannanase levels		Reference
	(nkat/ml)	(U/ml)	
Sclerotium rolfsii	2591	155.15	(Gubitz *et al.*, 1996)
S. rolfsii CBS 191.62	55	3.3	(Sachslehner *et al.*, 1998)
Aspergillus niger ATCC 46890	56	3.35	(Ademark *et al.*, 1998)
B. licheniformis NK-27	3310	198.2	(Feng *et al.*, 2003)
A. niger NCH-189	468	28	(Lin *et al.*, 2004)
A. fumigatus IMI 385708	668	40	(Puchart *et al.*, 2004)
T. lanuginosus CBS 218.34	247	15	(Puchart *et al.*, 1999)
S. Candida LMK004 (CBS 118736)	104	6.2	(Mudau *et al.*, 2006)
S. Candida LMK008	172	10.3	(Mudau *et al.*, 2006)
V. dahliae LMK006	56	3.35	(Mudau *et al.*, 2006)
B. subtilis WY34	18453	1105	(Jiang *et al.*, 2006)

* DNS method was used to determine the mannanase activities

Some recombinant mannanase activity levels in the literature are shown in Table 3.3. Mannanase activity of recombinant AsT1 (352 U/ml = 5876 nkat/ml) is at least 3 fold higher than the recombinant mannanases in the literature.

Table 3.3 Beta-mannanase activity levels measured from expression in different hosts (Setati *et al.*, 2001)

Donor strain	Gene	Expression host	Gene product name	Enzyme activity (nkat/ml)
Streptomyces lividans 1326	*manA*	*S. lividans* IAF10-164	ManA	1450
Streptomyces lividans IAF36	*manA*	*S. lividans* 1326	ManA	1917
Bacillus subtilis NM39		*E. coli* JM109	ManA	81.7
Caldocellum saccharolyticum	*manA*	*E. coli* RR28	ManA	1.33 *
Trichoderma reesei RutC30	*man1*	*S. cerevisiae* DBY746	ManA	0.22 *
Aspergillus aculeatus KSM510	*man1*	*A. oryzae*	Man1	nd
Aspergillus aculeatus Iizuka	*man1*	*S. cerevisiae* Y294	Man5A	521 *(ADH2p)*
Aspergillus aculeatus Iizuka	*man1*	*S. cerevisiae* Y294	Man5A	370 *(PGK1p)*

* Plate assay (congo red dye) was used instead of the standard DNS method.

A. sojae transformants had better growth rate than *A. fumigatus* confirming that *Aspergillus* species are promising hosts for the production of recombinant proteins due to their desirable growth characteristics (Ward *et al.*, 2006). As it is

seen from Figure 3.2, AsT1 showed the best growth rate among the other *A. sojae* transformants on 6th day of cultivation and the growth of *A. fumigatus* was not as high as the growth of transformants under the same conditions (Figure 3.2).

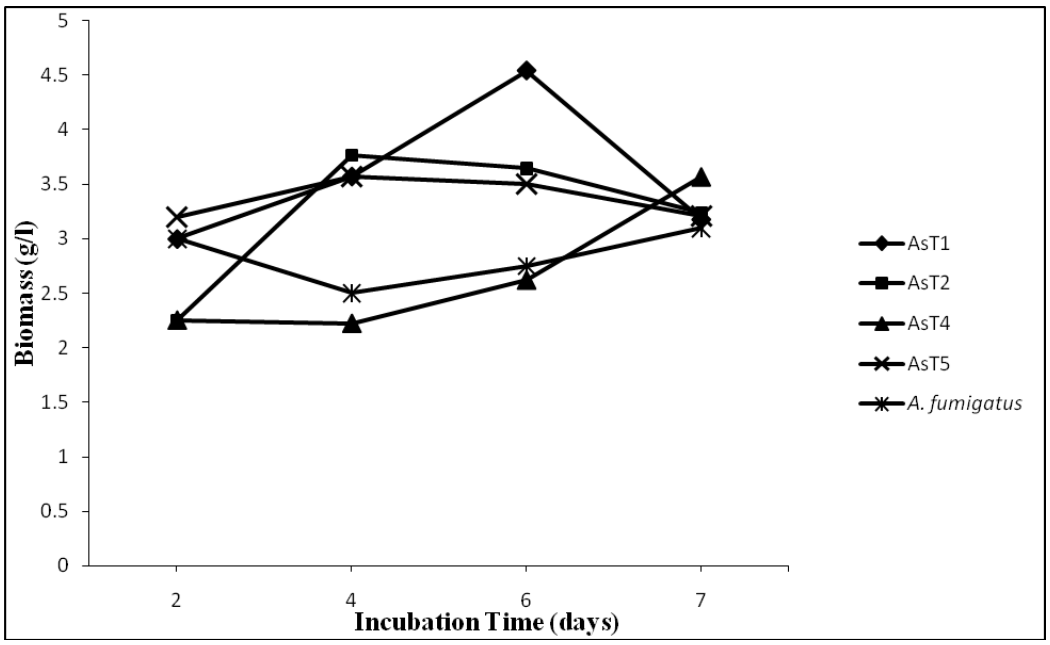


Figure 3.2 Growth of AsT1, AsT2, AsT4, AsT5 and *A. fumigatus* IMI 385708 on YpSs medium (Appendix B) containing 2% glucose (for *A. sojae* transformants) and 2% LBG (for *A. fumigatus*) as carbon source at 30 ºC, 155 rpm.

When mannanase productions were expressed as U/mg biomass, again *Aspergillus sojae* transformants showed higher activities than *A. fumigatus* (Figure 3.3).

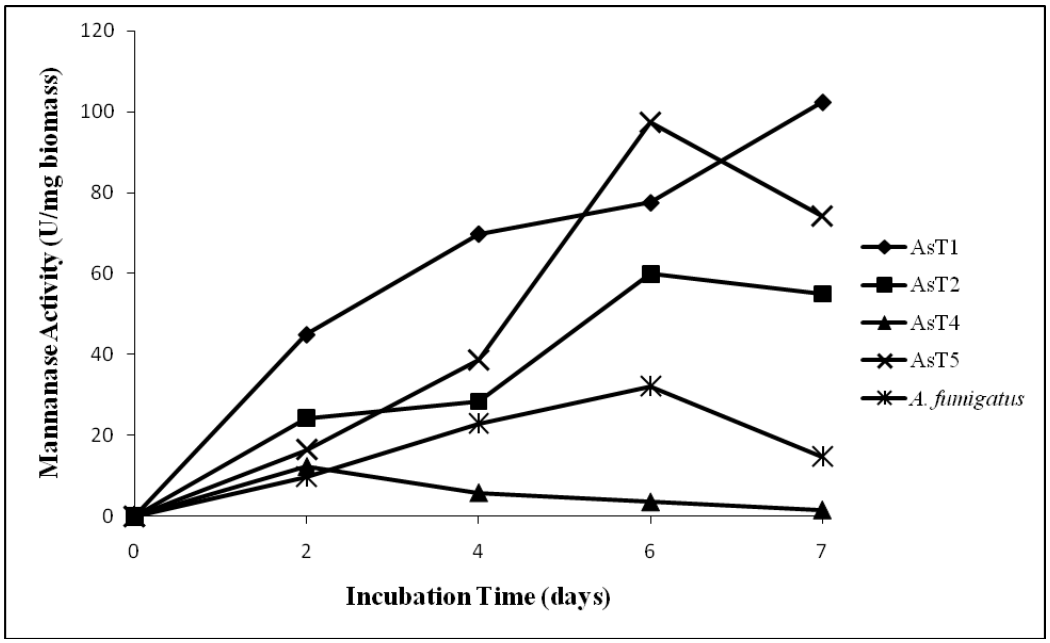


Figure 3.3 Comparison of mannanase production of *A. sojae* transformants and *A. fumigatus* IMI 385708 in terms of U/ mg biomass.

3.2.2 Comparison of Mannanase Production by using Guar Gum as the Substrate

Activities of *A.sojae* transformants were also compared by using guar gum as the substrate in enzyme assays. Accordingly, the highest production of mannanase was achieved on the 5th day of cultivation as 104.3 U/ml, 100 U/ml and 52.5 U/ml for AsT1, AsT2 and AsT5, respectively. Again the best results were obtained from AsT1.

It was seen that the mannanase activities measured by using guar gum are less than the activities measured by using locust-bean gum. This reveals that guar gum, which is a polysaccharide with higher degree of mannan backbone

galactosylation (Gal/Man ratio of 38:62) (Mc Cleary, 1979) was depolymerized with about less efficiency than locust bean gum with a less degree of mannan backbone galactosylation (Gal/Man ratio of 1:4) (Figure 3.4).

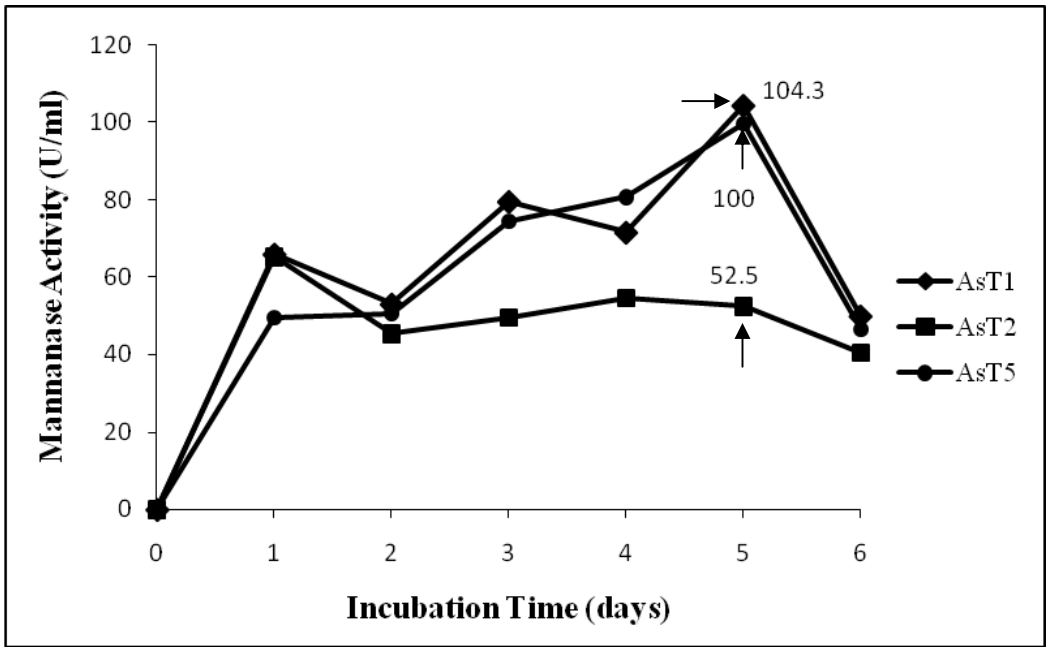


Figure 3.4 Mannanase activity comparison of *A. sojae* transformants on guar gum as the substrate.

3.2.3 Effect of Glucose Concentration on Mannanase Production

After AsT1 was determined as the best mannanase producing tranformant, optimization studies were carried out on this transformant.

The effect of different concentrations of glucose on mannanase production was investigated by adding different amounts of glucose into the growth medium. For this purpose, the fungus was grown in a shaker incubator (155 rev/min) at 30 ºC for 7 days.

AsT1 was grown in 1250 ml modified YpSs growth media containing 4 different concentrations of glucose (1%, 3%, 4% and 5%, w/v), and enzyme production in each medium was determined. As a result, the highest level of mannanase production was obtained in the medium containing 5% glucose. However the difference in mannanase activities obtained from 3%, 4% and 5% (w/v) were insignificant (Figure 3.5).

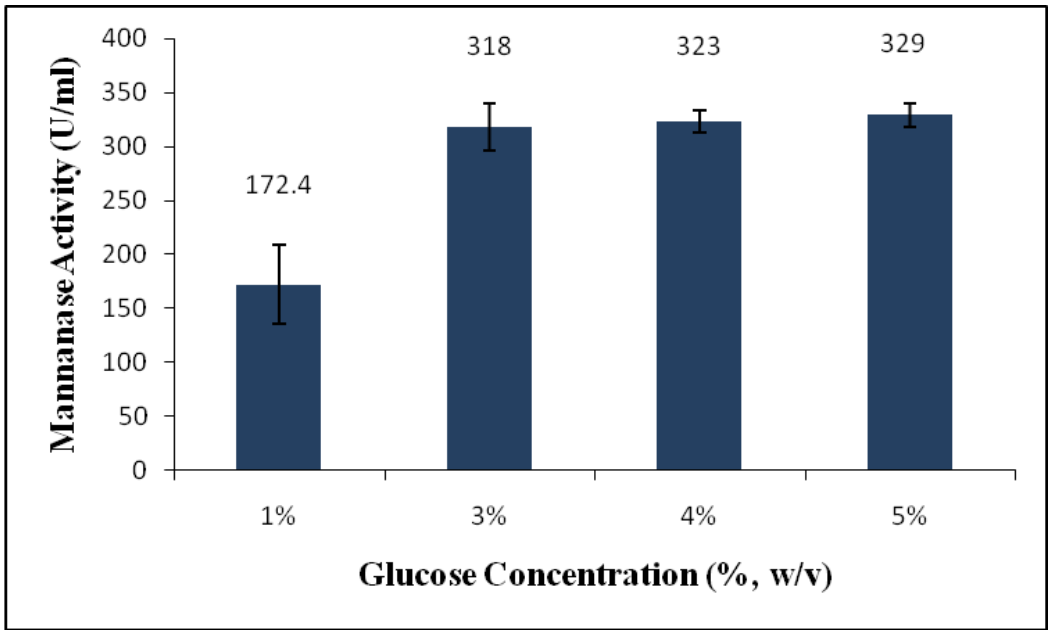


Figure 3.5 Mannanase activity at different glucose concentrations.

3.2.4 Effect of Alternative Carbon Sources on the Production of Mannanase Enzyme

In order to investigate the effect of alternative carbon sources on mannanase production by AsT1, 2% glucose in 1250 ml growth medium (Appendix B) was replaced with 2% maltose, 4% sugar beet molasses or 2% sucrose. Sugar beet molasses was added as 4% (g/ml) to the medium since it consists approximately 50% sucrose (http://en.wikipedia.org/wiki/Molasses; Pekin, 1983). Incubation was then carried out at 30 ºC for 10 days, and mannanase activities in culture supernatants were compared (Figure 3.6).

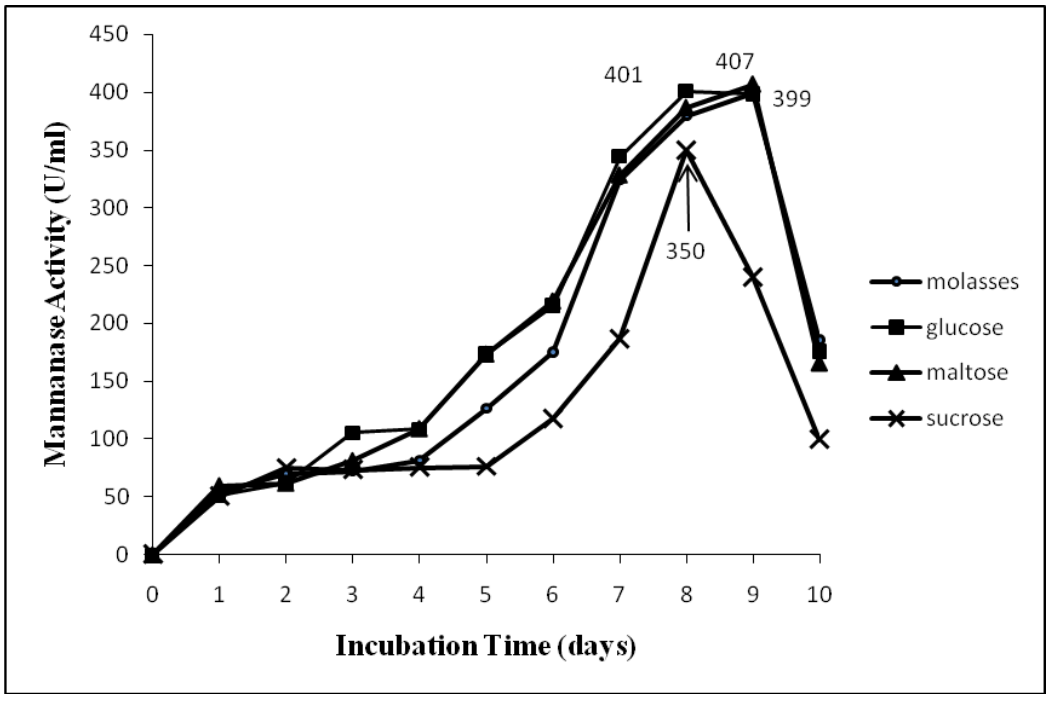


Figure 3.6 Mannanase activity of AsT1 in the presence of different carbon sources.

Maximum mannanase activities determined in the presence of different carbon sources are shown in Table 3.4.

Table 3.4 Maximum mannanase activity in the presence of different carbon sources

Type of carbon source	Maximum mannanase activity (U/ml)	Time of incubation (day)
Sugar beet molasses	399 ± 16.13	9
Glucose	401 ± 20.4	8
Maltose	407 ± 15.9	9
Sucrose	350 ± 9.9	8

Statistical analysis was performed for the interpretation of the results. Analysis of variance (ANOVA) results are shown in Appendix D. According to the results of the statistical analysis carried out between different carbon sources considering the activities on 8^{th} day of cultivation, p value was found as 0.203 showing that there was no a significant difference ($P > 0.05$) between the activities produced by the fungus grown in the media containing different carbon sources. Also, the multiple comparison tests (Tukey test) showed whether there is a significant difference between the carbon sources or not in a pairwise manner.

As a result, sugar beet molasses was found as the best carbon source tested because it is the most economical compared to pure carbohydrates. However, others may also be preferred if the impurities in the sugar beet molasses create a problem in downstream processing. Molasses is the main by-product of the sugar

industry, and is composed of inorganic and organic nutrients and vitamins as well as carbohydrates. Its nutritional value is beneficial for microorganisms, and molasses is used as raw material in distillery for alcohol production. However the effluents from molasses based distilleries contain large amounts of dark brown coloured molasses spent wash (MSW) which is a waste of alcoholic distillation. It contains high capacity of BOD, COD and dark pigment of melanoidin. Therefore, utilization of molasses in fermentations is advantageous because it consumes in a waste product for the production of a value-added product (Pant and Adholeya, 2007).

Also the growth of fungus AsT1 in the medium containing molasses was observed as the fittest growth, although the fungus showed the highest growth in both glucose and maltose containing media on the 6^{th} day of cultivation (Figure 3.7).

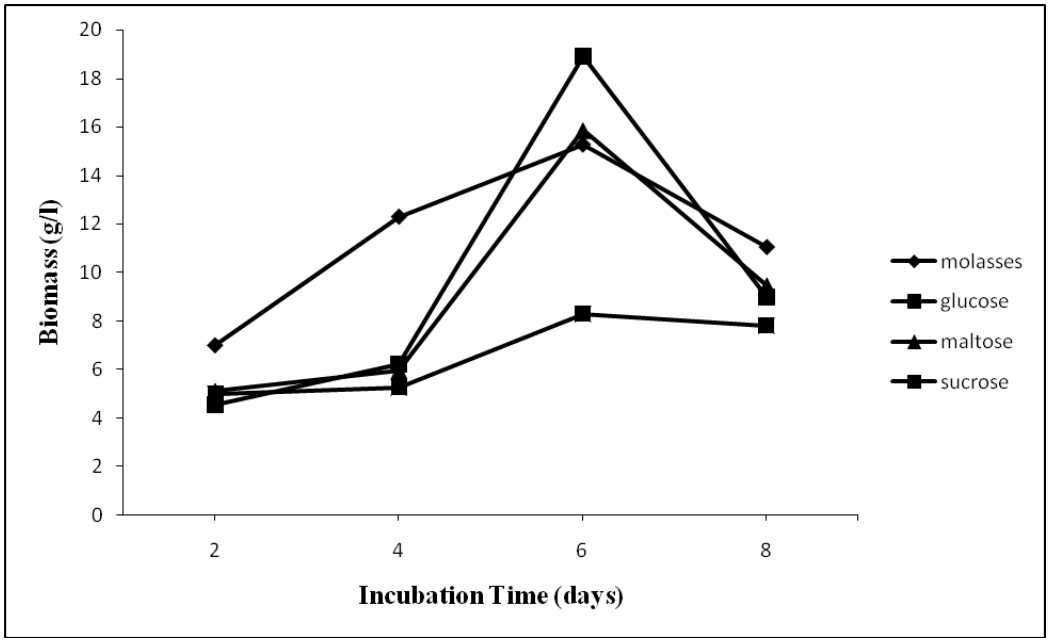


Figure 3.7 Effect of different carbon sources on the generation of biomass of AsT1.

3.2.5 Effect of Organic and Inorganic Nitrogen Sources on the Production of Mannanase Enzyme

In order to investigate the effect of different nitrogen sources, yeast extract in the YpSs medium (Appendix B) was replaced with equal amounts (0.4%, w/v) of $(NH_4)_2SO_4$, NH_4NO_3 and beef extract, and incubation was performed at 30 ºC for 9 days in a shaker incubator (155 rev/min).

In Figure 3.8, it is seen that beef extract is as the best nitrogen source among the others. However, beef extract is also a very expensive nitrogen source like the yeast extract. So NH_4NO_3, a cheap inorganic nitrogen source was chosen as a good nitrogen source for further use in optimization studies**.**

Statistical analysis was also performed for the interpretation of the results. Analysis of variance (ANOVA) results are shown in Appendix D. According to the results of the statistical analysis, p value was found as 0.002 showing that there is a significant difference ($P < 0.05$) between the activities produced by the fungus grown in the media containing different nitrogen sources. The multiple comparison test (Tukey test) was performed in order to find the significant difference between the N-sources. The results showed that the activities produced by the fungus grown in the media containing $(NH_4)_2SO_4$ showed this significant difference and the others did not show a significant difference from each other since their results were very close (Appendix D).

AsT1 grew best in medium containing yeast extract, but the biomass value of the fungus in the medium containing beef extract reached almost the same value as yeast extract on the 8^{th} day of growth (Figure 3.9). Despite this condition, AsT1 produced higher levels of mannanase in beef extract containing medium than in yeast extract containing medium (Figure 3.8). AsT1 did not produce high levels of mannanase in the medium containing $(NH_4)_2SO_4$ as nitrogen source although the growth of AsT1 was very similar in media containing NH_4NO_3 and $(NH_4)_2SO_4$ (Figure 3.9).

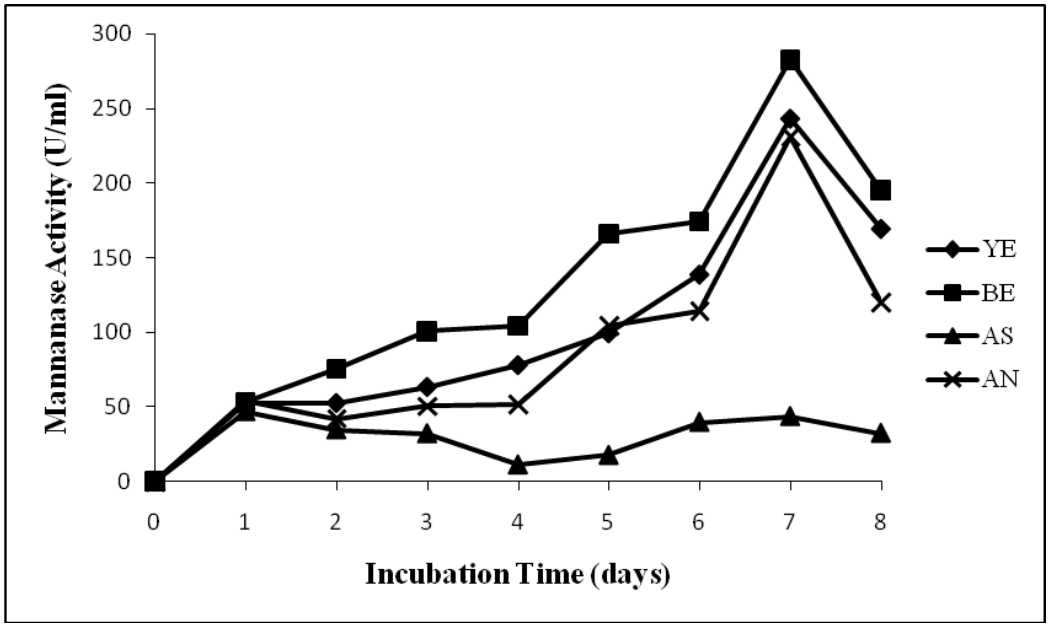


Figure 3.8 Mannanase activity in culture supernatants of AsT1 grown on different nitrogen sources; 0.4%, w/v of yeast extract (YE), beef extract (BE), $(NH_4)_2SO_4$ (AS) and NH_4NO_3 (AN).

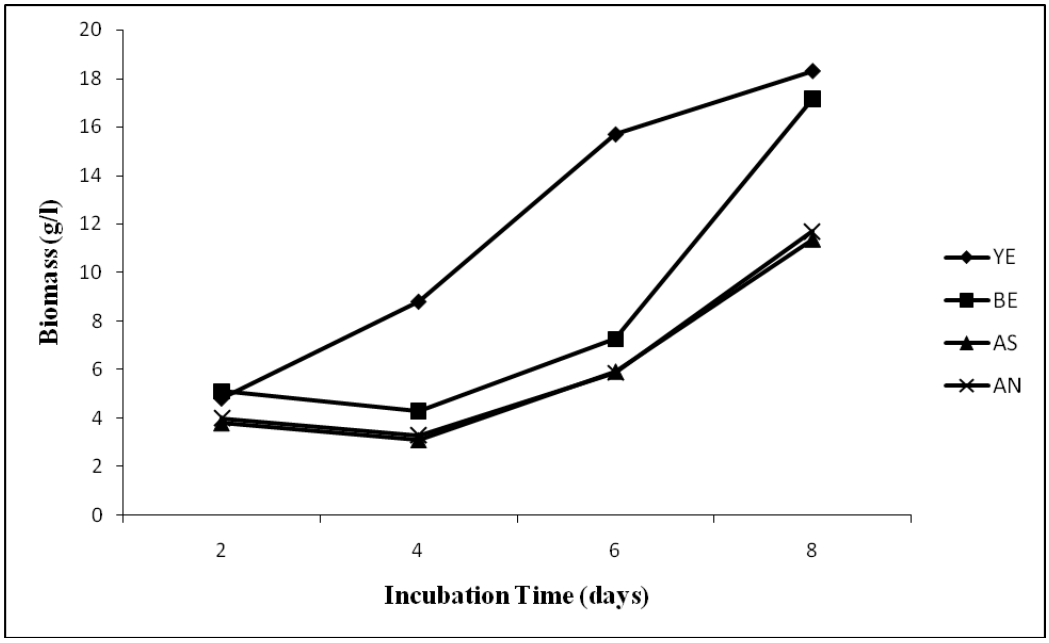


Figure 3.9 Growth of AsT1 in medium containing different nitrogen sources; 0.4%, w/v of yeast extract (YE), beef extract (BE), $(NH_4)_2SO_4$ (AS) and NH_4NO_3 (AN).

3.2.6 Effect of Number of Spores on Mannanase Production

In order to investigate the number of spores on the production of mannanase enzyme, AsT1 was cultivated in 100 ml modified YpSs medium by inoculating different number of spores ranging from $3.6*10^3$ to $3.6*10^7$. The samples were taken from the 4^{th} day of cultivation.

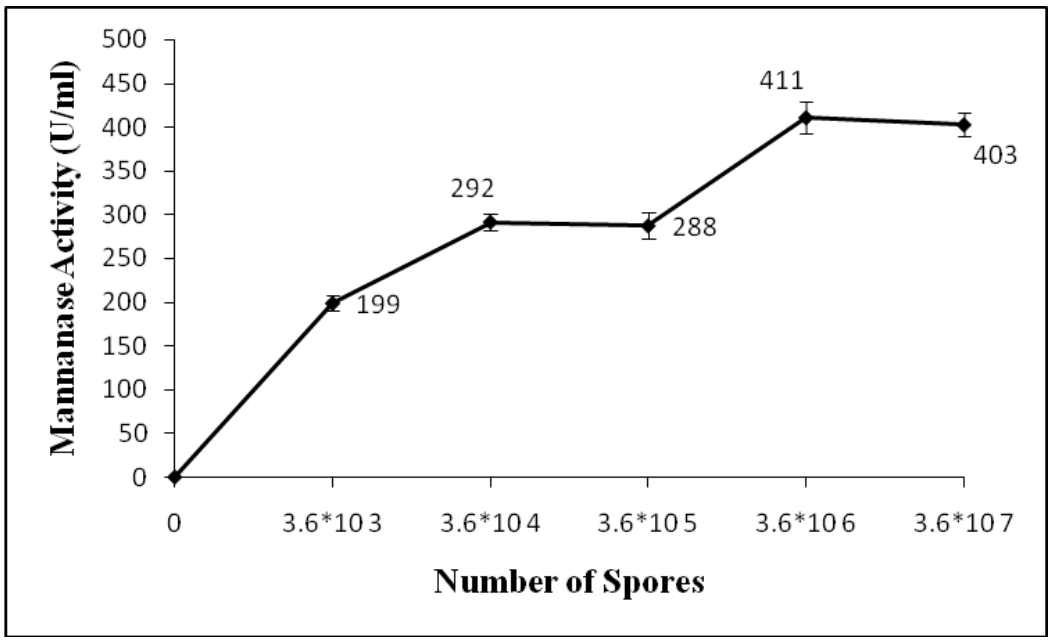


Figure 3.10 Effect of number of spores on mannanase production by AsT1 grown in the modified YpSs medium containing 2.24 % carbon, 0.43 % NH_4NO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4$, at 30 ºC, 210 rpm.

It was seen that low amounts of spore inoculation did not result in high production of mannanase. Although the highest yield was obtained when the number of $3.6*10^6$ spores was inoculated, there was no a big difference between the mannanase activities obtained when $3.6*10^6$ and $3.6*10^7$ spores were inoculated.

3.2.7 Effect of Initial Medium pH on Mannanase Production

In order to investigate effect of initial medium pH on the production of mannanase enzyme, AsT1 was cultivated in 100 ml modified YpSs medium

(Appendix B) with the initial medium pHs ranging from 3.0 to 8.0. The samples were taken from the 4th day of cultivation.

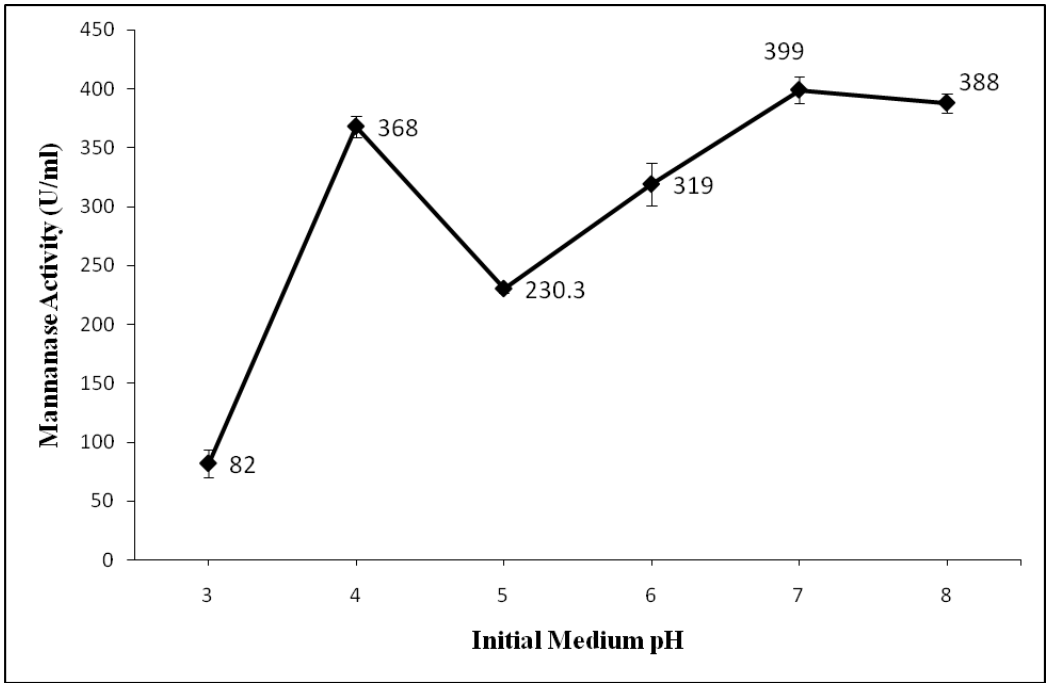


Figure 3.11 Effect of initial pH of medium on the mannanase production by AsT1 in the modified YpSs medium (2.24% sucrose), 0.43 % NH_4NO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4$, at 30 ºC, 210 rpm.

The highest mannanase activities were achieved at pHs 7.0, 8.0 and 4.0, respectively (Figure 3.11).

Production of manganese peroxidase in both recombinant *A. niger* strains carrying *mnp1* gene of *Phanerochaete chrysosporium* under the control of *gpd*A and *gla*A promoters was compared under different initial medium pHs (3.0 to

6.0). The *mnp1* gene under the control of *gpd*A promoter was highly expressed at pH 6, while the yield was very low at pH 5.0. The production yield was generally very low in the strain containing the gene under the control of *gla*A promoter, but higher yields were achieved at acidic pHs 3.0 and 4.0 (Figure 3.12). In the same study, production of human interleukin (IL-6) in recombinant *A. niger* carrying human interleukin (*hil6)* gene under the control of *A. nidulans gpd*A promoter, was the highest at initial medium pH 7.0 (Punt *et al*., 2002).

In this thesis, it was found that recombinant *A. sojae* carrying *man1* gene of *A. fumigatus* under the control of *gpd*A promoter showed the highest mannanase activity at initial medium pH of 7.0.

It can be concluded that there may be a difference in expression profiles of the two promoters at different medium pHs. The pH-dependent *gla*A promoter controls the highest level of gene expression under the acidic conditions. Although *gpd*A promoter provides a constitutive gene expression, higher protein production occurs under the conditions having a pH close to neutral.

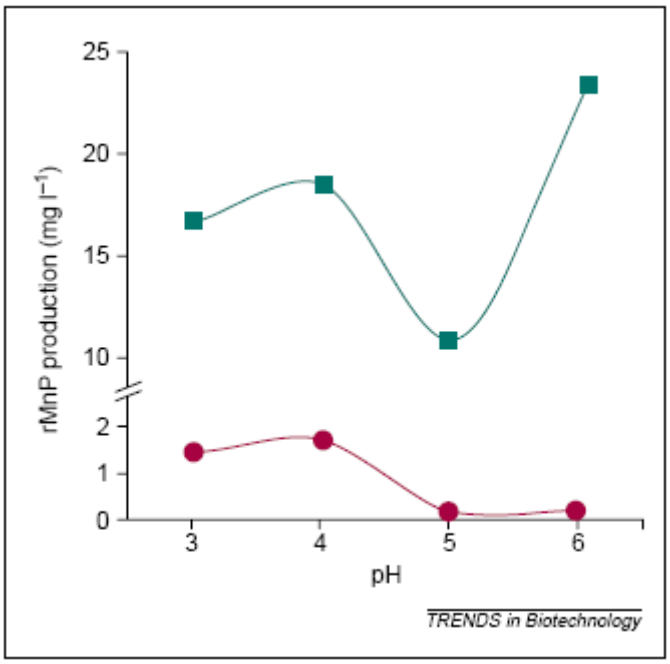


Figure 3.12 Production of manganase peroxidase (rMnP) in controlled batch fermentations at different pHs. Circles represent strain MGG029pMnp1.I#25 (*A. niger mng1* gene under the control of *gla*A promoter); squares represent strain MGG029pgpdMnP1.I#13 (*A. niger mng1* gene under the control of *gpd*A promoter) (Punt *et al.*, 2002).

3.3 Optimization of Growth Conditions for Mannanase Production by Response Surface Methodology (RSM)

RSM was used to investigate the influence of carbon and nitrogen sources, and agitation speed on the production of mannanase. Optimization studies were carried out by the following steps:

1. Preliminary experiments were carried out considering one-factor-at-a-time method to investigate the effects of variables of medium composition (carbon and nitrogen) and environmental factors (agitation speed) on mannanase production. In the second step, the concentration of the medium components and rate of the agitation were optimized using Response Surface Methodology.

2. For the optimization study, a Box–Behnken factorial design with three factors and three levels including six replicates at the centre point was used for fitting a second order response surface. A total of 30 runs were carried out to provide two replicates.

Table 3.5 gives the factors and their levels. The highest and lowest levels of the range for each variable were coded as plus and minus one, respectively, and the center point of the range was coded as zero. Table 3.6 describes the experimental design. This methodology allows the formulation of a second order equation that describes the process. A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response (mannanase activity) measured to the independent variables (M, N, agitation). A second order polynomial equation for a three factor system is:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

(Equation 3.1)

where Y is the predicted response, β_0 intercept, β_1, β_2, β_3 linear coefficients, β_{11}, β_{22}, β_{33} squared coefficients, β_{12}, β_{13}, β_{23} interaction coefficients and A, B, C, A^2, B^2, C^2, AB, AC, BC are levels of the independent variables. The response surface curves were obtained using the 'MINITAB 13' software for determining the optimum levels of the variables for maximal production of mannanase.

3.3.1 Preliminary Experiments (Selection of factor levels)

Medium optimization by the one-factor-at-a-time method involves changing one independent variable (molasses, NH_4NO_3 or agitation speed) while fixing the others at certain levels. This method was applied to find out the lowest and highest levels of the factors which would be used in the Box-Behnken design of the optimization study.

3.3.1.1 Effect of Different Carbon Concentrations

In order to determine the effect of total carbon in molasses on mannanase production, total sugar content of sugar beet molasses, that was used in this study, was determined by HPLC Analysis in the Central Laboratory of METU.

As a result of HPLC analysis, total sugar content of molasses was found as 3.2 mg in 10 mg/ ml solution of molasses (32%, w/w).

To determine the effect of different amounts of molasses on mannanase production, 1%, 2%, 4%, 6%, 8% and 10% molasses (w/v) containing 0.32%, 0.64%, 1.28%, 1.92%, 2.56%, 3.2% (w/v) sucrose contents respectively, were added separately to the 100 ml of modified YpSs media (Appendix B) and the fungus AsT1 was grown in a shaker incubator (155 rev/min) at 30 ºC for 7 days. The mannanase activities were compared in the media containing different amounts of molasses (Figure 3.13).

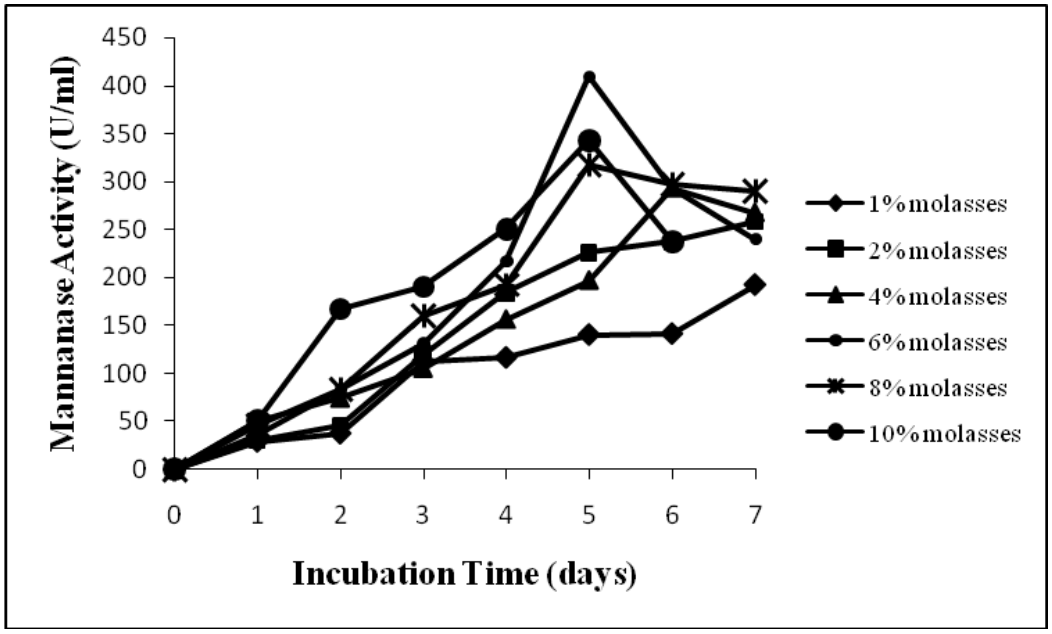


Figure 3.13 Mannanase production in the presence of different molasses concentrations (containing 32% (w/v) sucrose).

As it is seen from the graph, the highest amount of mannanase activity was obtained in the medium containing 1.92% (w/v) sucrose (6%, (w/v) molasses) and the lowest enzyme activity was seen in the medium containing lowest concentration of molasses (1%, w/v).

For the optimization studies, the safe range of the carbon level should be chosen according to the graph. In order to find out the safe range of the concentration level of sugar beet molasses, (1%) and (8%) molasses levels were found to be more efficient, including the point (6% molasses) in which highest level of mannanase obtained. The low level of molasses (0.1%) and high level of molasses (8%) were chosen in order to use in Box-Behnken experimental design.

3.3.1.2 Effect of Different Nitrogen Concentrations

NH_4NO_3 was found to be an economical and good nitrogen source for AsT1 growth and mannanase production (Section 3.2.5). To determine the effect of different amounts of NH_4NO_3 on mannanase production, 0.1%, 0.2%, 0.3%, 0.4% and 0.5% (w/v) NH_4NO_3 were added into the medium (Appendix B).

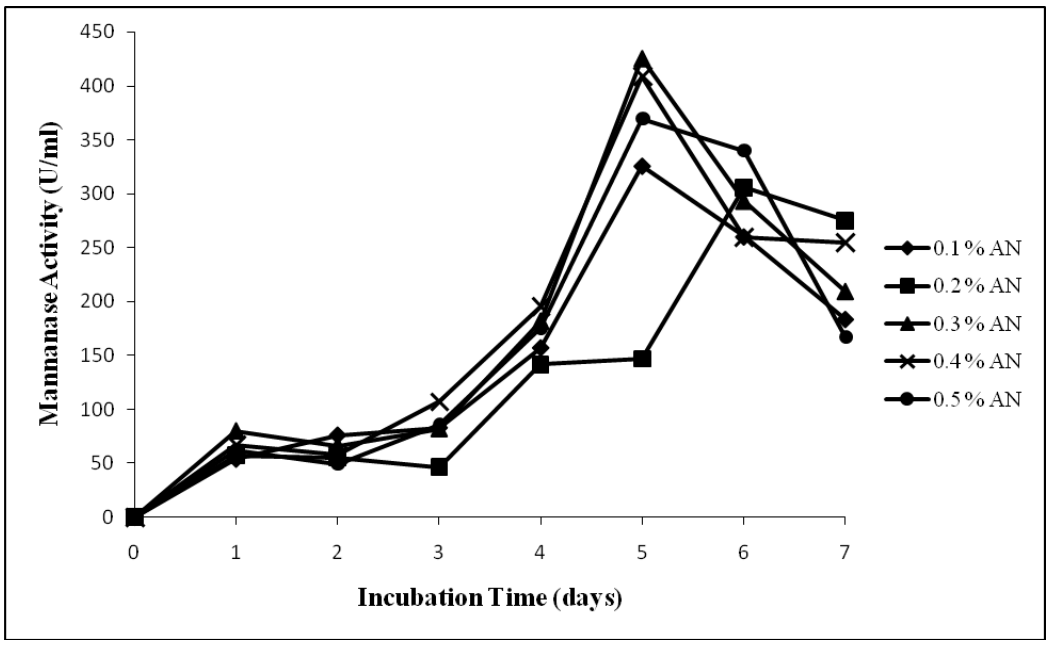


Figure 3.14 Mannanase activity in the culture medium of AsT1 grown under various NH_4NO_3 concentrations. AN: Ammonium nitrate.

From Figure 3.14, it is seen that highest mannanase activity was obtained in the growth medium containing 0.3% NH_4NO_3 and the lowest level of enyzme was

obtained in the medium containing 0.1% NH_4NO_3. It was observed that the mannanase activities were very close to each other in the media containing 0.3% NH_4NO_3, 0.4% NH_4NO_3 and 0.5% NH_4NO_3.

For the Box-Behnken design of optimization study, the low and high levels of ammonium nitrate were determined as 0.1% and 0.5 % (w/v), respectively. The safe range of ammonium nitrate level was chosen in order to include the point which resulted in the highest enzyme activity between the low and high levels.

3.3.1.3 Effect of Agitation Speed

Lastly, in many fungal fermentations, a high agitation rate is necessary to provide adequate mixing of the medium contents, and for efficient mass transfer for oxygen and nutrients. However, mechanical forces can cause mycelial damages. Thus, the agitation rate is limited to a range that avoids exerting high shear stresses on fungal mycelia (Feng *et al.*, 2003; Wang *et al.*, 2005). Thus the appropriate range of agitation speed was determined as 100-250 rpm. It was technically not possible to go over 250 rpm.

According to the results of the statistical analysis (ANOVA and Tukey Test), there was a significant difference between the highest value of agitation speed (250 rpm) and the others (Appendix D).

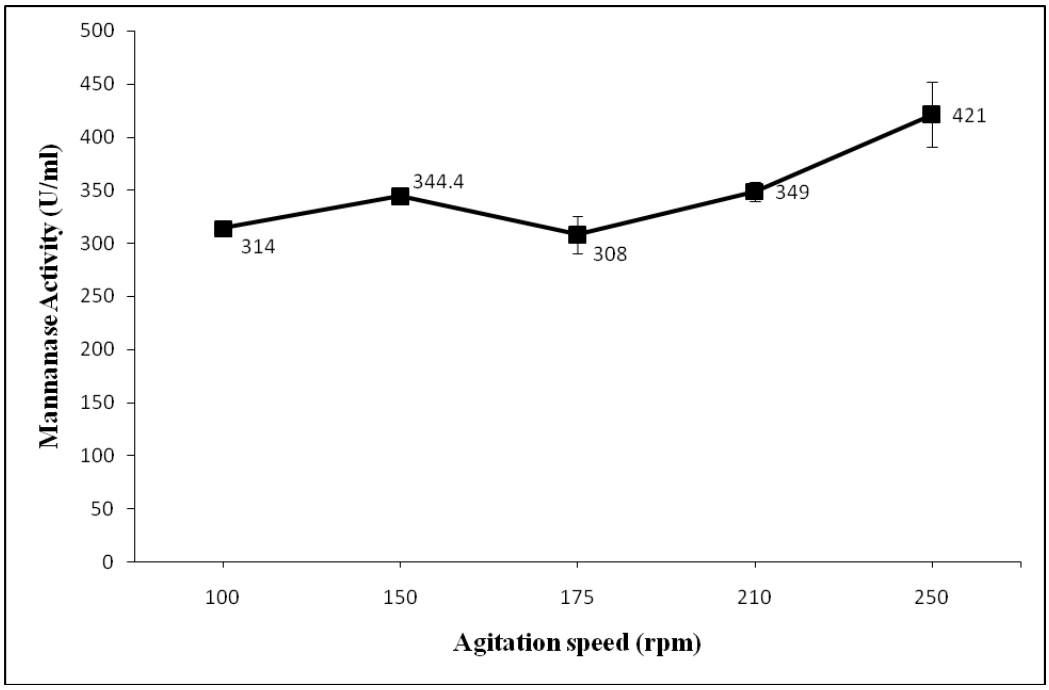


Figure 3.15 Mannanase activities in the modified YpSs medium containing (w/v), 2.24% sucrose, 0.43% NH_4NO_3 at 30 ºC, at different agitation rates.

Table 3.5 Factors and experimental design levels for response surface method selected according to one-factor-at-a-time approach

Factors	**Coded Symbols**	**Levels**		
		-1	0	+1
Molasses concentration % (w/v)	M	1	4.5	8
NH_4NO_3 concentration % (w/v)	N	0.1	0.3	0.5
Agitation speed (rpm)	A	100	175	250

3.3.2 RSM Analysis

Optimization of the growth conditions for *A. sojae* T1 for maximum mannanase production was studied by using Box-Behnken Design under the Response Surface Methodology. Growth period and temperature were kept constant at 4 days and 30 ºC, respectively.

As the factor levels for molasses and NH_4NO_3 contents of the medium and agitation speed were selected in Section 3.2.1, a three factor-two replicated Box-Behnken design was employed (Box and Behnken, 1960). In the design of the experiment, three levels (low-center-high points) of the factors were determined according to the preliminary experiments and the designed data was put into the

statistical program (MINITAB Release 13) and a total of 30 runs of experiments were generated by the program (Table 3.6). The main advantage of the design is that if the low and high levels of the factors are foreseen well, it enables one to study three variables simultaneously in a single experiment.

After measuring the activities for each run on the fourth day of incubation, the activity (response) data was put in the preformed worksheet and data analysis was performed. The design matrix and the fitness of the each term were analyzed by means of ANOVA. The regression coefficients and the appropriate model equation for mannanase activity were generated. According to the generated model equation, the predicted response (mannanase activity) values were calculated and compared with the experimental response values.

Table 3.6 Results of a three factor-two replicated Box-Behnken experimental design and mannanase production by AsT1 in a total of 30 runs of experiments.

RUN	INDEPENDENT VARIABLES			RESPONSE
	$C_{molasses}$ (%, w/v)	C_{NH4NO3} (%, w/v)	$A_{agitation\ speed}$ (rev/min)	Mannanase Activity (U/ml)
1	8	0.1	175	342.955
2	4.5	0.1	100	249.962
3	1	0.3	250	217.777
4	1	0.1	175	195.196
5	4.5	0.3	175	325.019
6	4.5	0.5	250	348.132
7	8	0.3	250	354.678
8	4.5	0.3	175	369.325
9	8	0.5	175	303.659
10	8	0.3	100	329.378
11	1	0.3	100	113.533
12	4.5	0.3	175	339.564
13	1	0.5	175	232.262
14	4.5	0.5	100	255.451
15	4.5	0.1	250	309.372
16	8	0.1	175	331.974
17	4.5	0.1	100	270.370
18	1	0.3	250	199.402
19	1	0.1	175	176.669
20	4.5	0.3	175	313.891
21	4.5	0.5	250	327.474
22	8	0.3	250	342.560
23	4.5	0.3	175	352.017
24	8	0.5	175	323.529
25	8	0.3	100	306.940
26	1	0.3	100	137.816
27	4.5	0.3	175	321.952
28	1	0.5	175	213.593
29	4.5	0.5	100	237.661
30	4.5	0.1	250	285.996

The data obtained for mannanase activity from the 30 experimental points were used for statistical analysis to optimize the medium components. The design matrix and the fitness of each term were analyzed by means of ANOVA (Table 3.7). The regression coefficients for each term are given in (Table 3.7). The second-order polynomial equation for the mannanase activity was found to be:

Mannanase activity (U/ml) = -165.1 + 75.2M + 241.056N + 2.417A – 4.4MM - 458.6NN - 0.006AA - 21.7MN - 0.05MA + 0.9NA **(Equation 3.2)**

The R^2 value 0.952 for mannanase production, points out the accuracy of the model. The ANOVA for the responses (Table 3.7) indicated that the model was significant. When expressed as a percentage, R^2 is interpreted as the percent variability in the response explained by the statistical model. This implies that the sample variation of 95.2 % for mannanase production, was attributed to the independent variables. This ensured a satisfactory adjustment of the quadratic model to the experimental data.

The adjusted R^2 corrects the R^2 values for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2 (Kaushik *et al.*, 2006). In this study, the adjusted R^2 value (93.1 %) is very close to the R^2 (95.2 %) value. The coefficients of regression equation were calculated by using statistical software MINITAB 13.

The 'Lack of fit P-value' 0.075 > 0.05 for mannanase activity implied that the lack of fit is insignificant and the model is adequate (Table 3.7).

Table 3.7 Response surface regression

Model coefficients estimated by multiple linear regression for activity

Term	Coef	SE Coef	T	P < 0.05
Constant	-165.1	50.954	-3.240	0.004
molasses	75.2	9.566	10.481	0.000
NH_4NO_3	241.1	133.445	1.806	0.086
agitation speed	2.4	0.452	5.347	0.000
molasses*molasses	-4.4	0.968	-8.042	0.000
NH_4NO_3* NH_4NO_3	-458.6	166.738	-2.751	0.012
agitation*agitation	-0.006	0.001	-4.958	0.000
molasses* NH_4NO_3	-21.7	12.205	-2.375	0.028
molasses*agitation	-0.05	0.033	-2.047	0.054
NH_4NO_3*agitation	0.9	0.427	2.096	0.049

S = 18.12 R-Sq = 95.2% R-Sq (adj) = 93.1%

Analysis of Variance for activity (ANOVA)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	130864	130864	14540.4	44.27	0.000
Linear	3	97624	39931	13310.3	40.52	0.000
Square	3	28568	28568	9522.7	28.99	0.000
Interaction	3	4671	4671	1557.2	4.74	0.012
Residual Error	20	6570	6570	328.5		
Lack-of-Fit	3	2143	2143	714.4	2.74	0.075
Pure Error	17	4426	4426	260.4		
Total	29	137434				

When the nitrogen and the two interaction terms are considered (Table 3.7), their effects seemed to be insignificant. However, according to the preliminary experiments nitrogen source was found to be effective on enzyme activity, and also the p-values of these terms are not so far from the confidence interval ($p < 0.05$) thus can be included in the model. Finally, coefficients of all the terms were integrated into the model equation in order to predict the response (Equation 3.2).

The plot of experimental values of mannanase activity versus those calculated from the equation (called as predicted) indicated a good correlation for mannanase activity with a coefficient of 0.951 (Figure 3.16).

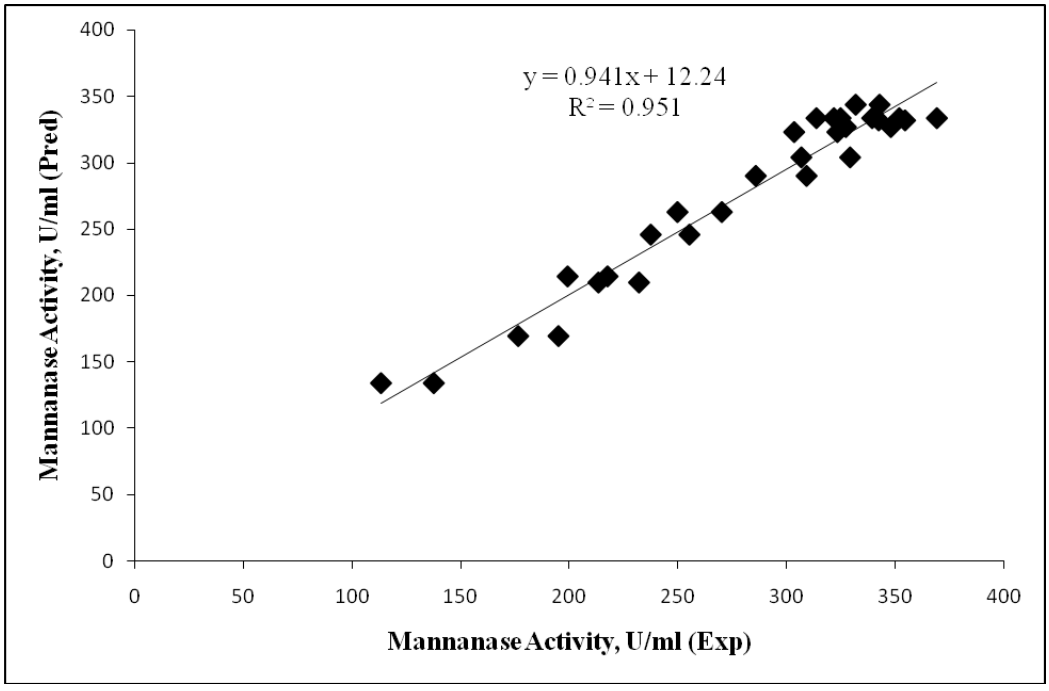


Figure 3.16 Correlation of predicted and experimental mannanase activity.

The contour plots and surface plots (variables vs response) of the designed experiment were created by using the program MINITAB 13. The effects of the independent variables on mannanase activity were represented via response surface plots (Figure 3.17, 3.18 and 3.19).

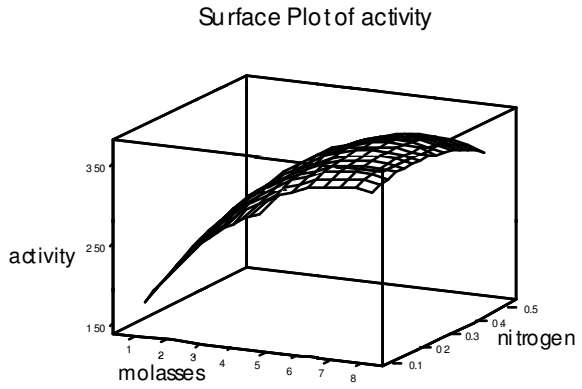


Figure 3.17 Response surface plot for molasses and nitrogen (NH_4NO_3) interaction at 175 rpm constant level of agitation speed.

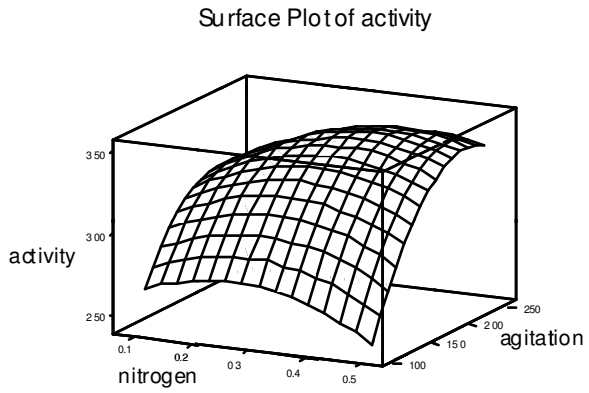


Figure 3.18 Response surface plot for nitrogen (NH_4NO_3) and agitation speed interaction holding the midlevel of molasses constant (4.5%, w/v).

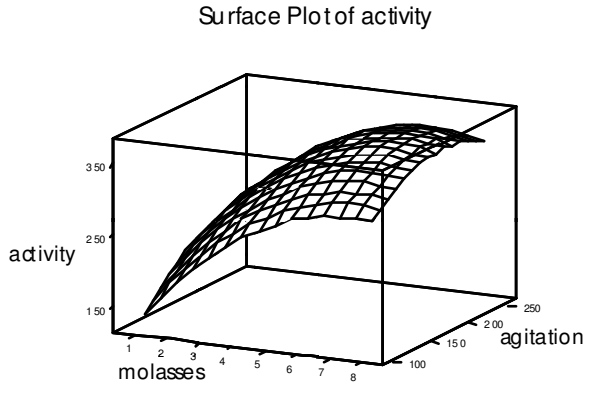


Figure 3.19 Response surface plot for molasses and agitation speed interaction holding the midlevel of nitrogen (NH_4NO_3) constant (0.3%, w/v).

The interaction of carbon, nitrogen concentrations and the agitation rate can also be observed from the contour plots (Figures 3.20, 3.21 and 3.22). In a contour plot, each contour represents a specific value for the height of the surface.

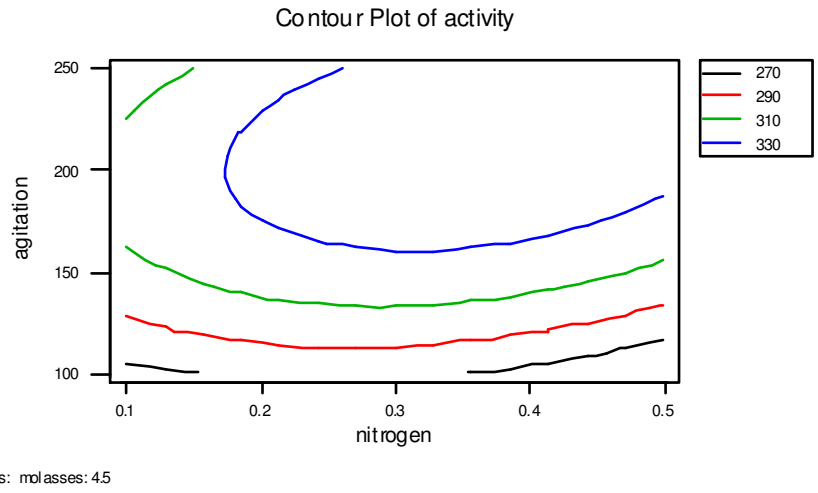


Figure 3.20 Contour plot for nitrogen (NH_4NO_3) and agitation speed interaction holding the midlevel of molasses constant (4.5%, w/v).

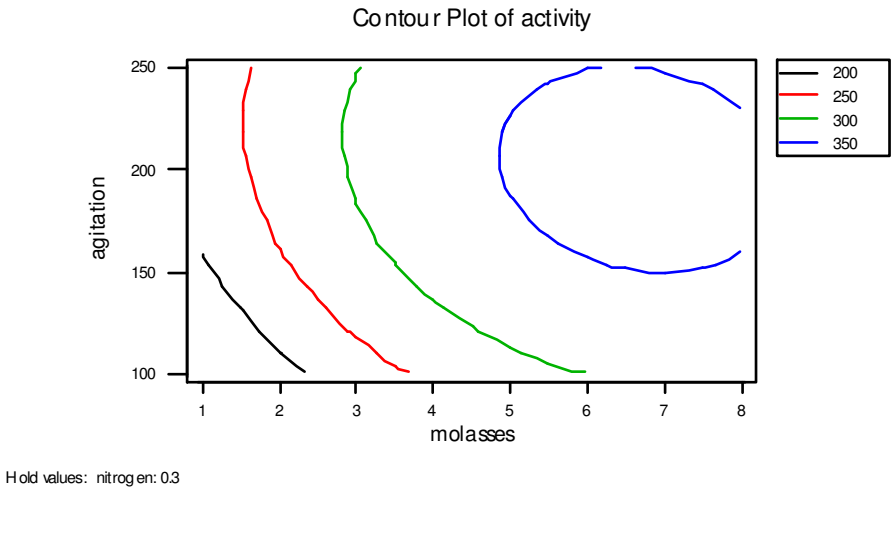


Figure 3.21 Contour plot for molasses and agitation speed interaction holding the midlevel of nitrogen (NH_4NO_3) constant (0.3%, w/v).

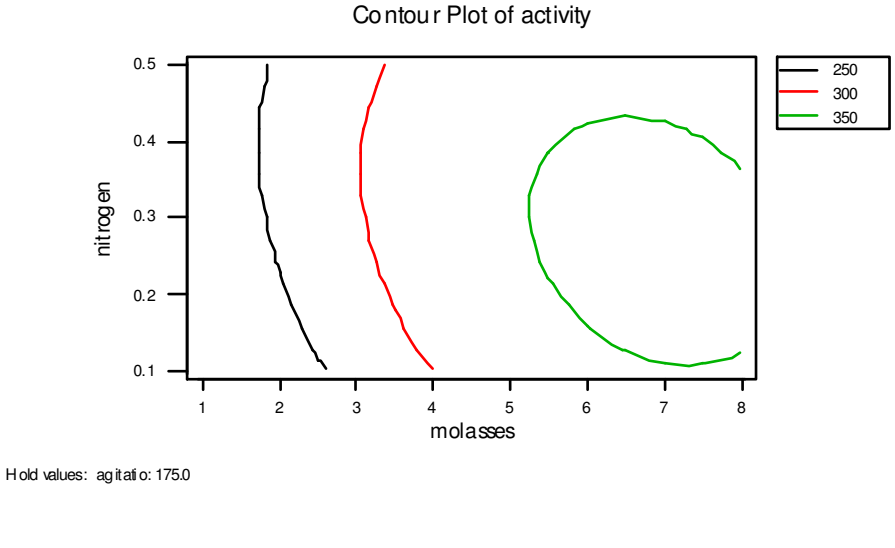


Figure 3.22 Contour plot for nitrogen (NH_4NO_3) and molasses interaction holding the midlevel of agitation speed constant (175 rpm).

3.3.2.1 Determination of Optimum Conditions

Optimum growth conditions were determined by using the response optimizer menu of the program.

The minimum obtained activity and the maximum obtained activity were put into the response optimizer section of the MINITAB 13 statistical program. According to the response optimizer results, the optimum values of the factors in the designed experiment were found as 7% molasses containing 2.24% sucrose, 0.43 % nitrogen (NH_4NO_3) and 207 rpm agitation speed in order to have the maximum activity. The maximum activity value in the optimum conditions was determined as 356 U/ml with a composite desirability of 1.0000 by the response optimizer.

In section 3.2.3, glucose concentration for maximum mannanase was found as 5% (w/v) but there was no a significant difference between the activities achieved at 3%, 4% and 5% glucose concentrations. The activity did not increase so much when the concentration of glucose increased. The reason of this can be carbon catabolite repression effect of glucose, since the mechanism occurs in the presence of higher amounts of readily utilizable carbon sources like glucose (Bailey and Arst, 1975). Because sucrose in the molasses is a disaccharide, carbon catabolite repression effect can be very low, so that optimum molasses content was found 7% (w/v) containing 2.24% sucrose for maximum mannanase production.

3.3.2.2 Validation of the Designed Model

In order to validate the model, five parallel experiments under the predicted optimum conditions were carried out at 30 ºC in a shaker incubator.

The statistical model was validated with respect to mannanase production under the optimum conditions predicted by the model in 100 ml modified YpSs medium containing 7% (w/v) molasses (2.24% sucrose), 0.43% (w/v) NH_4NO_3, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4$ with initial medium pH 7.0 and grown by shaking at 207 rpm and 30 ºC for four days. 5 parallel experiments were done. The average of the activities obtained from the 5 trials was 362.92 ± 13.9 U/ml on the fourth day of incubation. Incubation was continued until the 6th day as shown in Figure 3.23.

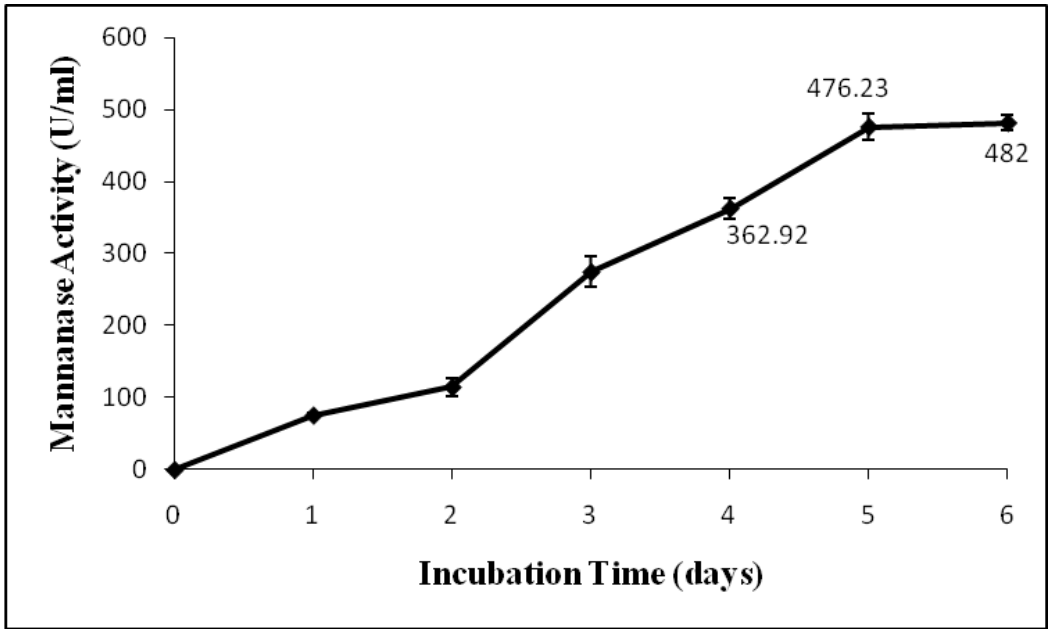


Figure 3.23 Time course of mannanase production by AsT1 under the predicted optimum conditions; modified YpSs medium (7% molasses, 0.43% NH_4NO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4$, w/v) at 30 ºC, 207 rpm.

3.4 TLC and HPLC Analysis of Hydrolysis Products of LBG

Thin-layer chromatography (TLC) was used to identify the products. Enzymatic hydrolysis of locust bean gum was carried out at 50 ºC in 50 mM Na-citrate buffer (pH 6.0). At appropriate times, aliquots (5 µl each) were removed and developed by TLC (silica gel 60 plates, 10 × 20 cm, Merck) with acetonitrile-H_20 (85:15, v/v) as a solvent system. The sugar spots were detected by spraying with 10% (v/v) H_2SO_4, and heating the plate at 100 ºC for 15 min. Mannose and the mannooligosaccharides M_2, M_3, GM_2, G_2M_5 were used as sugar standards.

The observed sugar spots were shown in Figure 3.24.

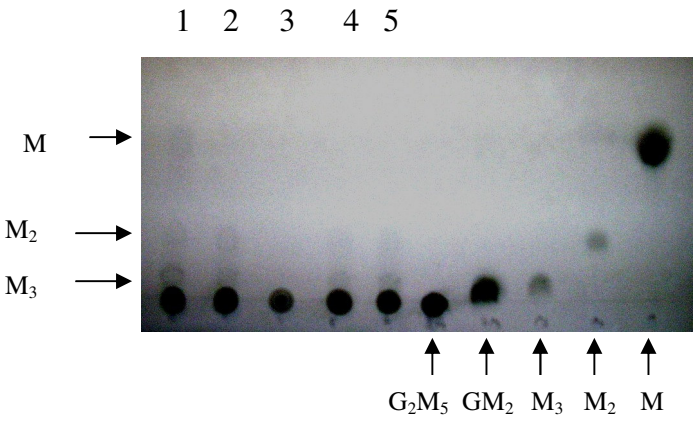


Figure 3.24 TLC Analysis of the LBG Hydrolysis Products; M, M2, M3 can be observed on the TLC plate as a result of incubation of 10 ml of (% 0,5, w/v) LBG with 2 ml crude mannanase enzyme (600 Unit) after: 1) 48 h; 2,3) 24 h; 4,5) 16 h.

In TLC diagram, 10 ml of % 0,5 w/v LBG was hydrolsed with 600 U of mannanase enzyme and after 16 hours and 24 hours (Lane 4,5,2), the products GM2 and M2 were observed on the plate. After 48 h of incubation, M, M2 M3 were observed as hydrolysis products.

The sample for HPLC analysis was prepared by incubating 900 µl of % 0,5 (w/v) locust-bean gum solution with 100 µl crude mannanase enzyme (40 Units) at 50 ºC for 48 hours. The standard mannooligosaccharides M_2, M_3, GM_2, G_2M_5 were prepared at the concentration of 10 mg/ml. The quantification of the hydrolysis products were performed by using standard curves of mannooligosaccharides (Appendix F).

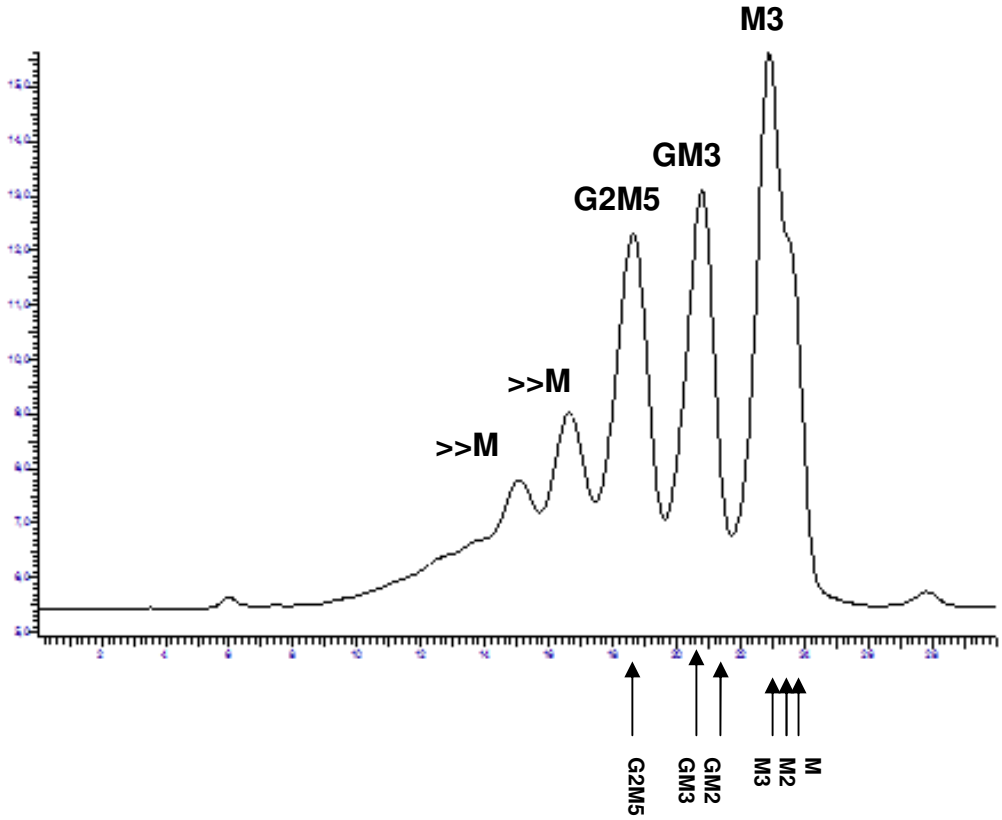


Figure 3.25 HPLC Chromatogram of the Hydrolysis Products. 900 µl of (% 0,5, w/v) locust-bean gum solution was incubated with 100 µl crude mannanase enzyme (40 Unit), 50 ºC for 48 hours.

According to the HPLC results, the major hydrolysis product was found as M3 (mannotriose) and GM3 and G2M5 were also found as the hydrolysis products. In the chromatogram, the peaks of mannose and mannobiose are integrated into the peak of mannotriose and so they could not be seperated well as a result of insufficient time of hydrolysis. After 48 h of incubation in TLC analysis,

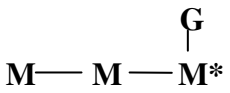
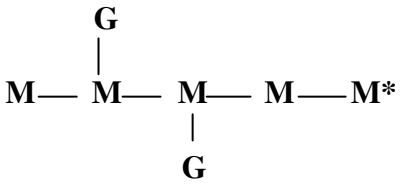
mannose and mannobiose were also detected. Although standard GM3 did not exist in the analysis, the fourth peak from the left side of the chromatogram shows the peak of GM3 since the retention time of 20.798 min does not match with the retention times of other standard sugars.

Hydrolysis of locust bean (carob) galactomannan with crude mannanase of AsT1 yielded mainly mannotriose (M3), 6^1-galactosyl-β-D-mannotriose (GM3) and 6^3,6^4-di-D-galactosyl-β-D-mannopentaose (G2M5) (Table 3.8) in accordance with data reported for the other fungal mannanases (Gubitz *et al*., 2000; McCleary *et al.,* 1983).

McCleary *et al*. (1983) studied the characterisation of the oligosaccharides produced on hydrolysis of galactomannan (carob) with β-D -mannanase from *A. niger*. 6^1-D-galactosyl-β-D-mannobiose (GM2), 6^1-D-galactosyl-β-D-mannotriose (GM3), 6^3,6^4-di-D-galactosyl-β-D-mannopentaose (G2M5) and 6^3,6^4-di-D-galactosyl-β-D-mannohexaose (G2M6) and 6^1,6^3,6^4-tri-D-galactosyl-β-D-mannopentaose (G3M5) and four nonasaccharides were characterized, but no penta- or hexa-oligosaccharides were produced.

GM3 was produced as the main product resulting from the hydrolysis of *Ceratonia siliqua* (locust bean) galactomannan using mannanases from *Schizophyllum commune* (Gubitz *et al*., 2000).

Table 3.8 The produced mannooligosaccharides and their structures after hydrolysis of locust bean gum (LBG).

Product	Rt_{prod} (min)	Rt_{stn} (min)	C_{prod} (mg/ml)	Structure
M3	22.904	22.899	1.6	**M — M — M***
GM3	20.798	a	0.8	
G2M5	18.649	18.725	0.73	
>> M	16.647	b	0.3	
>> M	15.093	b	0.2	

*Indicates the reducing terminal of the oligosaccharides.

[a] The retention time of GM3 standard is not known.

[b] Indicates the long chain oligosaccharides that have a degree of polymerization (DP) ≥ 8.

The investigate the prebiotic effects of the mannooligosaccharides, studies by using probiotic strains have been started in Ankara University.

CHAPTER 4

CONCLUSIONS

In this study, the following were concluded:

- Among the transformants of recombinant *Aspergillus sojae* ATCC11609, AsT1 was found to be the best mannanase producing transformant with the highest activity value of 352 U/ml on 6th day of cultivation. AsT5 and AsT2 followed AsT1 with mannanase activities of 341 U/ml and 218.4 U/ml, respectively.

- AsT1 had 12 fold increase in activity when compared to the mannanase activity of *A. fumigatus* IMI385708 grown at the same conditions, but on different carbon sources. The highest activity achieved was at least 3 fold higher than the other recombinant fungal mannanases in the literature (Section 3.2.1).

- The effects of different carbon sources on mannanase production were investigated and there was no statistically significant difference ($p=0.203$, $p > 0.05$) between the highest levels of mannanase activities in the medium containing maltose, glucose and molasses. Thus, molasses was chosen as the carbon source for further studies from the economical point of view.

- The effects of different nitrogen sources on mannanase production were investigated and highest level of mannanase was found in the medium

containing beef extract. However, there was no significant difference ($p=0.406$, $p > 0.05$) between the activities in beef extract and in NH_4NO_3, so NH_4NO_3 was chosen as the nitrogen source for further studies from the economical point of view.

- The effects of different initial medium pHs (3.0-8.0) on mannanase production was investigated and maximum mannanase activity was found as 399 U/ml at pH 7.0.

- The effect of inoculum number was investigated by inoculating different number of spores (3.6×10^3- 3.6×10^7) into modified YpSs medium. Maximum mannanase activity was found as 411 U/ml when 3.6×10^6 number of spores was inoculated into the medium at 4^{th} day of cultivation.

- The optimum conditions for maximum mannanase production were achieved when AsT1 was grown in the modified YpSs medium containing 7% (w/v) molasses (2.24% w/v sucrose), 0.43% (w/v) NH_4NO_3 and at 207 rpm of agitation rate by RSM.

- The validation of the model was done by performing 5 parallel experiments under the predicted optimum conditions. The average of mannanase activities achieved as the result of validation experiments (363 U/ml) was found to be very close to the predicted activity (356 U/ml) and showed a 2% increase in production. In time course of activitty analysis under the optimized medium, maximum activity was found as 482 U/ml on 6^{th} day of cultivation.Thus *c.* 1.4 fold of increase in activity was achieved related to the reference medium in Section 3.2.1 (352 U/ml).

- Hydrolysis of locust bean gum by endo-beta-mannanase resulted in different mannooligosaccharides. According to the results of TLC and HPLC analysis,

it was observed that mainly mannotriose, α-galatosyl-mannotriose and α-di-galactosyl-mannopentaose were obtained as the products of LBG hydrolysis by endo-beta-mannanase of AsT1.

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Table A.1 Chemicals that were used in the experiments and their suppliers.

Chemical	**Supplier**
Acetonitrile	MERCK
Beef Extract	SIGMA
Citric acid	SIGMA
DNS (3',5'-dinitrosalicylic acid)	SIGMA
Ethanol	GURUP DELTALAR
Guar gum	SIGMA
Glucose	MERCK
H_2SO_4	MERCK
K_2HPO_4	MERCK
Maltose	MERCK
Mannooligosaccharide standards	MEGAZYME
MgSO4.7H_2O	APPLICHEM
NaCl	MERCK
NH_4NO_3	SIGMA
$(NH_4)_2SO_4$	APPLICHEM
Phenol	MERCK
Potato Dextrose Agar	OXOID
Sucrose	MERCK
Tri-Na citrate-5,5-Hydrate	MERCK
Tween 20	SIGMA
Yeast Extract	MERCK

APPENDIX B

PREPARATIONS OF GROWTH MEDIA, BUFFERS AND SOLUTIONS

1) DNS (Dinitrosalicylic acid reagent), 1L

Dinitrosalicylic acid 10 g.

Phenol 2 g.

Sodium sulphite 0.5 g.

Sodium hydroxide 10 g.

The solution was then adjusted to 1 L by adding distilled water.

2) Modified YpSs Preculture medium

Yeast extract- 4% (w/v)

K_2HPO_4- 0.1% (w/v)

$MgSO_4.7H_2O$- 0.05 (w/v)

Glucose monohydrate- 1 % (w/v)

3) Modified YpSs Main culture medium

Yeast extract- 4% (w/v)

K_2HPO_4- 0.1% (w/v)

$MgSO_4.7H_2O$- 0.05 (w/v)

Glucose monohydrate- 2 % w/v, for *A. sojae* ATCC11906

Locust bean gum- 2% w/v, for *A. fumigatus* IMI385708

4) Modified YpSs Broth Media (for optimization studies)

NH_4NO_3-[0.1-0.5% w/v]

K_2HPO_4- [0.1% w/v]

$MgSO_4.7H_2O$- [0.05% w/v]

Sugar beet molasses- [1-8% w/v]

5) 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 L with H_2O. The solution is stored at room temperature.

6) Na-citrate Buffer Solution

50mM, pH: 6.0 Na-citrate buffer was prepared by mixing Na-citrate and citric acid solutions and adjusting pH to 6.0.

7) Potato Dextrose Agar

Suspend 39 g of mixture (Potato infusion 4.0 (infusion from 200 g potatoes); D(+)glucose 20.0; agar-agar 15.0) in 1L, autoclave (15 min at 121 °C).

8) Saline Tween (ST)

0.8 % NaCl

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

9) Substrate (0,5 % (w/v) LBG)

1g of locust-bean gum was dissolved in 160 ml, 50mM Na-citrate (pH 6.0) buffer. Homogenate was continuously stirred and heated till the boiling point and then left for cooling. And it was stirred at +4º C overnight and the next day it was completed to 200 ml by adding Na-citrate buffer.

APPENDIX C

MANNOSE STANDARD CURVE

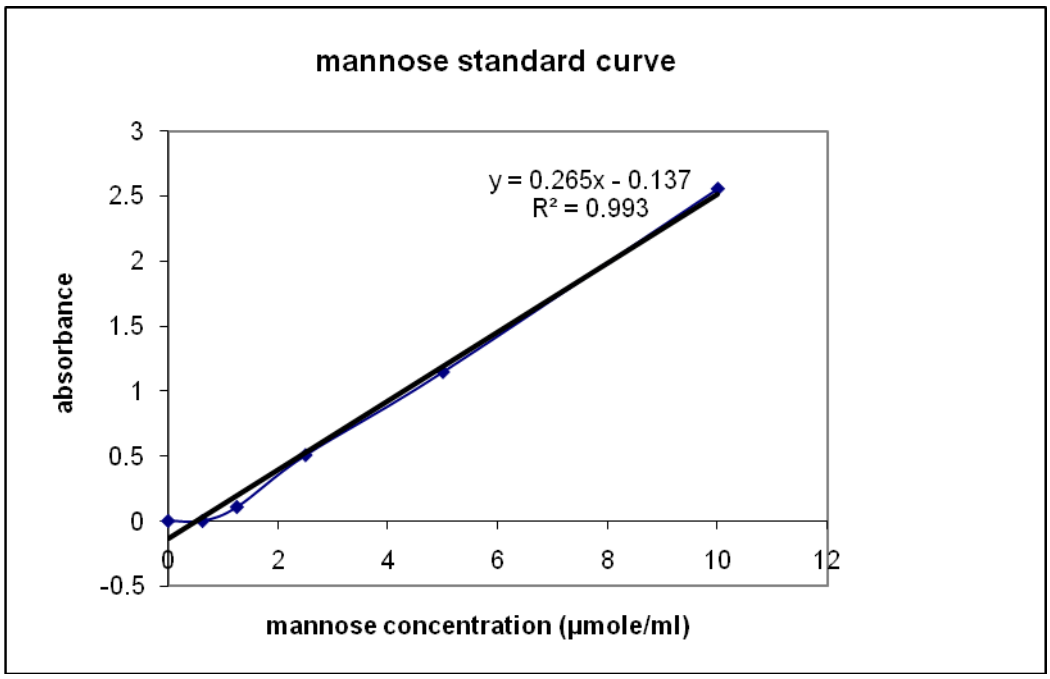

Figure C.1 Mannose standard curve

APPENDIX D

RESULTS OF ANOVA AND MULTIPLE COMPARISONS (TUKEY MODEL) TESTS

TABLE D.1 Results of ANOVA and Tukey tests for different carbon sources

ANOVA

ACTIVITY

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3063.000	3	1021.000	2.450	.203
Within Groups	1667.000	4	416.750		
Total	4730.000	7			

Multiple Comparisons

Dependent Variable: ACTIVITY

Tukey HSD

(I) CARBON	(J) CARBON	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
molasses	glucose	-22.0000	20.41446	.720	-105.1043	61.1043
	maltose	-7.5000	20.41446	.981	-90.6043	75.6043
	sucrose	31.5000	20.41446	.494	-51.6043	114.6043
glucose	molasses	22.0000	20.41446	.720	-61.1043	105.1043
	maltose	14.5000	20.41446	.888	-68.6043	97.6043
	sucrose	53.5000	20.41446	.177	-29.6043	136.6043
maltose	molasses	7.5000	20.41446	.981	-75.6043	90.6043
	glucose	-14.5000	20.41446	.888	-97.6043	68.6043
	sucrose	39.0000	20.41446	.351	-44.1043	122.1043
sucrose	molasses	-31.5000	20.41446	.494	-114.6043	51.6043
	glucose	-53.5000	20.41446	.177	-136.6043	29.6043
	maltose	-39.0000	20.41446	.351	-122.1043	44.1043

TABLE D.2 Results of ANOVA and Tukey tests for different nitrogen sources

ANOVA

ACTIVITY

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	64217.574	3	21405.858	6.306	.002
Within Groups	108619.6	32	3394.363		
Total	172837.2	35			

Multiple Comparisons

Dependent Variable: ACTIVITY

Tukey HSD

(I) NITROGEI	(J) NITROGEI	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
YE	BE	-25.9830	27.46457	.780	-100.3944	48.4285
	AS	87.9254*	27.46457	.016	13.5139	162.3369
	AN	17.3308	27.46457	.921	-57.0807	91.7422
BE	YE	25.9830	27.46457	.780	-48.4285	100.3944
	AS	113.9084*	27.46457	.001	39.4969	188.3198
	AN	43.3137	27.46457	.406	-31.0977	117.7252
AS	YE	-87.9254*	27.46457	.016	-162.3369	-13.5139
	BE	-113.9084*	27.46457	.001	-188.3198	-39.4969
	AN	-70.5946	27.46457	.068	-145.0061	3.8168
AN	YE	-17.3308	27.46457	.921	-91.7422	57.0807
	BE	-43.3137	27.46457	.406	-117.7252	31.0977
	AS	70.5946	27.46457	.068	-3.8168	145.0061

*. The mean difference is significant at the .05 level.

TABLE D.3 Results of ANOVA and Tukey tests for different agitation speeds

ANOVA

ACTIVITY

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16281.433	4	4070.358	14.730	.006
Within Groups	1381.677	5	276.335		
Total	17663.110	9			

Multiple Comparisons

Dependent Variable: ACTIVITY

Tukey HSD

(I) AGITAT	(J) AGITAT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
100 rpm	150 rpm	-30.7714	16.62334	.439	-97.4560	35.9132
	175 rpm	5.6205	16.62334	.996	-61.0641	72.3051
	210 rpm	-34.9962	16.62334	.342	-101.6808	31.6884
	250 rpm	-107.5160*	16.62334	.007	-174.2006	-40.8314
150 rpm	100 rpm	30.7714	16.62334	.439	-35.9132	97.4560
	175 rpm	36.3919	16.62334	.314	-30.2927	103.0765
	210 rpm	-4.2248	16.62334	.999	-70.9094	62.4598
	250 rpm	-76.7446*	16.62334	.029	-143.4292	-10.0600
175 rpm	100 rpm	-5.6205	16.62334	.996	-72.3051	61.0641
	150 rpm	-36.3919	16.62334	.314	-103.0765	30.2927
	210 rpm	-40.6167	16.62334	.242	-107.3013	26.0678
	250 rpm	-113.1366*	16.62334	.006	-179.8211	-46.4520
210 rpm	100 rpm	34.9962	16.62334	.342	-31.6884	101.6808
	150 rpm	4.2248	16.62334	.999	-62.4598	70.9094
	175 rpm	40.6167	16.62334	.242	-26.0678	107.3013
	250 rpm	-72.5198*	16.62334	.036	-139.2044	-5.8352
250 rpm	100 rpm	107.5160*	16.62334	.007	40.8314	174.2006
	150 rpm	76.7446*	16.62334	.029	10.0600	143.4292
	175 rpm	113.1366*	16.62334	.006	46.4520	179.8211
	210 rpm	72.5198*	16.62334	.036	5.8352	139.2044

*. The mean difference is significant at the .05 level.

APPENDIX E

VECTOR MAP

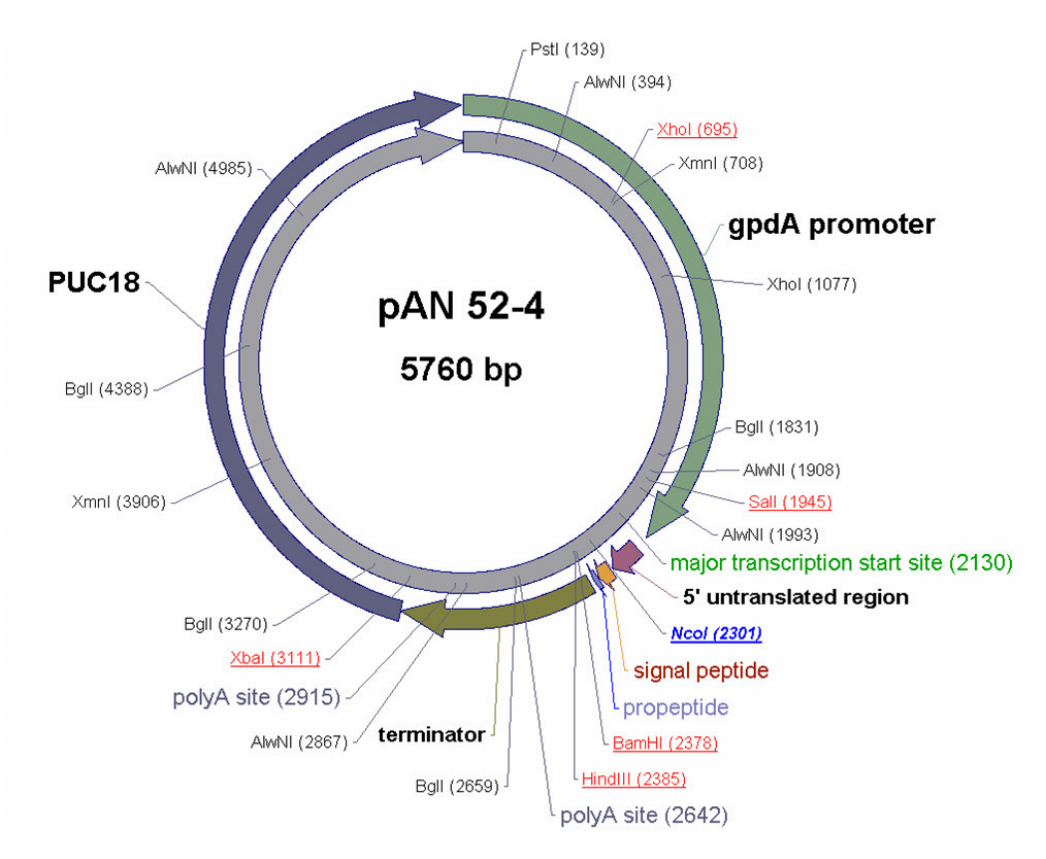

Figure E.1 Map of *Aspergillus sojae* expression vector, pAN52-4.

APPENDIX F

STANDARD CURVES OF OLIGOSACCHARIDES

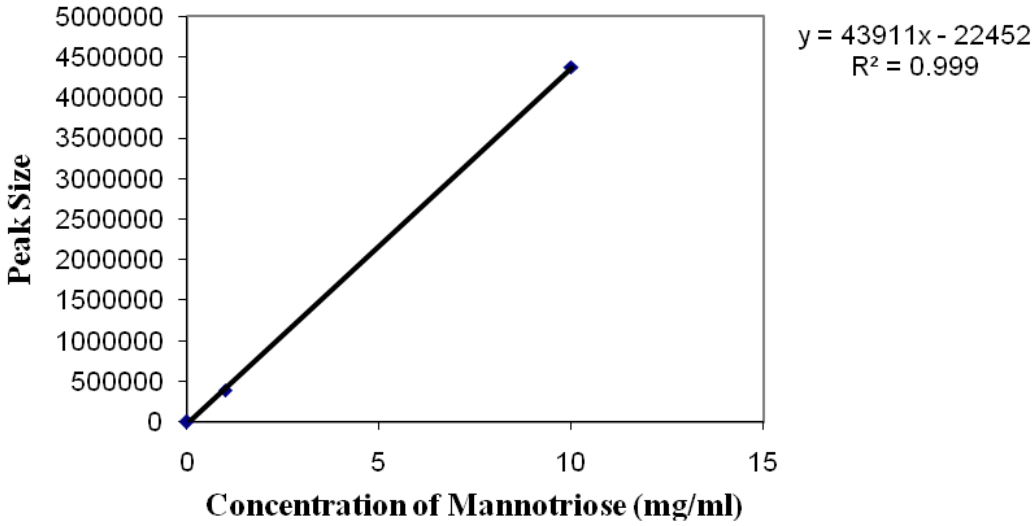

Figure F.1 Standard curve for M3 (Mannotriose).

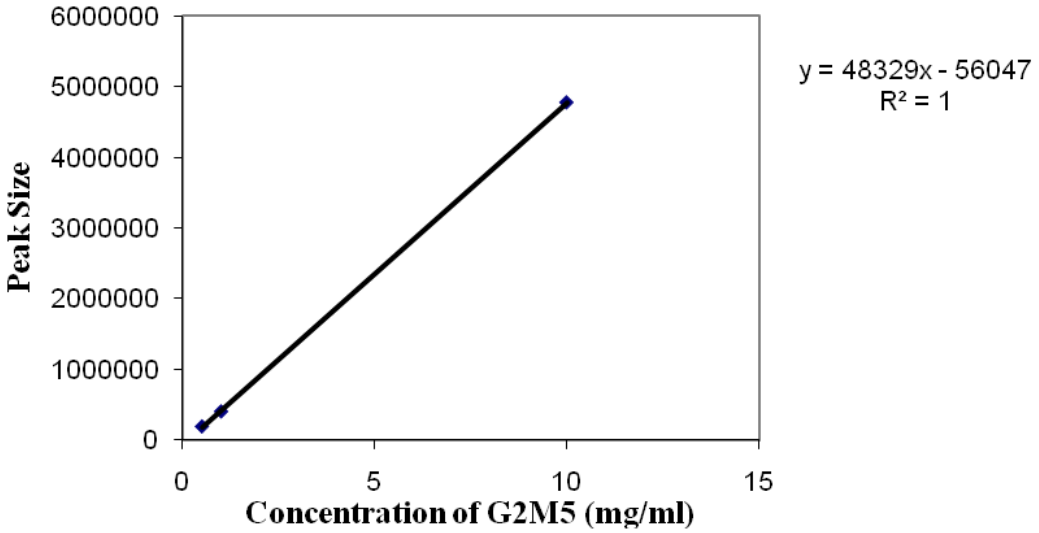

Figure F.2 Standard curve for G2M5 (6^3,6^4-di-α-galactosyl-β-1,4-mannopentaose).