Biochemistry Genetics and Microbiology

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

CLONING OF THE ENDOMANNANASE FROM *SCOPULARIOPSIS CANDIDA* LMK008 AND EVALUATION OF ITS EFFECT ON THE DIGESTIBILITY ON ANIMAL FEED

Ashant Pravin Gareeb

Submitted in fulfilment of the academic requirements for the degree of Master of Science in the Discipline of Microbiology, School of Biochemistry, Genetics and Microbiology, Faculty of Science and Agriculture at the University of KwaZulu-Natal, Durban.

As the candidate's supervisors, we have approved this dissertation for submission.

Signed: _____ **Name:** R. GOVINDEN **Date** 27 March 2012

Signed: _____ **Name:** ME SETATI **Date** 28 March 2012

This thesis is dedicated to my loving and caring parents, Pravin and Prathika Rajmun Gareeb Shah as well as my two very special and adorable little nephews, Yashad and Dhiran.

DECLARATION BY THE CANDIDATE

This MSc study was carried out in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Westville Campus, under the supervision of Dr. R. Govinden and Co-Supervision of Dr. M.E. Setati

1. The research reported in this dissertation, except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other scientists' writing unless specificially acknowledged as being sourced from other scientists. Where other written sources have been quoted, then their words have been re-written but the general information attributed to them has been referenced.
4. This dissertation does not contain text, graphics or tables taken from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References section.

_____

ASHANT PRAVIN GAREEB

TABLE OF CONTENTS

ABSTRACT

Present within the biodiverse hypersaline environment are a wide variety of halotolerant filamentous fungi. Many of these phytopathogens are capable of hydrolysing plant cell wall polysaccharides such as hemicellulose which are comprised of mannans and heteromannans which are polymers of the mannose sugars. Endoacting hydrolytic enzymes such as endo-β-1,4-mannanases are secreted into the extracellular environment and are involved in the catalysis of the random hydrolysis of β-1,4-mannosidic linkages within the backbone of mannan, galactomannan, glucomannan, and galactoglucomannan. Poultry are monogastric animals that are unable to efficiently digest high-fibre and mannan rich feeds such as soybean meals and this results in decreased or depressed animal performance. The use of feeds supplemented with β-mannanases has been shown to enhance the feeding value of mannan-based meals. In the current study, the degradation of β-mannan polysaccharides present in poultry feed by halotolerant *Scopulariopsis candida* LMK008 β-mannanase was investigated. SDS-PAGE, Native-PAGE in conjunction with zymogram analysis was used to assess the molecular weight of the endomannanases. At least three isozymes were detected: two of 56 kDa (pI 3.5 and 6.7) and one of 28 kDa. Anion exchange chromatography was used to purify the 28 kDa isozyme. Three mannan-based substrates, viz., locust bean gum, guar gum and soybean flour, were used to evaluate the hydrolysis capability of the crude as well as the purified β-mannanase via the release of reducing sugars and was detected using the DNS assay. The β-mannanase exhibited low activity with pure guar gum but high activity with locust bean gum galactomannan and soybean flour mannan. The hydrolysis activities of the crude and purified enzyme were then tested further on mannan-based soybean meals. In general it was found that more reducing sugars were released from the grower feed than the starter and layer feeds. Another common hydrolysis pattern observed in all feed types was that after prolonged incubation of 24 h there was a decrease in the amount of reducing sugars released which could be attributed to the presence of naturally-occurring microorganisms in the feed sample which metabolised the simple sugars resulting from the enzymatic hydrolysis of the mannan components in the feed samples. This was confirmed by standard plate count assays. The results obtained are encouraging and the purified β-mannanase could be applied as an industrial feed additive within the animal feed industry, however, further testing of the enzyme *in situ* is needed in order to prove its applicability. The cloning of the endomannanase has to date proven unsuccessful despite numerous techniques being employed and further research is also needed to accomplish this task.

ACKNOWLEDGMENTS

Bhagwan Shree Sathya Sai Baba for all his help and guidance.

I would like to thank my Supervisor, Dr. R. Govinden and Co-supervior, Dr. M.E. Setati for all their help, encouragement and for granting me the opportunity to complete my MSc. I am greatly appreciative for all that they have done for me during the course of my MSc study.

The National Research Foundation (NRF) for their financial assistance.

Dr. B. Masola (Discipline of Biochemistry, University of KwaZulu-Natal) for his help and use of his chromatography equipment.

My parents, Pravin and Prathika Gareeb as well as my brother and sister-in-law, Prashil and Natisha; for all their love, understanding and guidance.

And finally to my friends, colleagues and the Academic and Technical staff in the Disciplines of Microbiology and Biochemistry, UKZN (Westville campus) thanks for the support and encouragement when needed.

LIST OF FIGURES

Emericella nidulans FGSC A4(a); H: *Emericella nidulans* FGSC A4b (b); I: *Aspergillus nidulans* FGSC A4(a); J: *Aspergillus nidulans* FGSC A4(b); K: *Aspergillus nidulans* FGSC A4(c); L: *Phanerochaete ivhrysosporium* Man5C; M: *Phanerochaete ivhrysosporium* Man5D; N: *Armillariella tabescens* EJLY2098). Amino acid sequences obtained from the Carbohydrate Active Enzymes database (http://www.cazy.org/; Henrissat, 1991; Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Cantarel *et al.,* 2008). **19**

Figure 1.8(b): Sequence assembly of Family 5 Bacterial β-mannanase enzymes (A: *Acidothermus cellulolyticus;* B: *Acidothermus cellulolyticus* 11B ATCC43068; C*: Alicyclobacillus acidocaldarius;* D: *Bacillus agaradhaerens;* E: *Bacillus circulans* (a); F: *Bacillus circulans* (b); G: *Bacillus circulans* CGMCC1416; H: *Bacillus circulans* CGMCC1554; I: *Bacillus circulans* K-1; J: *Bacillus* sp.; K: *Bacillus* sp. JAMB-602; L: *Bacillus sp.* N16-5; M: *Caldibacillus cellulovorans;* N: *Caldicellulosiruptor saccharolyticus;* O: *Cellvibrio japonicas* Man 5C; P: *Cellvibrio japonicas* Man5B; Q: *Cellvibrio japonicas* Man 5A; R: *Clostridium cellulolyticum* Man5-K; S: *Clostridium cellulovorans* 743B; T: *Clostridium josui;* U: *Flammeovirga yaeyamensis* (a); V: *Flammeovirga yaeyamensis* (b); W: *Geobacillus stearothermophilus;* X: *Thermoanaerobacterium polysaccharolyticum;* Y: *Thermobifida fusca* YX; Z: *Thermotoga maritime* MSB8; AA: *Thermotoga maritime MSB8* Endoglucanase (mannanase); BB: *Vibrio sp. MA-138* Man5C; CC: *Vibrio sp. MA-138* ManA). Amino acid sequences obtained from the Carbohydrate Active Enzymes database (http://www.cazy.org/; Henrissat, 1991; Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Cantarel *et al.,* 2008). **20**

Figure 1.9(a): Sequence assembly of Family 26 Bacterial β-mannanase enzymes (A: *Bacillus subtilis* CICC 9011; B: *Bacillus amyloliquefaciens* B16; C: *Bacillus amyloliquefaciens* CCTCC AB94022; D: *Bacillus amyloliquefaciens* CICC20164; E: *Bacillus amyloliquefaciens* CICC22383; F: *Bacillus amyloliquefaciens* CICC23260; G: *Bacillus amyloliquefaciens* CICC23281; H: *Bacillus amyloliquefaciens* CICC23753; I: *Bacillus licheniformis* ATCC 14580; J: *Bacillus licheniformis* B30; *K: Bacillus licheniformis* CICC10084; *L: Bacillus licheniformis* CICC10181; M: *Bacillus licheniformis* CICC10182; N: *Bacillus licheniformis* CICC10266; O: *Bacillus licheniformis* CICC10335; P: *Bacillus licheniformis* HDY-04; Q: *Bacillus licheniformis* W10; R: *Bacillus sp.;* S: *Bacillus sp.* 5H; T: *Bacillus sp.* B23; U: *Bacillus sp.* CM3.1; V: *Bacillus sp.* JAMB750; W: *Bacillus sp.* AM-001; X: *Bacillus subtilis* A33; Y: *Bacillus subtilis* B6; Z: *Bacillus subtilis* B11). Amino acid sequences obtained from the Carbohydrate Active Enzymes database (http://www.cazy.org/; Henrissat, 1991; Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Cantarel *et al.,* 2008). **22**

Figure C7: Southern blotting hybridization of the mannanase gene from control fungal species using a high stringency wash step - 60°C with 0.5 ×SSC, 1% (w/v) SDS). (A) DNA electrophoresis of *Eco*RI restricted gDNA. (B) Southern blot analysis. (M, DNA molecular weight marker (O'Gene Ruler DNA Ladder mix: Fermentas, USA); lane 1, *Penicillium* spp; lane 2, *Trichoderma reseei;* lane 3, *Aspergillus niger;* lane 4, *Chaetomium indicum;* lane 5, *Fusarium oxysporum;* lane 6, *Sordaria fimicola;* lane 7, Positive control: *Aspergillus aculeatus* Man 1 gene). **172**

LIST OF TABLES

LIST OF ABBREVIATIONS

1. 2D-PAGE – Second dimensional-Polyacrylamide gel electrophoresis
2. cDNA – complementary Deoxyribonucleic acid
3. GG – Guar gum
4. LBG – Locust bean gum
5. MEA – Malt extract agar
6. NSPs – Non-starch polysaccharides
7. PCR – Polymerase chain reaction
8. RACE PCR – rapid amplification of cDNA ends Polymerase chain reaction
9. rpm – Revolutions per miniute
10. RT PCR – Reverse Transcription PCR
11. SBM – Soy bean meals
12. SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
13. sp. – species

CHAPTER 1:

INTRODUCTION &

LITERATURE REVIEW

1.1. INTRODUCTION

The main components of wood are cellulose, lignin and hemicellulose. The hemicellulose fraction contains many different sugar monomers such as xylose, mannose, galactose, rhamnose, and arabinose. Hemicelluloses contain most of the D-pentose sugars, and occasionally small amounts of L-sugars as well. The hemicellulose mannan is a polymer of the mannose sugar and is a major component of the cell walls of plants and some types of seeds. The mannan backbone comprises β-1,4-linked D-mannopyranose residues (Matheson and McCleary, 1985). Endoacting hydrolytic enzymes such as endo-β-1,4-mannanases (EC 3.2.1.78) catalyse the random hydrolysis of β-1,4-mannosidic linkages within the backbone of mannan, galactomannan, glucomannan, and galactoglucomannan.

The industrial use of β-mannanases has gained interest due to their ability to modify and degrade mannan-containing polysaccharides. They have applicaton in the feed industry (Wong and Saddler, 1993; Gübitz *et al*., 1997; Montiel *et al*., 1999; Jackson *et al.,* 1999a,1999b; Sachslehner *et al.*, 2000; Lee *et al*., 2003a; Daskiran *et al.,* 2004; Ferreira and Filho, 2004; Jackson *et al.,* 2004; Wu *et al*., 2005) as well as in paper production with the result of improved bleaching (Viikari *et al.,* 1994; Gübitz *et al.,* 1996b), oil and gas well stimulation (Adams *et al.,* 1995; Christoffersen, 2004), extraction of coffee beans (Sachslehner *et al.,* 2000; Nunes *et al.,* 2006). They have been shown to be involved in the bioconversion of non-utilized lignocellulosic substrates rich in mannan into added-value products (e.g., chemicals, prebiotic manno-oligosaccharides) (Kobayashi *et al.,* 1984, 1987; Puchart *et al.,* 2004) and in the food and pharmaceutical industries (Taubken *et al*., 1993; Kirk *et al.,* 2002).

Most of the fungal and other eukaryotic β-mannanases belong to the glycoside hydrolase (GH) family GH5 or GH26. The genes encoding for β-mannanase enzymes have been cloned from a variety of organisms (Christgau *et al.,* 1994; Stalbrand *et al.,* 1995; Tang *et al*., 2001; Xu *et al.,* 2002; Hrmová *et al.,* 2006; Zhang *et al.,* 2006). The determination and analysis of β-mannanase gene sequences has enabled the deduction of the primary structure of these β-mannanase enzymes. Therefore, the isolation and cloning of the β-mannanase gene represents an essential step in the engineering of the most efficient β-mannanase producing microorganism (Dhawan and Jagdeep, 2007).

Interest in the cloning and heterologous expression of β-mannan-degrading enzymes by *S. candida* has been triggered by the discovery of the ability of *Scopulariopsis* species to survive in hyper-saline environments. Monogastric animals such as poultry (chickens and turkeys) and pigs are unable to digest non-starch polysaccharide (NSPs) carbohydrates, some of which display anti-nutritional properties that depress animal performance. The use of different feeds supplemented with β-mannanases has been shown to reduce weight gain and feed conversion efficiency in poultry birds due to intestinal viscosity (Odetallah *et al.,* 2002; Pettey *et al.,* 2002; Lee *et al.,* 2003a; Daskiran *et al.,* 2004; Jackson *et al.,* 2004; Wu *et al.,* 2005).

1.2. LIGNOCELLULOSE

Lignocellulose, which is found in plant cell walls, is one of the most abundant organic substances on Earth, and is composed of three major constituents' viz., cellulose, hemicellulose and lignin (Fig. 1.1). Of these components, the hemicellulose fraction of the plant cell walls of hardwoods and softwoods consist of mannans, heteromannans and xylan (Erikkson *et al*., 1990) with occurrence in softwood varying between 5 - 15%, compared to

20 - 30% in hardwood (Coug hlan and Ha zlewood, 1993). S oftwood he micelluloses a re mostly ga lactoglucomannan, glucomannan a nd a rabinoglucoronoxylans (7 - 8%) whe reas acetylglucuronoxylan (15 - 30%) a nd glucomannan (3 - 5%) occur pr edominantly in hardwood (Coughlan a nd Hazlewood, 1993). Mannose is also found in the se eds of leguminous plants (Buckeridge *et al.,* 2000; Handford *et al.,* 2003) and in beans (Lundqvist *et al.,* 2002).

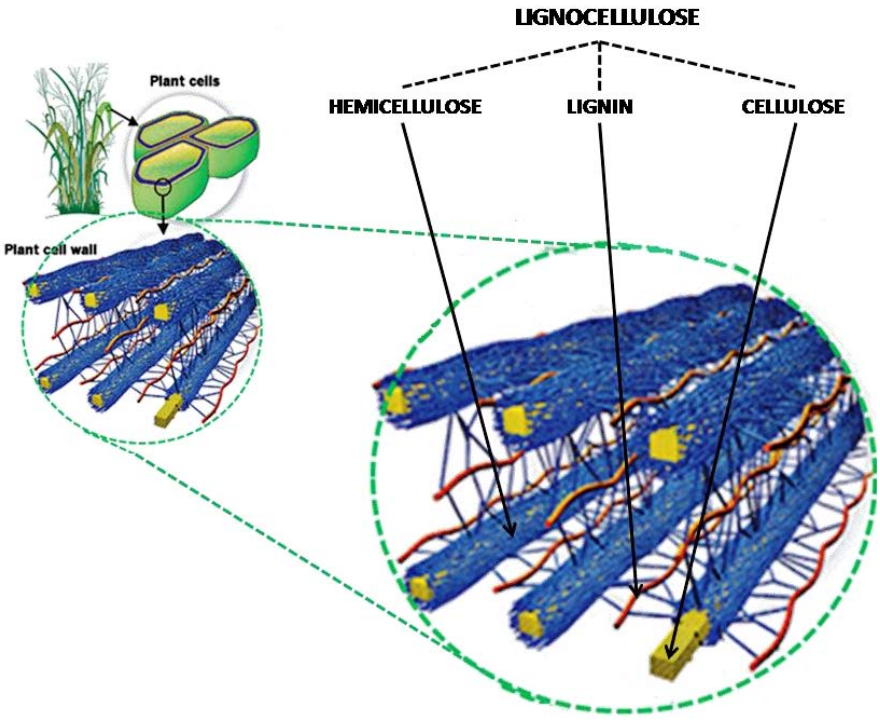

Figure 1.1: Lignocellulose structure. The hemicellulose fraction of lignocellulose consists of chains of xylose interspersed with side chains containing arabinose, galactose, mannose, glucose, acetyl, and other sugar groups (shown in blue), the lignin fraction is a cross-linked macromolecule composed of three types o f su bstituted phenol s (phenylpropanoids) (shown in r ed) a nd t he cellulose fraction (shown in yellow) is a linear chain of several hundred to more than 10,000 D-glucose units linked by β-1,4 bon ds. (Genomic Management I nformation Sy stem, Oak R idge National Labor atory: http://californiaagriculture.ucanr.org).

1.3. MANNAN-BASED POLYSACCHARIDES

Plant cell walls contain complex biopolymers known as mannan polysaccharides which are closely associated with cellulose and lignin (de Vries, 2003). These mannan biopolymers are present either as storage carbohydrates in the seed of plants, or as structural carbohydrates that cross link cellulose microfibrils (Puls and Schuseil, 1996). They are ubiquitous in nature and occur in different forms in plant cell walls (Table 1.1).

Table 1.1. Mannan polysaccharides: Occurrence and Structure (Nishinari *et al.,* 1992; Buckeridge *et al.,* 2000; Schröder *et al.,* 2001)

POLYSACCHARIDE	RESIDUES	BRANCHING RESIDUES	PLANT LOCALIZATION
Mannans	Mannose	None	Coffee and Palmae seeds
Galactomannans	Mannose	Galactose	Annonaceae, Convolvulaceae and Leguminosae,
Glucomannans	Mannose/Glucose	Galactose	Liliaceae, lettuce and tomato seeds
Galactoglucomannans	Mannose/Glucose/Galactose	Galactose	Secondary walls of Angiosperms, Gymnosperms. Ferns, Mosses

Variations of the β-mannan backbone (unsubstituted mannans), may be interrupted with D-glucose (glucomannans) and/or branched with α-1,6-linked galactose (galactomannans). The backbone could also have β-1,4-linked D-mannose and D-glucose residues which are branched by α-1,6-linked D-galactose (galactoglucomannan) and result in the formation of homo- and heteromannans (Fig. 1.2) (Tailford *et al.,* 2009).

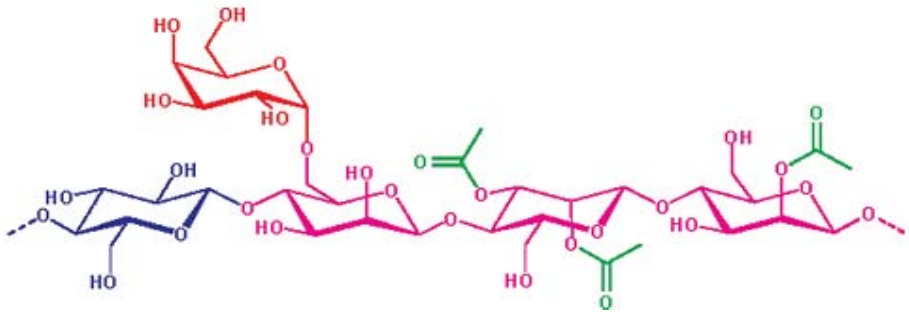

Figure 1.2: An example of the diversity displayed by mannans. The mannose backbone (magenta) of mannans may include glucose (blue) in glucomannans and may also be appended with 6-O α-galactosides (red) in galactomannans and glucogalactomannans. Acetate groups (green) may also be found on the O2 and O3 hydroxyls (Tailford *et al.,* 2009).

1.3.1. Mannan

A pure mannan is a polysaccharide, whose linear backbone comprises 90% or more β-1,4-mannopyranosyl residues with 10% or less of the mannose residues substituted by single units of α-1,6-linked galactoses (Fig. 1.3) (Buckeridge *et al.,* 2000). These mannans are self-interactive, insoluble in water and to a certain degree crystalline in the cell wall (Mulimani and Prashanth, 2002).

[→4)- β-D-Man*p*-1-4)- β-D-Man*p*-(1-4)- β-D-Man*p*-(1-4)-β-D-Man*p*-(1-4)-β-D-Man*p* (→1]

Figure 1.3: A mannan polymer showing mannopyranosyl residues linked to one another by β-1,4-mannosyl linkages (light blue) (Vinogradov *et al.,* 2000).

Unsubstituted β-1,4-mannan is an important structural component of some marine algae (Yamasaki *et al.,* 1998), and terrestrial plants such as ivory nut (Chanzy *et al.*, 2004) and coffee bean (Nunes *et al.,* 2006). In the bulbs and endosperm of some plants, mannans function as storage polysaccharides (Stoll *et al.,* 1999). Ivory nut mannan, from *Phytelphus macrocarpa* is an insoluble crystalline material comprising only mannose, with a backbone conformation very similar to that of cellulose (Stoll *et al.,* 1999).

1.3.2. Galactomannan

Galactomannan polysaccharides are composed of a β-1,4-D-mannopyranosyl backbone which contains a single D-galactopyranosyl residue linked to mannose C-6 sites (Fig. 1.4). Water-soluble galactomannan is the main storage carbohydrate in leguminous seed and comprises of up to 20% of the total dry weight (McCleary, 1988).

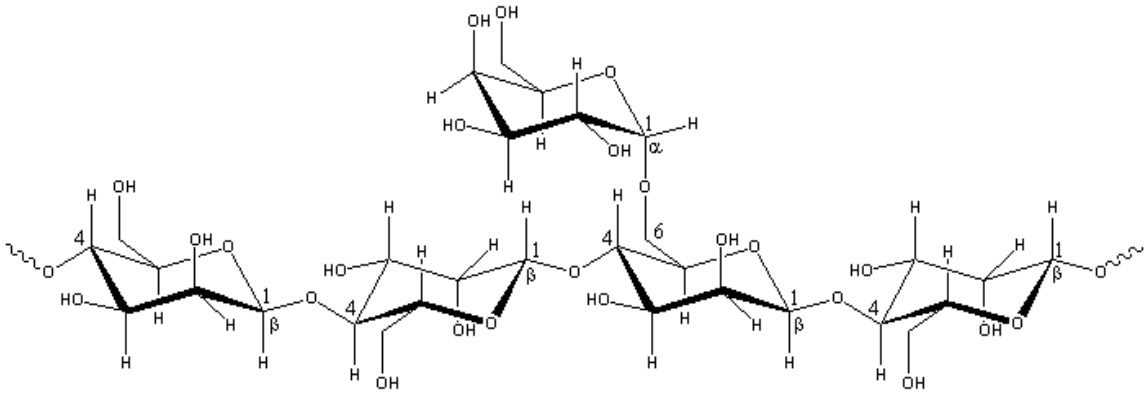

Figure 1.4: A schematic representation of a locust bean galactomannan. It consists of 1,4-linked β-D-mannose backbone with branch points from their 6-positions linked to α-D-galactose units (Chaplin, 2009).

The overall degree of polymerization of the backbone is 1000-1500 mannose units (Mikkonen *et al.,* 2007). Galactomannans differ from each other in their mannose/galactose ratio as well as their distribution pattern of galactose residues along the mannan chain. For example, the mannose/galactose ratios of commercially important galactomannans, namely, locust bean gum (LBG) (from *Ceratonia siliqua*) and guar gum (GG) (from *Cyamopsis tetragonolobus*), are approximately 3.5:1 and 1.5:1 units, respectively (Maier *et al.,* 1993; Daas *et al.,* 2000; Marracini *et al.,* 2005; Hsiao *et al.,* 2006 Mikkonen *et al.,* 2007) (Table 1.2).

Table 1.2. The distribution and ratio of galactose to mannose in different mannan polymers (Maier *et al.,* 1993; Daas *et al.,* 2000; Marracini *et al.,* 2005; Hsiao *et al.,* 2006 Mikkonen *et al.,* 2007)

POLYMER	GALACTOSE : MANNOSE RATIO
Ivory nut mannan	0 : 1
Locust bean gum	1 : 4
Tara gum	1 : 3
Guar gum	1 : 2
Fenugreek gum	1 : 1
Soybean gum	1 : 1.8

Studies on the distribution of galactose side groups along the mannan backbone are contradictory. Researchers in the past previously believed that side-chain units of LBG were distributed in uniform blocks, whereas the side-chain units of guar gum are distributed alternately along the mannan backbone (Baker and Whistler, 1975). However, a study conducted by Daas et al. (2000) proposed instead that LBG can show random, blockwise, and ordered distributions whereas GG has a blockwise distribution of galactose side groups. This property is widely used in the chemical characterization of legumes (Buckeridge and Dietrich, 1990; Buckeridge et al., 1995). Galactose substitutions in galactomannans differ in nature and have influences on the solubility of this polymer in water (Buckeridge et al., 2000). In a study conducted by Capek et al. (2000) it was shown that substituted galactomannans such as LBG have the ability to form viscous solutions with water (Marraccini et al., 2005), while pure unsubtituted mannans form insoluble polymers, as observed in ivory nuts.

The molecular size, Gal:Man ratio and the degree of branching of the polysaccharide has a direct influence on galactose substitutions in galactomannan (de Vries and Visser, 2001). The biological function of the mannan is more related to hardness as the galactose substitutions approaches zero. Polymers with higher degrees of galactosylation absorb excessive amounts of water and distribute it through the embryo which helps to protect the plants from conditions such as drought (Mulimani and Prashanth, 2002).

1.3.3. Glucomannans

Glucomannan polysaccharides are water-soluble and are wildly distributed in seeds of some *Liliaceae* and *Iridaceae* species where they have storage and structural functions (Buckeridge *et al.,* 2000). The glucomannan backbone is composed of D-glucose and D-mannose residues bonded together by β-1,4-linkages (Fig 1.5).

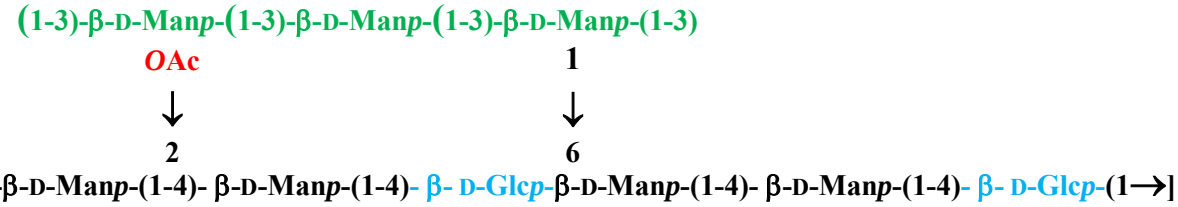

Figure 1.5: Glucomannan showing repeating units of mannose residues (blue and black) substituted by glucose residues (light blue) in the main chain and containing some branches of OAc: O-linked acetyl group (red), (Mudau, 2006).

They are made up of approximately 60% D-mannose and 40% D-glucose residues (Li *et al.,* 2005). D-Glucose and D-mannose residues are also acetylated at *O*-2 (Ratcliffe *et al.,* 2005). In some plant species short chains consisting of 11-16 hexose molecules may form branches which are linked to the main chain via β-(1-6) or β-(1-3) linkages (Hua *et al.,* 2004). In some plants, e.g., *Dendrobium officinale (Herba dendrobii)*, glucomannan may contain branches containing an acetyl group at carbon 2 or small mannooligosaccharide

chains (Hua *et al.,* 2004). The molar ratio of mannose to glucose varies from plant species to species, however, the mannose content is usually higher than that of glucose, e.g. the Man:Glc ratio is 1.6:1.0 in glucomannan from *Amorphophallus konjac* (Ratcliffe *et al.,* 2005) and 3.0:1.0 in glucomannan from *Orchis mascula* (Cescutti *et al.,* 2002).

1.3.4. Galacto-glucomannans

The complex mannan-based polysaccharide, galacto-glucomannan, is a major constituent of softwood hemicellulose (Capek *et al.,* 2002). Its backbone consists of β-1,4-linked D-mannose residues interspersed with glucose units with D-Galactose molecules attached to the mannose by α-(1-6)-linkages (Fig. 1.6). The mannose and glucose residues within the backbone are sometimes acetylated at C-2 or C-3. In some cases galacto-glucomannan polysaccharides, β-1,2-linked galactose disaccharides have been observed (Sims *et al.,* 1997).

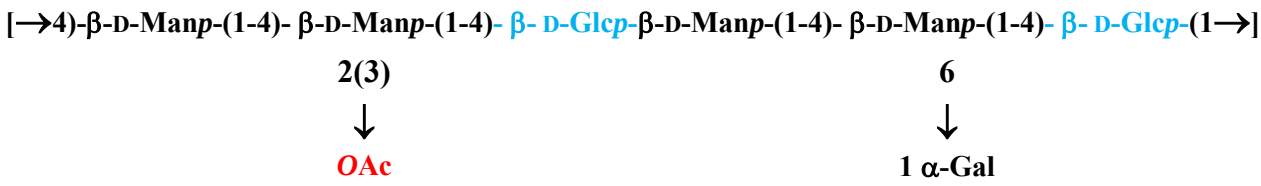

Figure 1.6: The structure of softwood O-acetyl galacto-glucomannan, OAc: O-linked acetyl group (red), Manp: Mannopyrannoside residues (black), Gal: Galactose residues, Glcp: Glucopyrannoside residues (light blue) (Mudau, 2006).

The water-insoluble galacto-glucomannan extracted from the secondary cell walls of gymnosperms and angiosperms has a Man:Glc ratio of 1: 4 or 1:3, and the acetylated water soluble one, a ratio of 1: 1.4 and 1:3 (Schröder *et al.,* 2001). In contrast galacto-glucomannans secreted into extracellular space by suspension cells, or those isolated from primary cell walls contain approximately equal amounts of galactose, glucose and mannose

residues. β-Acetylgalactoglucomannans comprise just over 25% of the dry weight and is the principal Hemicellulose in soft woods (Chen *et al.,* 2007). The backbone of this form of mannan comprises mannose and glucose in the ratio 3:1; in which the glucose residues may be distributed randomly (Puls and Schuseil, 1996; Tenkanen *et al.,* 1997).

1.4. ENZYMATIC HYDROLYSIS OF MANNAN

Due to the heterogeneity and complex chemical nature of mannan-based polysaccharides, a cocktail of enzymes is required for complete hydrolysis. The complete cleavage of β-1,4-mannan requires the concerted action of β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), and β-glucosidase (EC 3.2.1.21), as well as several debranching enyzmes such as α-galactosidase (EC 3.2.1.22) and acetyl esterase (EC 3.1.16) (Gúbitz *et al.,* 1996a; Hägglund *et al.,* 2003). β- Mannanases are the core enzyme involved in the random hydrolysis of β-1,4 mannosidic linkages within the backbones of mannans, galactomannans, and glucomannans (Fig. 1.7) (Stoll *et al.,* 1999).

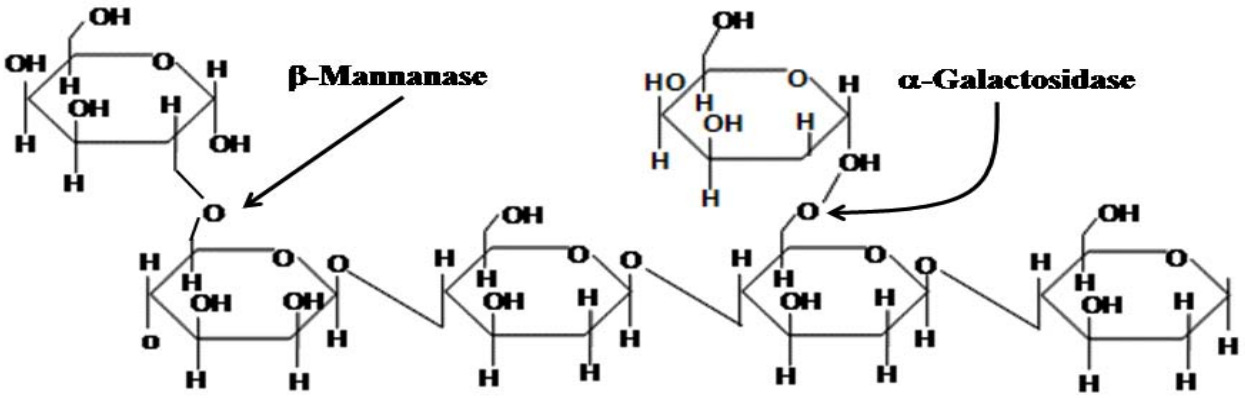

Figure 1.7: Scheme of enzymatic action on galactomannan. The β-(1-4) linked polymannose chain is substituted with α-(1-6) linked galactose residues. The arrows represent the glycosidic links recognized by β-mannanase and α-galactosidase (Dhawan and Jagdeep, 2007).

1.4.1. Structural organization

The hydrolysis of mannan polysaccharide is often affected by the pattern of distribution of D-glucosyl residues within the main chain in glucomannan and galactoglucomannan as well as the degree and pattern of substitution of the main chain by β-D-galactosyl residues in galactomannan and galactoglucomannan (McCleary, 1979). The pattern and distribution of O-acetyl groups contained within glucomannan affects the receptiveness of this polysaccharide to the hydrolysis process. β-mannosidases are indispensable for the complete hydrolysis of plant heteromannans. They convert the manno-oligosaccharides produced by β-mannanases to mannose (Franco *et al.,* 2004). During the hydrolysis of mannan by β-mannanases, the main release products are mannobiose and mannotriose. In general an uninterrupted sequence of 3 to 5 unsubtituted mannose residues is very often required for mannan cleavage to occur (Stålbrand *et al.,* 1993).

1.4.2. Biochemical properties of β-1,4-mannanases

Fungal endo-1,4-β-mannanases have been purified and characterized and it has been reported that they exhibit acidic to neutral pH optima, molecular weights ranging from 33 - 80 kDa and mesophilic to moderately thermophilic temperature optima as shown in Table 1.3. β-Mannanases isolated from fungi are often secreted into the culture liquid as multiple enzyme forms such as those from *A. fumigatus* (Puchart *et al.,* 2004); *S. rolfsii* (Gübitz *et al.,* 1996a) and *T. reesei* (Stålbrand *et al.,* 1993). Their ability to bind and degrade different substrates is a contributing factor to their multiplicity (Johnson *et al.,* 1990). The production of β-mannannase isoforms maybe regulated differently. For instance, *S. rolfsii* isoforms exhibited different functions on substrates of varying sizes (Grobwinder *et al.,* 1999), or they may be secreted as products of the same gene differing only in their post-translational modification as is in the case of a mannanases from a thermo-tolerant fungus *A. fumigatus*

(Purchart *et al.,* 2004).

Table 1.3: Biochemical properties of fungal β-1,4-mannanases

ORGANISMS	ENZYMES	MW (kDa)	TEMPERATURE OPTIMA (°C)	pH OPTIMA	pI	REFERENCE
Aspergillus aculeatus	Man 5A Man 5A [R]	45	50	3.0	NR	Setati *et al.* (2001)
Aspergillus niger	Mannanase	40	NR	3.5	3.7	Ademark *et al.* (1998)
Aspergillus awamori	Mannanase	NR	80	5	NR	Kurakake and Komaki (2001)
Aspergillus fumigatus	Man I Man II	60 63	60 60	4.5 4.5	5.2 4.9	Puchart *et al.* (2004)
Schlerotium rolfsii	Man I Man II	61.2 41.9	74 72	2.9 3.3	3.5	Gübitz *et al*. (1996a)
Trichoderma reesei	Man I Man II	53 51	70 70	3.5-4.0 3.5-4.0	5.4 4.6	Stålbrand *et al.* (1993)
Trichoderma harzianum	Man I	36.5	55	3.0	NR	Ferreira and Filho (2004)
Polyporus versicolor	Man (I-IV)	33.9-58	NR	NR	3.8-4.6	Johnson *et al.* (1990)
Thielavia terrestris	Man (I-IV)	30-89	NR	NR	4.5-5.5	Araujo and Ward (1990)

[R:] Recombinant, NR: Not reported

1.4.3. Mannanases sources

β-Mannanases are ubiquitous in nature and are found in a large number of microorganisms largely isolated from natural environments. Actinomycetes, bacteria, yeasts and fungi are well known mannan degraders (Talbot and Sygusch, 1990; Puchart *et al.,* 2004).The important β-mannanase producing organisms are listed in Table 1.4.

Table 1.4: Sources and Family classification of β-mannanases (Modified from Dharwan and Jagdeep, 2007)

FAMILY CLASSIFICATION	SOURCE		REFERENCES
	PROKARYOTES	EUKARYOTES	
	BACTERIA	FUNGI	
FAMILY 5	*Caldocellum saccharolyticum*		Gibbs *et al.* (1992)
	Caldibacillus cellulovorans.		Sunna *et al.* (2000)
	Vibrio sp.		Tamaru *et al.* (1995)
		Aspergillus aculeatus	Christgau *et al.* (1994); Setati *et al.* (2001); van Zyl *et al.* (2009)
		Trichoderma reesei	Stålbrand *et al,* (1995)
		Agaricus bisporus	Tang *et al.* (2001)
		Aspergillus tamarii	Civas *et al.* (1984)
		Aspergillus fumigatus	Puchart *et al.,*2004)
		Aspergillus niger	Ademark *et al.,*1998)
		Aspergillus oryzae NRRL	Regaldo *et al.,*2000)
		Aspergillus sulphureus	Chen *et al.* (2007)
		Aspergillus terreus	Huang *et al.* (2007)
	Caldocellum saccharolyticum		Bicho *et al.* (1991)
	Clostridium cellulolyticum		Perret *et al.* (2004)
	Bacillus agaradhaerens		Bettiol and Showell (2002)
	Thermoanaerobacterium polysaccharolyticum		Cann *et al.* (1999)
	Cellvibrio japonicus		Hogg *et al.* (2003)
	Thermomonospora fusca		Hilge *et al.* (1998)
FAMILY 26	*Rhodothermus marinus*		Politz *et al.* (2001)
	Pseudomonas fluorescens		Bolam *et al.* (1996)
	Clostridium thermocellum		Halstead *et al.* (1999)
		Orpinomyces sp. Strain P2	Ximenes *et al.* (1995)
	Bacillus brevis		Araujo and Ward (1990)

	Bacillus circulans K-1		Yosida *et al*. (1998)
	Bacillus polymyxa		Araujo and Ward (1990)
	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 168		Helow and Khattab (1996)
	Caldibacillus cellulovorans		Sunna *et al.* (2000)
	Cellulomonas fimi		Stoll *et al.* (1999, 2000)
	Clostridium thermocellum		Halstead *et al*. (1999)
	Dictyoglomus thermophilum		Gibbs *et al.* (1999)
	Paenibacillus curdlanolyticus		Pason *et al.* (2006)
	Paenibacillus polymyxa		Han *et al.* (2006)
	Pseudomonas fluorescens subsp. *Cellulose*		Braithwaite *et al.* (1995)
FAMILY 5 and 26	*Cellvibrio japonicus*		Hogg *et al.* (2001)
	Bacilli sp.		Hatada *et al.,* 2005); Sygusch *et al.* (1998)
	Caldicellulosiruptor saccharolyticus		Gibbs *et al.* (1992); Morris *et al.* (1995); Gibbs *et al.* (1996)
FAMILY CLASSIFICATION UNKNOWN	*Bacteroides ovatus*		Gherardini *et al.* (1987)
	Bacteroides ruminicola		Matsushita *et al*. (1991)
	Clostridium butyricum/beijerinckii		Nakajima and Matsuura (1997)
		Phanerochaete chrysosporium	Wymelenberg *et al.* (2005)

	Streptomyces galbus		Kansoh and Nagieb (2004)
	Streptomyces lividans		Arcand, *et al.* (1993)
	Thermotoga maritima		Parker *et al.* (2001)
	Thermotoga neapolitana		Duffaud *et al.* (1997)
		Trichoderma harzanium T4	Franco *et al.* (2004)

1.4.4. Family classification and modularity of β-mannanase enzymes

1.4.4.1.Family classification

Glycoside hydrolases (EC 3.2.1.-) such as β-mannanases degrade mannans and heteromannans via the hydrolysis of the glycosidic bonds present in these oligo-and polysaccharides. β-Mannanases have been grouped into two of the 85 sequence-based glycoside hydrolase families (GHs), 5 and 26 (Henrissat, 1991) as shown in Table 1.4. GHs are classified into these different families based on their amino acid sequence similarities (Henrissat and Bairoch, 1993, 1996). There is also a direct relationship between sequence and folding similarities. Structure conservation was shown to be much stronger than amino acid conservation and hence families can be grouped into "clans" according to their three-dimensional structure (Henrissat, 1990).

Family 5 includes a collection of highly divergent sequences, comprising not only fungal (Henrissat, 1991; Wang *et al.,* 1993; Christgau *et al.,* 1994; Stålbrand *et al.,* 1995, Yagüe *et al.,* 1997), but also bacterial sequences (Gibbs *et al.,* 1992; Arcand *et al.,* 1993; Morris *et al.*, 1995; Tamaru *et al.,* 1997). Specific examples of family 5 fungal β-mannanases and bacterial β-mannanases include fungal β-mannanases include *Aspergillus aculeatus, Trichoderma reesei* and *Agaricus bisporus* (Fig. 1.8a). Family 5 also comprises several bacterial β-mannanases including *Caldocellum saccharolyticum, Caldibacillus* sp*., Vibrio* species (Fig. 1.8b). Family 5 β-mannanases from higher eukaryotes include *Lycopersicon esculentum* and *Mytilus edulis.* β-mannanases in family 26 are of bacterial origin (Fig. 1.9a), with the exception of the anaerobic fungus *Orpinomyces* sp. strain P2, *Humicola insolens* and several *Piromyces* sp. (Fig. 1.9b). Mannan hydrolases in family 5, (Table 1.4), can be further divided into prokaryote and eukaryote classes by sequence analysis. Within the classes, there is at least 59% similarity among prokaryotic enzymes and 26 to 58% identity among

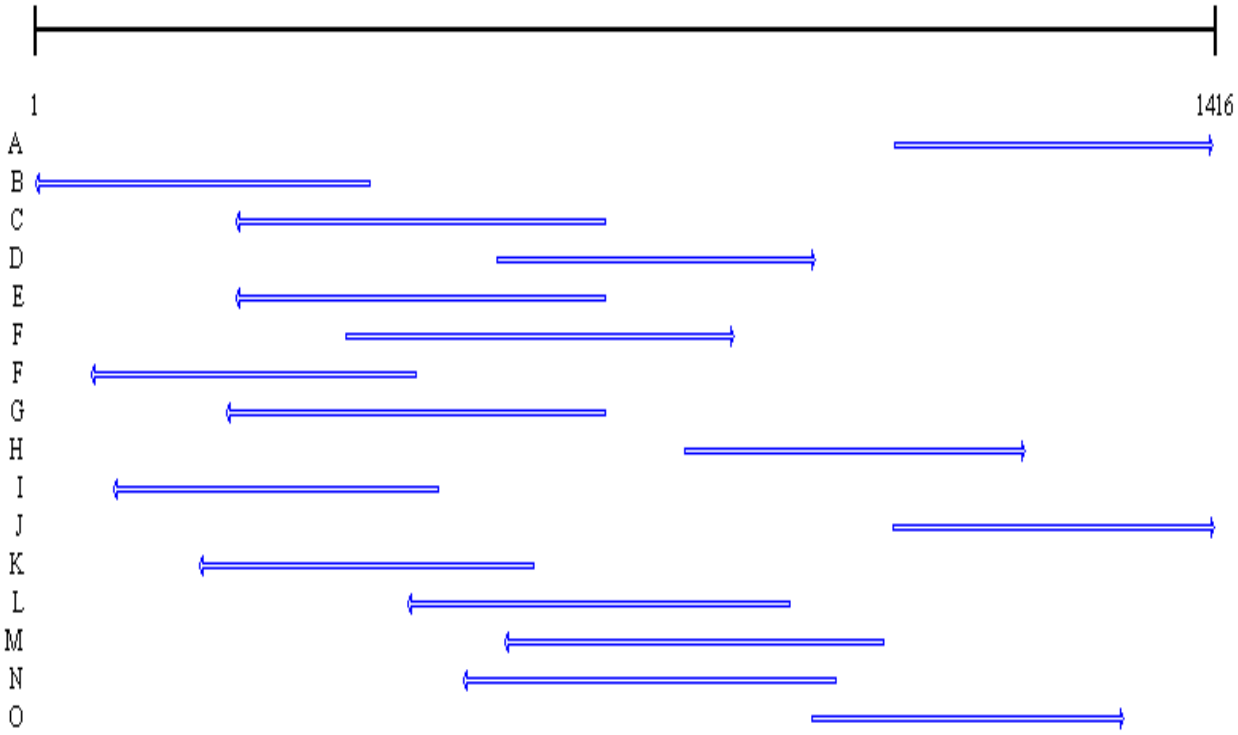

Figure 1.8(a): Sequence assembly of Family 5 Fungal β-mannanase enzymes (A: *Aspergillus aculeatus* ATCC16872; B: *Aspergillus sulphureus*; C: *Agaricus bisporus* C54-CARB8 (CEL4a); D: *Agaricus bisporus* D649 (CEL4); E: *Aspergillus fumigates* Af293; F: *Bospora sp*. MEY-1; G: *Emericella nidulans FGSC A4*(a); H: *Emericella nidulans* FGSC A4b (b); I: *Aspergillus nidulans* FGSC A4(a); J: *Aspergillus nidulans* FGSC A4(b); K: *Aspergillus nidulans* FGSC A4(c); L: *Phanerochaete 19hrysosporium* Man5C; M: *Phanerochaete 19hrysosporium* Man5D; N: *Armillariella tabescens* EJLY2098). Amino acid sequences obtained from the Carbohydrate Active Enzymes database (http://www.cazy.org/; Henrissat, 1991; Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Cantarel *et al.,* 2008).

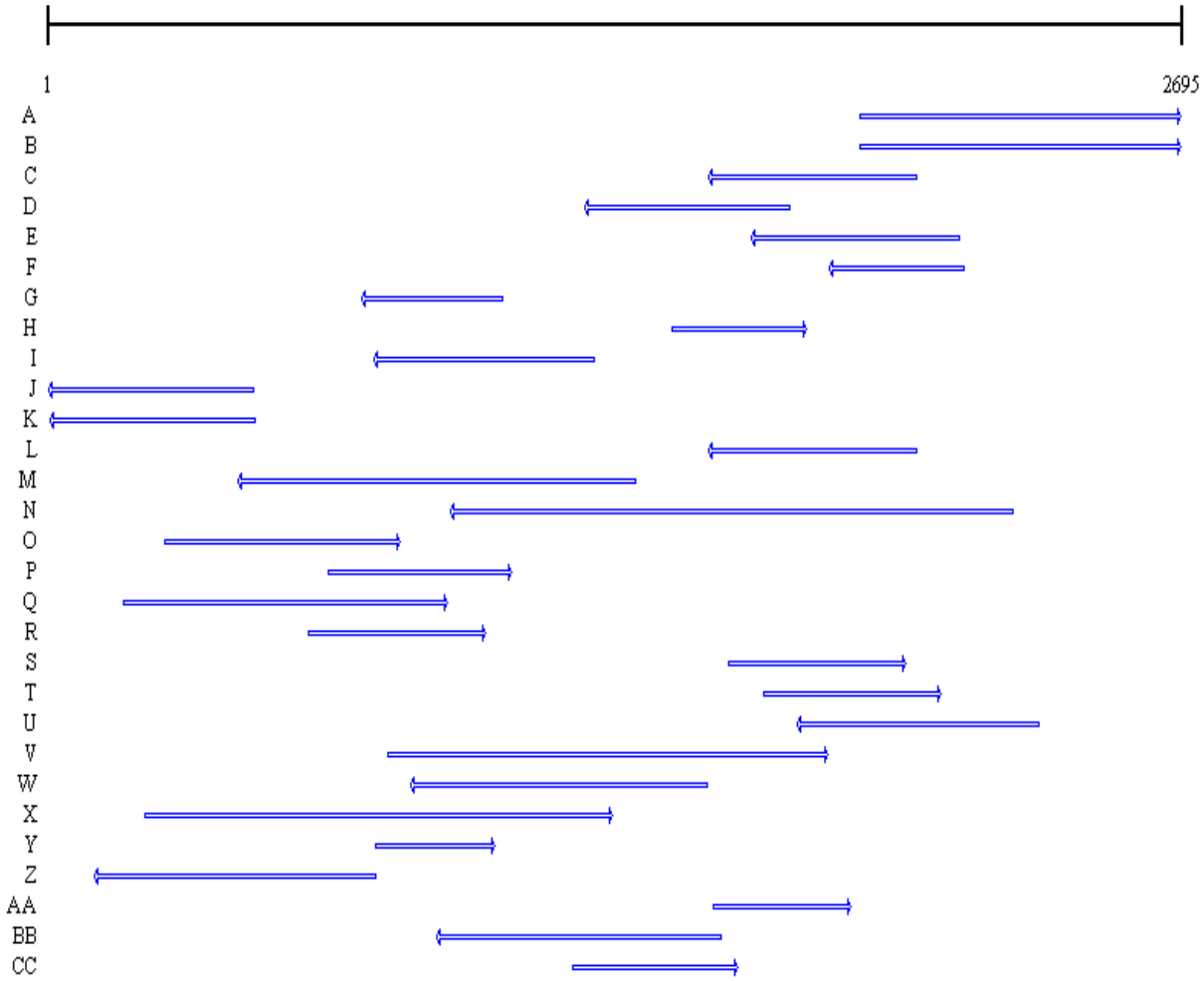

Figure 1.8(b): Sequence assembly of Family 5 Bacterial β-mannanase enzymes (A: *Acidothermus cellulolyticus;* B: *Acidothermus cellulolyticus* 11B ATCC43068; C*: Alicyclobacillus acidocaldarius;* D: *Bacillus agaradhaerens;* E: *Bacillus circulans* (a); F: *Bacillus circulans* (b); G: *Bacillus circulans* CGMCC1416; H: *Bacillus circulans* CGMCC1554; I: *Bacillus circulans* K-1; J: *Bacillus* sp.; K: *Bacillus* sp. JAMB-602; L: *Bacillus sp.* N16-5; M: *Caldibacillus cellulovorans;* N: *Caldicellulosiruptor saccharolyticus;* O: *Cellvibrio japonicas* Man 5C; P: *Cellvibrio japonicas* Man5B; Q: *Cellvibrio japonicas* Man 5A; R: *Clostridium cellulolyticum* Man5-K; S: *Clostridium cellulovorans* 743B; T: *Clostridium josui;* U: *Flammeovirga yaeyamensis* (a); V: *Flammeovirga yaeyamensis* (b); W: *Geobacillus stearothermophilus;* X: *Thermoanaerobacterium polysaccharolyticum;* Y: *Thermobifida fusca* YX; Z: *Thermotoga maritime* MSB8; AA: *Thermotoga maritime MSB8* Endoglucanase (mannanase); BB: *Vibrio sp. MA-138* Man5C; CC: *Vibrio sp. MA-138* ManA). Amino acid sequences obtained from the Carbohydrate Active Enzymes database (http://www.cazy.org/; Henrissat, 1991; Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Cantarel *et al.,* 2008).

eukaryotic enzymes, the low sequence similarity between the classes being attributed to evolution from unrelated ancestral proteins (Bewley *et al.,* 1997).

It has also been observed that β-mannanases belonging to the strains of the same species are also classified in different families. For example β-mannanases from different strains of *Caldicellulosiruptor saccharolyticus* have been classified in both families 5 and 26 and multiple β-mannanases in *Cellvibrio japonicus* have been classified in both families 5 and 26. β-Mannanases from different *Bacillius* species are also found in both families (Table 1.4) (Gibbs *et al.,* 1992, 1996; Morris *et al.,* 1996; Syguch *et al.,* 1998; Hog *et al.,* 2005; Hatada *et al.,* 2005).

Protein homology is often concluded on the basis of sequence similarity. If two or more enzymes have highly similar protein sequences, it is likely that they are homologous. For example it can be observed that bacterial β-mannanases belonging to *Acidothermus cellulolyticus* as well as *Clostridium cellulovorans* and *Clostridium josui* are homologous whereas amongst the other β-mannanase-producing strains very little or no sequence homology is observed (Fig. 1.8b). In Figures 1.9a and 1.9c it is also observed that β-mannanases belonging to various strains of *Bacillus amyloliquefaciens* have highly homologous protein structures, but when compared to β-mannanase sequences from other non-related bacteria such as *Bacillus licheniformis, Bacillus subtilis, Cellulomonas fimi*: *Cellvibrio japonicas*: *Clostridium* sp. *Dictyoglomus thermophilum, Paenibacillus* sp. *Pectobacterium carotovorum, Prevotella bryantii,* it can be observed that there is very little if any homology between the β-mannanase sequences. This phenomenon is also observed amongst fungal β-mannanases, where for example in Figure 1.8a it is observed that there is strong sequence homology amongst *Phanerochaete chrysosporium* and *Armillariella*

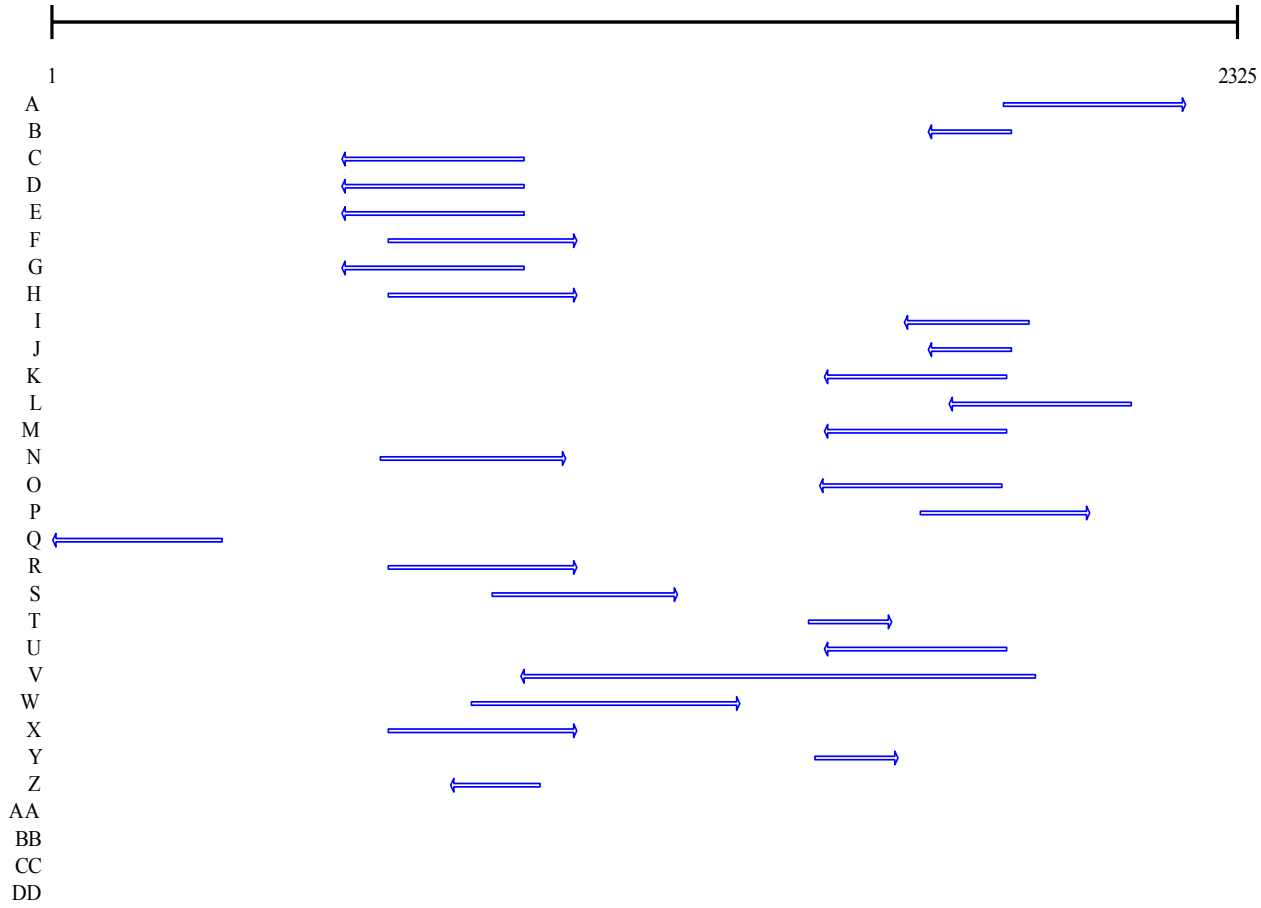

Figure 1.9(a): Sequence assembly of Family 26 Bacterial β-mannanase enzymes (A: *Bacillus subtilis* CICC 9011; B: *Bacillus amyloliquefaciens* B16; C: *Bacillus amyloliquefaciens* CCTCC AB94022; D: *Bacillus amyloliquefaciens* CICC20164; E: *Bacillus amyloliquefaciens* CICC22383; F: *Bacillus amyloliquefaciens* CICC23260; G: *Bacillus amyloliquefaciens* CICC23281; H: *Bacillus amyloliquefaciens* CICC23753; I: *Bacillus licheniformis* ATCC 14580; J: *Bacillus licheniformis* B30; K: *Bacillus licheniformis* CICC10084; *L: Bacillus licheniformis* CICC10181; M: *Bacillus licheniformis* CICC10182; N: *Bacillus licheniformis* CICC10266; O: *Bacillus licheniformis* CICC10335; P: *Bacillus licheniformis* HDY-04; Q: *Bacillus licheniformis* W10; R: *Bacillus sp.;* S: *Bacillus sp.* 5H; T: *Bacillus sp.* B23; U: *Bacillus sp.* CM3.1; V: *Bacillus sp.* JAMB750; W: *Bacillus sp.* AM-001; X: *Bacillus subtilis* A33; Y: *Bacillus subtilis* B6; Z: *Bacillus subtilis* B11). Amino acid sequences obtained from the Carbohydrate Active Enzymes database (http://www.cazy.org/; Henrissat, 1991; Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Cantarel *et al.,* 2008).

tabescens β-mannanase sequences whilst there is hardly any homology amongst the other fungal species belonging to Family 5. In Figure 1.9a it can be observed that β-mannanases from *Piromyces* sp. have strong sequence homology but there is very little homology between

the β-mannnanases from *Humicola insolens* and *Piromyces* sp (Henrissat and Davies, 1997; Cantarel *et al.,* 2008).

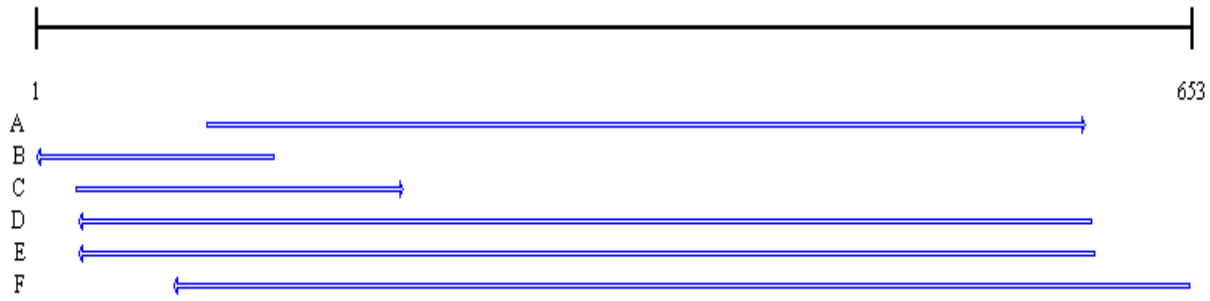

Figure 1.9(b): Sequence assembly of Family 26 Fungal β-mannanase enzymes (A: *Humicola insolens;* B: *Piromyces* sp. E2 ManA; C: *Piromyces* sp. E2 ManB; D: *Piromyces* sp. ManA; E: *Piromyces* sp. ManB; F: *Piromyces* sp. ManC). Amino acid sequences obtained from the Carbohydrate Active Enzymes database (http://www.cazy.org/; Henrissat, 1991; Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Cantarel *et al.,* 2008).

Sequence similarity may also arise without common ancestry, i.e., short sequences may be similar by chance, and sequences may be similar because both were selected to bind to a particular protein, such as a transcription factor. Such sequences are similar but not homologous. This information is useful for the analyzing genetic relatedness of proteins. Sequence regions that are homologous are also called conserved and are similar or identical sequences that occur within protein sequences, protein structures or polymeric carbohydrates across species or within different molecules produced by the same organism. Examples of conserved regions amongst the bacterial β-mannanases can be observed amongst various strains of *Bacillus amyloliquefaciens* (Fig. 1.9a) as well as amongst *Bacillus subtilis* (Fig. 1.9c). As a result of sequence information being transmitted from parents to progeny by genes, a conserved sequence implies that there is a conserved gene. Highly conserved proteins are often required for basic cellular function, stability or reproduction. Conservation

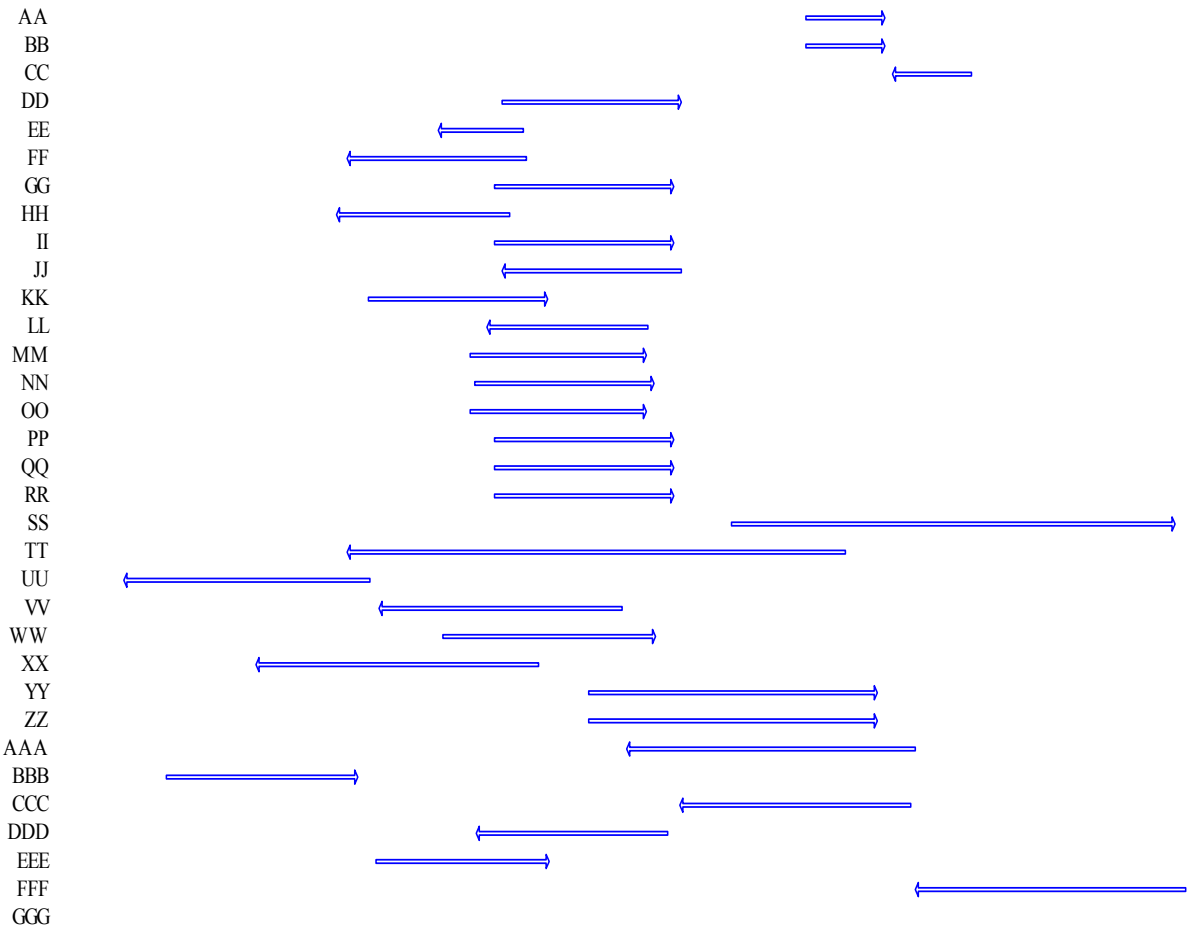

Figure 1.9(c): Sequence assembly of Family 26 Bacterial β-mannanase enzymes (AA: *Bacillus subtilis* B17; BB: *Bacillus subtilis* B21; CC: *Bacillus subtilis* B24; DD: *Bacillus subtilis* B28; EE: *Bacillus subtilis* B36; FF: *Bacillus subtilis* BME0437; GG: *Bacillus subtilis* CICC 10020; HH: *Bacillus subtilis* CICC 10076; II: *Bacillus subtilis* CICC 10260; JJ: *Bacillus subtilis* G1; KK: *Bacillus subtilis* HB002; LL: *Bacillus subtilis* MA139; MM: *Bacillus subtilis* MN-39; NN: *Bacillus subtilis* SDBZ4; OO: *Bacillus subtilis* WL-3; PP: *Bacillus subtilis* WL-7; QQ: *Bacillus subtilis* ydhT; RR: *Bacillus subtilis* Z2; SS: *Caldicellulosiruptor saccharolyticus* Rt8B.4; TT: *Cellulomonas fimi* ATCC 484; UU: *Cellulomonas fimi* Man26A; VV: *Cellvibrio japonicas* 26B; WW: *Cellvibrio japonicas* Ueda107; XX: *Clostridium cellulolyticum* H10; YY: *Clostridium thermocellum* ATCC27405; ZZ: *Clostridium thermocellum* F1; AAA: *Clostridium thermocellum* YS; BBB: *Dictyoglomus thermophilum* RT46B.1; CCC: *Paenibacillus sp* BME-14; DDD: *Pectobacterium carotovorum;* EEE: *Prevotella bryantii;* FFF: *Rhodothermus marinus* DSM4252). Amino acid sequences obtained from the Carbohydrate Active Enzymes database (http://www.cazy.org/; Henrissat, 1991; Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Cantarel *et al.,* 2008).

of protein sequences is indicated by the presence of identical amino acid residues at analogous parts of proteins. Conservation of protein structures is indicated by the presence of

functionally equivalent, though not necessarily identical, amino acid residues and structures between analogous parts of proteins (Henrissat, 1991; Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Cantarel *et al.,* 2008).

1.4.4.2. Modularity

Modularity refers to the concept that microbial enzymes are composed of modules. Various fungal and bacterial β-mannanases contain non-catalytic carbohydrate-binding modules that are linked to the catalytic domain via flexible linkers (Warren*,* 1996; Sunna *et al.,* 2001; Hägglund *et al.,* 2003). These non-catalytic carbohydrate-binding modules can either be a cellulose-binding or mannan-binding module and are thought to enhance enzyme activity by localizing and attaching the catalytic domain onto the surface of the substrate thereby improving enzyme-substrate association as well as local enzyme concentration (Henrissat *et al.,* 1995; Bolam *et al.,* 2004; Boraston *et al.,* 2004; Perret *et al.,* 2004). Family 5 and 26 β-mannanases belong to the 3-D structure group (β/α)z fold catalytic module characteristic of clan Glycoside hydrolase-A (Stoll *et al.,* 2005). The non- catalytic module consists of carbohydrate binding module which facilitates the targeting of the enzyme to the polysaccharide substrate.

CBM family classification is based on three dimensional structures as well as sequence similarities (Boraston *et al.,* 2004). β-Mannanases in aerobic fungi such as *Trichoderma reesei* are composed of a family 5 catalytic module linked to a family 1 CBM (Yague *et al.,* 1997; Tang *et al.,* 2001). Bacterial β-mannanases from families 5 and 26 have more complex structures. Examples of such complex enzymes are the family 5 β-mannanase enzyme isolated from *Thermoanaerobacterium polysaccharolyticum* which contains an S-layer homology-module or SLH-module and two internal family 16 CBMs (Cann *et al.,* 1999); and

the *Cellulomonas fimi* β-mannanase Man26A which contains a putative SLH-module, a mannan-binding family 23 CBM as well as a module of whose function has currently not been determined (Stoll *et al.,* 1999, 2000; Stålbrand *et al.,* 2006).

Several researchers including Halstead *et al.* (1999), Tamaru and Doi (2000), Shallom and Shoham (2003), and Perret *et al.* (2004) have isolated β-mannanases from both anaerobic bacteria as well as fungi that contain dockerin modules. The dockerin modules attach the β-mannanases either to the microbial cell surface or to multi enzyme complexes such as the cellulosome. *Caldicellulosiruptor saccharolyticus* (containing a family 26 β-mannanase) contains two family 27 mannan-binding modules (Sunna *et al.,* 2001) and *Paenibacillus polymyxa* contains a fibronectin domain type 3, CBM family 3 in addition to two catalytic domains (Han *et al.,* 2006). By comparing modular structures of different β-mannanases, CBMs have been envisaged for several β-mannanases from family 5 and family 26. This, however, has not been empirically determined with the exception of a few studies where researchers have determined that the β-mannanases from *Aspergillus aculeatus* and *Aspergillus niger* appear to lack CBMs (Christgau *et al.,* 1994; Ademark *et al.,* 1998), whereas β-mannanases from both families 5 and 26 appear to be sole catalytic modules. Some other family 5 β-mannanases have CBMs that have been classified into family 3, 16 and family 35. whilst some family 26 β-mannanases have family 23, 27 and 35 CBMs, which have been shown to bind to mannan (Stoll *et al.,* 2000; Sunna *et al.,* 2001). Thus it is difficult to ascertain any obvious patterns in modularity among β-mannanases.

1.5. CLONING AND EXPRESSION OF β-MANNANASE GENE

Gene cloning technology provides an excellent method for the manipulation and control of genes and is helpful in increasing our understanding and knowledge of the structure-function

relationship of genetic systems. It thus comes with no surprise that more than 50% of the industrially important enzymes are today produced from micro-organisms that have been genetically engineered (Dhawan and Jagdeep, 2007). The formation of genetically enhanced/ modified microbial strains with selected enzyme machinery is now possible with the use of recombinant DNA techniques. The sequence of some of the genes encoding *β*-1,4-mannanases have been determined and analysed to deduce the primary structure of mannanase enzymes. In this respect, isolation and cloning of the mannanase gene represents an essential step in the engineering of the most efficient microorganism. In the past decade several reports have been published on the isolation and manipulation of microbial mannanase genes with the aim of enzyme overproduction (Table 1.5) (Dhawan and Jagdeep, 2007). This involves studying the primary structure of the protein, its role in the secreting microorganism, and protein engineering to locate the active site residues and/or altering the enzyme properties to suit its commercial applications (Dhawan and Jagdeep, 2007).

The *Trichoderma reesei* man 1 gene was the first fungal β-mannanase gene characterized (Stålbrand *et al.,* 1995). In 2001, Tang and his collegues determined that the catalytic domain of CEL*4* from *Agaricus bisporus* had the most amino acid sequence similarity with Ascomycete β-mannanases from *A. aculeatus* and *T. reesei*, which belong to glycosyl hydrolase family 5 (43 and 42%, respectively). The design and construction of PCR primers to highly conserved regions has increased due to the availability of a number of family 5 and 26 β-mannanase sequences. This has provided the necessary sequence information for the isolation of the entire genes by the use of genetic engineering techniques such as genomic walking, Reverse Transcription PCR, RACE-PCR, etc., and this has allowed the identification of strains that carry β-mannanase genes from this family. In 1990 there were only two β-mannanase genes available for comparison, i.e., *ManA* from *C. saccharolyticus*

(Luthi *et al*., 1991) and *ManA* from a *Bacillus* sp. (Akino *et al*., 1989). Using these two sequences Henrissat (1990), compared then with 301 glycosyl hydrolases and subsequently classified them as type 5 and type 26 glycosyl hydrolases. There has since been an increase in the number of β-mannanase gene sequences being analysed and grouped into their respective families.

There have been several successful attempts at the cloning and expression of β-mannanases from bacteria such as *Bacillus stearothermophilus, Clostridium cellulovorans, Caldicellulosiruptor* sp. Rt8B*, Clostridium saccharolyticum* (Morris *et al.,* 1995; Sygusch *et al.,* 1998; Sunna *et al.,* 2000) into host strains of *E. coli*. There are however only a few reports on the molecular cloning, expression and sequencing of β-mannanase genes from alkaliphilic bacteria. These include several *Bacillus* sp. (Takeda *et al.,* 2004a, 2004b; Hatada *et al.,* 2005), *B. subtilis* B36 (Li *et al.,* 2006), *Bacillus* N16-5 (Yanhe *et al.,* 2004), *B. agaradhaerens* (Bettiol and Showell, 2002), *Bacillus* sp. *1633* (Kauppinen *et al.,* 2003) and *T. polysaccharolyticum* (Cann *et al.,* 1999).

The sizes of mannanase genes and their protein products are highly divergent (Table 1.5). This indicates that mannanases form a group of proteins that possess similar enzyme activities even though they have highly different primary protein structure. Family 5 includes a collection of highly divergent sequences, comprising not only endoglucanases (Henrissat, 1991, Wang *et al.,* 1993), but also bacterial (Gibbs *et al.,* 1992; Arcand *et al.,* 1993; Morris *et al.,* 1995; Tamaru *et al.,* 1997); fungal (Christgau *et al.,* 1994; Stålbrand *et al.,* 1995; Yagüe *et al.,* 1997) and plant (Bewley *et al.,* 1997) β-mannanases. Amino acid sequence alignments of members of family 5 rarely reveal levels of sequence identity

Table 1.5: Mannanase genes: Occurrence and their products (Dhawan and Jagdeep, 2007)

MICROBIAL SPECIES	PROTEIN	SIZE OF GENE (bp), ENZYME (aa /kDa), EXPRESSION HOST	MODULE ARRANGEMENT	REFERENCE
Aspergillus sulphureus	ManN	1345 bp/48 kDa (*P. pastoris* expressed)	CD5	Chen *et al*. (2007)
***Bacillus subtilis* B36**	Man 36B	1104 bp /367 aa, 38 kDa *(E. coli* expressed)	-	Li *et al*. (2006)
***Bacillus subtilis* B36**	Man	1080 bp /38 kDa (*E. coli* expressed)	-	Zhang *et al*. (2006)
Bacillus* sp. *JAMB-750	Man26A	2994 bp /997 aa (*B. subtilis* expressed)	CD26/CBM23/MBM/ CTIX/MAR	Hatada *et al*. (2005)
Clostridium cellulolyticum	Man5K	2994 bp /997 aa (*B. subtilis* expressed)	CD26/CBM23/MBM/ CTIX/MAR	Hatada *et al*. (2005)
***Bacillus sp.* N16-5**	ManA	1479 bp /461 aa, 50.7 kDa	CD5	Yanhe *et al*. (2004)
Cellvibrio japonicus	ManA, ManB, ManC	-	CD5/CBM2a, CD5/CBD5, CD5/CBD10/?	Hogg *et al*. (2003)
Cellvibrio japonicus	Man26B	-	CD26	Hogg *et al*. (2003)
Dictyoglomus thermophilum Rt46B	ManA	-	CD26	Gibbs *et al.* (1999)
Trichoderma reesei	Man1	1,440 bp; 410 aa, 44.3 kDa (*S. cerevisiae* expressed)	-	Stålbrand *et al.* (1995)
Trichoderma reesei	Man5A	-	CD5/LP/CBM1	Hägglund *et al*. (2003)
Thermotoga maritima	Man5	2,007 bp; 669 aa, 76.9 kDa (*E. coli* expressed)		Chhabra *et al*. (2002)
Agaricus bisporus	Cel4	*(S. cerevisiae, P. pastoris* expressed)	CD/CBM 1	Tang *et al.* (2001)
Clostridium thermocellum	Man26B	1773 bp/ 591 aa, 67.04 kDa *(E. coli* expressed)	CD26	Kurokawa *et al.* (2001)

Clostridium cellulovorans	ManA	1275 bp/ 425 aa, 47.15 kDa *(E. coli* expressed)	LP/DM/CD5	Tamaru and Doi (2000)
Caldibacillus cellulovorans	ManA	4567 bp *(E. coli* expressed)	ORF1/?/CBM3B/CD5/ CBM3b/ORF3	Sunna *et al.* (2000)
Rhodothermus marinus	ManA	997 aa /113 kDa	CD 26	Politz *et al.* (2000)
Cellulomonas fimi	Man26A	951 aa *(E. coli* expressed)	CD26/LP/CB M23	Stoll *et al.* (1999)
Clostridium thermocellum	Man26A	1767 bp /66.81 kDa *(E. coli* expressed)	CD26/LP/DM	Halstead *et al.* (1999)
Thermoanaerobacterium polysaccharolyticum	ManA	3291 bp/119.6 kDa *(E. coli* expressed)	LP/CD/CBM 16/SLH	Cann *et al.* (1999)
Bacillus stearothermophilus	Man F	2085 bp/76 kDa *(E. coli* expressed)	CD5/CBM27	Sygusch *et al.* (1998)
***Vibrio* sp.**	ManA	—	CD5	Tamaru *et al.* (1997)
***Piromyces* sp.**	ManA	—	CD/DM	Millward-Sadler, *et al.* (1996)
Paenibacillus polymyxa	Man26A	4056 bp/1352 aa *(E. coli* expressed)	CD44/FN3/CD26/CBM3	Han *et al.* (2006)
***Caldocellum saccharolyticum* Rt8B.4**	ManA	—	CD26/?	Gibbs *et al.* (1996)
Caldocellulosiruptor saccharolyticus	ManB in CelC	4098 bp	EG/CBM27/CD5	Morris *et al.* (1995)
***Pseudomonas fluorescens* GH26**	ManA	1257 bp /46.9 kDa *(E. coli* expressed)	CD26	Braithwaite *et al.* (1995)

CD: catalytic domain with family number; CBM: Carbohydrate binding module with family number; CTIX: collagen type IX alpha I chain; DM: dockerin module; EG: endoglucanase; FN3: fibronectin type III like repeat; LP: leader peptide; MAR: Membrane anchor region of Gram positive surface protein; MBM: mannan binding module; ORF: open reading frame; SLH: Surface layer like protein region: domain of unknown function; —: not available.

greater than 20%. Members of family 5 have been classified in eight subfamilies, subfamily A1–A8 (Béguin, 1990; Lo Leggio *et al.,* 1997; Hilge *et al*., 1998), with subfamily A7 being eukaryotic mannanases and A8, being bacterial mannanases.

The enzymes characterized within these subfamilies show at least 25% sequence identity and often display similar substrate specificities, for e.g. many enzymes of subfamily A3 additionally reveal lichenase activity, whereas some members of subfamily A4 show considerable xylanase activity (Béguin, 1990). It has also been determined that there is a high degree of sequence homology amongst bacterial β-mannanases with ranges of 34.4 to as much as 96.8% being deduced (Gibbs *et al.,* 1996; Yanhe *et al.,* 2004).

1.6. BIOTECHNOLOGICAL APPLICATION OF β-MANNANASES

In a rapidly expanding and competitive industrial enzyme biotechnology field, cellulases and hemicellulases have emerged as key enzymes. Due to their multiple properties, they find usage in a wide array of industrial applications (Hongpattarkere*,* 2002). Of the one billion US dollars that the world spends on the sales of industrial enzymes, 20% of this amount can be attributed to enzymes such as cellulases, hemicellulases and pectinases (Bhat, 2000). The focus on hemicellulases has until recently only been on xylanase enzymes which are capable of hydrolysing xylan – the primary constituent of hemicellulose in plant material, and has very rarely focused on one of the other constituents of hemicellulose, viz. mannan. Mannan hydrolysis has largely been disregarded despite its abundance. However, the utilization of mannanases for catalyzing the random hydrolysis of β-D-1,4 mannopyranoside linkages present in β-1,4 mannans is as important as the application and use of xylanases (Hongpattarkere*,* 2002).

Numerous industries worldwide use β-mannanases for a variety of applications and as result the worldwide requirement for enzymes for individual applications varies greatly. The use of enzymes in various traditional treatments such as the brewing, baking, food-processing, and leatherwork industries is well documented. However, knowledge about the roles that govern the diverse specificity of these enzymes is limited and, therefore, deciphering these secrets would enable us to exploit β-mannanases for their applications in biotechnology (Dhawan and Jagdeep, 2007).

1.6.1. β-mannanases in poultry feeds

In the animal feed industries enzymes such as β-mannanases are incorporated into feed to enhance its nutritional content (Odetallah *et al.,* 2002; Pettey *et al.,* 2002; Lee *et al.,* 2003a; Daskiran *et al.,* 2004; Jackson *et al.,* 2004; Wu *et al.,* 2005). These β-mannanases are sometimes isolated from extremophilic microorganisms and are likely to exhibit enzymatic properties suited to commercial applications such as feed processing because the manufacturing processes often involve harsh conditions such as those described below. These enzymes are vulnerable to the hydrothermal treatments used in feed processing such as pelleting, expansion and extrusion. The enzyme product has a specific 'stability curve' and a critical temperature point at which enzyme losses start to accelerate. Consequently enzyme producers are generally confronted with the need to guarantee the stability of their enzyme preparations, as well the amount of heat that their enzymes can withstand without deterioration. Commercial enzyme products usually contain several enzyme activities as well as auxiliary materials, such as coating substances, carriers, stabilizers and anti-dusting agents.

1.6.2. Pre-pelleting application of feed enzymes

Substances called carriers are used to immobilize and protect enzymes until their target is reached. Enzyme retrieval from these carriers can be incomplete which can be another of the causes of the loss of enzyme activity, although this type of problem seems to have been solved through the use of specific carriers (Kung, 1993; Gadient and Tristsch, 1995). Before the supplementation of feed with β-mannanases there are two factors which must be considered as they can alter the expected positive outcomes. On one hand there is the enzyme with its own biochemical properties and on the other the carrier or substance utilized to give the enzyme a solid form and adequate volume. One should, therefore, realize the importance of the nature of the carrier, along with its interaction with the enzyme it will "carry", not only from a commercial but from a functional standpoint (Mascrell and Ryan, 1997).

The feed-enzyme can be present as simply "carried on", absorbed, or micronized with each form supposedly an improved form from the previous one, from the standpoint of stability and reliability. In many cases this specification has been utilized in a general form as it is generally accepted that a purified enzyme can be transformed into solid forms on organic or inorganic carriers. In such cases one should refer to "absorbed enzymes" and these from a practical standpoint and are not normally thermally stable and generally have up to 50% of their enzyme activities destroyed when subjected to feed processing. The reason for this different behaviour can probably be explained as a result of the enzyme, which is absorbed on a solid product, normally remaining on the external surface of the carrier and is, therefore, more easily subjected to inactivation (Mascrell and Ryan, 1997).

Animal feed enzymes known as "thermo-resistant," "second generation", or "coated" enzymes are normally enzymes subjected to coating processes and are physically treated so

as to obtain a micronized enzyme, in a solid form, which later on can be incorporated onto a carrier subjected to a coating process with products such as fats, starches, etc. That is why the carrier utilized becomes a fundamental part of the product because it does not only contribute to the solid structure of the product but helps stabilize its enzyme activity, making it resistant to extreme temperatures and pH. This also explains why enzymes in solid form have a very good thermal stability, being capable of resisting temperatures close to 90 °C, even for periods over 30 min (Cowan, 1993).

1.6.3. Post-pelleting application of feed enzymes

Whenever temperatures are beyond the critical point of the enzyme, post-pelleting use of the β-mannanases in liquid form can be considered. Controlled tests and practical experience have shown that post-pelleting application is an efficient way to use enzymes. Losses of enzyme activity caused by the pelleting or extrusion processes are below 10% (Gadient, 1994). Several factors should be considered before this strategy is applicable. The first one is the system used to incorporate the product, i.e. a system that permits a liquid dosification which guarantees a homogenous distribution of the enzyme and, therefore, an intimate contact of the enzyme with the substrate. The requirements for dosification are very strict and therefore, define the spraying system to be used (Mascrell and Ryan, 1997). The spraying process is normally defined by the dose level required as well as the number, size and distribution of the spraying nozzles and the working procedures of the system, which can be either of the batch or continuous type. Trying to obtain a liquid spraying system that will reach the maximum number of feed particles is, of course, the main objective. In the continuous type system, the liquid is applied in the form of a liquid curtain, with turbulence movements, modifying is necessary the time that the feed is exposed to the enzyme. In the batch system it is only necessary to control both spraying equipment and time of application

on the other side one should consider the type of feed to be treated, as the type of pellet produced and the percentage of fines will definitely affect the homogenous distribution of the additive (Mascrell and Ryan, 1997).

In as far as the feed particle or pellet is concerned; the larger size of the pellet the greater the homogeneity of distribution, because it becomes easier to spray on each one of them. In the case of small size particles, it is more difficult to obtain a completely homogenous distribution of the enzyme over all particles. It is, therefore, important to contemplate both the type of feed to be produced and the animal to which it is going to be fed, because if reduced rations are to be fed homogeneity of the feed becomes particularly important (e.g., chickens and piglets). The percentage of fines is another aspect of feed quality that should be considered due to their great surface area it is possible that up to 20% of the enzyme may end up absorbed on that surface and, therefore, lost to the animal, if fines are not recovered (Mascrell and Ryan. 1997).

In view of the extended usage of heat producing processes such as pelleting, expansion, extrusion, etc, in the production of feeds for swine and poultry, thermal stability of the enzyme is an important factor. There is both scientific and practical evidence, that when temperatures range between 70 and 90°C, enzyme activity continues to be unaltered and significant improvements can be obtained (Mascrell and Ryan, 1997). Although criteria used to measure stability vary with products, it can be concluded that within temperatures between 50-80°C, any commercial enzyme should present good thermal stability. Also for the addition of β-mannanases to animal feed to result in noteworthy β-mannan decreases, these enzymes need to be capable of withstanding the temperatures that will be encountered within the animal gut environment. The pineal body regulates the chicken's body temperature and the

sentiment of temperature in the environment. Their body temperature is between 39.8 °C and 43.6 °C (Kiefer, 2001). During *in vivo* trials conducted on β-mannanase supplementation of animal feed, the enzyme is incubated together with the feed for 2 hours after which samples are removed for analysis of the amount of reducing sugar released due to the enzymatic hydrolysis of β-mannans (Vahjen *et al.,* 2005).

Fermentation processes such as solid state fermentation allows for increased purity and protein yields of β-mannanases which facilitates their use in animal feed applications. For a broad application, the cost of the enzyme is one of the main factors determining the economics of a process. Cost reduction of enzyme production by optimizing the fermentation medium is a basic principle for industrial production of feed enzymes and medium optimization was traditionally done by varying one factor while keeping the other factors at a constant level. This technique is incapable of detecting the true optimum especially due to interactions among factors and is also time-consuming **(**Triches-Damaso *et al.,* 2003)**.**

1.6.4. Feed processing

The enzymes used to hydrolyze the anti-nutritional components of animal feed are susceptible to degradation by various environmental factors such as microbial contamination, pH, and high temperatures. As discussed above, compounded feed manufacturing processes involve the exposure to high temperatures and pressure (pelleting, extrusion, expansion, etc.). These processes are considered as being very aggressive to the enzyme, from the standpoint of enzyme stability. Thus enzymes are often added to the feed mix in one of two accepted methods. Some companies prefer to add the enzyme in a solid form of a free flowing powder, as a component of the premix formulation. However, other companies prefer to add the enzyme once the pellets have been produced and cooled. In this case, the enzyme is supplied

as a liquid solution, which is sprayed onto the feed pellets and no risk is taken during the pelleting process. Other processes, such as milling and crumbling, even though they form part of feed processing, are not considered from the standpoint of enzyme stability, as milling is very rarely applied to already mixed feed and even in that case, as it is also the case with crumbling, the heat generated in those steps is not high enough to presumably affect enzyme stability. In this case it will pass through the pelleting process, assuming no significant losses will be produced during pelleting (Mascarell and Ryan, 1997).

One of the most commonly used mannanase enzyme within the animal-feed industry is Hemicell®, EC 3.2.1.78 or mannan endo-1,4-β-mannosidase, an endohydrolase enzyme, which is a fermentation product of *Bacillus lentus*. Hemicell® is available in two physical forms, dry powders and water soluble liquids. Powders are free-flowing, easy mixed and stable, whereas liquids are non-viscous and stabilized. Starter, grower and layer diets are supplemented with β-mannanases as either a liquid concentrate (0, 50, 80, 100, 110 and 720 MU (Hemicell®)/litre) or as a solid-dry-powder (140 MU Hemicell®/kg) of feed. The liquid Hemicell® is sprayed on to the feed prior to feeding or the powder form is incorporated into the feed (Odetallah *et al.,* 2002; Jackson *et al.,* 2004). The commonly applied dosage of the dry powder Hemicell® to feed is 300-500 grams /tonne feed, which is normally added to premixes, mixed together with the other components of a feed and further processed, by e.g. pelleting, whilst the dosage of liquid Hemicell® is 110 ml /tonne feed which is diluted with water and then sprayed (post-pelleting) onto the feed.

Once the enzyme-treated feed is fed to the animal, there must be sufficient time for the enzyme to degrade the anti-nutritive non-starch polysaccharide (NSP) feed components such as mannan, galactomannan, xylan and arabinoxylan in order to free and make available to the

animal most of the meal's starches and proteins which are trapped in the NSP. As the movement of feed from the mouth to the first part of the intestines (anterior duodenum) within the animal is time constrained, the amount of enzyme supplemented must be sufficient to digest the expected amount of target substrate in the feed within the transit time, or else, whatever substrate is digested after the duodenum will no longer be absorbed as desired. Thus, the dose of the enzyme supplement must be target ingredient inclusion rate dependent – meaning it is increased or decreased according to the incorporation level of target ingredient.

The analysis of enzyme activity as a quality control determination and evaluation of enzyme activity and stability is a fundamental point to consider, not only from the standpoint of enzyme definition but even from a commercial viewpoint since the potential user should be able to refer their selection of enzyme to objective qualities and practical aspects of the different available products. This explains the numerous attempts to standardize these analyses, through assays requiring easily available substrates and methods reflecting physiological reality, with the ultimate objective of obtaining simple and reliable data (Mascarell and Ryan, 1997). The dinitro salicilate method, which detects reducing sugars appearing as a direct effect of enzyme activity on carbohydrates, under known temperatures and pH (Bailey, 1988) is one of the most commonly used analytical methods when enzymes for animal feeding are considered.

1.6.5. Benefits of using β-mannanases in poultry feeds

1.6.5.1. Use of β-mannanases to improve the nutritional value of poultry feeds

Animal feed contains several anti-nutritional non-starch polysaccharide (NSPs) carbohydrates such as mannan, galactomannan, xylan, arabinoxylan, β-glucans, arabinogalactans, and

pectins which depress animal performance. Monogastric animals such as poultry (chickens and turkeys) and pigs are unable to digest these NSPs. Feed ingredients such as soybean meal (SBM), palm kernel meal (PKM), copra meal (CM), guar gum meal and sesame meal contain NSPs in the form of β-mannan (78%) which is found in the cell wall, and impair the digestibility and utilization of nutrients either by direct encapsulation of the nutrients or by increasing the viscosity of the intestinal contents (Sae-Lee, 2007). This causes a reduction in the rate of hydrolysis and the absorption of nutrients in the diet. Soybean meal and full fat soy are used in poultry feeds and swine diets (Pettey *et al.,* 2002) whereas PKM and CM are used as ruminant feed (Moss and Givens, 1994; Chandrasekariah *et al.,* 2001) and in rabbit diets (Aganga *et al.,* 1991). There is a commonality in the properties of all these feeds, namely high fibre content, low palatability, lack of several essential amino acids and high viscosity coupled with several anti- nutritional properties such as mannan, galactomannan, xylan and arabinoxylan, which all result their limited utilization (Sae-Lee, 2007).

β-Mannanases are able to digest the β-mannans and as a result release digestible sugars which can then be absorbed and metabolized by monogastric animals. The release of these digestible sugars has a positive effect on poultry health by reducing weight gain and glucose and water absorption and increases the feed conversion efficiency in poultry which is generally low due to intestinal viscosity caused by the consumption of mannan-based feeds. The beneficial effect of enzymatic degradation of β-mannan by addition of β-mannanase to diets containing SBM has been documented in broilers (Lee *et al.,* 2003a; Daskiran *et al.,* 2004; Jackson *et al.,* 2004), layers (Wu *et al.,* 2005), turkeys (Odetallah *et al.,* 2002) and swine (Pettey *et al.,* 2002).

1.6.5.2. Effect of β-mannanase on Growth Performance and immunity on broilers

In a study conducted by Zou *et al.* (2006), 204 broilers (one day old) were randomly allotted to 4 treatments. Each treatment (3 pens of 17 chicks per pen) was used to investigate the effects of β-mannanase based Hemicell® on growth performance and immunity. The chicks received the same basal diet based on corn-soybean meal and Hemicell® with an enzyme activity of > 165×10^6 U/kg was added to the basal diet at 0, 0.025, 0.05, and 0.075%, respectively. It was shown that the β-mannanase enzyme hydrolysed the β-mannan present within the feed and this generated an increase in insulin secretion and glucose absorption within the animals. The supplementation of the basal feed with Hemicell® increased relative immune organ weights (except for relative thymus weight of 3-week-old broilers provided with 0.075% Hemicell® and relative bursa weight of 6-week-old broilers provided with 0.025% Hemicell®). Supplementation also increased the concentration of serum IgM and T-lymphocyte proliferation of 6-wk-old broilers provided with 0.05% Hemicell®. This study has demonstrated that β-mannanases may improve the immunity of broilers (Zou *et al.,* 2006).

1.6.5.3. Effect of β-mannanase on the depression of growth in broiler chickens caused by the intestinal viscosity of guar meal

Guar gum is a highly viscous galactomannan polysaccharide composed of approximately 65% mannose and 35% galactose (Kok *et al.,* 1999). Guar meal is a by-product of guar gum extraction and has a protein content of 38% (Nagpal *et al.,* 1971). Guar meal comprises two fractions, germ and hull, which contain 45% and 35% protein, respectively. These fractions are produced during the extraction process. The hull and germ compose approximately 21

and 44% of the guar bean, respectively (Conner, 2002). It has been found that the use of high concentrations of guar meal reduces broiler chicken growth and feed efficiency presumably due to excessive digesta viscosity (Nagpal *et al.,* 1971; Conner, 2002) β-mannanase supplementation reduces intestinal viscosity and counteracts deleterious effects associated with increased viscosity of the intestinal contents (Bedford *et al.,* 1991; Almirall *et al.,* 1995; Lee *et al.,* 2003b).

1.6.5.4. Effects of β-mannanase on layer performance

β-mannanase has been shown to improve feed efficiency and weight gain in broilers and turkeys (Odetallah *et al.,* 2002). In an experiment conducted with 6400 Hyline W36 and W77 hens, Jackson *et al.* (1999a) determined the effect of β-mannanase (Hemicell®) at the recommended manufacturer's dose of 100 Mu/tonne of complete feed. They observed a 1% increase in egg production from 31 to 66 weeks of age. The use of the enzyme increased the egg weight in the early stages of production by 0.5 g/egg or 0.4 lb/case. The increase in egg weight is of particular interest with young hens where egg size is of greatest concern.

In experiment conducted by Jackson (1999b), 3200 Hyline W36 hens were supplied with four amino acid levels ranging from 80 to 110% of commercial requirements from 17 to 66 weeks of age. It was found that the energy levels were maintained constant between amino acid levels. This experiment is consistent with the previous study above by where egg production was increased by 0-2% as a result of enzyme addition over the production cycle. In this experiment, increasing the amino acid levels increased egg production up to the 100% level without enzyme treatment, while enzyme addition resulted in increased production up to 110% of commercial amino acid levels. This experiment demonstrated an increased utilization of protein at high densities.

1.6.5.5. Effect of β-mannanase on body weight uniformity

An important criterion in determining profits of farmers and animal breeders is body weight uniformity. A pen study conducted at the PARC Institute examined mixed-sex broilers at 21, 35, and 49 days of age with 12 replicate pens per treatment. Variability, as determined by coefficient of variation (CV) was considerably reduced at all ages with the β-mannanase enzyme. At 49 days of age, the CV was reduced from 13.4 to 11.3% as a result of the enzyme, representing a 16% reduction in body weight variability (McNaughton *et al.,* 1998). It is clear from the above study that the smallest animals are most positively affected by the addition of β-mannanase enzyme in the feed.

Manno-oligosaccharides, mannotriose and mannobiose as well as a small amount of mannose are generated when mannanases is included in poultry diet. Research has shown that the production of mannooligosaccharides improves a chicken's health. This is accomplished by either increasing the population of specific bacteria such as *Bifidobacteria* 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 (Lyons and Jacques, 2002). The use of β-mannanase in poultry research is widespread and has proven to be successful (Odetallah *et al.,* 2002; Pettey *et al.,* 2002; Lee *et al.,* 2003a; Daskiran *et al.,* 2004; Jackson *et al.,* 2004; Wu *et al.,* 2005; Zou *et al.,* 2006).

1.6.6. Other applications of β-mannanases

Other applications of β-1,4-mannanases include food and instant coffee processing, paper and pulp processing together with xylanases, as stain removal boosters in the laundry detergent industry, as non-nutritional food additives and degraders of thickening agents, as well as the

removal of oil from well bores during oil drilling (Kobayashi *et al.,* 1984, 1987; Viikari *et al.,* 1994; Adams *et al.,* 1995; Gübitz *et al.,* 1996a; McCoy, 2001; Schäfer *et al.,* 2002; Puchart, *et al.,* 2004; Nunes *et al.,* 2006).

1.7. CONCLUDING REMARKS

β-Mannanase biodiversity represents a precious resource for biotechnological advancement and plays an important role in the search for improved strains of microorganisms to be used in industry. Recently there has been an increase in the search for novel mannanases isolated from extreme conditions such as freezing Arctic waters, hot waters, saline waters, or extremely alkaline or acidic habitats. These mannanases are likely to imitate some of the unnatural properties of the enzymes that are desirable for their commercial applications and the exploitation of biodiversity to provide microorganisms that produce mannanases well suited for their diverse applications is considered to be one of the most promising future alternatives (Dhawan and Jagdeep, 2007).

A strain of the filamentous fungus *Scopulariopsis candida* was isolated from the solar salterns around the Florisbad salt pan in the Free State province, north-north west of the city of Bloemfontein (Mudau and Setati, 2008). They demonstrated that *S. candida* LMK008 is capable of secreting β-mannanase which shows potential as a feed additive for monogastric animals. *Microascus brevicaulis* and *M. manginii* which are holomorph species are documented to include anarmorphs *S. brevicaulis* and *S. candida* (Guègen) Vuillemin, respectively (Abbott and Sigler, 2001). The colonies of *Scopulariopsis* sp. are fast-growing, differ in colour from cream, white, grey, buff to brown, and black but are primarily light brown in colour. The conidia are subglobose, truncate, fine to coarsely roughened and the size ranging from 3.5-8.5 × 4-7.5 µm, (Lumley, 1999). Members belonging to the genus

Scopulariopsis are soil fungi and have been isolated from dairy products, paper, rice and cheese (Benguin and Nolard, 1999; Andrews *et al.,* 2000; Taligoola *et al.,* 2004). Some strains of *S. candida* have been found to be pathogenic. For example, studies conducted on immunocompromised host have found that some *Scopulariopsis* sp. causes onychomycosis and invasive sinosal infection (Kriesel *et al.,* 1994). Isolates such as *S. brumptii, S. candida* and *S. brevicaulis* which were obtained from nail lesions or outdoor aerosols have been shown to be keratinolytically active. *S. brevicaulis* which has been isolated from bird feathers have been reported as being associated with bird-borne illnesses (Camin *et al.,* 1998; Marchisio and Fusconi, 2001).

Moulds such as *S. candida, S. brevicaulis* and *S. brumptii* have been isolated from hypersaline environments including the Dead Sea (Buchalo *et al.,* 1998; Kis-Papo *et al.,* 2003; Grishkan *et al.,* 2004; Steiman *et al.,* 2004; Mudau and Setati, 2008). Currently there are a small number of reports on the production of polysaccharide-degrading enzymes by the genus *Scopulariopsis.* It has been reported that a *Scopulariopsis* sp. isolate produced two isoenzymes of endo-1,4-β-xylanase (Afzal *et al.,* 2005), *S. brevicaulis* TOF-1212 produced endoglucanase (Nakatani *et al.,* 2000) and *S. candida* LMK004 and LMK008 (used in the current study) produced β-mannanase (Mudau and Setati, 2006).

1.8. SCOPE OF THE STUDY

The isolation and cloning of β-mannanase genes represents an essential step in the engineering of the most efficient mannanase-producing microorganism. Genetic engineering technology today provides the possibility to over-express these individual enzymes in a host strain and as a result numerous protein expression systems have been utilized for the expression of heterologous proteins. It has been found that proteins expressed in *Escherichia*

coli lack eukaryotic post-translational modifications and are often insoluble and trapped in inclusion bodies and as a result of this may not be biologically active (Chen *et al.,* 2007).

In order to overcome the above disadvantages, eukaryotic yeast expression systems have been developed for the over-expression of heterologous proteins. They have many advantages, such as protein processing, protein folding, and post-translational modification as well as the fact that the yeast can be grown in inexpensive media (Chen *et al.,* 2007).

The industrial use of endo-acting hydrolytic enzymes such as endo-β-1,4-mannanases (EC 3.2.1.78) have gained increased interest due to their ability to modify and degrade mannan-containing polysaccharides in the feed industry (Jackson *et al.,* 1999a; Jackson *et al.,* 1999b). β-Mannanases catalyze the random hydrolysis of β-1,4-mannosidic linkages within the backbone of mannan, galactomannan, glucomannan, and galactoglucomannans which are major constituents of animal feed.

Interest in the cloning and heterologous expression of mannan-degrading enzymes by *S. candida* has been triggered by the discovery of the ability of *Scopulariopsis* species to produce mannanases which are active under hypersaline environments which are characterized by low water activity. It is thought that enzymes derived from halopohilic orgnanisms would be most suitable as animal feed additives since they could be added directly to the feed with low moisture input and still hydrolyse the target substrate. Monogastric animals such as poultry (chickens and turkeys) and pigs are unable to digest non-starch polysaccharide (NSPs) carbohydrates, due to various anti-nutritional properties that depress animal performance. The use of feeds supplemented with β-mannanases has been shown to reduce weight gain and feed conversion efficiency in poultry birds due to

intestinal viscosity and thus will eliminate the indigestible components and therefore enhance the feeding value of soybean meal.

1.9. HYPOTHESIS

It is hypothesised that the β-mannanases from *S. candida* LMK008 can efficiently hydrolyse mannan-containing poultry feed and thereby reduce the viscosity of the digesta and improve feed digestibility.

1.10. OBJECTIVES

1.10.1. To clone and express the β-mannanases from *S. candida* LMK008 in *E. coli*, and

1.10.2. To evaluate the potential of the β-mannanase from *S. candida* LMK008 as an animal feed additive.

1.11. AIMS

1.11.1. To monitor β-mannanase production by the filamentous fungus *S. candid*a LMK008 and assess if the fungus has multiple forms of the β-mannanase;

1.11.2. To synthesize full length *S. candid*a LMK008 β-mannanase cDNA and determine the gene sequence;

1.11.3. To clone the β-mannanase encoding gene in *E. coli*; and

1.11.4. To monitor the *in vitro* hydrolysis of mannan-based substrates as well as soybean meal by the β-mannanase from *S. candida* LMK008.

1.12. KEY QUESTIONS TO BE ANSWERED

1.12.1. How many mannanases does *S candida* LMK008 possess?

1.12.2. What effect does the β–mannanase have on the hydrolysis of mannan-based substrates and soy-bean animal feed?

CHAPTER 2:

PRODUCTION OF MANNANASE FROM *SCOPULARIOPSIS CANDIDA* LMK008 AND EVALUATION OF ITS EFFECT ON THE HYDROLYSIS OF MANNAN-BASED SUBSTRATES AND POULTRY FEED

2.1. INTRODUCTION

Poultry feeds are made up of a variety of different components, each designed to provide poultry with the maximum amount of nutrients required for their successful growth and improved egg laying capability. Poultry feed ingredients such as corn, wheat, oats, barley, sorghum, and milling by-products include concentrates which provide energy. Commonly used protein concentrates consist of soybean meal and other oilseed meals (peanut, sesame, safflower and sunflower), cottonseed meal, animal protein sources (meat and bone meal, dried whey, fish meal) grain legumes such as dry beans and field peas, and alfalfa. Grains are usually ground to improve digestibility (Mattocks, 2002).

Soybean flour is formed by the fine grinding of defatted soybeans and sized using a 0.149 mm sieve (Smith and Circle, 1972). Soybean β-mannans are composed of similar polysaccharides as locust bean gum. Soybean meal (SBM) is commonly used as a major protein source in the poultry and livestock feed industry (McEllhiney, 1994). SBM meal is a by-product of the vegetable and industrial oil industry, which removes the valuable oil, leaving a high-protein meal useful for livestock and poultry feed. It is produced by the toasting and blending of ground soybean hulls. SBM contains substantial amounts of carbohydrates (40%), of which 15 to 22% is polysaccharide comprising of acidic polysaccharides (8 to 10%), arabinogalactans (5%), and cellulosic materials (1 to 2%) (MacMasters *et al.,* 1941; Honing and Rackis, 1979), whereas the lesser-known portion (1 to 2%) are the heat-insensitive anti-nutritional β-mannans (Dierick, 1989).

β-Mannans which are mainly associated with the hull and fibre fraction of SBM are intensely anti-nutritional due to their highly viscous properties (Reid, 1985). This high viscosity reduces feed conversion and decreases the efficiency of carbohydrate utilization of

monogastric animals by partially blocking receptor sites on the intestinal surface (Dale, 1997). Other anti-nutritional properties such as ureases, goitrogens, antivitamins, phytates, saponins, and estrogens are deactivated by heat during the drying-toasting phase of the processing of soybean flour.

Enzymes such as endo-β-1,4-mannanases (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25) and α-galactosidase (EC 3.2.1.22) which are produced by plants, fungi and bacteria, are required for the complete degradation of mannan polymers (Ademark et al., 1998; Zakaria et al., 1998a, 1998b; Ferreira and Filho, 2004; Jiang et al., 2006). Research has shown that the addition of β-mannanases into animal feed generates manno-oligosaccharides, mannotriose and mannobiose as well as small amounts of mannose via the hydrolysis of mannan (Kensch, 2008). The mannose can then be absorbed in the intestine of mono-gastric animals. The production of mannobiose and mannooligosaccharides are, to some extent, irrelevant since these components of carbohydrates may not be absorbed and are therefore unable to supply energy to the animal. The production of mannooligosaccharides via enzyme degradation of mannan-based compounds improves poultry and swine health. (Nunes, and Malmlof, 1992; Jackson *et al.,* 1999a; Jackson, 2002; Odetallah *et al.,* 2002; Jackson *et al.,* 2003, 2004). The inclusion of β-mannanase into mannan-based animal diets results in decreased intestinal viscosity, which in turn improves both the weight gain of poultry and their feed conversion efficiency (Odetallah *et al*., 2002; Lee *et al*., 2003a; Daskiran *et al*., 2004; Jackson *et al*., 2004; Wu *et al*., 2005). In a study conducted by Tamaru *et al.* (1997), it was found that endo-mannanase from *Vibrio sp.* could not hydrolyse mannotriose but produced mannotriose. In another study by Kusakabe and Takashi (1988) it was found that the endo-mannanase from *Streptomyces* could hydrolyse mannotriose to form mannose and mannobiose. Industrial forms of mannanases are in the form of endo β-mannanase (e.g. Gamanase and Hemicell

mannanase), thus it can be conjectured that manno-oligosaccharides, mannotriose and mannobiose as well as a small amount of mannose are generated when this type of enzyme is included in poultry diets. Since only mannose can be absorbed in the intestine, the production of mannobiose and mannooligosaccharides are, to some extent, ineffective and these components of carbohydrates may not be absorbed and are therefore unable to supply energy to the host. It was also determined that the production of mannooligosaccharides can improve a chicken's health (Van Laere *et al*., 1999), 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 the current study, the production of mannan-degrading enzymes by *S. candida* strain LMK008 is reported with focus on the hydrolysis of mannan-based substrates and mannan-containing animal feed by this enzyme.

2.2. MATERIALS AND METHODS

2.2.1. Growth and Maintenance of culture

S. candida LMK008 previously isolated from solar salterns (Mudau and Setati, 2008) was maintained on Malt Extract Agar Plates (MEA) supplemented with 2.5% (w/v) NaCl. A stock solution of basal medium was prepared with the following mineral composition [(g/l) 7.0 KH_2PO_4, 2.0 K_2HPO_4, 0.5 $MgSO_4.7H_2O$, 1.0 $(NH_4)SO_4$] (Ferreira and Filho, 2004), which was further supplemented with 5.0 g locust bean gum (galactomannan), 0.6 g yeast extract and 100.0 g NaCl per litre. One centimetre mycelial pieces were cut out from the MEA stock plates and used to inoculate 100 ml of the basal medium in 500 ml shake flasks. The inoculated flasks were incubated at 25°C with shaking at 150 rpm for 7 days.

2.2.2. Plate enzyme assays

In order to detect cellulose, xylanase and mannanase activity, plate assays were performed using the crude enzyme extract (Teather and Wood, 1982) on MEA plates supplemented with 0.2% (w/v) carboxymethyl cellulose, 0.2% (w/v) oats spelt xylan, and 0.2% (w/v) locust bean gum, respectively. Fifty micro liters of the crude enzyme extract obtained from the culture filtrate was loaded in a well in the centre of the plate, and in the case of the control plates, 50 µl of phosphate buffer (pH 5.0) was added to the wells in place of the crude enzyme extract. The plates were then incubated at 37 °C for 16 h. After incubation, the plates were flooded with 0.1% (w/v) Congo red solution, incubated at room temperature for 15 min and washed several times with sterile 1 M NaCl solution to remove unbounded excess dye. A clearing zone surrounded by a red background was indicative of the hydrolysis of carboxymethyl cellulose, oats spelt xylan, or locust bean gum via cellulase, xylanase or β-mannanase enzymes respectively.

2.2.3. Determination of peak production of β-mannanase enzyme by *S. candida* LMK0008

One centimetre mycelial pieces were cut out from the MEA stock plates of *S. candida* LMK0008 and used to inoculate 100 ml of the basal medium (as prepared in section 2.2.1) in 500 ml shake flasks. The inoculated flasks were incubated at 25 °C with shaking at 150 rpm and sampled every 24 h for 6 days. The culture filtrates were collected by passing fungal cultures through a Mira cloth (Calbiochem, USA) to remove mycelia. The filtrate was analysed for β-mannanase activity using the DNS method for reducing sugars as indicated in section 2.2.4 below. Once the time for peak enzyme production was determined, flasks were re-inoculated and incubated for that period of time and the filtrate was harvested as described.

Three biological repeats were set-up for each experiment and analyses were done in triplicate for each sample.

2.2.3.1. Determination of β-mannanase activity

β-Mannanase activity was assayed using 0.5% (w/v) locust bean gum as a substrate. The substrate was prepared in 50 mM citrate buffer pH 5 by homogenizing at 80 °C and heating until the mixture boils. The mixture was cooled and left overnight with continuous stirring. The insoluble materials were removed by centrifugation at 3 840 × *g* for 5 min (Stålbrand *et al.,* 1993). The assay mixture contained 900 µl of the substrate and 100 µl of supernatant. The reaction mixture was incubated at 50 °C for 10 min. Reducing sugars produced due to enzyme activity were determined as mannose reducing equivalents using the modified dinitrosalicylic acid (DNS) method (Bailey *et al.,* 1992). 3,5-Dinitrosalicylic reagent was prepared without light exposure by first dissolving 10 g dinitrosalicylic acid (Sigma), followed by addition of 16 g NaOH which was also allowed to dissolve. Three hundred grams of Rochelle salt (Potassium-Sodium-Tartrate) was then slowly added and the solution was adjusted to 1 litre and then warmed briefly at 45 °C to dissolve all reagents. Mannose was used as a standard and β-mannanase activity was expressed in nkat (1 nkat = 0.06 International Unit defined as 1 µmol.min^{-1}).

2.2.4. Determination of protein concentration

Total protein levels in the crude enzyme extract and each extract during the purification stages were determined using the Micro BCA^{TM} protein assay reagent kit (Pierce, USA).

2.2.5. SDS-PAGE, Native-PAGE, and Zymogram analysis

Crude protein extracts, ammonium sulphate precipitates and purified proteins were analysed using SDS-PAGE, Native-PAGE and Zymogram analysis. One millilitre of the purified sample was freeze-dried, resuspended in 100 µl citrate-buffer pH 5 and dialysed for 2 h. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in conjunction with zymogram and native poly-acrylamide gel electrophoresis (Native-PAGE) were employed to assess the purity of the fractions and to determine the molecular mass of the protein. The crude enzyme extract was subjected to ammonium sulphate fractionization by increasing the concentration of the ammonium sulphate stepwise (20 to 80 % (w/v) by adding predetermined amounts of solid ammonium sulphate. The amount of ammonium sulphate added was determined from published nomograms (Dawson *et al.,* 1969). Each protein precipitate was dissolved individually in fresh 50 mM phospate buffer (pH 6.0) .The precipitated protein of interest was recovered by centrifugation (3 840 × *g* for 5 min) and dissolved in fresh buffer for the next stage of purification. Each of the protein fractions where assayed for β-mannanase activity and total protein concentration as described above in sections 2.2.3.1 and 2.2.4 above respectively. Thirty microliters of the concentrate was mixed with 30 µl Laemmli sample buffer and denatured by boiling for 10 min. Twenty microlitres of each sample was separated on 10% SDS-PAGE gels followed by Zymogram analysis to confirm activity and the molecular mass of the protein bands. The protein bands on the SDS-PAGE gel were visualized by staining with silver stain. The PageRuler Plus^{TM} prestained protein ladder (Fermentas, Hanover, Maryland, USA) was used to estimate the molecular mass of the proteins. Prior to the zymogram analysis, the SDS-PAGE gel was washed twice with 25% (v/v) isopropanol in 50 mM phospate buffer (pH 6.0) for 1 h each, followed by three washes with 50 mM phospate buffer (pH 6.0) for 1 h each. All washes were carried out with shaking at 150 rpm at room temperature. These washing steps served to re-nature the

proteins. The SDS-PAGE gel was then placed on a 0.5% (w/v) substrate gel which was prepared by adding 1% (w/v) agarose to a 0.5% locust bean gum solution and heating until the agarose dissolved. The mixture was cast into a Petri-dish and allowed to solidify. After electrophoresis, the gel was placed on the substrate gel and incubated at 50 °C for 1 h followed by staining of the substrate gel using 0.1% (w/v) Congo red solution for 20 min. The substrate gel was then destained using 1 M NaCl and then transferred to 0.5% acetic acid to enhance clarity of hydrolysis zones that indicated β-mannanase activity (Stålbrand *et al.,* 1993). To confirm the activity and molecular mass of the purified protein, undenatured protein samples were separated on a 10% Native-PAGE containing 1% (w/v) locust bean gum substrate which was then stained with 0.1% (w/v) Congo red solution for 20 min followed by destaining using 1 M NaCl. Bands exhibiting β-mannanase activity were shown as clear zones of hydrolysis against a dark background.

2.2.6. IEF and 2-Dimensional SDS-PAGE of crude 56 kDa band

S. candida LMK008 β-mannanase enzyme was produced as described in section 2.2.3 above. The 56 kDa β-mannanase band was excised and eluted out into Tris-HCl (pH 7.5) buffer. One milligram per millilitre of the eluted sample was loaded onto Criterion isoelectric focusing (IEF) precast gels (Bio-Rad) to determine the pI of the proteins and to identify which ion exchange column would be suitable for purification purposes**.** The PROTEAN Ready Gel precast gel together with the PROTEAN Plus Dodeca cell (Bio-Rad Laboratories**,** Hercules, California, U.S.A) were used to perform the the 2-D SDS-PAGE. The samples were run for 1 h: 30 min at 200 V, followed by 1 h 30 min at 400 V. All protocols used were as per manufacturer's instructions as described in the 2-D Electrophoresis for Proteomics: A Methods and Product Manual from Bio-Rad Laboratories **(**Hercules, California, U.S.A). A substrate gel was prepared by adding 1%

agarose to a 0.5% locust bean gum solution and heating until the agarose dissolved. The mixture was cast into a glass Petri-dish and allowed to solidify. After IEF the gel was placed on the substrate gel and incubated at 50 °C for 1 h followed by staining of the substrate gel using 0.1% (w/v) Congo red solution for 2 h. The substrate gel was then destained using 1 M NaCl and then transferred to 0.5% acetic acid to enhance clarity of hydrolysis zones that indicated mannanase activity (Stålbrand *et al.,* 1993).

2.2.7. β-Mannanase purification

S. candida LMK008 was cultivated until peak β-mannanase production as described in section 2.2.3. The ammonium sulphate precipitated dialysates were filtered through Cameo 0.45 μm nylon syringe filters (Micron Separations Incorporated, USA) and loaded onto an Bio-Scale™ Mini Macro-Prep High Q anion exchange cartridge (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were eluted with a linear gradient of 1 M NaCl in Tris-HCl buffer pH 7.5 at a flow rate of 1 ml min^{-1}. The fractions were assayed for β-mannanase activity using the DNS assay as described in section 2.2.3.1 above. Protein concentrations in the samples before and after purification were determined using the Bradford assay (Bradford, 1977).

2.2.8. Hydrolysis of mannan-based substrates by crude β-mannanase

Five milligram per millilitre stock solutions of either locust bean gum mannan, gaur gum mannan or soybean flour mannan (Sigma, USA) were prepared in 50 mM sodium-citrate buffer, pH 5.0, as previously described in section 2.2.3.1. These galactomannans differ from each other in their mannose/galactose ratio as well as the distribution pattern of galactose residues along the mannan chain. The mannose/galactose ratios of locust bean gum, guar gum

and soybean meal are approximately 3.5:1 and 1.5:1, 1.8:1 units, respectively (Peng *et al.,* 1991; Zhang and Tizzard, 1996; Daas *et al.,* 2000; Ross *et al.,* 2002; Mikkonen *et al.,* 2007).

The hydrolysis was carried out for 7 h at 45 °C with a final concentration of 2 nkat β-mannanase/mg substrate. Control reactions were set up whereby the substrates were incubated under the same conditions without the addition of enzyme. The resulting amount of reducing sugar released from the control reaction was subtracted from the resulting amount of reducing sugar released from the samples containing the enzyme in order to account for any background sugars that may be present within the samples. One millilitre samples were removed on an hourly basis and release of reducing sugars was determined using the dinitrosalicylic acid reagent (Bailey *et al.,* 1992). The samples were then cooled and the absorbance was read at 540 nm.

2.2.9. Hydrolysis of poultry feed by crude and purified β-mannanase

ALZU feed samples were obtained from Dr. M.J. Chimuka of the Agricultural Research Council of South Africa. The general composition of the feed starter-meal, grower-meal, and layer –meal samples is listed in Table 2.1. Stock solutions of 0.5 $g.ml^{-1}$ of feed samples were prepared in 50 mM sodium-citrate buffer, pH 5.0. Hydrolysis was carried out for 24 h at 45 °C. Initally 5 nkats crude enzyme extract was used. However, it was shown that the crude extract also had cellulase activity. The enzyme was purified and feed hydrolysis was determined using 5 nkats purified β-mannanase.

Table 2.1: General Composition of ALZU Starter, Grower and Layer Meals

ALZU BROILER MEAL TYPE	ALZU BROILER FEED COMPONENTS				
	ALZU Starter Broiler Concentrate (kg)	ALZU Grower Broiler Concentrate (kg)	ALZU Layer Broiler Concentrate (kg)	Maize (kg)	TOTAL (kg)
Starter meal	400	-	-	600	1000
Grower meal	-	400	-	600	1000
Layer meal	-	-	300	700	1000

2.3. RESULTS

2.3.1. Plate enzyme assays of crude β-mannanase

The screening of fungal isolates for hemicellulosic degrading enzyme production using qualitative assays such as plate enzyme assays gives a positive or negative indication of enzyme production. These assays are useful when screening for several classes of enzymes, where definitive quantitative data is not required. Zones of hydrolysis were observed on the locust bean gum (Fig. 2.1; A1) and cellulose (Fig. 2.1; B1) substrate plates, whilst no zones of hydrolysis were observed on the plates containing oat spelt xylan as a substrate (Fig. 2.1; C1). Also in the control plates, in which no enzyme was added, there are no zones of hydrolysis which can be observed in either the locust bean gum (Fig. 2.1(A2)), cellulose (Fig. 2.1; B2) or xylose (Fig. 2.1; C2). It can be deduced that *Scopulariopsis candida* LMK008 exhibited mannanase and cellulase activity but not xylanase activity.

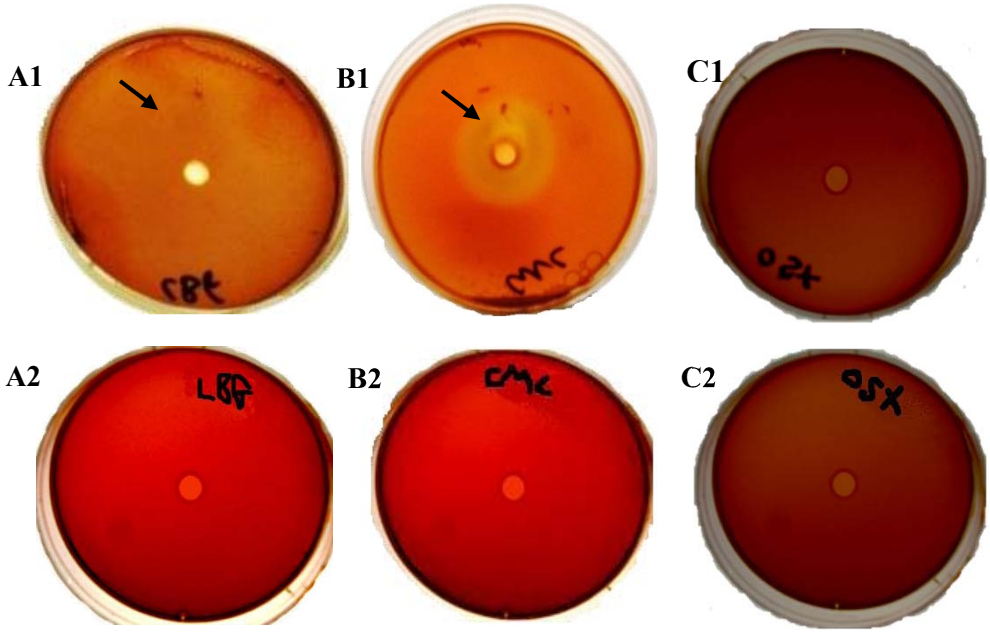

Figure 2.1: Zones of enzyme hydrolysis on MEA plates supplemented with either (A) locust bean gum, (B) carboxy-methyl cellulose or (C) oats spelt xylan substrate respectively. Enzyme assay: (A1) Positive β-mannanase hydrolysis, (B1) Positive cellulase hydrolysis, and (C1) Negative xylanase hydrolysis. Control assay: No zones of enzyme hydrolysis observed on the (A2) locust bean gum plate, (B2) carboxy-methyl cellulose plate or (C2) oats spelt xylan plate. Black Arrows indicate zones of hydrolysis.

2.3.2. Determination of peak production and activity of β-mannanase enzyme by *S. candida* LMK008

β-Mannanase activity of *S. candida* LMK008 was detected on day 2 (10.07±0.526 nkat.ml^{-1}) (Fig. 2.2) and increased exponentially up to day 4 (131.58±3.08 nkat ml^{-1}). Highest activity was detected on day 5 (171.78±3.05 nkat ml^{-1}) and this level of production was maintained for 24 hours after that.

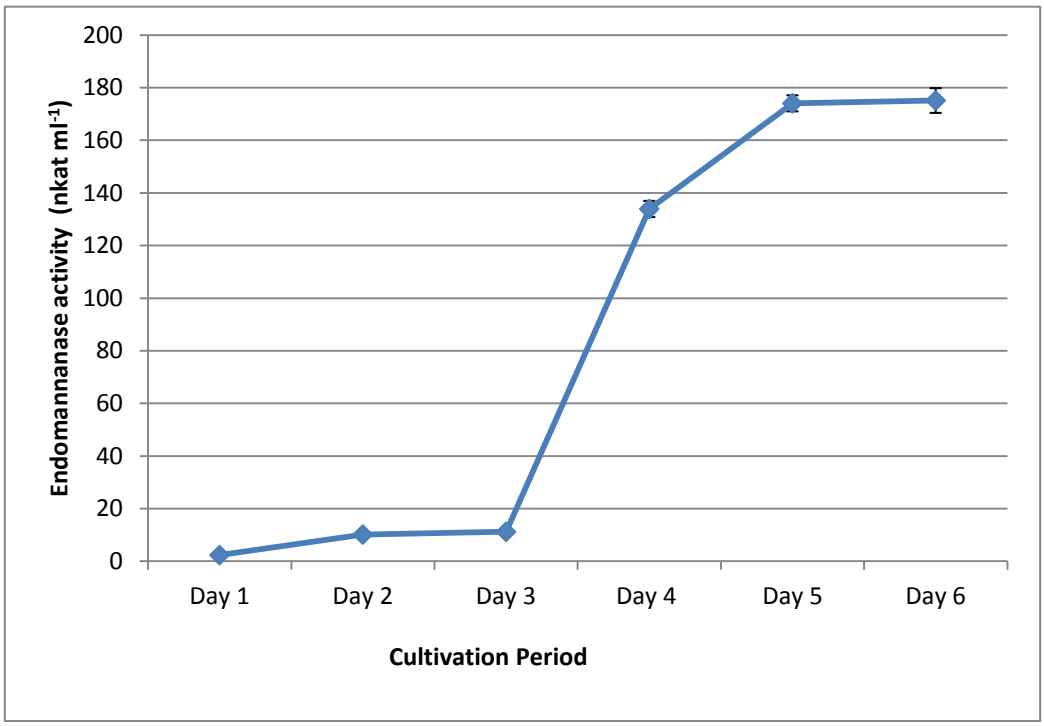

Figure 2.2: Time course for β-mannanase production by *Scopulariopsis candida* LMK008. Average values of independent triplicate experiments were used. n=3.

2.3.3. Determination of protein concentration of crude enzyme extract

It was determined that the crude enzyme extract had a total protein concentration of 533.70 μg.ml^{-1}.

2.3.4. SDS-PAGE of the crude LMK008 β-mannanase extract

SDS-PAGE analysis of the crude LMK008 β-mannanase extract revealed several protein bands with varying intensities and sizes (Fig. 2.3).

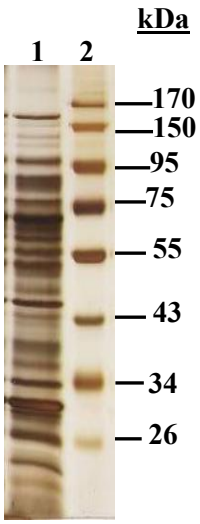

Figure 2.3: An SDS-PAGE gel of the crude β-mannanase from LMK 008 showing LMK008 β-mannanase (lane 1) (black arrow), and molecular weight marker - PageRuler Plus™ prestained protein ladder 10 to 170 kDa (Fermentas, Hanover, Maryland, USA) (lane 2)

2.3.5. SDS-PAGE and native gel electrophoresis of crude enzyme and ammonium sulphate precipitated protein fractions

SDS-PAGE analysis of the ammonium sulphate precipitated proteins revealed bands of approximately 56 kDa and 28 kDa (Fig, 2.4A). These were confirmed to be β-mannanase enzymes through Native-PAGE (Fig 2.4B).

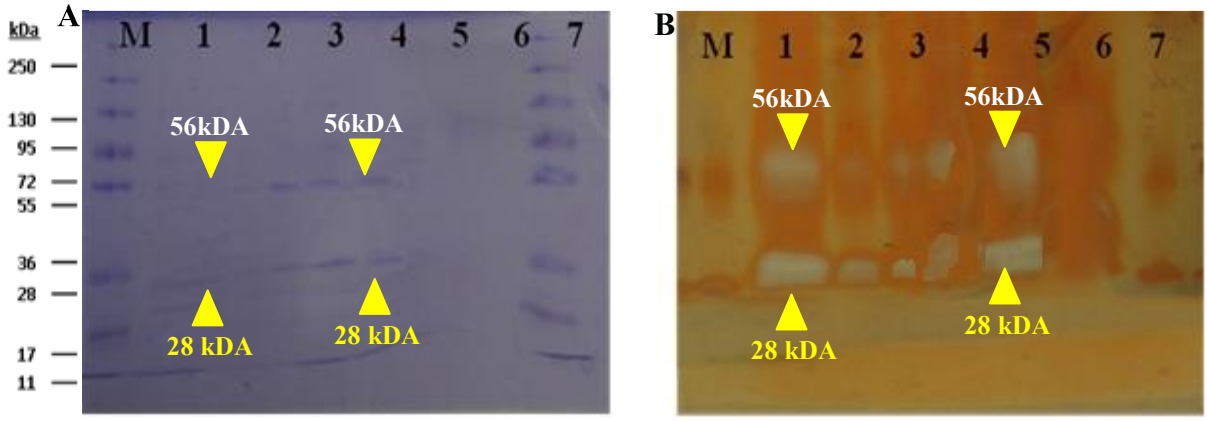

Figure 2.4: SDS-PAGE and Native gel of crude enzyme and ammonium sulphate precipitated protein fractions (A) SDS-PAGE Gel and (B) Native PAGE gel of Ammonium sulphate precipitated protein fractions. (Lane M: Protein marker; Lane 1: Crude enzyme extract; Lane 2: 20% (w/v) fraction; Lane 3: 40% (w/v) fraction; Lane 4: 60% (w/v) fraction; Lane 5: 80% (w/v) fraction; Lane 6: 80% (w/v) filtrate; Lane 7: Protein marker - PageRuler Plus ™ prestained protein ladder 10 to 250 kDa (Fermentas, Hanover, Maryland, USA).

2.3.6. Zymogram of 2D-PAGE analysis of 56 kDa β-mannanase

Two spots corresponding to β-mannanase activity were observed for the 56 kDa protein at two different pI values; 3.5 and 6.7 (Fig. 2.5).

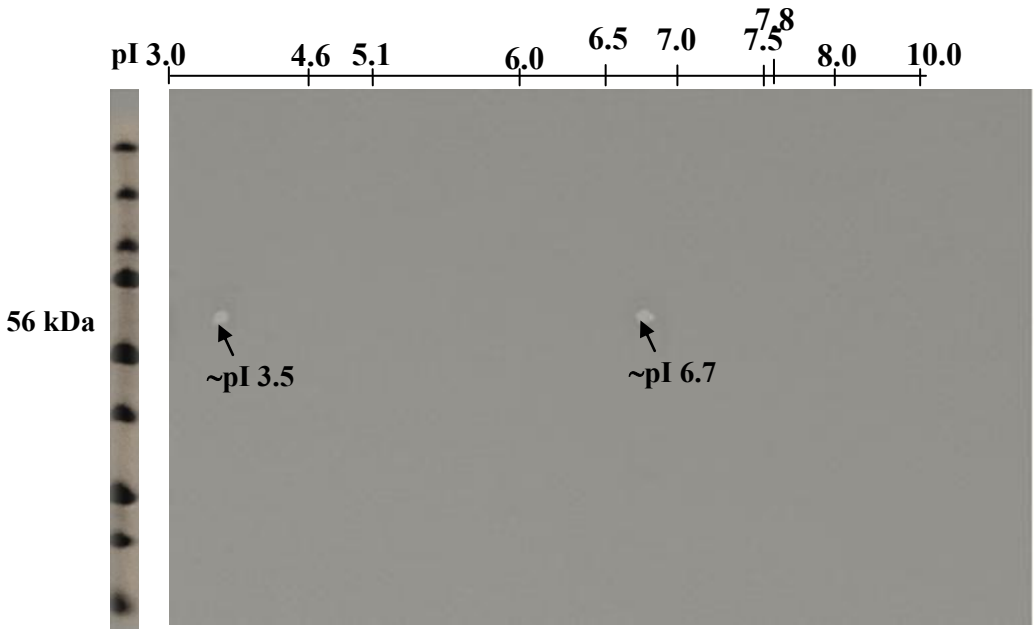

Figure 2.5: Zymogram gel of 2D-PAGE gel. Two clear spots indicate pI of the two 56 kDa isozymes (~3.5 and 6.7)

2.3.7. Purification of β-mannanase enzyme

The β-mannanase enzyme was purified from culture filtrate using ammonium sulphate precipitation and anion exchange chromatography. A 91.25-fold purity level was attained during purification of the β-mannanase resulting in an increase in specific activity from 1681.73 nkat.mg^{-1} to 1845.46 nkat.mg^{-1} (Table 2.2).

2.3.7.1. SDS-PAGE, NATIVE-PAGE and Zymogram analysis of purified β-mannanase

SDS-PAGE analysis of the purified β-mannanase revealed only one intense band of approximately 28 kDa (Fig, 2.6A). This was confirmed to be a β-mannanase through native-PAGE in conjunction with zymogram and activity staining (Fig 2.6B and 2.6C).

Table 2.2: Summary of purification of β-mannanase from *S. candida* LMK008

PURIFICATION STEP	VOLUME (ml)	ACTIVITY (nkat)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (nkat mg^{-1})	PROTEIN YIELD (%)	DEGREE OF PURIFICATION
Crude filtrate	100	192.76 ±0.006	0.11462 ±0.009	1681.73	100.00	1.00
$(NH_4)_2SO_4$ fraction	10	186.93 ±0.024	0.11053 ±0.006	1691.22	96.99	1.01
Anion Exchange Chromatography	5	178.18 ±0.001	0.09655 ±0.001	1845.46	91.25	1.09

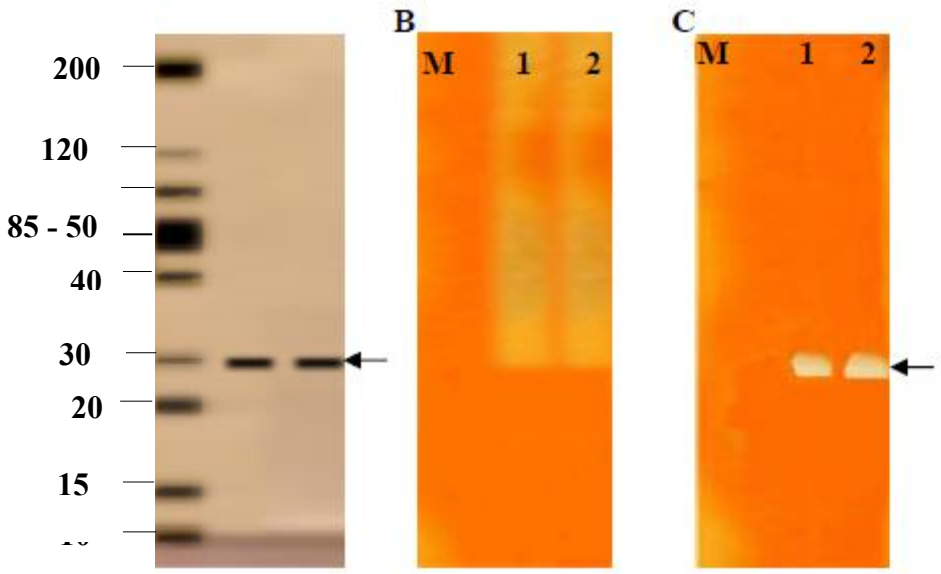

Figure 2.6: SDS-PAGE, Native-PAGE and Zymogram gels of the purified β-mannanase. (A) SDS-PAGE Gel, (B) NATIVE PAGE gel, (C) Zymogram of purified β-mannanase (Lane M: Protein marker - PageRuler Plus™ prestained protein ladder (Fermentas, Hanover, Maryland, USA); Lane 1 and Lane 2: 28 kDa β-mannanase [black arrow]).

2.3.8. Hydrolysis of mannan-based substrates by crude enzyme extract

Three mannan-based substrates; locust bean gum, guar gum and soybean flour, were used to evaluate the substrate specificity of crude enzyme i.e. its ability to hydrolyse the different mannan-based substrates. After 1 h of incubation there was a gradual increase in the

production of reducing sugars (Fig. 2.7). The hydrolysis of soybean flour and locust bean gum resulted in an increase of the production of reducing sugars which continued up until 4 h and 5 h respectively, where it peaked and thereafter began to decline. After 5 h of incubation the amount of reducing sugars produced from the hydrolysis of locust bean gum mannan (4.12 $mg.ml^{-1}$) and soybean flour mannan (7.31 $mg.ml^{-1}$) substrates began to decrease until after 6 hours of incubation. The hydrolysis of soybean flour mannan after 5 h of incubation decreased with a release of 4.76 $mg.ml^{-1}$ of reducing sugars and this trend continued up till 7 h of incubation.

The hydrolysis of soybean flour mannan resulted in the highest amount of reducing sugars being released with a 7.31 $mg.ml^{-1}$ release of reducing sugar after 5 h of incubation at 45 °C whilst the lowest release of reducing sugars was 2.12 $mg.ml^{-1}$after just 1 h of incubation. A similar trend of hydrolysis was observed with locust bean gum. However, the highest amount of reducing sugars released, i.e., 6.76 $mg.ml^{-1}$, was observed after 4 h incubation as compared to the soybean flour whose highest release of reducing sugars occurred after 5 h of incubation. The lowest levels of reducing sugars release amongst the three substrates were observed with guar gum hydrolysis with 3.88 $mg.ml^{-1}$of sugars being released after 4 h of incubation.

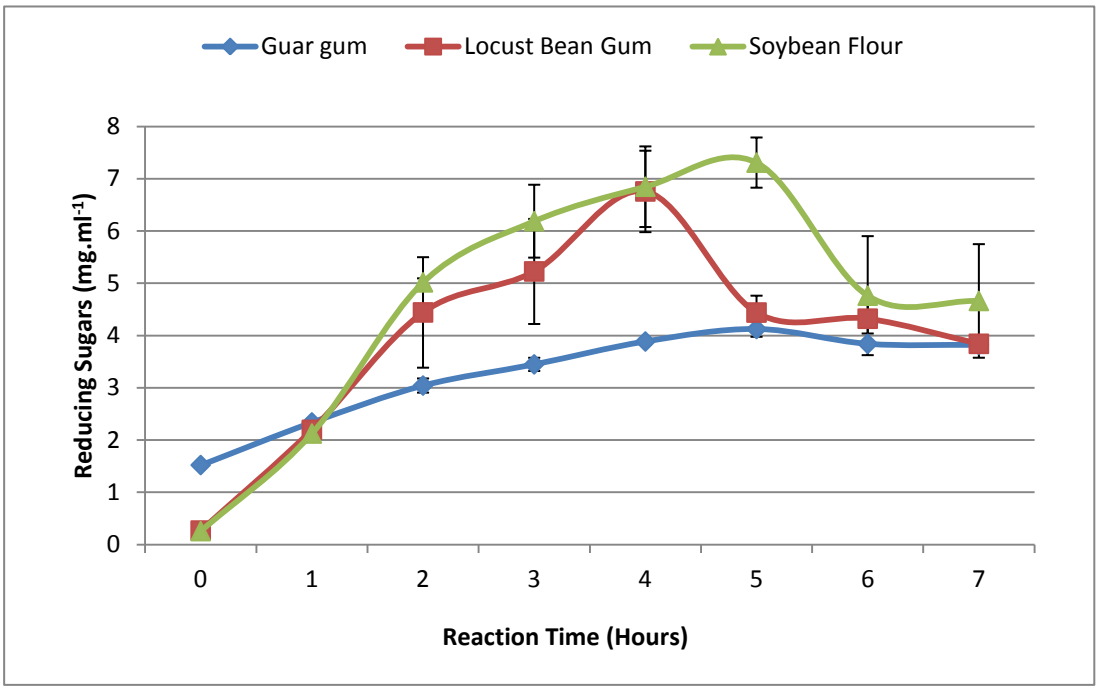

Figure 2.7: Hydrolysis of mannan-based substrates by β-mannanase crude enzyme extracts of *S. candida* LMK008 as determined by the DNS assay of reducing sugars. Average values of independent triplicate experiments were used. n=3.

2.3.9. Hydrolysis activity of *S. candida* LMK008 β-mannanase on animal feed

2.3.9.1. Hydrolysis activity of S. candida LMK008 crude enzyme extract on animal feed

Mannan-based animal feed was used to test the hydrolyzing activity of the crude β-mannanase extract via the release of reducing sugars using the DNS assay. A comparison of the three different feed types showed that there was a greater release of reducing sugars from the grower feed than the other two feed types (Fig. 2.8). It was observed that hydrolysis of the grower feed resulted in a 2.64 $mg.ml^{-1}$ release of reducing sugars after 2 h incubation whilst hydrolysis of starter and grower feeds resulted in a lower level of reducing sugars been released, i.e., 1.45 $mg.ml^{-1}$ and 1.69 $mg.ml^{-1}$, respectively

A general hydrolysis pattern was observed in that more reducing sugars were released from the grower feed than that of the other two diet s, with the least amount of re lease being observed with the starter feed hydrolysis. Another common hydrolysis pattern observed in all feed type was that after prolonged incubation of 24 h, there was a decrease in the amount of reducing sugars released (Fig. 2.8).

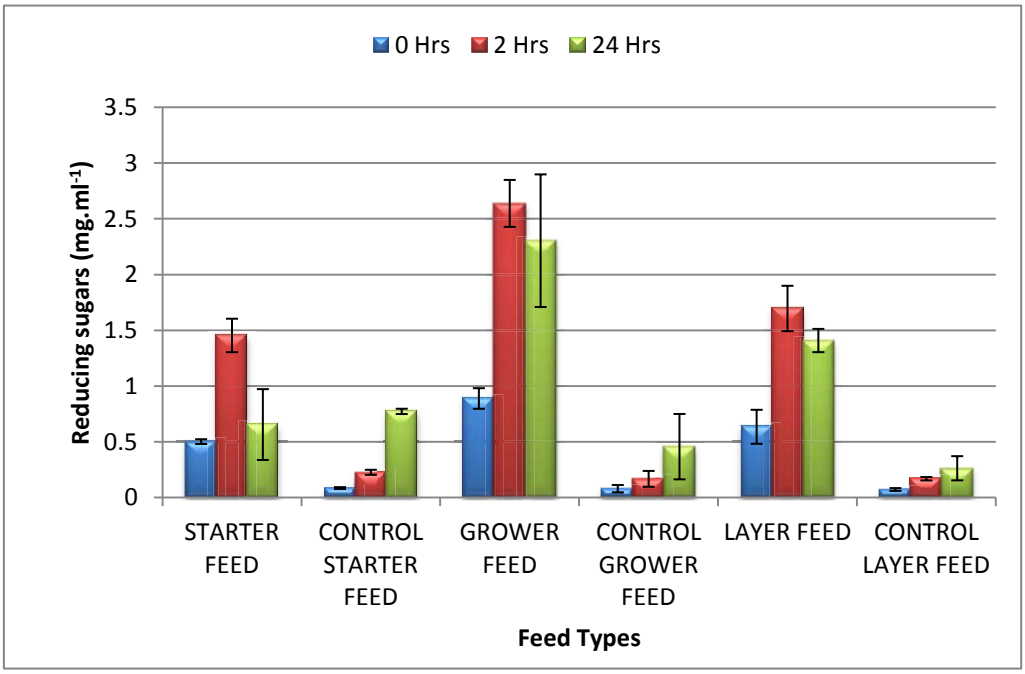

Figure 2.8: Hydrolysis of animal feed subs trates by β-mannanase crude en zyme ext racts of *S. candida* LMK008 as determined by the DNS assay of reducing sugars. Average values of independent triplicate experiments were used. n=3.

This decrease could be a result of the presence of naturally occurring microorganisms such as bacteria and fungi in the actual feed. To validate this, 24 h hydrolyses samples of the starter, grower and layer feed types were plated out on to nutrient agar plates and incubated for 24 h at 37 °C. Average plate counts (Table 2.3) revealed the presence of bacterial colonies (Fig. 2.9).

Table 2.3: Colony counts (cfu.ml^{-1}) of 24 h hydrolysis samples. Average values of independent triplicate experiments were used.

FEED TYPE	AVERAGE PLATE COUNTS (cfu.ml^{-1})
Starter Meal	$6.5 \times 10^2 \pm 2.251$
Grower Meal	$1.6 \times 10^2 \pm 1.366$
Layer Meal	$3.9 \times 10^2 \pm 1.789$

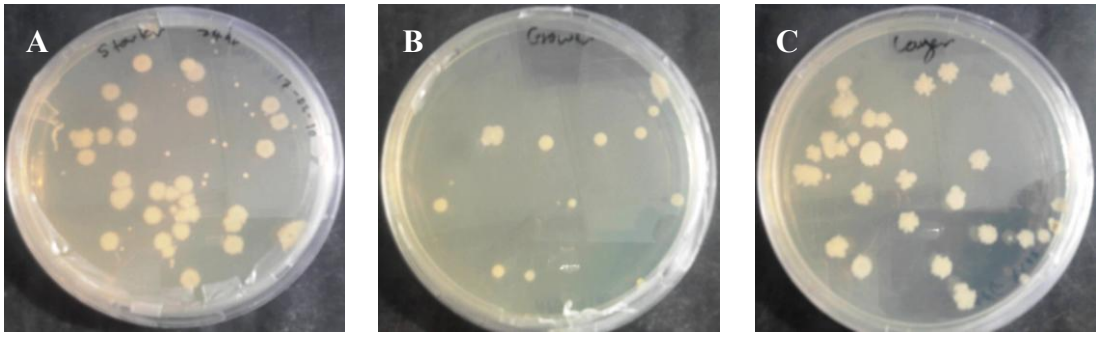

Figure 2.9: Colony plate counts of the 24 hour hydrolysis samples. (A) Starter Meal diet plate counts, (B) Grower Meal diet plate counts and (C) Layer Meal diet plate counts.

2.3.9.2. Hydrolysis activity of S. candida LMK008 purified enzyme extract on animal feed

Similar hydrolyis patterns were observed when the purified β-mannanase was used to hydrolyse the animal feed when compared to that of the crude enzyme hydrolysis patterns. In general it was observed that more reducing sugars were released from the grower feed than that of the other two diets, with the least amount been observed for the starter feed. Another common hydrolysis pattern observed in all feed types was that after prolonged incubation of 24 h there was a decrease in the amount of reducing sugars released (Fig. 2.10).

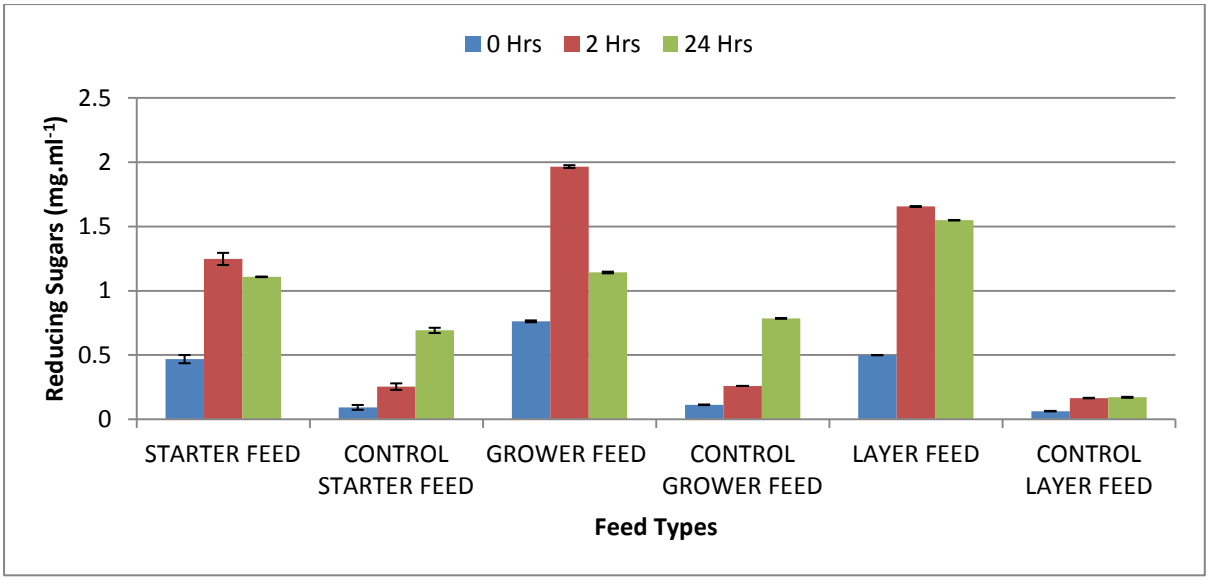

Figure 2.10: Hydrolysis of animal feed substrates by β-mannanase purified enzyme extracts of *S. candida* LMK008 determined by the DNS assay of reducing sugars. Average values of independent duplicate experiments were used. n=3.

2.4. DISCUSSION AND CONCLUSION

Filamentous fungi are capable of secreting considerable amounts of hydrolytic enzymes into their culture medium. This characteristic has been harnessed for industrial enzyme production (Nevalainen and Te'o, 2003; Peterson *et al.,* 2009). The *S. candida* strain LMK008 investigated in the current study was isolated from a hyper saline environment (Madau and Setati, 2006). In the current study, this *S. candida* strain LMK008, which was previously shown to produce β-mannanase was investigated to evaluate the potential of the enzyme in animal feed processing. The β-mannnase activity level (175 nkat.ml^{-1}; Fig. 2.2), obtained in the current study is within the ranges reported for other β-mannanase producers. Some of the known β-mannanase producers include *S. rolfsii* ATCC 46890 with a production of 2591 nkat.ml^{-1} (Gübitz *et al.,* 1996a), *A. fumigatus* IMI 385708 (668 nkat.ml^{-1}) (Puchart *et al.,* 2004), *T. lanuginosus* CBS 395 (247 nkat.ml^{-1}) (Puchart *et al.,* 2000), *A. niger* NCH189 (247 nkat.ml^{-1}) (Lin and Chen, 2004), *S. candida* LMK004 (CBS 118736)

(104 nkat.ml^{-1}) (Mudau and Setati, 2006), *A. niger* (56 nkat.ml^{-1}) (Ademark *et al.,* 1998), *V. dahliae* LMK006 (56 nkat.ml^{-1}) (Mudau and Setati, 2006) and *S. rolfsii* CBS 191.62 (55 nkat.ml^{-1}) (Sachslehner *et al.,* 1998).

SDS-PAGE analysis of the crude extract revealed multiple bands (Fig. 2.3). Prior to anion exchange chromatography, the crude enzyme preparation was subjected to 60% (w/v) ammonium sulphate precipitation, and 56 kDa and 28 kDa bands were observed for both the crude enzyme preparation and 60% ammonium sulphate precipitate (Fig. 2.4). The molecular weight of LMK008 β-mannanase was found to be in the range reported for most fungal β-mannanases, including those obtained from *Aspergillus oryzae* (42 kDa; Eriksson and Winell, 1968), *Aspergillus tamarii* (56 kDa, 57 kDa; Civas *et al.,* 1984), *Polyporus versicolor* (34 kDa, 57 kDa; Johnson *et al.,* 1990), *Trichoderma reesei* (46 kDa, 51 kDa, 53 kDa; Stalbrand *et al.,* 1993), *Trichoderma reesei* (53 kDa; Arisan-Atac *et al.,* 1993), *Sclerotium rolfsii* (42 kDa, 61.2 kDa; Gübitz *et al.,* 1996a, 1996b), *Aspergillus niger* (40 kDa; Ademark *et al.,* 1998) and *Aspergillus aculeatus* (50 kDa; Setati *et al.,* 2001). The IEF and 2D-analysis in conjunction with zymogram analysis of the crude enzyme preparation revealed two zones (spots) of clearing corresponding to a molecular weight size 56 kDa but with differing pI values. The first spot had a pI value of 3.5 whilst the second spot had a pI value of around 6.7 (Fig. 2.5). The 2D-PAGE zymogram results obtained indicate that there are multiple isoforms of the 56 kDa β-mannanase.

It was then decided that since both protein spots had an acidic pI, anion exchange chromatography would be suitable for purification purposes**.** A 1.09-fold purity level was obtained during the purification of the β-mannanase with a specific activity of 1845.46 nkat.mg^{-1} being obtained, which is 8.87% more than that of a specific activity of 1681.73 nkat.mg^{-1} of the culture filtrate (Table 2.2). Although several fractions tested positive for

proteins, only one displayed β-mannanase activity. This was confirmed to be a β-mannanase through Native-PAGE in conjunction with zymogram and activity staining and was determined to have a molecular weight of 28 kDa. The purification of the 56 kDa β-mannanase was unsuccessful. This could be due to differences in the binding properties of the two β-mannanases towards the anion exchange column. The result obtained in the current study is consistent with the previous study by Mudau and Setati (2008) in which the authors partially purified the 28 kDa β-mannanase.

Numerous fungal endo-1,4-β-mannanases have been purified and characterized and it has been reported that they exhibit wide molecular weights ranging from 33 - 80 kDa, e.g., the Man (I-IV) and Man (I-IV) isolated from *Polyporus versicolor* (Johnson *et al.,* 1990) and *Thielavia terrestris* (Araujo and Ward, 1990) have molecular weight range of between 30-89 kDa and 33.9-58 kDa respectively, whilst enzymes such as the Man I from *Trichoderma harzianum* has a molecular weight of approximately 36.5 kDa (Ferreira and Filho, 2004). The 28 kDa molecular weight of the purified β-mannanase obtained from *S. candida* strain LMK008 is much lower than those that have been previously isolated and purified. However, the molecular weight of this β-mannanase is similar to that obtained by Blibech *et al.* (2010) who purified and characterised an 18 kDa ManIII from *Penicillium occitanis* Pol6z.

In the previous study by Mudau (2006) the partially purified 28 kDa β-mannanase from *S. candida* LMK008 exhibited varying degrees of halotolerance. It could tolerate higher NaCl concentrations with ≥ 60% of its activity remaining even at 20% (w/v) NaCl. In addition, removal of MgSO4 by dialysis resulted in loss of activity which could signify inactivation of this enzyme at lower salt concentrations, a behaviour common amongst halophilic enzymes (Madern *et al*., 2000). It can therefore, be inferred that the LMK008 β-mannanase would be

more suitable for reactions performed at low water activity such as in the animal feed environment.

Three mannan-based substrates, viz; locust bean gum, guar gum and soybean flour, were used to evaluate the hydrolyzing capability of the crude β-mannanase isolated from *S. candida* LMK008 via the release of reducing sugars. The amount of reducing sugars was influenced by the kind of substrates tested. It was determined that the highest amount of reducing sugars release by the action of the crude β-mannanase on the various substrates was from soybean flour, followed by locust bean gum – indicitative of the enzyme having a higher activity on these substrates, whilst the least amount of reducing sugars resulting from the hydrolysis was from guar gum, which is indicative the enzyme exhibiting low activity with the substrate (Fig. 2.7). A possible reason for the decrease in galactomannan activity in guar gum could be that the hydrolysis yield of the β-mannanases depends on the degree of galactose substitution, as well as the nature of the substitution sugar in the mannan backbone (Sunna *et al.,* 2000). The levels of β-mannan in the mannan-based substrates differ based on their galactose to mannan ratios. Guar gum and soybean flour have a similar galactose to mannose ratio of 1:2 and 1:8 respectively, whilst locust bean gum has a ratio of 1:4 (Hsiao *et al.,* 2006; Maier *et al.,* 1993; Marracini *et al.,* 2005).

It has been reported in the literature that the degree of hydrolysis of galactomannan decreases with increasing substitution by galactose, which suggests that mannanase cleavage of the mannan backbone is obstructed by the sugar residue (McCleary, 1983). In the current study the β-mannanase from *S. candida* LMK008 was shown to exhibit high specificity with galactomannans such as locust bean gum and soybean flour mannan while the specificity decreased with the increase in galactose substitution in the galactomannan guar gum (Fig.

2.7). Similar sets of results were obtained by Bicho *et al.* (1991) who examined hydrolysis of the galactomannans, locust bean gum and guar gum by the recombinant family 5 ManA of *Caldicellulosiruptor saccharolyticus*. They found that ManA exhibited high specificity with the galactomannan locust bean gum, while the specificity decreased with the increase in galactose substitution in galactomannans such as gaur gum. They suggested that α-1,6-galactose branches such as those found in guar gum mannan may sterically hinder the mannanase, preventing enzyme-substrate binding.

The hydrolysis of soybean flour resulted in the highest amount of reducing sugars being released with a 7.31 $mg.ml^{-1}$ release of reducing sugar after 5 h of incubation at 45°C whilst the lowest release of reducing sugars was 2.12 $mg.ml^{-1}$ after just 1 h of incubation (Fig. 2.7). A similar trend of hydrolysis was observed with locust bean gum. However, the highest amount of reducing sugars released from locust been gum i.e., 6.76 $mg.ml^{-1}$, was observed after 4 h incubation as compared to the soybean flour whose highest release of reducing sugars occurred after 5 h of incubation. The lowest levels of reducing sugars release was observed with guar gum hydrolysis with 3.88 $mg.ml^{-1}$ of sugars been released after 4 h of incubation. The results obtained in the current study is comparative to the results obtained by Songsiriritthigul *et al.* (2010) who observed that a β-mannanase isolated from *Bacillus licheniformis* exhibited a slightly larger increase in release of reducing sugars (17.5 $mg.ml^{-1}$) from the hydrolysis of the galactomannan locust bean gum than that of the highly substituted galactomannan from guar gum which according to the authors was negligible when using the standard DNS assay. Also, in a study conducted by Talbot and Sygusch (1990) it was found that when the hydrolysis of locust bean gum and guar gum by the β-mannanase from *Bacillus stearothermophilus* was determined, guar gum exhibited lower mannananase activity (i.e., lower amounts of mannan oligosaccharides being released when compared to the amount of mannan oligosaccharides released from the hydrolysis of locust bean gum (1.5 $mg.ml^{-1}$) (i.e.

higher mannanase activity). The specificity of mannanase for β-1,4 mannosidic linkages is consistent with hydrolysis of locust bean gum, soybean flour and guar gum. The lower β-mannanase activity towards guar gum compared with locust bean gum or soybean flour supports the hypothesis that the enzyme activity is limited by the number of branched α-galactose residues (Gherardini *et al.,* 1987). This same reduction has also been noted for other mannanases, using a galactomannan substrate possessing the same percentage of galactose residues as guar gum (McCleary, 1983). Talbot and Sygusch (1990) determined that *B. licheniformis* ManB prefers soluble and low-substituted mannan substrates which was evident from a comparison of the relative activity on soluble locust bean gum with a mannose-to-galactose ratio of 4:1, and soluble guar gum with a mannose-to-galactose ratio of 2:1 (Gubitz *et al.,* 2001), while the former is a good substrate, the activity on the latter is negligible during the 10-min standard assay. Based on this, it can be concluded that the crude β-mannanase from the current study also prefers soluble and low-substituted mannan substrates such as locust bean gum and soybean mannan.

The degree of digestibility of animal feed (starter, grower and layer diets) subjected to an *in vitro* digestion was indirectly assessed by measuring the total sugars released. The beneficial effects of feed enzymes are primarily the release of sugars (Malathi and Devegowda, 2001). Release of sugars caused by exogenous enzymes is due to the breakdown of the non-starch polysaccharides in the feed which can lead to release of their respective monosaccharides. A comparison of the three different feed types showed that there was a greater release of reducing sugars from the hydrolysis of the grower feed by the crude β-mannanase preparation than the other two feed types (Fig. 2.8). It was observed that hydrolysis of the grower feed by the crude β-mannanase preparation resulted in a 2.64 $mg.ml^{-1}$ release of reducing sugars after

2 h incubation whilst the starter and layer feeds, resulted in a lower level of reducing sugars been released, i.e., 1.45 $mg.ml^{-1}$ and 1.69 $mg.ml^{-1}$, respectively.

A general hydrolysis pattern was observed in that more reducing sugars were released from the grower feed than that of the other two diets, with the least amount of release resulting from the starter feed. Another common hydrolysis pattern observed in all feed types was that after prolonged incubation of 24 h there was a decrease in the amount of reducing sugars released (Fig. 2.8). This decrease could be a result of the presence of naturally occurring microorganisms such as bacteria and fungi in the feed samples. Twenty four hour hydrolyses samples of the starter, grower and layer feed types were plated out onto nutrient agar plates and incubated for 24 h at 37 °C. Average plate counts (Table 2.3) revealed the presence of bacterial colonies (Fig. 2.9), which could account for the decrease in hydrolysis rates after 24 h of incubation. The reason for this decrease could be attributed to the microorganisms utilizing the simple sugars; mannose, mannobiose, mannotriose, mannotetraose and mannopentose, which are a result of the enzymatic breakdown of mannan; as carbon sources. This possible breakdown of the mannan polymers could have resulted in the lower release of reducing sugars by the *S. candida* LMK008 crude β-mannanase preparation after 24 h of incubation.

The hydrolysis of the animal feed by the purified β-mannanase showed similar hydrolysis patterns to that obtained with the crude enzyme. However, it was noted that the amount of reducing sugars released were lower then that of the crude enzyme hydrolysis. It was observed in Figs. 2.1A1 - 2.1B1, that the enzymes present in crude enzyme extract had hydrolysed the locust bean gum mannan-based substrate as well as the cellulose-based carboxymethylcellulose. This was indicative of the presence of β-mannanases and cellulases,

respectively. When the crude enzyme was used for the hydrolysis experiments it was observed that there was a higher release of reducing sugars which could be a result of the action of cellulase, mannanase and other hydrolytic enzmes. This is in comparison to the purified β-mannanase preparation (in which no other hydrolytic enzymes were present – as evidenced by the single band that was observed on the native-PAGE and zymogram gels (Fig. 2.6B and 2.6C), in which there was a lower release of reducing sugars. The lower release of reducing sugars could be the result of only the purified β-mannanse acting on the mannan content of the feed. Also the purification of the crude β-mannanase resulted in only one isoform been present as evidenced in Fig. 2.6 above. As a result of this, there could have been fewer β-mannanase isoforms hydrolysing the mannan-based feed samples as compared to the crude extract, in which a larger proportion of β-mannanase isoforms were present as observed in Fig. 2.5 in which the 56 kDa isoform was observed. The absence of the 56 kDa isomer form the purified enzyme could have contributed to the lower release of reducing sugars as only one of the isomers were present in order to hydrolyse the mannan-based feed.

A comparison of the three different feed types showed that there was a greater release of reducing sugars from the hydrolysis of the grower feed by the crude β-mannanase preparation than the other two feed types (Fig. 2.10). It was observed that hydrolysis of the grower feed by the purified β-mannanase resulted in a 1.97 $mg.ml^{-1}$, release of reducing sugars after 2 hours incubation whilst the starter and layer feeds, resulted in a lower level of reducing sugars been released i.e. 1.25 $mg.ml^{-1}$ and 1.65 $mg.ml^{-1}$ respectively (Fig. 2.10). The results obtained in the current study are comparative to the results obtained by Cho *et al.* (2006) who investigated the application of a β-mannanase isolated from *Bacillus* sp. strain WL1 (with a dosage of 4 $nkat.mg^{-1}$ soybean meal being applied). It was determined that the *Bacillus* mannanase was able to reduce the mannan content within the soybean meal tested and a level

of 1.7 mg.ml^{-1} of reducing sugars after 2 h of incubation was obtained. In another study by Vahjen *et al.* (2005), the application of a β-mannanase isolated from a commercially available strain *A. aculeatus* SP570 (Energex) was tested. They determined that when the enzyme was applied onto soybean meal, 1.46 mg.ml^{-1} of reducing sugars was released after 2 h of incubation. The amount of reducing sugars obtained in their study was more then the amount obtained in the current study. It must be noted that the enzyme activity of the dosage of mannanase used in the study by Vahjen *et al.* (2005) was determined to be 2 nkat.mg^{-1}soybean meal, whilst in the current study an enzyme dosage of 5 nkat.mg^{-1} was used.

Due to the direct relationship that exists between target substrate and enzyme (i.e. arabinoxylan–xylanase, mannan-mannanase, phytate–phytase), the use of single enzyme preparations such as xylanases, mannanases, glucanases or phytases has proven to be reasonably successful in the feed industry (Rosin *et al.,* 2007). The results obtained in the present study suggest the possibility of utilizing the β-mannanase isolated from *S. candida* LMK008 as a feed additive in poultry feed in order to alleviate the deleterious effects of β-mannan present in the feed. In order to obtain a better understanding of the effect that this enzyme has on β-mannan containing feed, it is necessary to conduct *in vivo* trials and this should include studies on the intestinal stability of the enzyme *in vivo*. It would also be important to determine the end products of enzymatic hydrolysis of soya bean non-starch polysaccharides in order to exclude possible undesirable effects.

CHAPTER 3:

CLONING OF

SCOPULARIOPSIS CANDIDA

LMK008 β-MANNANASE GENE

3.1. INTRODUCTION

Heteropolysaccharides such as hemicelluloses vary in chemical composition and are abundant in plant cell walls. The hemicellulose mannan is a major component of the cell walls of plants and some types of seeds backbone comprises β-1,4-linked D-mannopyranose residues (Matheson and McCleary, 1985). Endoacting hydrolytic enzymes such as endo-β-1,4-mannanases (EC 3.2.1.78) catalyse the random hydrolysis of β-1,4-mannosidic linkages within the backbone of mannan, galactomannan, glucomannan, and galactoglucomannan.

β-Mannanases are grouped into the glycoside hydrolase (GH) families 5 and 26 based on their amino acid sequences (Henrissat and Bairoch, 1993). Amongst the microorganisms bacteria, fungi and yeasts are known β-mannanases producers. The following fungal β-mannanases have received the most attention due to their potential for practical applications in food, feed and fruit industries: *Aspergillus aculeatus* (Christgau *et al.,* 1994; Setati *et al.,* 2001), *Aspergillus niger* (Regalado *et al.,* 2000), *Aspergillus sulphureus* (Chen *et al.,* 2007a), *Sclerotium rolfsii* (Gubitz *et al.,* 1996a, 1996b), and *Trichoderma reesei* (Stålbrand *et al.,* 1993; 1995). However, despite the success of these fungal enzymes in industry, their use is still limited by low yields and high production costs. Therefore, identification and exploration of new β-mannanases with good properties and high-level yield is highly sought-after. Gene cloning offers a relatively cheaper alternative to other methods of protein mass production such as optimization of culture conditions, fermentation, etc. Many of the prospective donor microorganisms are reported to be pathogenic and are screened for genetic sequences to be inserted into non-pathogenic hosts (Surgey *et al.,* 1996). Enzyme-producing organisms can also be improved using classical methods of hybridization (Solis *et al.,* 1997; Soetan *et al.,* 2010).

3.2. MATERIALS AND METHODS

3.2.1. Genomic DNA preparation

S. candida LMK008 was cultivated as described previously in chapter two, section 2.2.1. The mycelia were harvested by passing the fungal culture through a Mira cloth (Calbiochem, USA) after which the mycelia was stored at -20 °C until further use. Genomic DNA was prepared from the mycelia of *S. candida* LMK008 using the method of Raeder and Broda (1985). Frozen mycelia were ground into a fine powder in liquid nitrogen using a mortar and pestle. Freeze-dried ground material (50 mg) was re-suspended in 400 µl extraction buffer in an Eppendorf tube by stirring with a pipette tip. The slurry was mixed homogeneously with 300 µl phenol. Then 150 µl chloroform was added and mixed and the suspension was centrifuged for 40 min at 10 000 rpm. The upper aqueous phase was taken off immediately, transferred to an Eppendorf tube containing 25 µl RNAse A solution (became turbid) and incubated for 10 min at 37 °C. The solution was extracted with one volume of chloroform and centrifuged as before but for only 10 min. The upper phase was transferred into a sterile Eppendorf tube and mixed with about 0.54 volume isopropanol (250 µl). DNA precipitated visibly into a lump which was allowed to settle. As much liquid as possible was taken off with a pipette without disturbing the precipitate. The tube was spun for about 5 s and any remaining liquid was drawn out with a Pasteur pipette. The DNA pellet was rinsed with 70% (v/v) ethanol. The ethanol was then carefully decanted and the pellet was dried for 30 min at 37 °C. The DNA pellet was then resuspended in 30 µl sterile nuclease-free water and stored at -20 °C until further use.

3.2.2. PCR amplification of the *S. candida* LMK008 β-mannanase gene

The genomic DNA from *S. candida* LMK008 was amplified using PCR. The following degenerate primers were used: Forward primer MANSU-F (17 bases) (5'-

TTCAACGACGTCAACAC-3') and Reverse primer MANSU-R (17 bases) (5'-AYCCAGCCRTTGCCCCA-3'). The primers used were designed based on sequence alignments of numerous mannanase genes of fungal origin. The genomic DNA was amplified using the following conditions: Initialization step at 95 °C for 2 min; 40 cycles of [Denaturation step: 95 °C for 1 min; Annealing step at 60 °C for 1 min; Extension/elongation step at 72 °C for 1.5 min]; and a Final elongation step at 72 °C for 5 min. The reaction conditions for the PCR were as follows: 1× Supertherm Taq polymerase buffer; 1.0 mM $MgCl_2$; 200 μM dNTPs; 1 U Taq Supertherm polymerase; 0.125 μM Forward primer; 0.125 μM Reverse primer). The PCR product was then run on a 1% (w/v) agarose gel in order to determine if amplification had occurred. Several modifications were attempted to optimise PCR conditions: (i) the use of varying concentrations of $MgCl_2$ (1.5 mM, 2.0 mM, and 2.5 mM) and (ii) changing annealing temperatures (53°C, 54°C, 55°C, and 56°C). The following PCR conditions were used during these subsequent PCR runs: Initialization step at 94 °C for 2 min; 40 cycles of [Denaturation step: 94 °C for 30 s; Annealing step at 53 °C, 54 °C, 55 °C, or 56 °C for 1 min; Extension/elongation step at 72 °C for 1.5 min]; and a Final elongation step at 72°C for 10 min. None of these attempts proved successful.

3.2.3. Construction of a genomic DNA library

The genomic DNA prepared in section 3.2.1 was partially digested with *Xba*I and the digested DNA was then fractionated by agarose gel electrophoresis. The excised ~ 1000 bp fragments were purified with a Zymoclean gel extraction kit (Sigma-Aldrich, USA) according to manufacturer's recommendations. The fragments were ligated into *Xba*I-digested plasmid pUC19 (dephosporylated with alkaline phosphatase) and then transformed into *E. coli* DH5α using the $CaCl_2$ transformation protocol (Sambrook *et al.,* 1989). The transformants were selected on LB-Amplicillin-IPTG-X-gal plates containing 100 $\mu g.ml^{-1}$

Ampicillin, 20 mg.ml^{-1} X-Gal and 200 mg.ml^{-1} IPTG. The plates were incubated overnight at 37 °C and then the white transformants were picked up the plates and grown overnight at 37 °C on LB-Ampicillin (100 μg.ml^{-1}) plates further supplemented with 0.5% locust bean gum for the screening of possible β-mannanase activity by transformants. The transformants were flooded with 0.1% (w/v) Congo-red and destained with 1M NaCl in order to observe zones of clearing which would indicate a transformant with β-mannanase activity (Teather and Wood, 1982).

3.2.4. RNA extraction, cDNA synthesis, cloning

S. candida LMK008 was cultivated, and mycelia harvested as previously described in section 3.2.1. Total RNA was then isolated using the phenol/freeze method of Schmitt *et al.* (1990). The quality of the RNA was determined by running a sample of the RNA on a 2% (w/v) agarose gel in 1× Tris-Borate-EDTA buffer. To generate sufficient quantities of the cDNA from *S. candida* LMK008 RNA, SMART™ RACE cDNA Amplification Kit (Clonetech, CA, USA) or RevertAid™ First Strand cDNA Synthesis Kit **(**Fermentas™, Hanover, USA) were used on separate occasions with approximately 1000 ng of total RNA as per manufacturer's instructions. Degenerate primers MANSU-Forward primer, MANSU-Reverse primer (for the SMART™ RACE cDNA synthesis), Random Oligo(dT)$_{18}$ primer and the Random Hexamer primer (RevertAid™ First Strand cDNA synthesis) were used for each of the cDNA synthesis attempts.

The cDNA fragments generated from the RNA using the RevertAid™ First Strand cDNA Synthesis Kit were then ligated into the pBlueScript vector. The construct was then transformed by electroporation (2.5 kV, 25 μF, 200 Ω) into *E. coli* DH5α electrocompetent cells. The transformed cells were then transferred to a sterile Eppendorf tube and incubated at

37 °C for 1h with moderate shaking. One hundred microlitres of transformed cells was then spread-plated onto LB-Ampicillin (100 mg.ml^{-1}) plates and incubated overnight at 37 °C (Sambrook *et al.,* 1989). Sixteen hours later, the plates were observed for presence of blue-white colonies indicative of transformants (white colonies). cDNA generated from the human GAPDH control RNA (1.3 kb) (supplied with the RevertAid™ First Strand cDNA Synthesis Kit was used as a positive control. The white transformants were picked up from the plates and grown overnight at 37 °C on LB-Ampicillin (100 mg.ml^{-1}) plates further supplemented with 0.5% locust bean gum for screening of possible β-mannanase activity of the transformants. The transformants were flooded with 0.1% (w/v) Congo-red and destained with 1M NaCl in order to observe zones of clearing which would indicate a transformant with β-mannanase activity (Teather and Wood, 1982).

Since none of the cDNA clones produced an active mannanase enzyme, it was decided to ascertain whether the β-mannanase cDNA sequences were harboured in one of the clones. To achieve this, Southern hybridization was done.

3.2.5. Southern blot analysis of *S. candida* LMK008 and control fungi

3.2.5.1. Growth of cultures

S. candida LMK008, *Aspergillus niger, Sordaria fimicola, Trichoderma reesei*, *Fusarium oxysporum, Chaetomium indicum,* and *Penicillium* spp were obtained from the Discipline of Microbiology, University of Kwazulu-Natal culture collection. Agar pieces (cm^2) were cut out from all the MEA stock plates and used to inoculate 10 ml of malt extract and in the case of *S. candida* LMK008, basal medium, in 50 ml shake flasks. The inoculated flasks were incubated at 25 °C with shaking at 150 rpm for 6d.

3.2.5.2. Southern blot analysis

Genomic DNA was prepared from the mycelia of *S. candida* LMK008 and the six control fungi using the method of Raeder and Broda (1985) as described in section 3.2.1. *S. candida* LMK008 genomic DNA was digested with *Eco*RI, *Bam*HI and *Xba*I and the six control samples were digested with *Eco*RI. Thereafter, all samples were separated on a 1% (w/v) agarose gel, transferred onto a Hybond-N nylon membrane and probed with the labeled *man1* gene fragment from *A. aculeatus.* The β-mannanase probe was prepared by restricting the pBS-Man1 isolated from *Aspergillus aculeatus* with *Eco*RI in order to separate the *man1* gene fragment from the pBlueScript (pBS) vector. The restriction product was then separated on a 1% (w/v) / agarose gel and the resulting *man1* gene fragment was excised from the gel and purified using a gel extraction kit. The DNA probe was labeled using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany) as per manufacturer's conditions.

All hybridization procedures were performed as described by the DIG Application Manual for Filter Hybridization (Roche, Germany). Two stringency washes were employed for the hybridization of the β-mannanase *man1* probe to the *S. candida* LMK008 β-mannanase and/or control organisms viz., high stringency ~ 60 °C with 0.5× SSC, 1.0% (w/v) SDS or low stringency ~ 50 °C with 0.2× SSC, 1.0% (w/v) SDS).

3.3. RESULTS

3.3.1. PCR amplification of the LMK008 β-mannanase gene

Attempts to amplify of the LMK008 β-mannanase gene were unsuccessful. Attempts to optimise PCR conditions by using $MgCl_2$ at various concentrations and reducing annealing temperatures also proved unsuccessful (Figs.C1 and C2 in Appendix C). The failure of the

degenerate primers to amplify the gene could be due the lack of homology between the target *S. candida* LMK008 mannanase and other family 5 mannanases.

3.3.2. Construction of a genomic DNA library

Attempts at the construction of a genomic DNA library proved unsuccessful. Although genomic DNA clones were obtained, the mannanase gene was not isolated.

3.3.3. cDNA synthesis and cloning

RACE PCR to generate cDNA from the RNA using either the SMART™ RACE cDNA Amplification Kit or RevertAid™ First Strand cDNA Synthesis Kit was also unsuccessful. Several cDNA clones were obtained and spotted onto substrate plates. None of the clones tested positive for mannanase activity (Fig. 3.1 below, Fig C5, C6 in Appendix C).

3.3.4. Southern Hybridization using the *A. aculeatus man1* gene as a probe

Southern hybridization with a Family 5 mannanase encoding gene from *A. aculeatus* as a probe against genomic DNA from *S. candida* LMK008 (Fig. 3.2B, 3.2C) and other fungi (Fig. C7 in Appendix C) did not produce and positive signals. The technique was carried out successfully as evidenced by a postive signal when the probe hybridised to the positive control (plasmid harbouring the *A. aculeatus* man1 gene) (lane 4, Figs 3.2B, 3.2C).

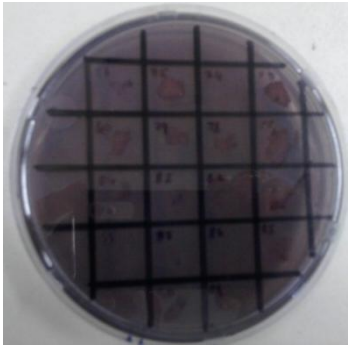

Figure 3.1: Mannanase activity screening of cDNA library bacterial clones.

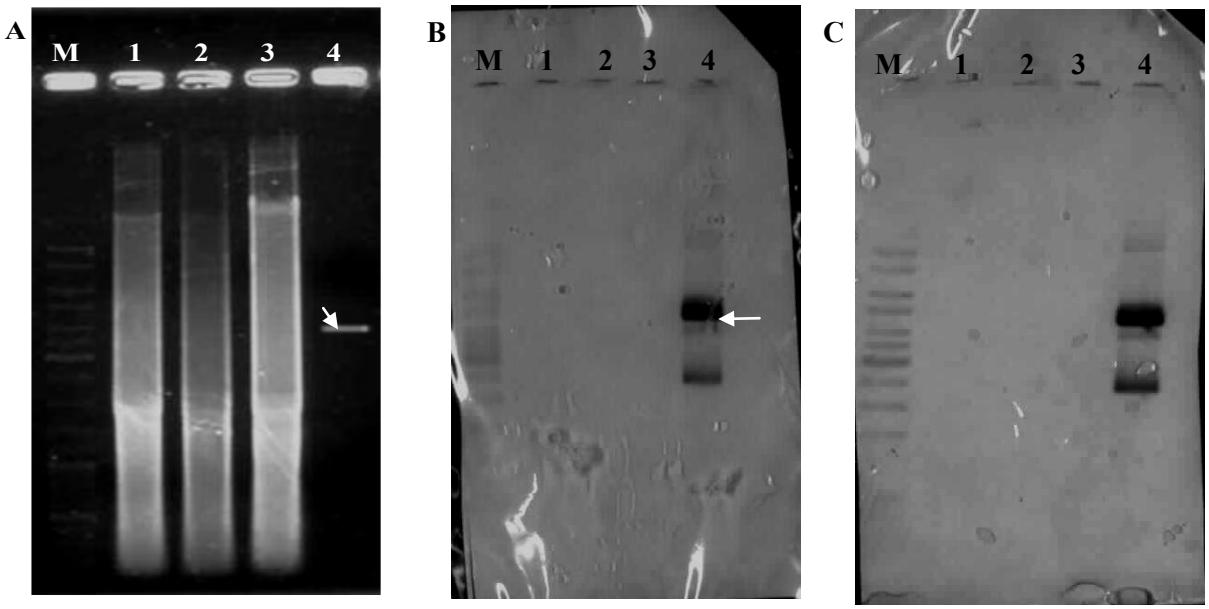

Figure 3.2: DNA electrophoresis of genomic DNA of *S. candida* LMK008 restricted with: *Eco*RI (lane 1); *Bam*HI (lane 2); *Xba*I (lane 3); Positive control: 1180 bp *A. aculeatus man1* gene (white arrows) (lane 4), M: DNA molecular weight marker (O'Gene Ruler DNA Ladder mix: Fermentas, USA). (B) Southern hybridization using the *A. aculeatus man1* gene as a probe. High stringency wash step: 60°C with 0.5× SSC, 1% (w/v) SDS) and (C) Low stringency wash step: 50 °C with 0.2× SSC, 1% (w/v) SDS.

3.4. DISCUSSION AND CONCLUSION

In the current study, attempts were made to amplify the partial cDNA fragment of *S. candida* LMK008 β-mannanase by reverse transcription PCR. To obtain the full length cDNA, 5′ and 3′ rapid amplification of cDNA ends (RACE) was performed using the SMART™ RACE cDNA Amplification Kit (Clonetech, CA, USA) as well as the RevertAid™ First Strand cDNA Synthesis Kit as per manufactures instructions. These attempts, however, were unsuccessful. One of the reasons for the failure of these techniques could be the lack of homology between the target mannanase of *S. candida* LMK008 and family 5 mannanases. At the outset, after conducting several searches of the database for amino acid and nucleotide sequences of the mannanase enzymes and genes, respectively, it was demonstrated that there

was not a great deal of homology. This was also reported by Bewley *et al.* (1997) who found that there was only 26 - 58% identity among eukaryotic mannanases. For most proteins, homology is often concluded on the basis of sequence similarity, for example if two or more enzymes have highly similar protein sequences, it is likely that they are homologous (Hilge *et al.,* 1998; Sabini *et al.,* 2000). Fungal β-mannanase sequence alignments conducted for Family 5 and 26 have revealed very little if not no homology amongst the sequences aligned. For example it was observed that there is strong sequence homology between *Phanerochaete chrysosporium* and *Armillariella tabescens* β-mannanase sequences whilst there is hardly any homology amongst the other fungal species belonging to Family 5 (http://www.cazy.org/; Henrissat and Davies, 1997; Cantarel *et al.,* 2008). It is, therefore, not surprising that the β-mannanase from *S. candida* was not able to be isolated based on sequence homology.

Using degenerate primers: forward primer MANSU-F and reverse primer MANSU-R; which were designed based on β-mannanase DNA sequence alignments from several fungal species; the genomic DNA from *S. candida* LMK008 was subjected to PCR amplified in an attempt to amplify the β-mannanase gene. However, after examination of the PCR products on a 1% (w/v) agarose gel, it was observed that the PCR amplification of the β-mannnanase gene was unscuccessful. When designing the degenerate primers used in the current study, it was assumed that the β-mannanase from the test organism would have sufficient homology with other fungal β-mannanases despite them being of different species and genera. One possible reason for the unsuccessful PCR amplification could be that the use of degenerate primers can greatly reduce the specificity of the PCR amplification (Kramer and Coen, 2006).

Despite clones been obtained from the genomic DNA library, the β-mannanase gene might not have been expressed in recombinant *E. coli*. The prokaryotic host would not have

transcriptional system necessary to process eucaryotic genes and mRNA, ie., remove introns, and, therefore, no protein would have been produced. However, the presence of the genes in of the clones could not be confirmed due to the lack of homology in the nucleotide sequence between the probe and the *S. candida* gene (Lin *et al.,* 2006). Also, the failure to generate a fully functional β-mannanase enzyme from the recombinant *E. coli* clones generated from the cDNA library could be due a lack of a properly glycosylated enzyme (Stålbrand *et al.,* 1995).

Another possible reason for the unsuccessful attempts at cloning could be the formation of primer dimers of approximately 300 bp which were observed on the gel images (Fig. B5). A possible reason for the formation of these primer dimers could be the annealing of the 3' end of one primer to itself or the second primer may cause primer extension which could result in the formation of so-called primer dimers which are visible as low-molecular-weight bands on PCR gels (Kramer and Coen, 2006). Primer dimer formation often competes with formation of the DNA fragment of interest. Using the OligoAnalyzer 3.1 from Intergrated DNA technologies (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/Default.aspx), the degenerate MANSU-Forward and Reverse primers were analysed for the formation of self as well as hetero-dimers. According the OligoAnalyzer, the maximum delta g refers to the energy needed to break apart a fully complementary set of oligos. The delta g values above each self-dimer refers to the amount of energy needed to break apart that particular self dimer. If the delta g for the self dimer is more negative than -9kcal/mol, or if it is more than 10-30% of the maximum delta g, one should consider redesigning the oligo because the self dimer could interfere with the PCR reaction. Based on the data obtained, the MANSU-Forward primer produced 6 self dimers (Fig. 3.3A) with its Delta G value being -9.45 kcal/mole and its maximum delta G value being -30.35 kcal/mole. This means that the delta G value is more negative than -9kcal/mol and the delta G value is 68.9% higher than that of

the maximum delta G value, whilst the MANSU-Reverse primer produced 2 self dimers (Fig. 3.3B) with its delta G value being -3.14 kcal/mole and its maximum Delta G value being -38.95 kcal/mole. This means that the delta G value is 91.9% higher than that of the maximum delta G value. Based on this information there is a possibility that primers dimers observed on the gels could have interfered with the RACE-PCR reactions and were unable to amplify the target mannanase gene.

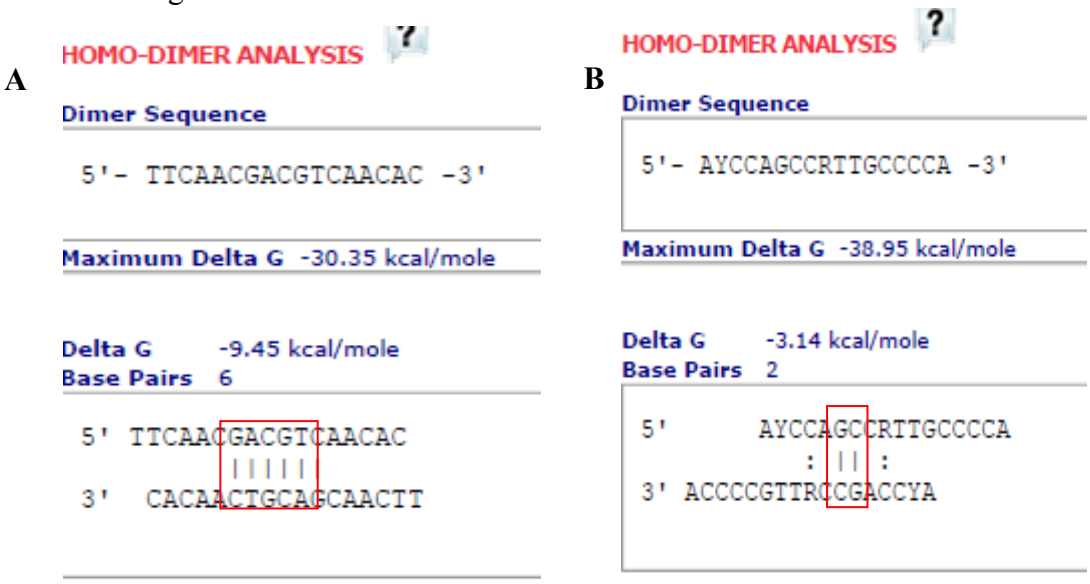

Figure 3.3: Homo/Self-Dimer analysis of (A) MANSU-Forward and (B) MANSU-Reverse Degenerate primers (Red block indicates Self primer dimers).

Despite numerous attempts at cloning the β-mannanase encoding genes from *S. candida* strain LMK008 using a variety of different molecular biology techniques, this has proved difficult to accomplish. One of the ways to accomplish this will be to sequence the purified β-mannanase and obtain the protein sequence, thereafter, reverse translating said protein sequence and obtaining the DNA sequence. Thereafter, it would be possible to design primers based on the DNA sequence which would be more specific for the β-mannanase from *S. candida* strain LMK008.

CHAPTER 4:

GENERAL DISSCUSSION AND CONCLUSIONS

4.1. GENERAL DISCUSSION

Halotolerant filamentous fungi such as *Aspergillus* sp., *Chaetomium globosum*, *Emericella nidulans, Eurotium* sp., *Gymnascella marismortui*, *Penicillium* sp., and *Scopulariopsis candida*, are just some of the mycobiota inhabiting hypersaline environments (Kis-Papo *et al.,* 2003; Mudau and Setati, 2008). Also found within this biodiverse niche are a wide variety of lignicolous phytopathogens. Many of these phytopathogens are capable of hydrolysing plant cell wall polysaccharides such as hemicellulose (Mudau, 2006).

The hemicellulose fraction of the plant cell walls of hardwoods and softwoods are composed of mannans and heteromannans which are polymers of the mannose sugar and are a major component of the cell walls of plants and some types of seeds (Matheson and McCleary, 1985; Erikkson *et al*. 1990). Endoacting hydrolytic enzymes such as endo-β-1,4-mannanases (EC 3.2.1.78) are secreted into the extracellular environment and are involved in the catalysis of the random hydrolysis of β-1,4-mannosidic linkages within the backbone of mannan, galactomannan, glucomannan, and galactoglucomannan. Poultry are monogastric animals and are thus unable to efficiently digest high-fibre containing feeds such as soybean, guar, palm and coconut kernel meals due to their inability to degrade or use these mannan-based meals. Ingestion of such feeds result in decreased or depressed animal performance (Wu *et al.,* 2005; Kim *et al.,* 2006; Chong *et al.,* 2008; Jackson *et al.,* 1999a; 1999b). The use of feeds supplemented with β-mannanases has been shown to reduce weight gain and feed conversion efficiency in poultry birds due to intestinal viscosity and thus eliminates the indigestible β-mannan and enhances the feeding value of mannan based meals.

In the current study, the degradation of β-mannan polysaccharides present in poultry feed by halotolerant *Scopulariopsis candida* LMK008 β-mannanase was investigated. The hydrolysis

of the animal feed by both the crude and purified β-mannanase showed similar hydrolysis patterns, where after 2 hours of incubation considerable levels of reducing sugars were released. However, hydrolysis of the feed by the purified enzyme was lower than that of the crude enzyme hydrolysis. Despite numerous attempts at cloning the β-mannanase encoding genes from *S. candida* strain LMK008 using a variety of different molecular biology techniques, all attempts were unsuccessful. One of the ways to accomplish this could be to sequence the purified β-mannanase and obtain the protein sequence, thereafter reverse translating said protein sequence and obtaining the DNA sequence. Thereafter, it would be possible to design primers based on the DNA sequence which would be more specific for the β-mannanase from *S. candida* strain LMK008.

4.2. CONCLUSIONS

The use of enzymes in particular β-mannanases as feed supplements has over the years considerably increased. However, additional research is still needed in order to fully exploit this enzyme as a feed additive. The following areas of research and development should be investigated include:

1. Development of more sensitive and accurate assays;
2. Specific identification of the catalytic properties of the purified β-mannanase most suited to poultry;
3. A better understanding of the effects that this particular β-mannanase will have on the physiological and endocrine responses of poultry which have been fed β-mannanase supplemented mannan-based diets;

4. A better understanding of the ways that the β-mannanase produces its beneficial effects and of the nature of the interactions of such an enzyme with other dietary components; and
5. Development of simple models (*in vitro* and *in vivo*) to predict responses to β-mannanase treatment.

In the future numerous new enzymes products are expected to be developed and in many cases this will be accomplished through the use of recombinant DNA technology. The advantages of the use of such technologies include the fact that these enzymes are expected to have increased stability and superior catalytic properties and are available at a much lower cost. These enzymes could find wide application in the diets of many different classes of poultry. As previously mentioned, enzymes not only improve poultry performance but also have many other beneficial effects, including reduced pollution of the environment. Enzymes as feed additives have had a very great impact on the poultry industry and will continue to provide an ever-growing range of benefits.

REFERENCES

Abbott, S.P., and L. Sigler. 2001. Heterothallism in the Microascaceae demonstrated by three species in the *Scopulariopsis brevicaulis* series. *Mycologia* **93:** 1211-1220.

Adams, M. W., and R.M. Kelly. 1995. Enzymes from microorganisms in extreme environment. *Chemical and Engineering News* **18:** 32-42.

Ademark P., A. Varga, J. Medve, V. Harjunpaa, D. Torbjorn, F. Tjerneld, and H. Stalbrand. 1998. Softwood hemicellulose-degrading enzymes from *Aspergillus niger*: Purification and properties of a β-mannanase. *Journal of Biotechnology* **63:** 199-210.

Afzal, A.J., S. Ali, F. Latif, M.I. Rajoka, and K.S. Siddiqui. 2005. Innovative kinetic and thermodynamic analysis of a purified superactive xylanase from *Scopulariopsis* sp. *Applied Biochemistry and Biotechnology* **120:** 51-70.

Aganga, A. A., A.O. Aduku, M. Abdulmalik, and A. Sekoni. 1991. Effect of different protein sources and their levels on the reproduction of breeding rabbits. *Journal of Applied Rabbit Research* **14:** 30-33.

Akino, T., C. Kato, and K. Horikoshi. 1989. The cloned *β*–mannanase gene from alkalophilic *Bacillus sp. AM-001* produces two β-mannanases in *Escherichia coli. Applied Biochemistry and Biotechnology* **152:** 10–15.

Akita, M., N. Takeda, K. Hirasawa, H. Sakai, M. Kawamoto, M. Yamamoto, W.D. Grant, Y. Hatada, S. Ito, and K. Horikoshi. 2004. Crystallization and preliminary X-

ray study of alkaline mannanase from an alkaliphilic *Bacillus* isolate. *Acta Crystallographica* **60:** 1490–1492.

Almirall, M., M. Francesch, A. M. Perez-Vendrell, J. Brufau, and E. Esteve-Garcia. 1995. The difference in intestinal viscosity produced by barley and β-glucanase alter digesta enzyme activities and ileal nutrient digestibilities more in broiler chicks than in cocks. *Journal of Nutrition* **125:** 947–955.

Andrews, P., W.R. Culle, and E. Polishchuk. 2000. Antimony biomethylation by *Scopulariopsis brevicaulis*: characterization of intermediates and methyl donor. *Chemosphere* **41:** 1717-1725.

Araujo, A., and O.P. Ward. 1990. Hemicellulases of *Bacillus* species: preliminary comparative studies on production and properties of mannanases and galactanases. *Journal of Applied Bacteriology* **68:** 253–261.

Arcand, N., D. Kluepfel, F. D. Paradis, R. Morosoli, and F. Shareck. 1993. β-Mannanase of *Streptomyces lividans* 66: Cloning and DNA sequence of the *manA* gene and characterization of the enzyme. *Biochemical Journal* **290:** 857–863.

Arisan-Atac, I., R. Hodits, D. Kristufek, and C.P. Kubicek. 1993. Purification, and characterization of β-mannanase of *Trichoderma reesei* C-30. *Applied Microbiology and Biotechnology* **39:** 58-62.

Arvill, A., and L. Bodin. 1995. Effect of short-term ingestion of konjac glucomannan on

serum cholesterol in healthy men. *American Journal of Clinical Nutrition.* **61:** 585–589.

Bailey, M.J. 1988. A note on the use of dinitrosalicylic acid for determining the products of enzymatic reactions. *Applied Microbiology and Biotechnology* **29:** 494–496.

Bailey, M.J., P. Biely, and K. Potanen. 1992. Interlaboratory testing of methods for assay of xylananse activity. *Journal of Biotechnology.* **23:** 257-270.

Baker, **C. W., and R. L. Whistler.** 1975**.** Distribution of D-galactosyl groups in guaran and locust-bean gum. *Carbohydrate Research* **45:** 237-243**.**

Bedford, M. R., H. L. Classen, and G. L. Campbell. 1991. The effect of pelleting, salt, and pentosanase on the viscosity on intestinal contents and the performance of broilers fed rye. *Poultry Science* **70:** 1571–1577.

Béguin, P. 1990. Molecular biology of cellulose degradation. *In* Annual Review of Microbiology. **44:** (Ornston, N., ed), pp. 219-248, Annual Reviews Inc., Palo Alto.

Beguin, H., and N. Nolard. 1999. Relationship between mycobiota in wall-to-wall carpet dust and age of carpet. *Aerobiologica* **15:** 2999-306.

Bettiol, J. P., and M.S. Showell. 2002. Detergent compositions comprising a mannanase and a protease. *US Patent* 6376445.

Bewley, J.D., R.A. Burton, Y. Morohashi, and G.B. Fincher. 1997. Molecular cloning of a cDNA encoding a (1-4)-β-mannan endohydrolase from the seeds of germinated tomato (*Lycopersicon esculentum*). *Planta* **203:** 454–459.

Bhat, M. K. 2000. Cellulases and related enzymes in biotechnology. *Biotechnology Advances* **18:** 355-383.

Bicho, P. A., T. A. Clarke, K. Mackie, H. W. Morgan, and R. M. Daniel. 1991. The characterisation of a thermostable endo-β-1,4-mannanase cloned from *"Caldocellum saccharolyticum." Applied Microbiology and Biotechnology* **36:** 337–343.

Blibech, M., R. E. Ghorbel, I. Fakhfakh, P. Ntarima, K. Piens, A.B. Bacha and S.E. Chaabouni. 2010. Purification and characterization of a low molecular weight of β-mannanase from *Penicillium occitanis* Pol6. Applied Biochemistry and Biotechnology **160:** 1227–1240.

Bolam, D.N., H. Xie, G. Pell, D. Hogg, G. Galbrainth, B. Hensrissat, and H.J. Gilbert. 2004. X4 modules represents a new family of carbohydrate-binding modules that display novel properties. *Journal of Biological Chemistry* **279:** 22953-22963.

Bolam, D.N., N. Hughes, R. Virden, J.H. Lakey, G.P. Hazlewood, B. Henrissat, K.L. Braithwaite, and H.J. Gilbert. 1996. Mannanase A from *Pseudomonas fluorescens sp. cellulosa* is a retaining glycosyl hydrolase in which E212 and E320 are the putative catalytic residues. *Biochemistry* **35:** 16195-16204.

Boraston, A.B., D.N. Bolam, H.J. Gilbert and, G.J. Davies GJ. 2004. Carbohydrate-binding modules: fine-tuning polysaccharides recognition. *Biochemical Journal* **382:** 769-781.

Bradford, M.M. 1977. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72:** 248–254.

Braithwaite, K. L., G.W. Black, G.P. Hazlewood, B.R. Ali, and H.J. Gilbert. 1995. A non-modular endo-β-1,4-mannanase from *Pseudomonas fluorescens* subspecies *cellulosa. Biochemical Journal.* **305:** 1005–1010.

Buchalo, A.S., E. Nevo, S.P. Wasser, A. Oren, and H.P. Molitoris. 1998. Fungal life in the extremely hypersaline water of the Dead Sea. *Proceedings of the Royal Society B: Biological Science* **265:** 1461-1465.

Buckeridge, M.S., and S.M.C. Dietrich. 1990. Galactomannan from Brasilian legume seeds. *Revista Brasileira de Botânica* **13:** 109-112.

Buckerigde, M.S., V.R. Panegassi, D.C. Rocha, and S.M.C. Dietrich. 1995. Seed galactomannan in the classification and evolution of the leguminosae. *Phytochemistry* **38:** 871-875.

Buckeridge, M.S., H.P. dos Santos, and M.A.S. Tine. 2000. Mobilization of storage cell wall polysaccharides in seeds. *Plant Physiology and Biochem*istry **38:** 141-156.

Camin, A.M., D. Chabasse, and C. Guigen. 1998. Keratinophilic fungi associated with starlings (*Sturnus vulgaris*) in Brittany, France. *Mycopathologia* **143:** 9-12.

Cann, I. K., S. Kocherginskaya, M.R. King, B.A. White, and R.I. Mackie. 1999. Molecular cloning, sequencing, and expression of a novel multidomain mannanase gene from *Thermoanaerobacterium polysaccharolyticum. Journal of Bacteriology* **181:** 1643–1651.

Cantarel, B.L., P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, and B. Henrissat. 2008. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Research* **37:** D233-238.

Capek, P., M. Kubačková, and J. Alföldi. 2000. Galactomannan from the secondary cell wall of *Picea abies* L. karst. *Carbohydrate Research* **329:** 635-645.

Capek, P., J. Alföldi, and D. Lišková D. 2002. An acetylated galactoglucomannan from *Picea abies* L. Karst. *Carbohydrate Research* **337:** 1033-1037.

Carbohydrate Active Enzymes database. (http://www.cazy.org/)

Cescutti, P., C. Campa, F. Delben, and R. Rizzo. 2002. Structure of the oligomers obtained by enzymatic hydrolysis of the glucomannan produced by the plant *Amorphophallus konjac. Carbohydrate Research* **337:** 2505-2511.

Chandrasekharaiah, M., K.T. Sampath, A. Thulasi, and S. Anandan. 2001. *In situ*

protein degradability of certain feedstuffs in the rumen of cattle. *Indian Journal of Animal* Sciences **71:** 261-264.

Chanzy, H., M. Dube, and R.H. Marchessault. 2004. Single crystals and oriented crystallization of ivory nut mannan. *Biopolymers* **18:** 887-898.

Chanzy, H., and R. Vuong. 1985. Ultrastructure and morphology of crystalline polysaccharides. *In:* Polysaccharides Topics in structure and morphology. E. D. T. Atkins, (ed.) VCH Publishers, Bristol, UK.

Chaplin, M. 2006. Water Structure and Science: Guar Gum. London South Bank University, www.lsbu.ac.uk/water/hygua.html Date accessed: 08 February 2010.

Chaplin, M. 2009. Locust bean gum, London South Bank University, http://www1.lsbu.ac.uk/water/hyloc.html**.** Date accessed: 08 February 2010.

Chen, X., Y. Cao, Y. Ding, W. Lu and D. Li. 2006. Cloning, functional expression and characterization of *Aspergillus sulphureus* β-mannanase in *Pichia pastoris*. *Journal of Biotechnology* **128:** 452-461.

Chen, Y., J. Long, L. Liao, Y. Zhan, and J.Yang. 2000. Study on the production of β-mannanase by *Bacillus* sp. M50. *Wei Sheng Wu Xue Bao* **40:** 62–68.

Chesson, A. 1987. Supplementary enzymes to improve the utilization of pig and poultry diets. In: Recent Advances in Animal Nutrition. pp. 71-89. Butterworths, London.

Chhabra, S. R., K.R. Shockley, D. Ward, and R.M. Kelly. 2002. Regulation of endo-acting glycosyl hydrolases in the hyperthermophilic bacterium *Thermotoga maritima* grown on glucan and mannan based polysaccharides. *Applied and Environment Microbiology* **68:** 545–554.

Cho, K., K. Yoon, D. Kim, H. Oh and Y. Oh. 2006. *Bacillus* sp. WL1 strain producing mannanase. *U.S. Patent* 6984406B2.

Chong, C.H., I. Zulkifli, and R. Blair. 2008. Effects of dietary inclusion of palm kernel cake and palm oil, and enzyme supplementation on performance of laying hens. *Asian-Australasain Journal of Animal Science* **21:** 1053–8.

Christgau, S., S. Kauppinen, J. Vind, L.V. Kofod, and H. Dalbøge. 1994. Expression, cloning, purification and characterization of a β-1,4- mannanase from *Aspergillus aculeatus. Biochemistry and Molecular Biology International* **33:** 917–925.

Christoffersen, L. 2004. Products and applications that promote sustainability. *In:* REBIO (DIVERSA), Boca Chica, Dominican Republic.

Civas, A., R. Eberhard, P. le Dizet, and F. Petek. 1984. Glycosidases induced in *Aspergillus tamarii* secreted *α*-D-galactosidase and β- D-mannanase. *Biochemical Journal* **219:** 857–863.

Conner, S. 2002. Characterization of guar meal for use in poultry rations. Texas A and M University, College Station, TX. *Ph.D. Dissertation*.

Couch, J.R., Y.K. Bakashi, T.M. Ferguson, E.B. Smith, and C.R. Greger. 1967. The effect of processing on the nutritional value of gaur meal for broiler chicks. *British Journal of Poultry Science* **8:** 243-250.

Coughlan, M.P., and G.P. Hazlewood. 1993. Hemicellulose and Hemicellulases. Portland Place Press Ltd., London, UK.

Cowan, W.D. 1993. The stability of enzymes in animal feed. *Feed International* **3:** 22-25.

Cuevas, W.A., A.. Kantelinen, P. Tanner, P., B. Bodie, and S. Leskinen. 1996 Purification and characterization of novel mannanases used in pulp bleaching. Proc. 6th Int. Conf. Biotechnol. Pulp and paper industry, Vienna, Austria, 123-126.

Cui, F., J. Shi, and Z. Lu. 1999. Production of neutral β-mannanase by *Bacillus subtilis* and its properties. *Wei Sheng Wu Xue Bao*. **39:** 60–63.

Daas, P.J.H., H.A. Schols, and H.H.J. de Jongh. 2002. On the galactosyl distribution of commercial galactomannans. *Carbohydrate Research* **329:** 609–619.

Dale, N. 1997. Current status of feed enzymes for swine. Hemicell, Poultry and Swine Feed Enzyme. ChemGen Crop. Gaithersburg, MD.

Daskiran, M., and R. G. Teeter. 2001. Effect of endo-β-D-mannanase (Hemicell) supplementation on growth performance of two-week-old broilers fed diets varying in β-mannan content. Animal Science Research Report. Publication P986. Oklahoma Agriculture Experiment Station, Stillwater, OK.

Daskiran, M., R.G. Teeter, D.W. Fodge, and H.Y. Hsiao. 2004. An evaluation of endo-β-D-mannanase (Hemicell®) effects on broiler performance and energy use in diets varying in β-mannan content. *Poultry Science* **83:** 662–668.

Davies, G., and B. Henrissat. 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* **3:** 853-859.

Dawson, R.M.C., D.C. Elliot, W.H. Elliot, and K.M. Jones, eds. 1969. Precipitiation techniques. *In* Data for Biochemical Research, 2nd Ed. Oxford University Press, London. pp. 291.

de Vries, R.P. 2003. Regulation of *Aspergillus* genes encoding plant cell wall polysaccharide-degrading enzymes; relevance for industrial production. *Applied Microbiology and Biotechnology* **61:** 10-20.

de Vries, R.P., and J. Visser . 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiology and Molecular Biology Reviews* **65:** 497-522.

Demirjian, D.C., F. Moris-varas, and C.S. Cassidy. 2001. Enzymes from

extremophiles. *Current Opinion in Chemical Biology* **5:** 144-151.

Dhawan, S. and K. Jagdeep. 2007. Microbial mannanases: An overview of production and applications. *Critical Reviews in Biotechnology* **27:** 197-216.

Dierick, N. A. 1989. Biotechnology aids to improve feed and feed digestion: Enzyme and fermentation. *Archives of Animal Nutrition (Berlin)* **3:** 241–246.

Duffaud, G. D., C.M. McCutchen, P. Leduc, K.N. Parker, and R.M. Kelly. 1997. Purification and characterization of extremely thermostable *β*-mannanase, *β*-mannosidase, and *α*-galactosidase from the hyperthermophilic eubacterium *Thermotoga neopolitana* 5068. *Applied and Environmental Microbiology* **63:** 169–177.

Duruksu, G., B. Ozturk, P. Biely, U. Bakir, and Z.B. Ogel. 2009. Cloning, expression and characterization of endo-β-1,4-mannanase from *Aspergillus fumigatus* in *Aspergillus sojae* and *Pichia pastoris***.** *Biotechnology Progress* **25:** 271-276.

Eriksson, K.E., and M.J. Winell. 1968. Purification and characterization of a fungal β-mannanase. *Inorganica Chimica Acta* **22:** 1924-1934.

Eriksson, K.E.L., R.H. Blanchette, and P. Ander. 1990. Biodegradation of lignin. *In:* Eriksson K-EL, R.H. Blanchette, and P. Ander (eds) Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, Berlin Heidelberg New York.

Ethier, N., G. Talbot, and J. Sygusch. 1998. Gene cloning, DNA sequencing, and expression of thermostable beta-mannanase from *Bacillus stearothermophilus. Applied and Environmental Microbiology* **64:** 4428-4432.

Fanutti, C., T. G.W. Ponyi, G.P. Black, G.P. G.P. Hazlewood, and H.J. Gilbert. 1995. The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *Journal of Biological Chemistry* **270:** 29314–29322.

Ferreira, H.M., and E.X.F. Filho. 2004. Purification and characterization of a β-mannanase from *Trichoderma harzianum* strain T4. *Carbohydrate polymers* **57:** 23-29.

Filho, E.X.F. 1998. Hemicellulases and biotechnology. *Recent Research Developments in Microbiology* **2:** 165-176.

Food and Agriculture Organization of the United Nations (FAO). 2006. Guar gum. Joint FAO/WHO Expert Committee on Food Additives, *Monographs* **3:** 1-4.

Franco, P.F., H.M. Ferreira, and E.X.F. Filho. 2004. Production and characterization of hemicellulose activities from *Trichoderma harzianum* strain T4. *Biotechnology and Applied Biochemistry* **40:** 255-259.

Gadient, M. 1994. Use of liquid carbohydrases and other additives for animals. *Proceedings of the 3rd International Kahl Symposium.,* Hamburg, November 29th.

Gadient, M. and J.C. Tristsch. 1995. Product formulations for enzyme used in animal nutrition. *ln: Enzymes in Animal Nufrifion,* Proceeding of the 2" Symposium Noordwijkerhout, Netherlands, *Technology and Analysis* **1:** 70-75.

Genomic Management Information System, Oak Ridge National Laboratory. Structure of lignocellulose. http://californiaagriculture.ucanr.org. Date accessed: 23 June 2010.

Gherardini, F., M. Babcock, and A. Salyers. 1987. Purification and characterization of a cell-associated, soluble mannanase from *Bacteroides ovatus*. *Journal of Bacteriology* **169:** 2038–2043.

Gibbs, M. D., A.U. Elinder, R.A. Reeves, and P.L. Bergquist. 1996. Sequencing, cloning and expression of the β-1,4-mannanase gene *manA* from the extremely thermophilic anaerobic bacterium *Caldicellulosiruptor* Rt8B.4. *FEMS Microbiology Letters* **141:** 37-43.

Gibbs, M. D., R.A. Reeves, A. Sunna, and P.L. Bergquist. 1999. Sequencing and expression of a *β*-mannanase gene from the extreme thermophile *Dictyoglomus thermophilum* Rt46B.1, and characteristics of the recombinant enzyme. *Current Microbiology* **39:** 351–357.

Gibbs, M.D. D. J. Saul, E. Luthi, and P. L. Bergquist. 1992. The β-mannanase from "*CaldocellSSum saccharolyticum*" is part of a multidomain enzyme. *Applied and Environmental Microbiology* **58:** 3864–3867.

Greenberg, N. A. 2000. The chemical, physical, and physiological properties of partially hydrolyzed guar gum. AACC 2000 Annual Meeting Kansas City, Missouri.

Grishkan, I, E. Nevo, and S.P. Wasser. 2004. *Micromycetes* from the saline Arubataim cave: Mount Sedom, The Dead Sea south-western shore, *Israel Journal of Arid Environments* **57:** 431-443.

Grobwinder, C., A. Sachslehner, B. Nidetzky, and D. Haltrich. 1999. Endo-β-1,4-mannananase is efficiently produced by *Sclerotium (Athelia) rolfsii* under depressed conditions. *Journal of Biotechnology* **67:** 189-203.

Gübitz, G.M., D. Haltrich, B. Latal, and W. Steiner. 1997. Mode of depolymerisation of hemicellulose by various mannanases and xylanases in relation to their ability of bleach softwood pulp. *Applied Microbiology and Biotechnology* **47:** 658-662.

Gübitz, G.M., H. Hayn, G. Urbanz, and W. Steiner. 1996a. Purification and properties of an acidic β-mannanase from *Sclerotium rolfsii. Journal of Biotechnology* **45:** 165-172.

Gübitz, G.M., M. Hayn, M. Sommerauer, W. Steiner. 1996b. Mannan degrading enzymes from *Sclerotium rolfsi*: characterization and synergism of two endo β-mannanases and a β-mannosidase. *Bioresource Technology* **58:** 127-135.

Gubitz G, A. Sachslehner, and D. Haltrich. 2001. Microbial mannanases: substrates, production and application**.** *Glycosyl Hydrolases for Biomass Conversion: 2001; Washington, DC: ACS Symposium Series, American Chemical Society* **769:** 239-262.

Hägglund, P., T. Eriksson, A. Collen, W. Nerinckx, M. Claeyssens and H. Stalbrand. 2003. A cellulose-binding module of *Trichoderma reesei* β- mannanase Man5A increases the mannan-hydrolysis of complex substrates. *Journal of Biotechnology* **101:** 37-48.

Halstead, J. R., P.E. Vercoe, H.J. Gilbert, K. Davidson, and G.P. Hazlewood. 1999. A family 26 mannanase produced by *Clostridium thermocellum* as a component of the cellulosome contains a domain which is conserved in mannanases from anaerobic fungi. *Microbiology* **145:** 3101–3108.

Han, Y., C. Kye, S. Hong, L. Sun, K. Yong, K. Goon, and K. Hoon. 2006. A cel44C-man26A gene of endophytic *Paenibacillus polymyxa* GS01 has multi-glycosyl hydrolases in two catalytic domains. *Applied Microbiology and Biotechnology* **73:** 618–630.

Handford, M.G., T.C. Baldwin, and F. Goubet F. 2003. Localization and characterization of cell wall mannan polysaccharides in *Arabidopsis thaliana. Planta* **218:** 27-36.

Harjunpää, V., A. Teleman, M. Siika-aho, and T. Drakensberg. 1995. Kinetic and stereochemical studies of manno-oligosaccharide hydrolysis catatlysed by β-mannnanase from *Trichoderma reesei. European Journal of Biochemistry* **243:** 278-283.

Hashimoto, T., and Y. Nakata. 2003. Synergistic degradation of arabinoxylan with α-L-arabinofuranosidase, xylanase and β-xylosidase from soy sauce koji mold, *Aspergillus oryzae,* in high salt condition. *Journal of Bioscience and Bioengineering* **95:** 164-169.

Hatada, Y., N. Takeda, K. Hirasawa, Y. Ohta, R. Usami, Y. Yoshida, S. Ito, and K. Horikoshi. 2005. Sequence of the gene for a high-alkaline mannanase from an alkaliphilic *Bacillus* sp. strain JAMB-750, its expression in *Bacillus subtilis* and characterization of the recombinant enzyme. *Extremophiles* **9:** 497–500.

Haytowitz, D.B., and R.H. Matthews. 1986. Composition of Foods: Legume & Legume Products. USDA, Human Nutrition Information Service Agriculture Handbook No. 8-16. US Government Printing Office, Washington, DC.

Helow, E. R., and A.A. Khattab. 1996. The development of a *B. subtilis* 168 culture condition for enhanced and accelerated β-mannanase production. *European Journal of Biochemistry* **43:** 289–299.

Henrissat, B. 1990**.** A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **280:** 304–316.

Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino-acid sequence similarities. *Biochemical Journal.* **280:** 309-316.

Henrissat, B., and A. Bairoch. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **293:** 781–788.

Henrissat, B., and A. Bairoch. 1996. Updating the sequence based classification of glycosyl hydrolases. *Biochemical Journal* **316:** 695–696.

Henrissat, B., and G.J. Davies. 1997. Structural and sequence-based classification of glycoside hydrolases. *Current Opinion in Structural Biology*. **7:** 637-644.

Henrissat, B., I. Callebaut, S. Fabrega, P. Lehn, J.P. Mornon, and G. Davies. 1995. Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. *Proceedings of the National Academy of Sciences U.S.A.* **92:** 7090–7094.

Henrissat, B., T. Teeri, and R.A.J. Warren. 1998. A scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants. *FEBS Letters* **425:** 352–354.

Hilge, M., S.M. Gloor, W. Rypniewski, O. Sauer, T.D. Heightman, W. Zimmerman, K. Winterhalter and K. Piontek. 1998. High-resolution native and complex structures of thermostable *β*-mannanase from *Thermomonospora fusca*-substrate specificity in glycosyl hydrolase family 5. *Structure* **6:** 1433–1444.

Hogg, D., E.J. Woo, D.N. Bolam, V.A. McKie, H.J. Gilbert, and R.W. Pickersgill. 2001. Crystal structure of mannanase 26A for *Pseudomonas cellulosa* and analysis of residues involved in substrate binding. *Journal of Biological Chemistry* **276:** 31186-31192.

Hogg, D., G. Pell, P. Dupree, F. Goubet, S.M. Martin-Orue, S. Armand and H.J. Gilbert. 2003. The modular architecture of *Cellvibrio japonicus* mannanases in glycoside hydrolase families 5 and 26 points to differences in their role in mannan degradation. *Biochemical Journal* **371:** 1027–1043.

Hongpattarkere, T. 2002. Hyperthermostable cellulolytic and hemicellulolytic enzyme and their biotechnological applications. *Songklanakarin Journal of Science and Technol*ogy **24:** 481-49.

Honing, D.H., and J.J. Rackis. 1979. Determination of total pepsin pancreatic indigestibility content (dietary fiber) of soybean products, wheat bran, and corn bran. *Journal of Agricultural and Food Chemistry* **27:** 1262-1266.

Horton, D. 1997. Advances in Carbohydrate Chemistry and Biochemistry. Vol. 52 Academic Press, London, UK.

Hrmová, M., R.A. Burton, P. Biely, J. Lahnstein, and G.B. Fincher. 2006. (1,4)-β-D-Mannan endohydrolases from barley (*Hordeum vugare* L.): catalytic properties, primary structure, molecular model and gene expression patterns. *Biochemical Journal* **399:** 77–90.

Hsiao, H.Y., D. M. Anderson, and N. M. Dale. 2006. Levels of *β*-mannan in soybean meal. *Poultry Science* **85:** 1430–1432.

Hua, Y.F., M. Zhang, C.X. Fu, Z.H. Chen, and G. Y.S. Chan. 2004. Structural characterization of a 2-*O*-acetylglucomannan from *Dendrobium officinale* stem. *Carbohydrate Research* **339:** 2219-2224.

Huang, S. P., C.L. Wang, G.M. Zhang, and L.X. Ma. 2007. Construction of a double functional recombinant strain of *Pichia pastoris* co-expressing phytase and mannanase and the enzymatic analysis. *Wei Sheng Wu Xue Bao* **47:** 280–284.

Intergrated DNA technologies. OligoAnalyzer 3.1. (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/Default.aspx). Date accessed: 30 November 2011.

Jackson, M.E. 2002. Potential of NSP enzymes in pullet and layer corn-soybean meal based diets. Multi-state Poultry Meeting, May 14-16, 2002, Indianapolis, Indianna, USA *Presentation.*

Jackson, M.E., D.M. Anderson, H.Y. Hsiao, G.F. Mathis, and D.W. Fodge. 2003. Beneficial effect of β-Mannanase feed enzyme on performance of chicks challenged with Eimeria sp. and Clostridium perfringens. *Avian Diseases* **47:** 759-763.

Jackson, M. E., D. W. Fodge, and H. Y. Hsiao. 1999a. Effects of β-mannanase in corn-soybean meal diets on laying hen performance. *Poultry Science* **78:** 1737-1741.

Jackson, M. E., D. W. Fodge, and H. Y. Hsiao. 1999b. Effects of Hemicell® in corn-soybean meal diets with varying amino acid densities on laying hen performance. *Poultry Science* **78:** Supplement 1: 136.

Jackson, M. E., K. Geronian, A. Knox, J. McNab, and E. McCartney. 2004. A dose-response study with the feed enzyme β-mannanase in broilers provided with corn-soybean meal based diets in the absence of antibiotic. *Poultry Science* **83:** 1992–1996.

Jiang, Z., Y. Wei, D. Li, L. Li, P. Chai, and I. Kusakabe. 2006. High-level production, purification and characterization of a thermostable β-mannanase from the newly isolated *Bacillus subtilis* WY34. *Carbohydrate Polymers* **66:** 88-96.

Johnson, K.G., N.W. Ross, and H. Schneider. 1990. Purification and some properties of β-mannanase fr.om *Polyporus versicolor. World Journal of Microbiology and Biotechnology* **6:** 245-25.

Kansoh, A. L., and Z.A. Nagieb. 2004. Xylanase and mannanase enzymes from *Streptomyces galbus* NR and their use in biobleaching of softwood kraft pulp. *Anton. van. Leeuwonhoek.* **85:** 103–114.

Kataoka, N., and Y. Tokiwa. 1998. Isolation and characterization of an active mannanase-producing anaerobic bacterium, *C. tertium* KT-5A from lotus soil. *Journal of Applied Microbiology* **84:** 357–367.

Kauppinen, M. S., M. Schulein, K. Schnorr, L.N. Andersen, and M.E. Bjørnvad. 2003. Mannanases. *US Patent* 6,566,114.

Keithley, J., and B. Swanson B. 2005. Glucomannan and obesity: a critical review. *Alternative Therapies in Health and Medicine* **6:** 30–34.

Kensch, O. 2008. Mannanase engineering for fibre degradation. *Speciality Chemicals Magazine* **28:** 18-19.

Kiefer, L. 2001. Behavioural research on chickens. http://www.chicken-yard.net/general/behavior.html. Date accessed: 28 February 2010.

Kim, S.W., J.H. Zhang, K.T. Soltwedel, and D.A. Knabe. 2006. Use of carbohydrases in corn-soybean meal based grower-finisher pig diets. *Animal Research* **55:** 563–578.

Kirk, O., V. Borchert, and C.C. Fuglsang. 2002. Industrial enzyme applications. *Current Opinions in Biotechnology* **13:** 345-351.

Kis-Papo, T., A. Oren, S.P. Wasser, and E. Nevo. 2003. Survival of filamentous fungi in hypersaline Dead Sea water. *Microbial Ecology* **45:** 183-190.

Kobayashi, Y., R. Echigen, M. Mada, and M. Mutai. 1984. Effect of hydrolyzate of Konjac mannan and soybean oligosaccharides on intestinal flora in man and rats, *In:* T.Mitsuoka (Ed.),Intestinal flora and dietary factors. Proceedings of 4th RIKEN symposium on intestinal flora, Japan Scientific Societies Press, Tokyo, pp. 69–90.

Kobayashi, Y., R. Echigen, M. Mada, and M. Mutai. 1987. Effects of hydrolyzates of konjac mannan and soybean oligosaccharides on intestinal flora in man and rats, *In:* T.

Mitsuoka (Ed.), Intestinal flora and food factors, Gakkai Shuppan Centre, Tokyo, pp. 79–97.

Kok, M. S., S. E. Hill, and J. R. Mitchell. 1999. Viscosity of galactomannans during high temperature processing: Influence of degradation and solubilisation. *Food Hydrocolloids* **13:** 535–542.

Kooyk, Y. V. and T.B.H. Geijtenbeek. 2003. DC-Sign: Escape mechanism for pathogens. *Nature Review / Immunology* **3:** 697-709.

Koyuncu, S., and P. Haggblom. 2009. A comparative study of cultural methods for the detection of *Salmonella* in feed and feed ingredients. *BMC Veterinary Research* **5:** 1-10.

Kraehenbuhl, J.P., and M. Corbett. 2004. Keeping the gut microflora at bay. *Science* **303:** 1624-1625.

Kramer M.F., and D.M. Coen. 2006. Enzymatic amplification of DNA by PCR: standard procedures and optimization. *Current Protocols in Cytometry* **37:** A.3K.1–A.3K.15.

Kriesel, J.D., E.E. Adderson, W.M. Gooch III, and A.T. Pavia. 1994. Invasive sinonasal disease due to *Scopulariopsis candida*: Case report and review of *Scopulariopsis. Clinical Infectious Diseases* **19:** 317-319.

Kung, L. 1993. Direct-fed microbial and enzyme feed additives. *ln:* Direct-fed microbial, enzymes, and forage additive compendium, National feed Ingredients Assn., West Des Moines, Iowa, pp. 17-21.

Kurakake, M., and T. Komaki. 2001. Production of β-mannanases and β-mannosidase from *Aspergillus awamori* K4 and their properties. *Current Microbiology* **42:** 377-380.

Kurokawa, J., E. Hemjinda, T. Arai, S. Karita, T. Kimura, and K. Sakka. 2001. Sequence of the *Clostridium thermocellum* mannanase gene *man26B* and characterization of the translated product. *Bioscience, Biotechnology and Biochemistry* **65:** 548–554.

Kusakabe, I., and R. Takashi. 1988. Enzymatic preparation *β*-1,4-mannooligosaccharides and *β*-1,4-glucomannooligosaccharides. *Methods in Enzymology*. **160:** 518–523.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.

Lee, J.T., C.A. Bailey, and A.L. Cartwright. 2003a. Guar meal germ and hull fractions differently affect growth performance and intestinal viscosity of broiler chickens. *Poultry Science* **82:** 1589- 1595.

Lee. J.T., C. A. Bailey and A. L. Cartwright. 2003b. β-Mannanase ameliorates viscosity-associated depression of growth in broiler chickens fed guar germ and hull fractions. *Poultry Science* **82:** 1925-1931.

Lee, J.T., S. Connor-Appleton, C.A. Bailey and A.L. Cartwright. 2005. Effects of guar meal byproduct with and without β-mannanase Hemicell on broiler performance. *Poultry Science* **84:** 1261-1267.

Leeds, A. R., S. S. Kang, A. G. Low, and I. E. Sambrook. 1980. The pig as a model for studies on the mode of action of guar gum in normal and diabetic man. *Proceedings of the Nutrition Society* **39:** 44.

LeNours, D.J., L. Anderson, H. Stalbrand, and L. LoLeggio. 2005. The structure and characterization of a modular endo-β-1,4-mannanase from *Cellulomonas fimi. Biochemistry* **44:** 12700–12708.

Li, B., B. Xie, J.F. Kennedy. 2005. Studies on the molecular chain morphology of konjac glucomannan. *Carbohydrate Polymers* **64:** 501-612.

Li, Y. N., K. Meng, Y.R. Wangm, and B. Yao. 2006. A β-mannanase from *B. subtilis* B36: purification, properties sequencing, gene cloning and expression in *E. coli. Z Naturforsch C* **61:** 840–846.

Lin, S.L., J.D. Miller, and S.Y. Ying. 2006. Intronic MicroRNA (miRNA). *Journal of Biomedicine and Biotechnology* **26818:** 1–13.

Lin, T.S, and C. Chen. 2004. Enhanced mannanase production by submerged culture of *Aspergillus niger* NCH-189 using defatted copra based media. *Process Biochemistry* **39:** 1103–1109.

Litchfield, C.D. and P.M. Gillevet. 2002. Microbial diversity and complexity in hypersaline environments: A preliminary assessment. *Journal of Industrial Microbiology and Biotechnology* **28:** 48-55.

Lo Leggio, L., N.J. Parry, J. van Beeumen, M. Claeyssens, M.K. Bhat, and R.W. Pickersgill. 1997. Crystallization and preliminary X-ray analysis of the major endoglucanase from *Thermoascus aurantiacus*. *Acta Crystallographica Section D: Biological Crystallography* **53:** 599-604.

Lumley, T.C. 1999. Microfungus communities of rotting wood in the boreal mixed-wood region of northern Alberta, Canada. University of Alberta, Department of Biological Sciences. PhD Thesis.

Lundqvist, J., A. Teleman, L. Junel, G. Zaachi, O. Dahlman, F. Tjerneld, and H. Stålbrand. 2002. Isolation and characterization of galactoglucomannan from spruce *(Picea abies). Carbohydrate Polymers* **48:** 29–39.

Luthi, E., J.N. Bhana, R.A. Grayling, D.R. Love, and P.L. Bergquist. 1991. Cloning, sequence analysis and expression in *Escherichia coli* of a gene coding for a β-mannanase from the extremely thermophilic bacterium *Caldocellum saccharolyticum*. *Applied and Environmental Microbiology* **57:** 694–700.

Lyons, T. P., and Jacques, K. A. (eds). 2002. Nutritional Biotechnology in the Feed and Food Industries. Nottingham University Press, Nottingham, UK.

MacMasters, M. M., S. Woodruff, and H. Klaas. 1941. Studies on soybean carbohydrates. *Industrial and Engineering Chemistry Research* **13:** 471–474.

Madern, D., C. Ebel, and G. Zaccai. 2000. Halophillic adaptation of enzymes. *Extremophiles* **4:** 91-98.

Maier, H., M. Anderson, C. Karl, K. Magnuson, and R.L. Whistler. 1993. Guar, locust bean, tara and fenugreek gums. *In:* R.L. Whistler, and J.N. Bemiller (eds) Industrial gums: Polysaccharides and their derivatives. Academic Press, Inc. New York, pp. 215-218.

Malathi, V., and G. Devegowda. 2001. *In vitro* evaluation of non starch polysaccharide digestibility of feed ingredients by enzymes. *Poultry Science* **80:** 302–305.

Marchisio, V.F., and A. Fusconi. 2001. Morphological evidence for keratinolytic activity of *Scopulariopsis* spp. isolates from nail lesions and the air. *Medical Mycol*ogy **39:** 287-294.

Marraccini, P., W.J. Rogers, V. Caillet, A. Deshayes, D. Granato, F. Lausanne, S. Lechat, D. Pridmore, and V. Pètiard. 2005. Biochemical and molecular characterization of α-D-galactosidase from coffee beans. *Plant Physiology and Biochemistry* **45:** 909-920.

Marzio, L., R. Del Bianco, M.D. Donne, O. Pieramico, and F. Cuccurullo. 1989. Mouth-to-cecum transit time in patients affected by chronic constipation: effect of glucomannan. *American Journal of Gastroenterology* **84:** 888–891.

Mascrell, K. and M. Ryan. 1997. Technical aspects of enzyme utilization: Dry vs liquid enzymes, *In:* Morand-Fehr, P. (Ed.) Feed manufacturing in Southern Europe: New challenges and options. Méditerranéennes Spain, pp. 161-174.

Matheson, N.K. and B.V. McCleary. 1985. Enzymes metabolising polysaccharides and their application to the analysis of the structure and function of glucans. *In*: The Polysaccharides, Volume 3. (G.O. Aspinall, Ed.), Academic Press, pp. 1-105.

Matsushita, O., J.B. Russell, and D.B. Wilson. 1991. A *Bacteroides ruminicola β*-1,4-endoglucanase is encoded in two reading frames *Journal of Bacteriology* **173:** 6919–6926.

Mattocks, J. 1992. Pastured-raised poultry nutrition. *Heifer International Project Report* **1:** 1-36.

McCleary, B. V. 1979. Modes of action of *β*-mannanase enzymes of diverse origin on legume seed galactomannans. *Phytochemistry* **18:** 757–763.

McCleary, B. V. 1983. Enzymic interactions in the hydrolysis of galactomannan in germinating guar: the role of exo-β-mannanase. *Phytochemistry* **22:** 649–658.

McCleary, B.V. 1988. β-D-mannanase. *Methods in Enzymology* **160:** 596-615.

McCoy, M. 2001. Soaps and detergents: An update on the latest developments within the detergent industry also introducing the latest new enzyme, a mannanase. *Chemical and Engineering News* **20:** 19-23.

McEllhiney, R. R., ed. 1994. Feed Manufacturing Technology IV. American Feed Industry Association. Washington, DC.

McNaughton, J. L., H. Hsiao, D. Anderson, and D. W. Fodge. 1998. Corn/soy/fat diets for broilers, β-mannanase and improved feed conversion. *Poultry Science* **77:** 153-159.

Mendoza, N. S., M. Arai, T. Kawaguchi, T. Yoshida, and L.M. Joson. 1994. Isolation of mannan utilizing bacteria and culture conditions for mannanase production. *World Journal of Microbiology and Biotechnology* **10:** 51–54.

Mikkonen, K.S., H. Rita, H. Helen, R.A. Talja, L. Hyvönen, and M. Tenkanen. 2007. Effect of polysaccharide structure on mechanical and thermal properties of galactomannan-based films. *Biomacromolecules* **8:** 3198-3205.

Millward-Sadler, S. J., J. Hall, G.W. Black, G.P. Hazlewood, and H.J. Gilbert. 1996. Evidence that the *Piromyces* gene family encoding endo-β-1,4-mannanase arose through gene duplication. *FEMS Microbiology Letters* **141:** 183–188.

Montiel, M.D., J. Rodriguez, M.I. Perel-Leblic, M. Hernandez, M.E. Arias, and J.L. Copa-Patino. 1999. Screening of mannanases in actinomycetes and their potential

application in the biobleaching of pine kraft pulps. *Applied Microbiology and Biotechnology* **52:** 240-245.

Morris, D. D., R.A. Reeves, M.D. Gibbs, D.J. Saul, and P. Bergquist. 1995. Correction of the the β-mannanase domain of the *celC* pseudogene from *Caldocellulosiruptor saccharolyticus* and activity of the gene product on kraft pulp. *Applied and Environmental Microbiology* **61:** 2262–2269.

Moss, A.R. and D.I Givens. 1994. The chemical composition, digestibility, metabolisable energy content and nitrogen degradability of some protein-concentrates. *Animal Feed Science and Technology* **47:** 335–351.

Mudau, M.M. 2006. The production, purification and characterization of endo1,4-β-mannanase from newly isolated strains of *Scopulariopsis candida*. University of the Free-State. *MSc. Thesis*.

Mudau, M.M. and M.E. Setati. 2006. Screening and identification of endomannanase producing microfungi from hypersaline environments. *Current Microbiology* **56:** 477-481.

Mudau, M.M., and M.E. Setati. 2008. Partial purification and characterization of endo-β-1,4-mannanases from *Scopulariopsis candida* strains isolated from solar salterns. *African Journal of Biotechnology* **7:** 2279-2285.

Mulimani, V.H., and S. Prashanth. 2002. Investigating plant galactomannans. *Biochemistry and Molecular Biology Education* **30:** 101-103.

Nagpal, M. L., O. P Agrawal, and I. S. Bhatia. 1971. Chemical and biological examination of guar-meal (*Cyampsis tetragonoloba* L.). *Indian Journal of Animal Sciences* **4:** 283–293.

Nakajima, N., and Y. Matsuura. 1997. Purification and characterization of konjac glucomannan degrading enzyme from anaerobic human intestinal bacterium, *Clostridium butyricum-Clostridium beijerinckii* group. *Bioscience, Biotechnology and Biochemistry* **61:** 1739–1742.

Nakatani F, T. Kawaguchi, G. Takada, J. Sumitani, Y. Moriyama, and M. Arai. 2000. Cloning and sequencing of an endoglucanase gene from *Scopulariopsis brevicaulis* TOF-1212, and its expression in *Saccharomyces cerevisiae*. *Bioscience, Biotechnology and Biochemistry* **64:** 1238-1246.

Nevalainen, K.M.H., and V.S.J. Te'o. 2003. Enzyme production in industrial fungi: molecular genetics strategies for integrated strain improvement. *In:* Arora,D.K.,Khachatourians, G.G. (Eds.), Applied Mycology and Biotechnology. Fungal Genomics, vol. 3. *Elsevier*, Amsterdam, pp. 241–260.

Nicolas, P., E. Raetz, S. Reymond, and J.L. Sauvageat. 1998. Hydrolysis of galactomannans of coffee extract with immobilized β-mannanase. *U.S. Patent* 5714183.

Ninawe, S., M. Kapoor, and R.C. Kuhad. 2008. Purification and characterization of extracellular xylanase from *Streptomyces cyaneus* SN32. *Bioresource Technology* **99:** 1252–1258.

Nishinari, K., P.A. Williams and G.O. Phillips. 1992. Review of the physiochemical characteristics and properties of konjac mannan. *Food Hydrocolloids* **6:** 1238-1246.

NRC. 1994. Nutrient requirements of poultry. 9th Revised Edition. National Academy of Sciences Press, Washington, D.C..

Nunes, C. S. and K. Malmlof. 1992. Effects of guar gum and cellulose on glucose absorption, hormonal release and hepatic metabolism in the pig. *British Journal of Nutrition* **68:** 693-700.

Nunes, F.M., A. Reis, M.R.M. Domingues, and M.A. Coimbra. 2006. Characterization of galactomannan derivatives in roasted coffee beverages. *Journal of Agricultural and Food Chemistry* **54:** 3428 3439.

Odetallah, N. H., P. R. Ferket, J. L. Grimes, and J. L. McNaughton, 2002. Effect of Mannan-endo-1,4-B-Mannosidase on the growth performance of turkeys fed diets containing 44% and 48% C.P. soybean meal. *Poultry Science* **81:** 1322–1331.

O'Neill, M.A., and R.R. Selvendran. 1985. Hemicellulosic complexes from the cell walls of runner bean (*Phaseolus coccineus*). *Biochemical Journal* **227:** 475-481.

Parker, K. N., S.R. Chhabra, D. Lam, W. Callen, G.D. Duffaud, M.A. Snead, J.M. Short, E.J. Mathur, and R.M. Kelly. 2001. Galactomannanases Man2 and Man5 from *Thermotoga* species: growth physiology on galactomannans, gene sequence analysis, and biochemical properties of recombinant enzymes. *Biotechnology and Bioengineering.* **75:** 322–333.

Pason, P., K.L. Kyu, and K. Ratanakhanokchai. 2006. *Paenibacillus curdlanolyticus* Strain B-6 xylanolytic-cellulolytic enzyme system that degrades insoluble polysaccharides. *Applied and Environmental Microbiology* **72:** 2483–2490.

Peng, S.Y., J. Norman, G. Curtin, D. Corrier, H.R. McDaniel, and D. Busbee. 1991. Decreased mortality in Norman murine sarcoma in mice treated with the immunomodular, acemannan. *Molecular Biotherapy* **3:** 79-87.

Perret, S., A. Belaich, H.P. Fierobe, J.P. Belaich, and C. Tardi. 2004. Towards designer cellulosomes in *Clostridia*: mannanase enrichment of the cellulosomes produced by *Clostridium cellulolyticum. Biotechnology and Applied Biochemistry* **40:** 255–259.

Peterson, R., J. Grinyer, J. Joss, A. Khan, and H. Nevalainen. 2009. Fungal proteins with mannanase activity identified directly from a Congo Red stained zymogram by mass spectrometry. *Journal of Microbiology Methods* **79:** 379-377.

Pettey, L. A., S. D. Carter, B. W. Senne, and J. A. Shriver. 2002. Effects of β-mannanase addition to corn-soybean meal diets on growth performance, carcass traits,

and nutrient digestibility of weanling and growing-finishing pigs. *Journal of Animal Science* **80:** 1012–1019.

Politz, O., M. Krah, K. K. Thomsen and R. Borris. 2001. A highly thermostable endo-(1,4)-β-mannanase from the marine bacterium *Rhodothermus marinus. Applied Microbiology and Biotechnology* **53:** 715-721.

Puchart, V., M. Vršanská, M. K. Bhat, P. Biely. 2000. Purification and characterisation of α-galactosidase from a thermophilic fungus *Thermomyces lanuginosus*. *Biochimica et Biophysica Acta.* **1524:** 27–37.

Puchart, V., M. Vrsanska´, P. Svoboda, J. Pohl, Z.B. Ogel, and P. Biely. 2004. Purification and characterization of two forms of endo-b-1,4- mannanase from a thermatolerant fungus *Aspergillus fumigatus* IMI 385708 (formerly *Thermomyces lanuginosus* IMI 158749). *Biochimica et Biophysica Acta.* **1674:** 239–250.

Puls, J., and J. Schuseil. 1996. Chemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis, pp. 1–28. *In* M. P. Coughlan and G. P. Hazlewood (ed.), Hemicelluloses and hemicellulases. Portland Press, London, England.

Raeder, U and P. Broda. 1985. Rapid DNA isolation from filamentous fungi. *Letters in Applied Microbiology* **1:** 17-20.

Rainbird, A. L., A. G. Low, and T. Zebrowska. 1984. Effect of guar gum on glucose and water absorption from isolated loops of jejunum in conscious growing pigs. *British Journal of Nutrition* **52:** 489-498.

Ratcliffe, I., P.A. Williams, C. Viebke, and J. Meadows. 2005. Physicochemical characterization of konjac glucomannan. *Biomacromolecules* **6:** 1977-1986.

Ray, S., M.H. Pubols, and J. McGinnis. 1982. The effects of purified guar degrading enzyme on chick growth. *Poultry Science* **61:** 489-498.

Regalado, C., B.E. Garcia-Almendarez, L.M. Venegas-Barrera, A. TellezJurado, G. Rodriguez-Serrano, S. Huerta-Ochoa, and J.R. Whitaker. 2000. Production, partial purification and properties of *β*mannanases obtained by solid substrate fermentation of spent soluble coffee wastes and copra paste using *Aspergillus oryzae* and *Aspergillus niger*. *Journal of the Science of Food and Agriculture* **80:** 1343–1350.

Reid, J. S. G. 1985. Cell wall storage carbohydrate in seeds- Biochemistry of the seed "gums" and "hemiecllulоses." *Advances in Botanical Research* **2:** 125–155.

Rosin, E.A., G. Blank, B.A Slominski and R.A Holley. 2007. Enzyme supplements in broiler chicken diets: *in vitro* and *in vivo* effects on bacterial growth. *Journal of the Science of Food and Agriculture* **87:** 1009–1020.

Ross, S. A., C. J.G. Duncan, D.S. Pasco, and N. Pugh. 2002. Isolation of a galactomannan that enhances macrophage activation from the edible fungus *Morchella esculenta*. *Journal of Agricultural and Food Chemistry* **50:** 5683-5685.

Sabini, E., H. Schubert, G. Murshudov, G.S. Wilson, M. Siika-Aho, and M. Penttilä. 2000. The three-dimensional structure of a *Trichoderma reesei* β-mannanase from glycoside family 5. *Acta Crystallographica Section D* **56:** 3-13.

Sachslehner, A., G. Foildl, N. Foidl, G. Gübitz, and D. Haltrich. 2000. Hydrolysis of isolated coffee mannan and coffee extract by mannanases of *Sclerotium rolfsii. Journal of Biotechnology* **80:** 127-134.

Sachslehner, A., B. Nidetzky, K.D. Kulbe, and D. Haltrich. 1998. Induction of mannanase, xylanase and endoglucanase activities in *Sclerotium rolfsii*. *Applied and Environmental Microbiology* **64:** 594–600.

Sae-Lee, N. 2007. The production of fungal mannanase, cellulase and xylanase using palm kernel meal as a substrate. *Walailak Journal of Science and Technology* **4:** 67-82.

Sakon, J., W.S. Adney, M.E. Himmel, S.R. Thomas, and P.A. Karplus. 1996. Crystal structure of thermostable family 5 endocellulase E1 from *Acidothermus cellulolyticus* in complex with cellotetraose. *Biochemistry* **35:** 10648–10660.

Sambrook, I.E., and A.L. Rainbird. 1985. The effect of guar gum and level and source of dietary fat on glucose tolerance in growing pigs. *British Journal of Nutrition* **54:** 27-35.

Sambrook, J., E.J. Fritsche, and T. Maniatis. 1989. *In:* Molecular Cloning, A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press.

Schäfer, T., O. Kirk, T.V. Borchert, C.C. Fuglsang, S. Pedersen, S. Salmon, H.S. Olsen, R. Deinhammer, and H. Lund. 2002. Enzymes for technical applications. *In:* Fahnestock, S.R, and S.T. Steinbüchel (eds) Biopolymers, Wiley VCH, pp. 377-437.

Schmitt, M.E., T.A. Brown, and B.L. Trumpower. 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Research* **18:** 3091-3092.

Schröder, R., P. Nicolas, S.J.F. Vincent, M. Fischer, S. Reymond, and R.J. Redgewell. 2001. Purification and characterization of galactoglucomannan from kiwifruit *(Actinidia deliciosa). Carbohydrate Research* **331:** 291-306.

Schröder, R., R.G. Atkinson and R. J. Redgwell. 2009. Re-interpreting the role of endo-β-mannanases as mannan endotransglycosylase/hydrolases in the plant cell wall. *Annals of Botany* **104:** 197–204.

Setati, M.E. P. Ademark, and W.H. van Zyl. 2001. Expression of the *Aspergillus aculeatus* endo-β-1,4-mannanase encoding gene (man1) in *Saccharomyces cerevisiae* and characterization of the recombinant enzyme. *Protein Expression and Purification* **21:** 105–114.

Shallom, D., and Y. Shoham. 2003. Microbial hemicellulases. *Current Opinions in Microbiology* **6:** 219–228.

Sims, I., D.J. Craik, and A. Basic A. 1997. Structural characterization of glucomannan secreted by suspension-cultured cells of *Nicotiana plumbaginifolia. Carbohydrate Research* **303:** 79-92.

Singh, S., A.M. Madlala, and B.A. Prior. 2003. *Thermomyces lanuginosus:* properties of strains their hemicellulases. *FEMS Microbiology Reviews* **27:** 3–16.

Smith, A.K., and S.J. Circle. 1972. Protein products as food ingredients. *In* A.K. Smith and S.J. Circle, Soybeans: Chemistry and Technology, Vol. 1, Proteins, Avi Publishing, Westport, Connecticut.

Soetan, K. O., O.O. Aiyelaagbe and C.O. Olaiya. 2010. A review of the biochemical, biotechnological and other applications of enzymes. *African Journal of Biotechnology* **9:** 382-393.

Solis S, M.E. Flores, and C. Huitron. 1997. Improvement of pectinase production by interspecific hybrids of Aspergillus strains. *Letters in Applied Microbiology* **24:** 77-81.

Solis, S., M.E. Flores, and C. Huitron. 1997. Improvement of pectinase production by interspecific hybrids of *Aspergillus* strains. *Letters in Applied Microbiology* **24:** 77-81.

Songsiriritthigul, C., B. Buranabanyat, D. Haltrich and M. Yamabhai. 2010. Efficient recombinant expression and secretion of a thermostable GH26 mannan endo-1,4-β-mannosidase from *Bacillus licheniformis* in *Escherichia coli*. *Microbial Cell Factories* **9:** 1-13.

Sreekrishna, K., K. Johnstone, C. Saunders, and J. Bettiol. 2000. Enzyme exhibiting mannase activity, cleaning compositions, and methods of use. *US Patent* 6060299.

Stålbrand, H., M. Antov, L. Anderson, A. Andersson, and F. Tjerneld. 2006. Affinity partitioning of a *Cellulomonas fimi* beta-mannanase with a mannan-binding module in galactomannan/starch aqueous two-phase system. *Journal of Chromatography A* **1123:** 53–59.

Stålbrand, H., A. Salaheimo, J. Vehmaanperä, B. Henrissat, and M. Penttilä. 1995. Cloning and gene expression in *Saccharomyces cerevisiae* of a *Trichoderma reesei* β-mannanase gene containing a cellulose binding domain. *Applied and Environmental Microbiology* **61:** 1090-1097.

Stålbrand, H., M. Siika-aho, and L. Viikari. 1993. Purification and characterisation of two β-mannanases from *Trichoderma reesei*. *Applied and Environmental Microbiology* **29:** 229-242.

Steiman, R., L. Ford, V. Ducros, J.L. Lafond, and P. Guiraud. 2004. First survey of fungi in hypersaline soil and water of Mono Lake area (California). *Antonie van Leeuwenhoek* **85:** 69-83.

Steiman, R., P. Guiraud, L. Sage, F. Seigle-Murandi. 1997. Soil mycoflora from the Dead Sea oases of Ein Gedi and Einot Zuqim (Israel). *Antonie van Leeuwenhoek* **72:** 261-270.

Stoll, D., A. Boraston, H. Stålbrand, B.W. McLean, D.G. Kilburn, and R.A. Warren. 2000. Mannanase Man26A from *Cellulomonas fimi* has a mannan-binding module. *FEMS Microbiology Letters* **183:** 265–269.

Stoll, D., J. LeNours, L. Anderson, H. Stålbrand, and L. LoLeggio. 2005. The structure and characterization of a modular endo-*β*-1,4-mannanase from *Cellulomonas fimi*. *Biochemistry* **44:** 12700–12708.

Stoll, D., H. Stålbrand, and R.A.J. Warren. 1999. Mannan-degrading enzymes from *Cellulomonas fimi*. *Applied and Environmental Microbiology* **65:** 2598–2605.

Sun Y. M., H.Y. Yu, W.J. Wang, Y.S. Yang, and Y.H. Yang. 2003. Purification and properties of *Bacillus subtilis SA–22* endo-1,4-β-D-mannanase. *Sheng Wu Gong ChengXue Bao.* **19:** 327–330.

Sunna, A., M.D. Gibbs, and P.L. Bergquist. 2001. Identification of Glu-519 as the catalytic nucleophile in β-mannosidase 2A from *Cellulomonas fimi*. *Biochemical Journal* **356:** 791-798.

Sunna, A., M.D. Gibbs, C.W.J. Chin, and P.J. Nelson. 2000. A gene encoding a novel multidomain β-1,4-mannanase from *Caldibacillus cellulovorans* and action of the

recombinant enzyme on kraft pulp. *Applied and Environmental Microbiology* **66:** 664–670.

Surgey, N., J. Robert-Baudouy, and G. Condemine. 1996. The *Erwinia chrysanthami* pec T gene regulates pectinase gene transfer. *Journal of Bacteriology* **178:** 1593-1599.

Sygusch, J., N. Ethier, and G. Talbot. 1998. Gene cloning, DNA sequencing, and expression of thermostable *β*-mannanase from *Bacillus stearothermophilus. Applied and Environmental Microbiology* **64:** 4428– 4432.

Tailford, L.E., V.M.A. Ducros, J.E. Flint, S.M. Roberts, C. Morland, Z.L. Zechel, N. Smith, M.E. Bjøernvad, T.V. Borchert, K.S. Wilson, G.J. Davies, and H.J. Gilbert. 2009. Understanding how diverse β mannanases recognize heterogeneous substrates. *Biochemistry* **48:** 7009–7018.

Takeda, N., K. Hirasawa, K. Uchimura, Y. Nogi, Y. Hatada, R. Usami, Y. Yoshida, W.D. Grant, and S. Ito. 2004b. Purification and enzymatic properties of a highly alkaline mannanase from alkaliphilic *Bacillus* sp.strain JAMB-750**.** *International Journal of Biological Macromolecules* **4:** 67–74.

Takeda, N., K. Hirasawa, K. Uchimura, Y. Nogi, Y. Hatada, M. Akita, R. Usami, Y. Yoshida, and W.D. Grant. 2004a. Alkaline mannanase from a novel species of alkaliphilic *Bacillus. Journal of Applied Glycoscience* **51:** 229–236.

Talbot, G., and J. Sygusch. 1990. Purification and characterization of thermostable *β*-mannanase and β-galactosidase from *Bacillus stearothermophilus. Applied and Environmental Microbiology* **56:** 3505–3510.

Taligoola, H.K., M.A. Ismail, and S.K. Chebon. 2004. Mycobiota associated with rice grains marketed in Uganda. *Journal of Biological Science* **4:** 271-278.

Tamaru, Y. T. Araki, H. Amagoi, H. Mori, and T. Morishita. 1995. Purification and characterization of an extracellular β-1,4-mannanase from a marine bacterium, *Vibrio* sp. strain MA-138. *Applied and Environmental Microbiology* **61:** 4454–4458.

Tamaru, Y., T. Araki, T. Morishita, T. Kimura, K. Sakka, and J. Ohmiya. 1997. Cloning, DNA sequencing, and expression of the beta1,4-mannanase gene from a marine, *Vibrio* sp. strain MA-138. *Journal of Fermentation and Bioengineering* **83:** 201–205.

Tamaru, Y., and R.H. Doi. 2000. The *engL* gene cluster of *Clostridium cellulovorans* contains a gene for cellulosomal ManA. *Journal of Bacteriology* **182:** 244–247.

Tang, C.M., L.D. Waterman, and M.H. Smith. 2001. The *cel4* gene of *Agaricus bisporus* encodes a β-mannanase. *Applied and Environmental Microbiology* **67:** 2298–2303.

Taubken, N., B. Sauerbrei, and J. Thiem. 1993. Synthesis of β-mannopyranosides by enzymatic approaches. *Journal of Carbohydrate Chemistry* **12:** 651- 667.

Teather, R.M., and P.J. Wood. 1982. Use of Congo red-polysccharide interactions in enumeration and characterization of cellulolytic bacteria from bovine rumen. *Applied and Environmental Microbiology* **43:** 777-780.

Tenkanen, M., M. Makkonen, M. Perttula, L. Viikari and A. Teleman. 1997. Action of *Trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. *Journal of Biotechnology* **57:** 191-204.

Triches-Damaso, M.C., M.S. Almeida, E. 2005**Kurtenbach, O.B. Martins, N. Pereira, Jr., C.M.M.C. Andrade, and R. M. Albano.** 2003. Optimized expression of a thermostable xylanase from *Thermomyces lanuginosus* in *Pichia pastoris*. *Applied and Environmental Microbiology* **69:** 6064–6072.

Vahjen, W., T. Busch and O. Simon. 2004. Study on the use of soya bean polysaccharide degrading enzymes in broiler nutrition. *Animal Feed Science and Technology* **120:** 259-276.

van den Burg, B. 2003. Extremophiles as a source for novel enzymes. *Current Opinions in Microbiology* **6:** 213-218.

Van Laere, K. M. J., R. Hartemink, G. Beldman, S. Pitson, C. Dijkema, H. A. Schols and A. G. J. Voragen. 1999. Transglycosidase activity of *Bifidiobacterium adolescentis* DSM 20083 galactosidase. *Applied Microbiology and Biotechnology* **52:** 681–688.

van Zyl, P.J., V. Moodley, S. H. Rose, R. L. Roth and W. H. van Zyl. 2009. Production of the *Aspergillus aculeatus* endo-1,4-β-mannanase in *A. niger*. *Journal of Industrial Microbiology and Biotechnology* **36:** 611-617.

Verma, S.V.S., and J.M. McNab. 1982. Guar meal in diets for broiler chickens. *British Journal of Poultry Science* **23:** 95-105.

Viikari, L., A. Kantelinen, J. Sundquist, and M. Linko. 1994. Xylanases in bleaching: from an idea to an industry. *FEMS Microbial. Reviews* **13:** 335-350.

Viikari, L., M. Tenkanen, J. Buchert, M. Rättö, M. Bailey, M. Siika-aho, and M. Linko. 1993. Hemicellulases for industrial applications, pp. 131–182. *In:* J. N. Saddler (ed.) Bioconversion of forest and agricultural plant residues. CAB International, Wallingford, United Kingdom.

Vinogradov, E., B. O. Petersen, and J. Ø. Duus. 2000. Isolation and characterization of non-labeled and ^{13}C-labeled mannans from *Pichia pastoris* yeast. *Carbohydrate Research* **325:** 216–221.

Wainø, M., and K. Ingvorsen. 2003. Production of β-xylanase and β-xylosidase by extremely halophilic archaeon *Halorhabdus utahensis*. *Extremophiles* **7:** 87-93.

Walsh, D.E., V. Yaghoubian, and A. Behforooz. 1984. Effect of glucomannan on obese patients: a clinical study. *International Journal of Obesity* **8:** 289–293.

Walsh, G.A.; R.F. Power, and D.R. Headon. 1993. Enzymes in the animal-feed industry. *Trends in Biotech*nology **11:** 424–429.

Wang, Q., D. Tull, A. Meinke, N. R. Gilkes, R. A. J. Warren, R. Aebersold, and S. G. Withers. 1993. Glu 280 is the nucleophile in the active site of *Clostridium thermocellum* CelC, a family A endo-β-1,4-glucanase. *Journal of Biological Chemistry* **268:** 14096–14102.

Warren, R. A. J. 1996. Microbial hydrolysis of polysaccharides. *Annual Review of Microbiology* **50:** 183–212.

Wejse, P.L., K. Ingvorsen, K.K. Mortensen. 2003. Purification and characterization of two extremely halotolerant xylanases from a novel halophilic bacterium. *Extremophiles* **7:** 423 – 431.

Whistler, R.L., and J. Saarnio. 1957. Galactomannans in soybean hulls. *Journal of the American Chemical Society* **79:** 6055-6057.

Wong, K. K. Y., and J. N. Saddler. 1993. Applications of hemicellulases in the food, feed, and pulp and paper industries, p. 127–143. *In:* M. P. Coughlan and G. P. Hazlewood (ed.), Hemicellulose and hemicellulases. Portland Press Ltd., London.

Wu, G., M.M. Bryant, R.A. Voitle, and D.A. Roland, Sr. 2005. Effects of β-Mannanase in corn-soy diets on commercial Leghorns in second-cycle hens. *Poultry Science* **84:** 894-897.

Wymelenberg, A. V., G. Sabat, D. Martinez, A.S. Rajangam, T.T. Teeri, J. Gaskell, P.J. Kersten and D. Cullen. 2005. The *Phanerochaete chrysosporium* secretome: Database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium. *Journal of Biotechnology* **118:** 17–34.

Ximenes, E.A., H. Chen, I.A. Kataeva, M.A. Cotta, C.R. Felix, L.G. Ljungdahl, and X. Li. 1995. A mannanase, ManA, of the polycentric anaerobic fungus *Orpinomyces* sp. strain PC-2 has carbohydrate binding and docking modules. *Canadian Journal of Microbiology* **51:** 559–568.

Xu, B., Hagglund, P., Stalbrand, H., Janson, J.C., 2002. Endo-beta- 1,4-mannanases from blue mussel, *Mytilus edulis*: purification, characterization and mode of action *Journal of Biotechnology* **92:** 267–277.

Yagüe, E., M. Mehak-Zunic, L. Morgan, D.A. Wood, and C.F. Thurston. 1997. Expression of CEL2 and CEL4, two proteins from *Agaricus bisporus* with similarity to fungal cellobiohydrolase I and *β*-mannanase, respectively, is regulated by carbon source. *Microbiology* **143:** 239–244.

Yamasaki, T., Y. Miyazaki, and Y. Kamei. 1998. Isolation of bacteria that decompose major polysaccharides in the cell wall of the marine red alga *Porphyra yezoensis* and their application for protoplast production. *Canadian Journal Microbiology* **44:** 789–794.

Yanhe, M., Y. Xue, Y. Dou, Z. Xu, W. Tao, and P. Zhou. 2004. Characterization and gene cloning of a novel beta-mannanase from alkaliphilic *Bacillus sp.* N16-5. *Extremophiles* **8:** 447–454.

Yoon, S., D. Chu, and L.R. Juneja. 2008. Chemical and physical properties, safety and application dietary fiber of partially hydrolyzed guar gum. *Journal of Clinical Biochemistry and Nutrition* **42:** 1-7.

Yoshida, S., Y. Sako, and A. Uchida. 1998. Cloning, sequence analysis, and expression in *Escherichia coli* of a gene coding for an enzyme from *Bacillus circulans* K-1 that degrades guar gum. *Bioscience, Biotechnology and Biochemistry* **62:** 514–520.

Zakaria, M. M., M. Ashiuchi, S. Yamamoto, and T. Yagi. 1998a. Optimization for beta-mannanase production of a psychrophilic bacterium, *Flavobacterium sp. Bioscience, Biotechnology and Biochemistry* **62:** 655–660.

Zakaria, M.M., S. Yamamoto, T. Yagi. 1998b. Purification and characterization of an endo-1, 4- β-mannanase from *Bacillus subtilis* KU-1. *FEMS Microbiology Letters* **158:** 25 – 31.

Zhang, L. and I.R. Tizzard. 1996. Activation of a mouse macrophage cell line by acemannan: the major carbohydrate fraction from aloe vera gel. *Immunopharmacology* **35:** 119-128.

Zhang, Q., X. Yan, L. Zhang, and W. Tang. 2006. Cloning, sequence analysis, and heterologous expression of a β-mannanase gene from *B. subtilis* Z-2. *Molecular Biology* **40:** 418–424.

Zou, X.T., X. J. Qiao, and Z. R. Xu. 2006. Effect of β-mannanase (Hemicell®) on growth performance and immunity of broilers. *Poultry Science* **85:** 2176–2179.

APPENDIX

APPENDIX A: MICROBIOLOGICAL MEDIA AND BUFFERS

1. MICROBIOLOGICAL MEDIA

1.1. Difco™ Malt Extract Agar Plates (supplemented with 2.5% (w/v) NaCl

Maltose, Technical……. 12.75 g

Dextrin……... 2.75 g

Glycerol……. 2.35 g

Peptone……..... 0.78 g

NaCl…….. 25.0g

Agar……15.0 g

1.2. Mineral Basal medium (Ferreira and Filho, 2004), (with supplements)

KH_2PO_4…… 7.0 g

K_2HPO_4…….……2.0 g

$MgSO_4.7H_2O$……. 0.5 g

$(NH_4)SO_4$…….…….. 1.0 g

} Basal Mineral medium

Locust bean gum (Galactomannan).5.0 g

Yeast Extract Powder……0.6 g

NaCl……..100.0 g

} Supplement

1.3. Luria-Bertani Broth

Yeast Extract powder……5.0 g

Bacto-Tryptone Powder……10.0 g

NaCl……..10.0 g

1.4. Super Optimal broth with Catabolite repression (SOC medium)

Yeast Extract powder……5.0 g

Bacto-Tryptone Powder……20.0 g

NaCl……0.5 g

KCl…….....0.186 g

Glucose…….. 3.603 g

2. MICROBIOLOGICAL BUFFERS

2.1. 50 mM Citrate buffer pH 5.0

2.1.1. Solution A: 0.1 M Citric Acid

21.06 g citric acid powder ($C_6H_8O_7$) in 1 L doubled distilled H_2O

2.1.2. Solution B: 0.1 M Sodium citrate

20.41 g Sodium citrate powder ($Na_3C_6H_5O_7$) in 1 L doubled distilled H_2O

Mix 205 ml of Solution A and 295 ml of Solution B with 500 ml of doubled distilled H_2O. Check that pH of citrate buffer and adjust accordingly to pH 5.0. Autoclave to sterilise.

2.2. 50 mM Phospate buffer pH 6.0

2.2.1. Solution A: 0. 2M Sodium phosphate, mono-sodium salt

27.60 g Sodium phosphate powder (NaH_2PO_4) in 1 L doubled distilled H_2O

2.2.2. Solution B: 0.2 M Sodium phosphate, di-sodium salt

53.62 g Sodium citrate powder (Na_2HPO_4) in 1 L doubled distilled H_2O

Mix 438.5 ml of Solution A and 61.5 ml of Solution B with 500 ml of doubled distilled H_2O. Check that pH of citrate buffer and adjust accordingly to pH 6.0. Autoclave to sterilise.

3. BUFFERS AND SOLUTIONS FOR SOUTHERN HYBRIDIZATION

- **Depurination solution (250 mM HCl)**

  20.66 ml HCl in 979.34 ml doubled distilled H_2O

- **Denaturation solution (0.5 M NaOH, 1.5 M NaCl)**

  20.0 g NaOH, 87.66 g NaCl in I L doubled distilled H_2O

- **Neutralization solution (0.5 MTris-HCl [pH 7.5], 1.5 M NaCl)**

  78.8 g Tris base, 87.6 g NaCl in doubled distilled H_2O (adjusted to pH 7.5 with few drops HCl)

- **20× SSC buffer (3 M NaCl, 300 mM sodium citrate, pH 7.0)**

175.4 g NaCl, 88.2 g Sodium citrate in 1 L doubled distilled H_2O (adjusted to pH 7.0 with few drops HCl).

- **High stringency wash buffer - 0.5× SSC, 1.0% (w/v) SDS**

  25.0 ml 20× SSC buffer, 10.0 ml 10.0% (w/v) SDS in 965.0 ml doubled distilled H_2O

- **Low stringency wash buffer - 0.2× SSC, 1.0% (w/v) SDS**

  10.0 ml 20× SSC buffer, 10.0 ml 10.0% (w/v) SDS in 980.0 ml doubled distilled H_2O

APPENDIX B: STANDARD CURVES AND RAW DATA

Table B1: Absorbance values used to generate standard curve for the determination Endomannanase activity (nkat.ml^{-1}).

MANNOSE SUGAR DILUTION	ABSORBANCE (540 nm)				MANNOSE UNITS (nkat/ml)
	SET 1	SET 2	SET 3	AVERAGE	
1:1	0.34	0.546	0.289	0.392	16.67
1:2	0.178	0.136	0.219	0.178	8.33
1:3	0.109	0.106	0.084	0.099	5.55
1:4	0.129	0.042	0.035	0.069	4.17
1:5	0.09	0.059	0.097	0.082	3.33

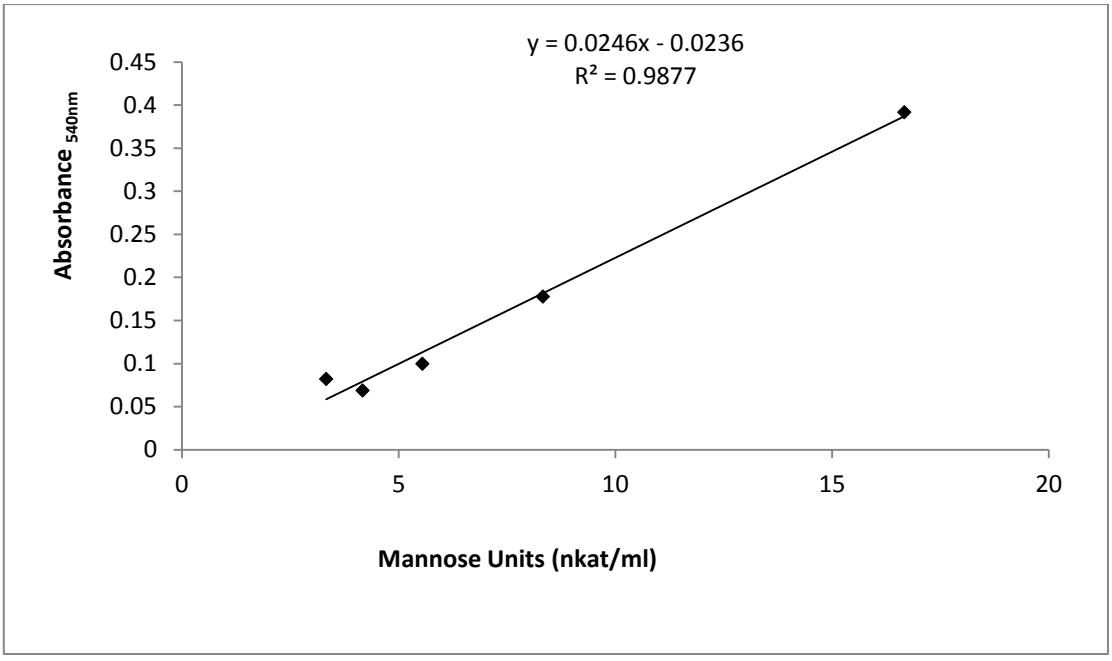

Figure B1: Mannose standard curve used for the determination Endomannanase activity (nkat.ml^{-1}).

Table B2: Absorbance values used to generate standard curve of Mannose sugars for the determination of substrate specificity and hydrolysis of poultry feed.

MANNOSE SUGAR DILUTION	ABSORBANCE (540 nm)				MANNOSE UNITS (nkat/ml)
	SET 1	SET 2	SET 3	AVERAGE	
1:1	0.366	0.378	0.369	0.371	16.67
1:2	0.135	0.139	0.141	0.138	8.33
1:3	0.081	0.085	0.081	0.082	5.55
1:4	0.045	0.043	0.043	0.044	4.17
1:5	0.033	0.032	0.026	0.031	3.33

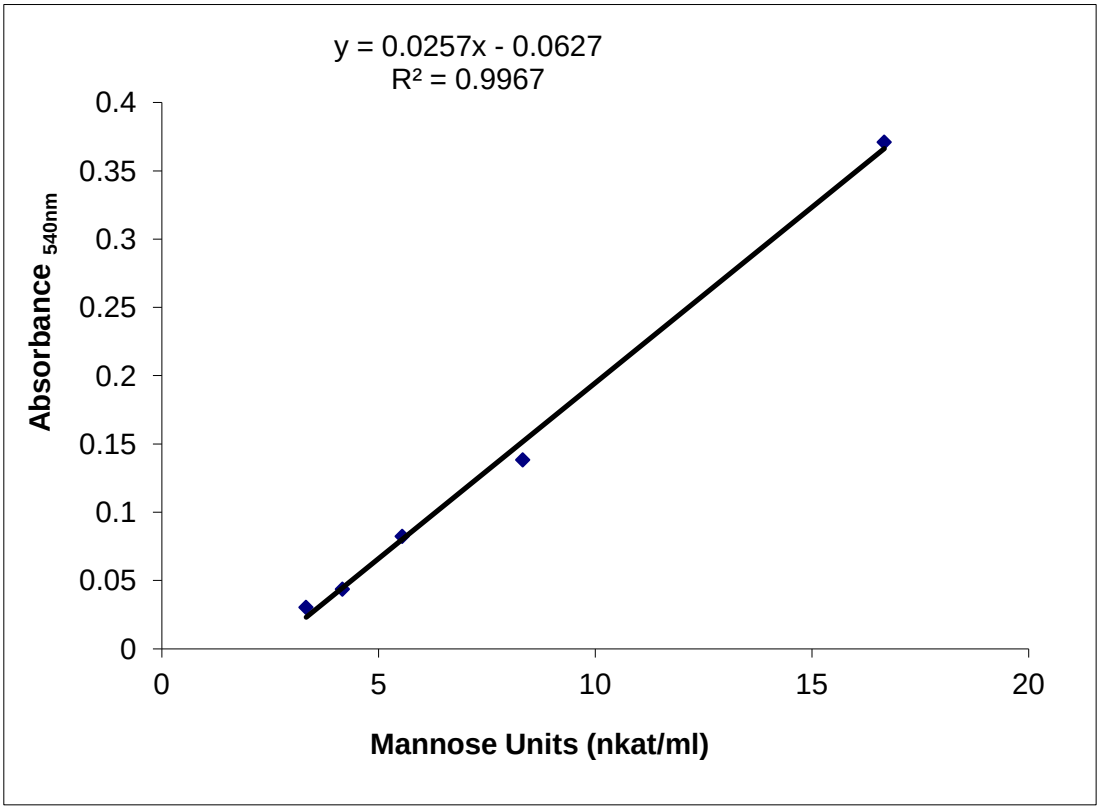

Figure B2: Mannose standard curve used for determination of substrate specificity and hydrolysis of poultry feed.

Table B3: Absorbance values used to generate standard curve for the determination of protein concentration (µg.ml^{-1}).

Conc(µg/ml)	Absorbance values OD_{595}			Average Absorbance values OD_{595}
	1	2	3	
10	0.021	0.026	0.025	0.024
20	0.027	0.028	0.021	0.025
30	0.065	0.062	0.065	0.064
40	0.1	0.103	0.094	0.099
50	0.107	0.126	0.121	0.118

60	0.123	0.122	0.125	0.123
70	0.155	0.153	0.16	0.156
80	0.17	0.169	0.175	0.171
90	0.171	0.178	0.189	0.179
100	0.215	0.213	0.21	0.213

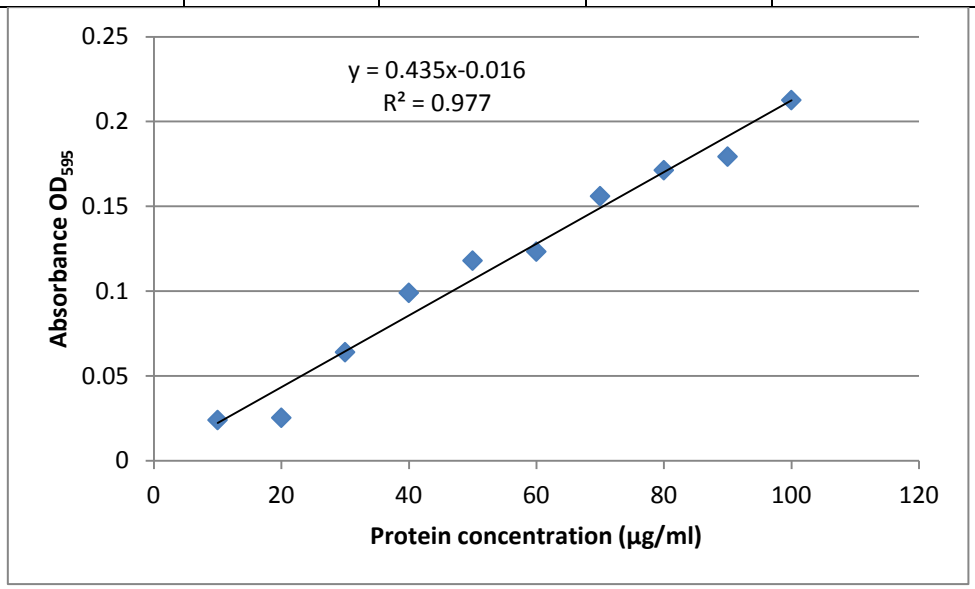

Figure B3: Protein concentration standard curve.

DETERMINATION OF PEAK PRODUCTION OF β-MANNANASE BY *S. CANDIDA* LMK008

Table B4: Run 1 of β-mannanase enzyme activity by *S. candida* LMK008

DAY	ABSORBANCE 540nm			AVERAGE ABSORBANCE 540nm	ENZYME ACTIVITY (nkat/ml)
	FLASK 1	FLASK 2	FLASK 3		
Day 1	0.043	0.065	0.074	0.052	2.153
	0.003	0.063	0.076		
	0.005	0.066	0.072		
Day 2	0.221	0.241	0.101	0.229	9.502
	0.22	0.516	0.124		
	0.221	0.301	0.116		
Day 3	0.234	0.19	0.176	0.197	8.170
	0.243	0.191	0.154		
	0.253	0.178	0.153		
Day 4	0.256	0.333	0.395	0.316	130.935
	0.245	0.334	0.383		
	0.223	0.332	0.339		
Day 5	0.502	0.412	0.323	0.411	170.401

	0.502	0.415	0.311		
	0.502	0.418	0.311		
Day 6	0.501	0.409	0.31	0.407	169.018
	0.499	0.409	0.31		
	0.5	0.418	0.31		

Table B5: Run 2 of β-mannanase enzyme activity by *S. candida* LMK008

DAY	**ABSORBANCE $_{540nm}$**			**AVERAGE ABSORBANCE $_{540nm}$**	**ENZYME ACTIVITY (nkat/ml)**
	FLASK 1	**FLASK 2**	**FLASK 3**		
Day 1	0.044	0.068	0.074	0.053	2.199
	0.004	0.066	0.076		
	0.007	0.066	0.072		
Day 2	0.218	0.241	0.101	0.239	9.912
	0.22	0.516	0.124		
	0.313	0.301	0.116		
Day 3	0.185	0.19	0.176	0.194	8.063
	0.249	0.191	0.154		
	0.273	0.178	0.153		
Day 4	0.266	0.333	0.395	0.318	131.904
	0.268	0.334	0.383		
	0.211	0.332	0.339		
Day 5	0.506	0.412	0.314	0.411	170.447
	0.504	0.415	0.312		
	0.504	0.418	0.312		
Day 6	0.503	0.409	0.311	0.409	169.709
	0.504	0.409	0.311		
	0.504	0.418	0.312		

Table B6: Run 3 of β-mannanase enzyme activity by *S. candida* LMK008

DAY	**ABSORBANCE $_{540nm}$**			**AVERAGE ABSORBANCE $_{540nm}$**	**ENZYME ACTIVITY (nkat/ml)**
	FLASK 1	**FLASK 2**	**FLASK 3**		
Day 1	0.07	0.069	0.071	0.057	2.347
	0.004	0.069	0.076		
	0.007	0.071	0.072		
Day 2	0.314	0.239	0.111	0.260	10.793
	0.313	0.51	0.114		
	0.313	0.312	0.115		
Day 3	0.285	0.192	0.169	0.206	8.557
	0.252	0.191	0.163		
	0.253	0.182	0.169		
Day 4	0.266	0.333	0.395	0.318	131.904
	0.268	0.334	0.383		
	0.211	0.332	0.339		
Day 5	0.501	0.416	0.323	0.421	174.504

	0.503	0.415	0.356		
	0.503	0.415	0.353		
Day 6	0.504	0.412	0.423	0.423	175.473
	0.504	0.419	0.31		
	0.504	0.418	0.312		

Table B7: Average of triplicate β-mannanase enzyme activity by *S. candida* LMK008

DAY	ENZYME ACTIVITY			AVERAGE ENZYME ACTIVITY	STANDARD DEVIATION
	RUN 1	RUN 2	RUN 3		
Day 1	2.153	2.199	2.347	2.237	0.086
Day 2	9.502	9.912	10.793	10.069	0.526
Day 3	8.170	8.064	8.557	8.263	0.129
Day 4	130.936	131.904	131.904	131.581	3.083
Day 5	170.401	170.447	174.504	171.784	3.050
Day 6	169.018	169.710	175.473	169.018	4.708

HYDROLYSIS OF MANNAN BASED SUBSTRATES

1. HYDROLYSIS OF 0.5% (w/v) GUAR GUM

Table B8: Absorbance values $_{560\ nm}$ Run 1 hydrolysis of 0.5% (w/v) guar gum

INCUBATION PERIOD	ABSORBANCE $_{540nm}$			AVERAGE ABSORBANCE $_{540nm}$	REDUCING SUGARS (mg.ml^{-1})
	SET 1	SET 2	SET 3		
0 Hrs	0.35	0.242	0.295	0.296	1.520
1 Hrs	0.488	0.522	0.504	0.504	2.405
2 Hrs	0.655	0.587	0.643	0.643	2.991
3 Hrs	0.764	0.75	0.760	0.760	3.490
4 Hrs	0.856	0.829	0.863	0.863	3.924
5 Hrs	0.948	0.827	0.903	0.903	4.096
6 Hrs	0.843	0.841	0.842	0.842	3.838
7 Hrs	0.835	0.834	0.836	0.836	3.811

Table B9: Absorbance values $_{560\ nm}$ Run 2 hydrolysis of 0.5% (w/v) guar gum

INCUBATION PERIOD	ABSORBANCE $_{540nm}$			AVERAGE ABSORBANCE $_{540nm}$	REDUCING SUGARS (mg.ml^{-1})
	SET 1	SET 2	SET		

			3		
0 Hrs	0.356	0.354	0.353	0.354	1.769
1 Hrs	0.486	0.484	0.484	0.485	2.322
2 Hrs	0.696	0.692	0.696	0.695	3.212
3 Hrs	0.778	0.777	0.776	0.777	3.561
4 Hrs	0.865	0.861	0.864	0.863	3.927
5 Hrs	0.951	0.951	0.952	0.951	4.300
6 Hrs	0.841	0.841	0.843	0.842	3.835
7 Hrs	0.839	0.839	0.841	0.840	3.826

Table B10: Absorbance values $_{560\ nm}$ Run 3 hydrolysis of 0.5% (w/v) guar gum

INCUBATION PERIOD	ABSORBANCE $_{540nm}$			AVERAGE ABSORBANCE $_{540nm}$	REDUCING SUGARS (mg.ml^{-1})
	SET 1	SET 2	SET 3		
0 Hrs	0.355	0.356	0.357	0.356	1.776
1 Hrs	0.478	0.474	0.474	0.475	2.282
2 Hrs	0.623	0.623	0.633	0.626	2.922
3 Hrs	0.712	0.714	0.713	0.713	3.290
4 Hrs	0.834	0.834	0.834	0.834	3.802
5 Hrs	0.876	0.874	0.872	0.874	3.972
6 Hrs	0.845	0.843	0.846	0.845	3.848
7 Hrs	0.839	0.837	0.839	0.838	3.821

2. HYDROLYSIS OF 0.5% (w/v) LOCUST BEAN GUM

Table B11: Absorbance values $_{560\ nm}$ Run 1 hydrolysis of 0.5% (w/v) locust bean gum

INCUBATION PERIOD	ABSORBANCE $_{540nm}$			AVERAGE ABSORBANCE $_{540nm}$	AVERAGE ABSORBANCE $_{540nm}$ (*Dilution Factor)	REDUCING SUGARS (mg.ml^{-1})
	SET 1	SET 2	SET 3			
0 Hrs	0.145	0.15	0.146	0.147	0.735	3.383
1 Hrs	0.233	0.231	0.257	0.240	1.202	5.361
2 Hrs	0.375	0.366	0.379	0.373	1.867	8.180
3 Hrs	0.409	0.427	0.412	0.416	2.080	9.084
4 Hrs	0.472	0.485	0.485	0.481	2.403	10.455
5 Hrs	0.339	0.336	0.338	0.338	1.688	7.424
6 Hrs	0.334	0.334	0.333	0.334	1.668	7.339

Table B12: Absorbance values $_{560\ nm}$ Run 2 hydrolysis of 0.5% (w/v) locust bean gum

INCUBATION PERIOD	ABSORBANCE $_{540nm}$	AVERAGE ABSORBANCE	AVERAGE ABSORBANCE	REDUCING SUGARS

	SET 1	SET 2	SET 3	$_{540nm}$	$_{540nm}$ (*Dilution Factor)	(mg.ml^{-1})
0 Hrs	0.144	0.145	0.144	0.144	0.722	0.267
1 Hrs	0.243	0.243	0.244	0.243	1.217	2.365
2 Hrs	0.385	0.386	0.389	0.387	1.933	5.403
3 Hrs	0.419	0.417	0.413	0.416	2.082	6.032
4 Hrs	0.482	0.482	0.482	0.482	2.410	7.424
5 Hrs	0.347	0.345	0.344	0.345	1.727	4.527
6 Hrs	0.341	0.346	0.343	0.343	1.717	4.485

Table B13: Absorbance values $_{560\ nm}$ Run 3 hydrolysis of 0.5% (w/v) locust bean gum

INCUBATION PERIOD	ABSORBANCE $_{540nm}$			AVERAGE ABSORBANCE $_{540nm}$	AVERAGE ABSORBANCE $_{540nm}$ (*Dilution Factor)	REDUCING SUGARS (mg.ml^{-1})
	SET 1	SET 2	SET 3			
0 Hrs	0.141	0.141	0.146	0.143	0.713	0.267
1 Hrs	0.239	0.234	0.234	0.236	1.178	2.238
2 Hrs	0.278	0.276	0.278	0.277	1.387	3.121
3 Hrs	0.312	0.317	0.319	0.316	1.580	3.941
4 Hrs	0.403	0.402	0.402	0.402	2.012	5.771
5 Hrs	0.353	0.355	0.354	0.354	1.770	4.746
6 Hrs	0.343	0.343	0.344	0.343	1.717	4.520

3. HYDROLYSIS OF 0.5% (w/v) SOYBEAN FLOUR

Table B14: Absorbance values $_{560\ nm}$ Run 1 hydrolysis of 0.5% (w/v) soybean flour

INCUBATION PERIOD	ABSORBANCE $_{540nm}$			AVERAGE ABSORBANCE $_{540nm}$	AVERAGE ABSORBANCE $_{540nm}$ (*Dilution Factor)	REDUCING SUGARS (mg.ml^{-1})
	SET 1	SET 2	SET 3			
0 Hrs	0.149	0.146	0.149	0.148	0.740	0.000
1 Hrs	0.245	0.246	0.245	0.245	1.227	2.063
2 Hrs	0.381	0.381	0.381	0.381	1.905	4.939
3 Hrs	0.405	0.404	0.409	0.406	2.030	5.468
4 Hrs	0.445	0.441	0.443	0.443	2.215	6.253
5 Hrs	0.476	0.471	0.476	0.474	2.372	6.917
6 Hrs	0.335	0.336	0.339	0.337	1.683	3.999
7 Hrs	0.332	0.332	0.332	0.332	1.660	3.899

Table B15: Absorbance values $_{560\ nm}$ Run 2 hydrolysis of 0.5% (w/v) soybean flour

INCUBATION PERIOD	ABSORBANCE $_{540nm}$			AVERAGE ABSORBANCE $_{540nm}$	AVERAGE ABSORBANCE $_{540nm}$ (*Dilution	REDUCING SUGARS (mg.ml^{-1})
	SET	SET	SET			

	1	2	3		Factor)	
0 Hrs	0.144	0.145	0.144	0.144	0.722	0.000
1 Hrs	0.244	0.245	0.245	0.245	1.223	2.127
2 Hrs	0.386	0.386	0.384	0.385	1.927	5.108
3 Hrs	0.48	0.408	0.405	0.431	2.155	6.076
4 Hrs	0.449	0.449	0.448	0.449	2.243	6.450
5 Hrs	0.479	0.479	0.478	0.479	2.393	7.086
6 Hrs	0.334	0.336	0.337	0.336	1.678	4.055
7 Hrs	0.334	0.334	0.334	0.334	1.670	4.020

Table B16: Absorbance values $_{560\ nm}$ Run 3 hydrolysis of 0.5% (w/v) soybean flour

INCUBATION PERIOD	**ABSORBANCE $_{540nm}$**			**AVERAGE ABSORBANCE $_{540nm}$**	**AVERAGE ABSORBANCE $_{540nm}$ (*Dilution Factor)**	**REDUCING SUGARS (mg.ml^{-1})**
	SET 1	**SET 2**	**SET 3**			
0 Hrs	0.141	0.142	0.142	0.142	0.708	0.000
1 Hrs	0.242	0.246	0.246	0.245	1.223	2.183
2 Hrs	0.376	0.379	0.378	0.378	1.888	5.002
3 Hrs	0.472	0.473	0.473	0.473	2.363	7.016
4 Hrs	0.51	0.513	0.511	0.511	2.557	7.835
5 Hrs	0.516	0.516	0.514	0.515	2.577	7.920
6 Hrs	0.435	0.436	0.436	0.436	2.178	6.231
7 Hrs	0.429	0.425	0.429	0.428	2.138	6.061

Table B17: Average of Triplicate hydrolysis of locust bean gum, guar gum and soybean flour

INCUBATION PERIOD	**REDUCING SUGARS (mg.ml^{-1})**			**STANDARD DEVIATION**		
	GUAR GUM	**LOCUST BEAN GUM**	**SOYBEAN FLOUR**	**GUAR GUM**	**LOCUST BEAN GUM**	**SOYBEAN FLOUR**
0 Hours	1.520	0.267	0.257	0.000	0.000	0.000
1 Hours	2.336	2.194	2.124	0.056	0.177	0.054
2 Hours	3.042	4.441	5.016	0.135	1.057	0.077
3 Hours	3.445	5.224	6.187	0.126	1.005	0.697
4 Hours	3.884	6.756	6.846	0.063	0.779	0.771
5 Hours	4.122	4.438	7.308	0.148	0.323	0.480
6 Hours	3.840	4.321	4.762	0.006	0.282	1.139
7 Hours	3.819	3.840	4.661	0.007	0.000	1.087

4. CRUDE β-MANNANASE HYDROLYSIS OF ANIMAL FEED

Table B18: Control samples run 1 crude β-mannanase hydrolysis of animal feed

INCUBATION PERIOD	ABSORBANCE VALUES 540nm									AVERAGE ABSORBANCE VALUES 540nm		
	STARTER FEED			GROWER FEED			LAYER FEED			STARTER FEED	GROWER FEED	LAYER FEED
	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3			
0 Hrs	0.097	0.097	0.096	0.122	0.123	0.123	0.061	0.069	0.06	0.0967	0.123	0.063
2 Hrs	0.257	0.255	0.257	0.266	0.258	0.257	0.169	0.177	0.169	0.256	0.260	0.172
24 Hrs	0.755	0.89	0.756	0.805	0.748	0.755	0.125	0.158	0.136	0.800	0.769	0.140

Table B19: Absorbance values $_{560\ nm}$ Crude β-mannanase supplemented samples

INCUBATION PERIOD	ABSORBANCE VALUES 540nm									AVERAGE ABSORBANCE VALUES 540nm		
	STARTER FEED			GROWER FEED			LAYER FEED			STARTER FEED	GROWER FEED	LAYER FEED
	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3			
0 Hrs	0.155	0.158	0.161	0.242	0.269	0.265	0.107	0.108	0.112	0.158	0.259	0.109
2 Hrs	0.592	0.549	0.591	0.772	0.775	0.861	0.556	0.578	0.569	0.577333333	0.803	0.568
24 Hrs	0.981	0.982	0.98	1.039	1.355	1.195	0.431	0.438	0.32	0.981	1.196	0.396

Table B20: Corrected Absorbance values $_{560\ nm}$ enzyme absorbance – control absorbance)

INCUBATION PERIOD	AVERAGE ABSORBANCE 540 nn		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.061	0.136	0.046
2 Hrs	0.321	0.542	0.396
24 Hrs	0.180	0.427	0.257

Table B21: Reducing sugars ($mg.ml^{-1}$) crude β-mannanase supplemented samples

INCUBATION PERIOD	REDUCING SUGARS ($mg.ml^{-1}$)		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.527	0.844	0.461
2 Hrs	1.628	2.566	1.946
24 Hrs	1.033	2.077	1.355

Table B22: Absorbance values $_{560\ nm}$ Run 2 control samples crude β-mannanase hydrolysis of animal feed

INCUBATION PERIOD	ABSORBANCE VALUES 540nm									AVERAGE ABSORBANCE VALUES 540nm		
	STARTER FEED			GROWER FEED			LAYER FEED			STARTER FEED	GROWER FEED	LAYER FEED
	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3			
0 Hrs	0.066	0.097	0.079	0.06	0.057	0.057	0.065	0.065	0.069	0.081	0.058	0.066
2 Hrs	0.194	0.229	0.205	0.116	0.128	0.126	0.15	0.149	0.156	0.209	0.123	0.152
24 Hrs	0.719	0.774	0.748	0.114	0.113	0.12	0.267	0.268	0.272	0.747	0.115	0.269

Table B23: Absorbance values $_{560\ nm}$ Run 2 crude β-mannanase supplemented samples

INCUBATION PERIOD	ABSORBANCE VALUES 540nm									AVERAGE ABSORBANCE VALUES 540nm		
	STARTER FEED			GROWER FEED			LAYER FEED			STARTER FEED	GROWER FEED	LAYER FEED
	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3			
0 Hrs	0.122	0.148	0.122	0.19	0.183	0.19	0.151	0.159	0.156	0.131	0.188	0.155
2 Hrs	0.44	0.453	0.461	0.643	0.643	0.628	0.443	0.441	0.443	0.451	0.638	0.442
24 Hrs	0.792	0.844	0.845	0.473	0.477	0.47	0.509	0.528	0.522	0.827	0.473	0.520

Table B24: Corrected Absorbance values $_{560\ nm}$ (enzyme absorbance – control absorbance)

INCUBATION PERIOD	AVERAGE ABSORBANCE 540 nn		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.05	0.130	0.089
2 Hrs	0.242	0.515	0.291
24 Hrs	0.08	0.358	0.251

Table B25: Reducing sugars ($mg.ml^{-1}$)

INCUBATION PERIOD	REDUCING SUGARS ($mg.ml^{-1}$)		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.479	0.817	0.644
2 Hrs	1.293	2.449	1.499
24 Hrs	0.606	1.783	1.330

Table B26: Absorbance values $_{560\ nm}$ Control samples run 3 crude β-mannanase hydrolysis of animal feed

INCUBATION PERIOD	ABSORBANCE VALUES 540nm									AVERAGE ABSORBANCE VALUES 540nm		
	STARTER FEED			GROWER FEED			LAYER FEED			STARTER FEED	GROWER FEED	LAYER FEED
	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3			
0 Hrs	0.081	0.078	0.085	0.054	0.065	0.064	0.093	0.088	0.087	0.081	0.061	0.089
2 Hrs	0.217	0.216	0.216	0.114	0.129	0.122	0.184	0.188	0.186	0.216	0.122	0.186
24 Hrs	0.775	0.766	0.78	0.5	0.487	0.473	0.38	0.386	0.38	0.773	0.487	0.382

Table B27: Absorbance values $_{560\ nm}$ Crude β-mannanase supplemented samples run 3

INCUBATION PERIOD	ABSORBANCE VALUES 540nm									AVERAGE ABSORBANCE VALUES 540nm		
	STARTER FEED			GROWER FEED			LAYER FEED			STARTER FEED	GROWER FEED	LAYER FEED
	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3	FLASK 1	FLASK 2	FLASK 3			
0 Hrs	0.137	0.137	0.136	0.225	0.236	0.246	0.216	0.215	0.215	0.137	0.24	0.215
2 Hrs	0.502	0.489	0.491	0.743	0.738	0.749	0.504	0.507	0.522	0.494	0.743	0.511
24 Hrs	0.786	0.801	0.777	1.142	1.16	1.13	0.686	0.698	0.665	0.788	1.144	0.683

Table B28: Corrected Absorbance values $_{560\ nm}$ (enzyme absorbance – control absorbance) run 3

INCUBATION PERIOD	AVERAGE ABSORBANCE 540 nn		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.055	0.175	0.126
2 Hrs	0.278	0.622	0.325
24 Hrs	0.014	0.657	0.301

Table B29: Run 2 crude β-mannanase supplemented samples reducing sugars ($mg.ml^{-1}$)

INCUBATION PERIOD	REDUCING SUGARS ($mg.ml^{-1}$)		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.502	1.007	0.802
2 Hrs	1.444	2.902	1.645
24 Hrs	0.328	3.053	1.543

Table B30: Triplicate crude β-mannanase supplemented animal feed

INCUBATION PERIOD	REDUCING SUGARS ($mg.ml^{-1}$)									AVERAGE REDUCING SUGARS ($mg.ml^{-1}$)		
	Run 1			Run 2			Run 3					
	STARTER FEED	GROWER FEED	LAYER FEED	STARTER FEED	GROWER FEED	LAYER FEED	STARTER FEED	GROWER FEED	LAYER FEED	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.527	0.844	0.461	0.479	0.817	0.644	0.502	1.007	0.801	0.503	0.889	0.635
2 Hrs	1.628	2.566	1.946	1.292	2.449	1.499	1.444	2.902	1.645	1.455	2.639	1.697
24 Hrs	1.033	2.078	1.355	0.606	1.783	1.330	0.328	3.053	1.543	0.656	2.305	1.409

Table B31: Triplicate hydrolysis of animal feed with crude enzyme extract (with standard deviation)

INCUBATION PERIOD	AVERAGE REDUCING SUGARS ($mg.ml^{-1}$)			STANDARD DEVIATION		
	STARTER FEED	GROWER FEED	LAYER FEED	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.503	0.889	0.635	0.021	0.092	0.152
2 Hrs	1.455	2.639	1.697	0.150	0.211	0.204
24 Hrs	0.656	2.3045	1.409	0.318	0.595	0.104

5. PURIFIED β-MANNANASE HYDROLYSIS OF ANIMAL FEED

Table B32: Absorbance values $_{560\ nm}$ control samples run 1

CONTROL (No Enzyme)	ABSORBANCE 540 nn								
	STARTER FEED	STARTER FEED	STARTER FEED	GROWER FEED	GROWER FEED	GROWER FEED	LAYER FEED	LAYER FEED	LAYER FEED
0 Hrs	0.091	0.091	0.091	0.112	0.112	0.113	0.062	0.063	0.062
2 Hrs	0.254	0.256	0.254	0.261	0.259	0.254	0.167	0.169	0.166
24 Hrs	0.694	0.695	0.695	0.789	0.787	0.785	0.173	0.175	0.176

Table B33: Absorbance values $_{560\ nm}$ enzyme added run 1

ENZYME ADDED	ABSORBANCE 540 nn								
	STARTER FEED	STARTER FEED	STARTER FEED	GROWER FEED	GROWER FEED	GROWER FEED	LAYER FEED	LAYER FEED	LAYER FEED
0 Hrs	0.147	0.145	0.143	0.222	0.229	0.225	0.114	0.118	0.115
2 Hrs	0.495	0.497	0.495	0.664	0.665	0.663	0.497	0.497	0.497
24 Hrs	0.893	0.892	0.896	0.998	0.997	0.998	0.476	0.478	0.473

Table B34: Average absorbance values $_{560\ nm}$ controls run 1

CONTROL (No Enzyme)	AVERAGE ABSORBANCE 540 nn		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.091	0.112	0.06
2 Hrs	0.254	0.258	0.167
24 Hrs	0.695	0.787	0.175

Table B35: Average absorbance values $_{560\ nm}$ purified β-mannanase supplemented samples run 1

ENZYME ADDED	AVERAGE ABSORBANCE 540 nn		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.145	0.225	0.116
2 Hrs	0.496	0.664	0.497
24 Hrs	0.894	0.998	0.476

Table B36: Corrected absorbance values $_{560\ nm}$ values (enzyme absorbance – control absorbance) run 1

CORRECTED enz absorbance - control absorbance	AVERAGE ABSORBANCE 540 nn		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.054	0.113	0.053
2 Hrs	0.241	0.406	0.330
24 Hrs	0.199	0.211	0.301

Table B37: Reducing sugars from purified β-mannanases ($mg.ml^{-1}$) run 1

	Release of reducing sugars ($mg.ml^{-1}$)		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.496	0.746	0.493
2 Hrs	1.289	1.988	1.665
24 Hrs	1.111	1.160	1.543

Table B38: Absorbance values $_{560\ nm}$ control samples run 2

CONTROL (No Enzyme)	ABSORBANCE 540 nn								
	STARTER FEED	STARTER FEED	STARTER FEED	GROWER FEED	GROWER FEED	GROWER FEED	LAYER FEED	LAYER FEED	LAYER FEED
0 Hrs	0.095	0.095	0.095	0.115	0.115	0.113	0.066	0.064	0.067

2 Hrs	0.252	0.255	0.256	0.263	0.261	0.262	0.164	0.164	0.163
24 Hrs	0.689	0.689	0.689	0.783	0.784	0.783	0.169	0.168	0.167

Table B39: Absorbance values $_{560\ nm}$ purified enzyme added run 2

ENZYME ADDED	ABSORBANCE 540 nn								
	STARTER FEED	STARTER FEED	STARTER FEED	GROWER FEED	GROWER FEED	GROWER FEED	LAYER FEED	LAYER FEED	LAYER FEED
0 Hrs	0.139	0.135	0.134	0.232	0.235	0.238	0.121	0.12	0.125
2 Hrs	0.475	0.477	0.476	0.654	0.659	0.66	0.489	0.489	0.489
24 Hrs	0.886	0.886	0.889	0.988	0.984	0.985	0.473	0.469	0.473

Table B40: Average absorbance values $_{560\ nm}$ purified β-mannanase supplemented samples run 2

CONTROL (No Enzyme)	AVERAGE ABSORBANCE 540 nn		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.095	0.114	0.066
2 Hrs	0.254	0.262	0.164
24 Hrs	0.689	0.783	0.168

Table B41: Average absorbance values $_{560\ nm}$ purified β-mannanase supplemented samples run 2

ENZYME ADDED	AVERAGE ABSORBANCE 540 nn		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.136	0.235	0.122
2 Hrs	0.476	0.658	0.489
24 Hrs	0.887	0.986	0.472

Table B42: Absorbance values $_{560\ nm}$ values (enzyme absorbance – control absorbance) run 2

CORRECTED enz abs - control abs	AVERAGE ABSORBANCE 540 nn		
	ENZYME ADDED		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.041	0.121	0.056
2 Hrs	0.222	0.396	0.325
24 Hrs	0.198	0.202	0.304

Table B43: Reducing sugars from purified β-mannanases ($mg.ml^{-1}$) run 2

	Release of reducing sugars ($mg.ml^{-1}$)		
	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.441	0.779	0.506
2 Hrs	1.207	1.944	1.646
24 Hrs	1.106	1.125	1.554

DUPLICATE AVERAGE ANIMAL FEED HYDROLYSIS REDUCING SUGARS RELEASED (mg.ml^{-1})

Table B44: Duplicate Average values of reducing sugars released (mg.ml^{-1}) obtained from the purified β-mannanase hydrolysis of animal feed with standard deviation

	Run 1			**Run 2**		
	STARTER FEED	GROWER FEED	LAYER FEED	STARTER FEED	GROWER FEED	LAYER FEED
0 Hrs	0.496±0.032	0.747±0.019	0.493±0.007	0.441±0.002	0.779±0.001	0.506±0.002
2 Hrs	1.289±0.047	1.988±0.025	1.664±0.011	1.207±0.000	1.944±0.002	1.646±0.002
24 Hrs	1.111±0.002	1.160±0.020	1.543±0.007	1.106±0.003	1.125±0.002	1.554±0.004

Enyme activities and protein concentrations of Pre-Purification products

Table B45: Enzyme activity assay (DNS assay) and protein concentration of Ammonium sulphate precipitated protein fractions

ENZYME ACTIVITY				**PROTEIN CONCENTRATION**		
Triplicate Absorbance values (540 nm)	**Average Absorbance values (540 nm)**	**Average Absorbance values × DF (10) for crude enzyme and 60% AS pellet)**	**ENZYME ACTIVITY (nkat.ml^{-1})**	**Triplicate Absorbance Values (540 nm)**	**Average Absorbance Values (540 nm)**	**PROTEIN CONCENTRATION (µg/ml)**

Crude Enzyme: 0.457 0.456 0.468	0.460	4.603	192.763	Crude Enzyme: 2.530 2.525 2.510	2.522	114.620
20 % Ammonium sulphate fraction: 0.005 0.017 0.007	0.009	0.009	1.361	20 % Ammonium sulphate fraction: 0.113 0.113 0.111	0.112	5.120
40 % Ammonium sulphate fraction: 0.019 0.020 0.022	0.020	0.020	1.806	40 % Ammonium sulphate fraction: 0.131 0.139 0.136	0.135	6.150

60 % Ammonium sulphate fraction: 0.436 0.477 0.426	0.446	4.460	186.931	60 % Ammonium sulphate fraction: 2.430 2.435 2.430	2.432	110.530
80 % Ammonium sulphate fraction: 0.123 0.056 0.015	0.065	0.065	3.653	80 % Ammonium sulphate fraction: 0.395 0.396 0.394	0.395	17.950
80 % Ammonium sulphate filtrate: 0.087 0.004 0.041	0.044	0.044	2.791667	80 % Ammonium sulphate filtrate: 0.289 0.289 0.275	0.284	12.92

Table B46: Summary of the calculations of the values obtained from pre-purification of β-mannanase from *S. candida* LMK008

PURIFICATION STEP	VOLUME (ml)	TOTAL ACTIVITY (nkat ml^{-1})	TOTAL PROTEIN (mg ml^{-1})	SPECIFIC ACTIVITY (nkat mg^{-1})	PROTEIN YIELD (%)	DEGREE OF PURIFICATION
Crude Enzyme	100	192.760	0.115	1681.730	100.00	1.00
20% $(NH_4)_2SO_4$ Fraction	95	1.360	0.005	265.810	0.71	0.16
40% $(NH_4)_2SO_4$ Fraction	110	1.810	0.006	293.580	0.94	0.17
60% $(NH_4)_2SO_4$ Fraction	106	186.930	0.111	1691.220	96.99	1.01
80% $(NH_4)_2SO_4$ Fraction	130	3.650	0.002	203.500	1.89	0.12
80% $(NH_4)_2SO_4$ Filtrate	132	2.000	0.013	216.070	1.45	0.13

APPENDIX C: ELECTROPHOREIS GEL IMAGES

PCR

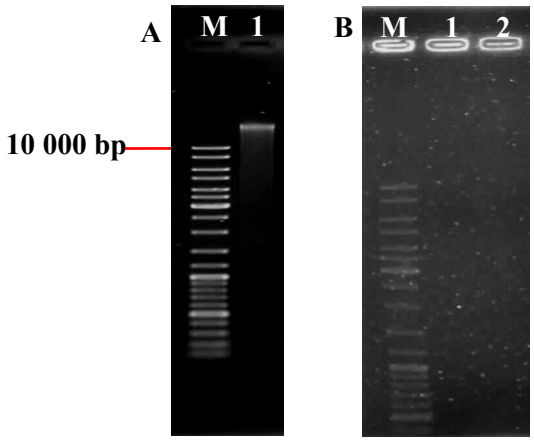

Figure C1: PCR of genomic DNA isolated from *S. candida* LMK008. (A) Lane M, DNA Ladder Marker; lane 1: genomic DNA. (B) Lane M, DNA Ladder Marker - (O'Gene Ruler DNA Ladder mix: Fermentas, USA); lane 1, PCR Sample; lane 2, Negative control (No template added)

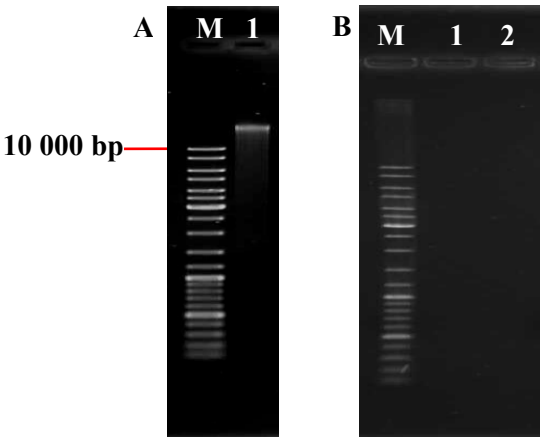

Figure C2: PCR of genomic DNA isolated from *S. candida* LMK008. (A) Lane M, DNA Ladder Marker; lane 1: genomic DNA. (B) Lane M, DNA Ladder Marker: (O'Gene Ruler DNA Ladder mix: Fermentas, USA); lane 1, PCR Sample; lane 2, Negative control (No template added)

RT-PCR

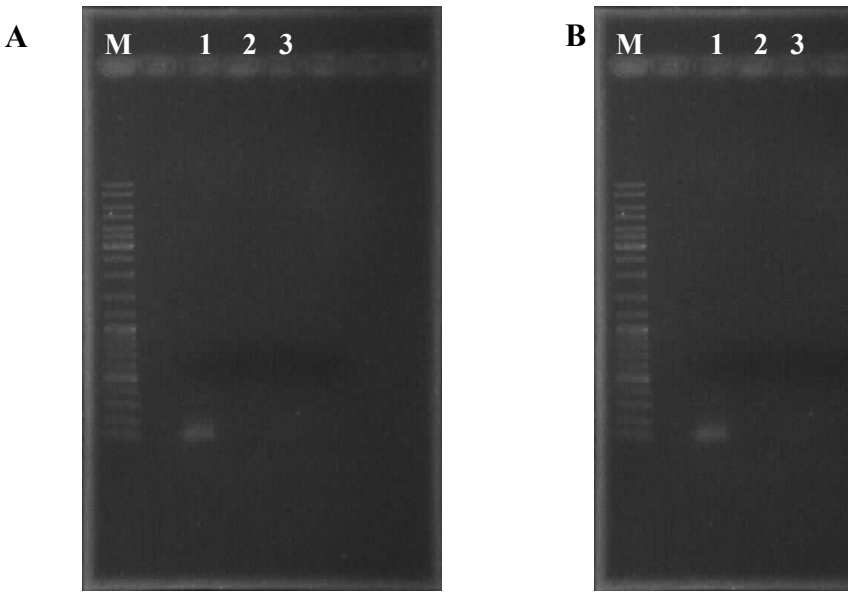

Figure C3. RT-PCR of RNA isolated from *S. candida* LMK008. A: Lane M, DNA Ladder Marker; lane 1, Positive control (pAW109 RNA); lane 2, Negative control (no template); lane 3, *S. candida* LMK008 RNA. B: Lane M, DNA Ladder Marker: (O'Gene Ruler DNA Ladder mix: Fermentas, USA); lane 1, Positive control (pAW109 RNA); lane 2, Negative control (no template); lane 3, *S. candida* LMK008 RNA.

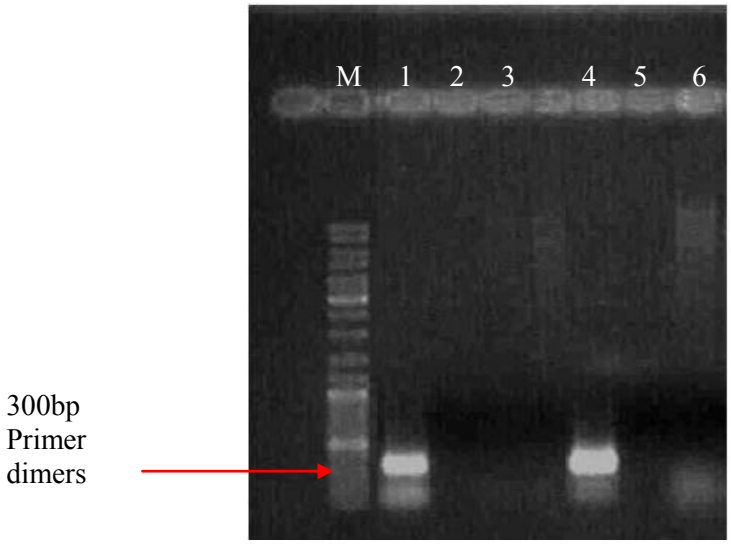

Figure C4: RT-PCR of RNA isolated from *S. candida* LMK008. Lane M, DNA Ladder Marker: (O'Gene Ruler DNA Ladder mix: Fermentas, USA); lane 1, Positive control (2.5 µl pAW109 RNA |300bp|); lane 2, Negative control (no template); lane 3, 2.5 µl *S. candida*

LMK008 RNA; lane 4, Positive control (5.0 µl pAW109 RNA); lane 5, Negative control (no template); lane 6, 5.0 µl *S. candida* LMK008 RNA.

cDNA Synthesis

Touch Down PCR for 5′-RACE

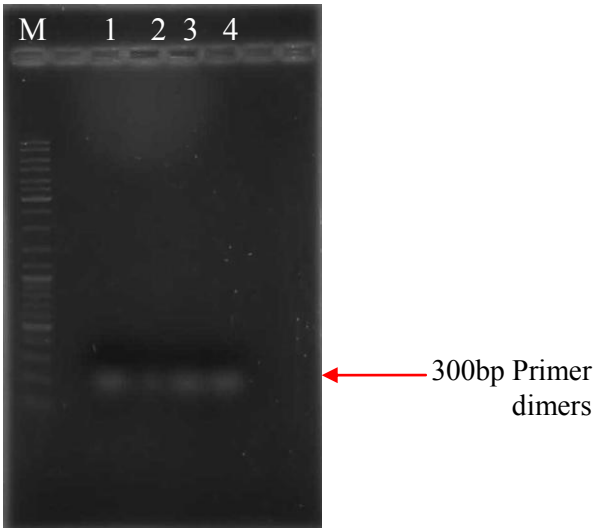

Figure C5: 5′-RACE-PCR : Lane M , D NA Ladder: (O'Gene Ruler DNA Ladder mix: Fermentas, USA); lane 1, 5′-RACE Sample; lane 2: GSP 1 + 2 (Positive control); lane 3, UPM only (Negative control); lane 4, GSP1 only (Negative control).

Southern Hybridization

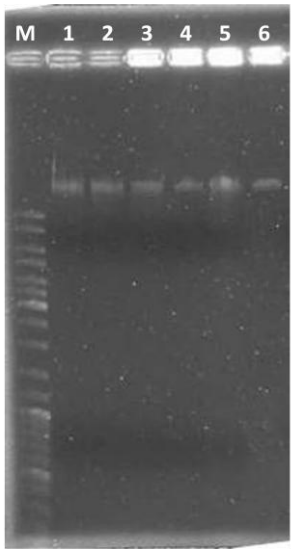

Figure C6: Genomic DNA isolated from 6 control fungi used for Southern hybridisation (M, DNA molecular weight marker (O'Gene Ruler DNA Ladder mix: Fermentas, USA); lane 1, *Penicillium* spp; lane 2, *Trichoderma reseei;* lane 3, *Aspergillus niger;* lane 4, *Chaetomium indicum;* lane 5, *Fusarium oxysporum;* lane 6, *Sordaria fimicola*)

Southern blot of Control fungi

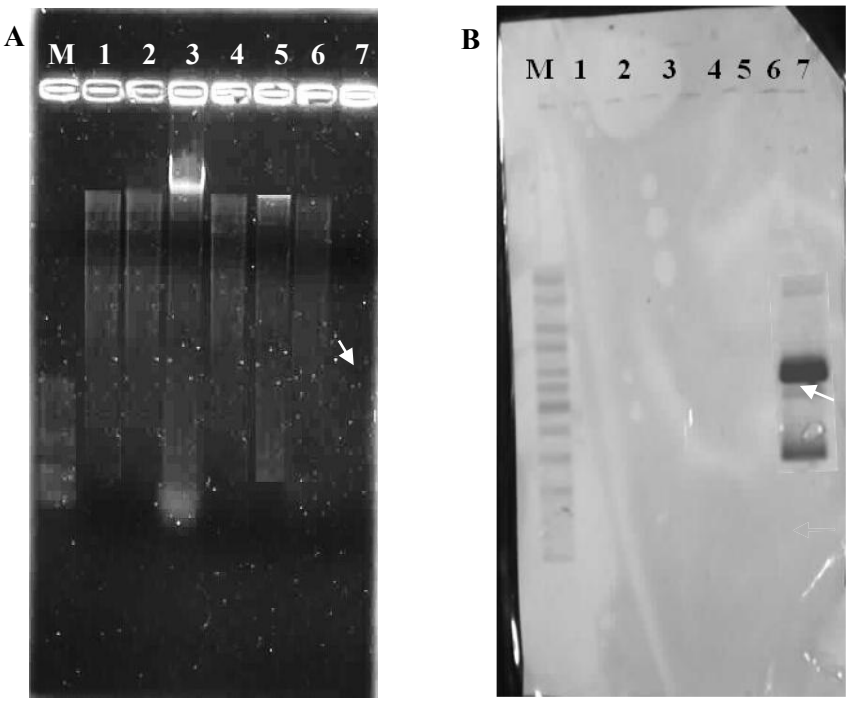

Figure C7: Southern blotting hybridization of the mannanase gene from control fungal species using a high stringency wash step - 60°C with 0.5 ×SSC, 1% (w/v) SDS). (A) DNA electrophoresis of *Eco*RI restricted gDNA. (B) Southern blot analysis. (M, DNA molecular weight marker (O'Gene Ruler DNA Ladder mix: Fermentas, USA); lane 1, *Penicillium* spp; lane 2, *Trichoderma reseei;* lane 3, *Aspergillus niger;* lane 4, *Chaetomium indicum;* lane 5, *Fusarium oxysporum;* lane 6, *Sordaria fimicola;* lane 7, Positive control: *Aspergillus aculeatus* Man 1 gene).