THE PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ENDO-1,4-B-MANNANASE FROM NEWLY ISOLATED STRAINS OF *SCOPULARIOPSIS CANDIDA*

BY

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DECLARATION

I, THE UNDERSIGNED, HEREBY DECLARE THAT THE WORK CONTAINED IN THIS DISSERTATION IS MY OWN ORIGINAL WORK AND HAS NOT PREVIOUSLY IN ITS ENTIRETY OR IN PART BEEN SUBMITTED AT ANY UNIVERSITY OR OTHER INSTITUTION FOR A DEGREE.

SIGNATURE: _____

MM MUDAU

DATE: _____

THIS DISSERTATION IS DEDICATED TO MY SIBLINGS, LYDIA AND REFILWE AND MY PARENTS MRS JANE MUDAU AND MR PATRICK MUDAU

ACKNOWLEDGEMENTS

DEAR **LORD, GOD ALMIGHTY**, I WOULD LIKE TO THANK YOU FOR BEING THE SOURCE OF MY SUCCESS.

I WOULD LIKE TO EXTEND MY GRATITUDE TO:

DR SETATI ME FOR BELIEVING IN ME AND ACCEPTING ME TO BE HER STUDENT, FOR HER GUIDANCE AND PATIENCE THROUGHOUT THE STUDY. THE DEVOTION AND TIME TO HER STUDENTS.

DR POHL C FOR NEVER HESITATING TO HELP AND FOR THE TRANSLATION OF THE SUMMARY

GODFREY AND DIRK FOR HELPING ME WITH BIOCHEMISTRY

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Chapter One

Introduction and literature review

1.1 Introduction

Plant cell walls typically contain 38 - 50% cellulose, 15 - 25% lignin as well as 23 - 32% hemicellulose (Puls and Schuseil, 1993). Mannans together with xylans are major constituents of the hemicellulose fraction (de Vries and Visser, 2001). Depending on their source, mannan polysaccharides may appear as pure mannans with no substitutions in their backbone, or with their backbone containing galactose and/or glucose residues (de Vries, 2003). A cocktail of enzymes including β-1,4-mannanase, β-mannosidase and α-galactosidases is required for the complete degradation of these polysaccharides (Ademark *et al*., 1998). β-Mannanase randomly

hydrolyses the mannan backbone to produce oligosacharides of various lengths. Further hydrolysis by α-galactosidase removes the side groups while β-mannosidase and β-glucosidase catalyse the removal of terminal mannose and glucose residues, respectively (Franco *et al.,* 2004; Stålbrand *et al.,* 1993). Several fungi and bacteria secrete these enzymes simultaneously when growing on mannan-based polysaccharides. However, research has mainly been focused on enzyme systems from fungal species such as *Trichoderma*, *Aspergillus*, *Sclerotium* and to a lesser extent *Penicillium* (Puchart *et al*., 2004; Ademark *et al*., 1998; Gübitz *et al.,* 1996 a,b; Stålbrand *et al*., 1993).

There has been growing interest over the years in the industrial potential of mannan degrading enzymes especially β-mannanase. This enzyme is widely applied in poultry feed to reduce the anti-nutritional factor of mannan polymers found in corn-soy based feeds (Wu *et al*., 2005), in the detergent industry (Schäfer *et al*., 2002; McCoy, 2001) and in processing of instant coffee (Sachslehner *et al*., 2000). β-Mannosidase and α-galactosidase are mainly used in the food and pharmaceutical industries (Taubken *et al*., 1993; Kirk *et al.,* 2002). Until recently, extreme environments such as high temperature, pH and salt were thought to be uninhabitable for fungi. However, several researchers have shown that fungi can tolerate such conditions and have developed adaptation mechanisms to survive these extremes. A wide variety of moulds have been isolated from hypersaline environments including the Dead Sea (Buchalo *et al*., 1998; Kis-Papo *et al*., 2003). The ability of moulds to survive in hypersaline environments has opened new avenues of research including isolation of new fungi that may produce different biological products of industrial interest.

1.2 Objective and aims of the project

1.2.1 Objective

Investigate mannan degrading enzyme system in *Scopulariopsis candida*

- Production of β-mannanase, β-mannosidase, α-galactosidase
- Optimization of conditions for endo-1,4-β-mannanase production
- Purification and characterization of β-mannanase

1.2.2 Aims

i. A literature review on the biological degradation of mannan and mannan-based polysaccharides

ii. Determination of the effect of NaCl concentration, medium composition and carbon sources on growth and enzyme production

iii. Purification of the endo-1,4-β-mannanases produced by two *Scopulariopsis candida* strains LMK004 and LMK008

iv. Characterization of the purified enzymes with focus on their pH and temperature optima and stability and the effect of NaCl on enzyme stability

1.3. Literature review

1.3.1 The genus *Scopulariopsis candida*

The genus *Scopulariosis* is classified as follows:

Kingdom: Fungi

Phylum: Ascomycota

Class: Euascomycetes

Order: Microascales

Family: Microascaceae

Genus: *Scopulariopsis*

Scopulariopsis anamorphs are known for many species of the genus *Microascus* (Ascomycota, Microascaceae). Holomorph species, *Microascus brevicaulis* and *Microascus manginii* are recognized to include anarmorphs *Scopulariopsis brevicaulis* and *Scopulariopsis candida* (Guègen) Vuillemin, respectively (Abbott and Sigler, 2001).

The colonies are fast growing, vary in color from white, cream, grey, buff to brown, black but are predoninantly light brown. Conidia are subglobose, truncate, fine to coarsely roughened and the size ranging from 3.5-8.5 x 4-7.5 μm, (Lumley, 1999)

Most members of the genus *Scopulariopsis* are soil fungi. In addition they have been isolated from the dairy products, paper, rice and cheese (Benguin and Nolard, 1999; Taligoola *et al*., 2004; Andrews *et al*., 2000). Pathogenic capacity of *S. candida* was found in immunocompromised host where it causes onychomycosis and invasive sinosal infection (Kriesel *et al*., 1994). Some isolates of *S. brumptii*, *S.candida* and *S. brevicaulis* obtained from nail lesions or outdoor aerosols are keratinolytically active. In addition *S. brevicaulis* has been isolated from bird feathers and was reported to be associated with bird-borne illness (Marchisio and Fusconi, 2001; Camin *et al*., 1998).

A wide variety of moulds including *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; Mudau and Setati, 2006; Grishkan *et al*., 2004; Steiman *et al*., 2004). There are currently few reports on the production of polysaccharide degrading enzymes by the genus *Scopulariopsis*. Amongst those reported was a *Scopulariopsis* species that produced two isoenzymes of endo-1,4-β-xylanase (Afzal *et al.,* 2005) and *Scopulariopsis brevicaulis* TOF-1212 which produces endoglucanase (Nakatani *et al*., 2000). The ability of *Scopulariopsis* species to survive in hypersaline environments triggered the interest in studying the production of mannan-degrading enzymes by *Scopulariopsis candida*.

1.3.2 Structure and occurrence of mannan polysaccharides

Mannan polysaccharides are complex biopolymers that are commonly found in plant cell walls where they are closely associated with cellulose and lignin (de Vries, 2003). These biopolymers

are present either as structural carbohydrates that cross-link cellulose microfibrils or as storage carbohydrates in seeds of various plants (Puls and Schuseil, 1993). Homo- and heteromannans are based on variations of a β-mannan backbone (unsubstituted mannans), which might be interrupted with D-glucose (glucomannans) and/or branched with α-1,6-linked galactose (galactomannans), while some have a backbone containing β-1,4-linked D-mannose and D-glucose residues which are branched by α-1,6-linked D-galactose (galactoglucomannan). Mannan-based polysaccharides are ubiquitous in nature and occur in different forms in plant cell walls (Table 1).

Table 1. Structure and occurrence of mannan polysaccharides (Nishinari *et al*., 1992; Buckeridge *et al*., 2000; Schröder *et al*., 2001)

Polysaccharide	Residues	Branching residues	Plant localization
Mannans	Mannose	None	Palmae and Coffee seeds
Galactomannans	Mannose	Galactose	Leguminosae, Annonaceae and Convolvulaceae
Glucomannans	Mannose/Glucose	Galactose	Liliaceae, lettuce and tomato seeds
Galactoglucomannan	Mannan/Glucose/Galactose	Galactose	Secondary walls of Gymnosperms and Angiosperms. Ferns, Mosses etc.

Depending on their structure and branching degree, these cell wall polysaccharides may play distinct roles in plants, from hardness in Palmae to water-related control in Leguminosae, but all having in common the storage function of carbohydrates (Handford *et al*., 2003).

1.3.2.1 Mannans

Mannans are a group of plant carbohydrates that consists of mannose molecules linked together to form a polymer (Fig 1). Pure mannans are regarded as those polysaccharides whose 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 (Buckeridge *et al*., 2000). They are insoluble in water, self-interactive and to some extent crystalline in the cell wall (Mulimani

and Prashanth, 2002). Since pure mannans impart hardness to the seeds of monocotyledons and dicotyledons, mannan-containing seeds are very hard and resistant to mechanical change (Buckeridge *et al*., 2000).

[→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. An example of a mannan polymer showing mannopyranosyl residues linked to one another by β-1,4-mannosyl linkages

1.3.2.2 Galactomannans

Galactomannans consist of a linear backbone of β-1,4-linked D-mannose residues to which D-galactose residues are attached by α-1,6-linkages (Fig 2). These polysaccharides are typically present in large amounts in the endosperm of seeds of leguminous plants (Buckeridge *et al*., 2000) where they play a major role as storage carbohydrates. The galactose to mannose (Gal:Man) ratio and the distribution of galactosyl residues along the mannan backbone vary from species to species, and is widely used in chemical characterization of legumes (Buckeridge and Dietrich, 1990; Buckeridge *et al*., 1995).

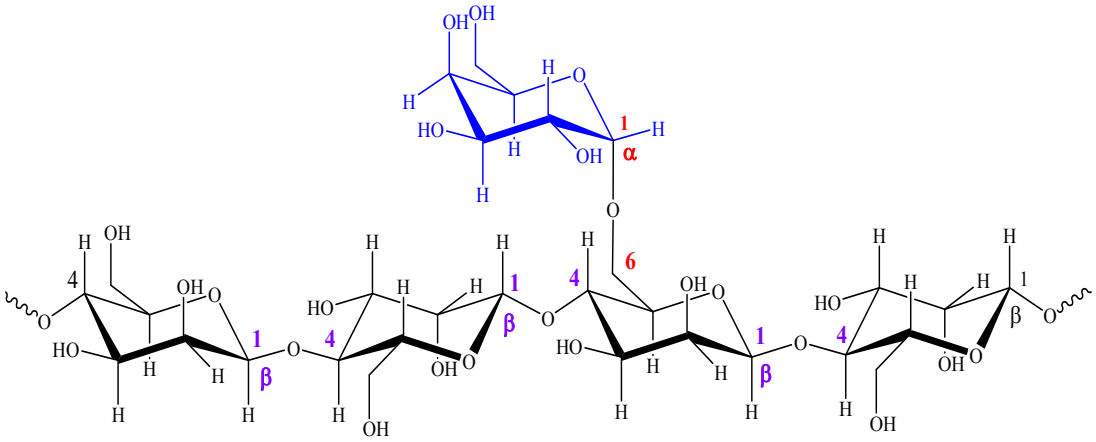

Figure 2: A schematic representation of a locust bean galactomannan consisting of 1,4-linked β-D-mannose backbone with branch points from their 6-positions linked to α-D-galactose units.

The most studied galactomannans within the family Leguminosae are guar gum (*Cyamopsis tetragonolobus*), fenugreek (*Trigonella foenum-graecum*), locust bean gum or carob (*Ceratonia siliqua*) and more recently tara gum (*Caesalpinia spinos*), which has been receiving acceptance as an alternative product to locust bean and guar gum (Table 2).

Table 2. The distribution and ratios of galactose to mannose in different mannan polymers (Maier *et al*., 1993; Marraccini *et al.,* 2005).

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

The degree of galactose substitutions in galactomannans varies widely in nature and has direct influence on the solubility of the polymer in water (Buckeridge *et al.*, 2000). For example, pure unsubtituted mannans form insoluble polymers, as observed in ivory nuts (*Phytelephas macrocarpa*) (de Vries and Visser, 2001; Capek *et al*., 2000), while substituted galactomannans such as locust bean gum have the ability to form viscous solutions with water (Marraccini *et al.*, 2005). This property depends on the molecular size, Gal:Man ratio and the degree of branching of the polysaccharide (de Vries and Visser, 2001). If the galactose substitutions approaches zero the biological function of the polymer is more related to hardness. In contrast, polymers, with higher degrees of galactosylation (fenugreek being the extreme case), imbibe high amounts of water and distribute it through the embryo, this helps to protect the plants from harsh conditions such as droughts (Mulimani and Prashanth, 2002).

1.3.2.3 Glucomannans

Glucomannans are widely distributed in seeds of some Liliaceae and Iridaceae species where they act as both structural and storage polymers (Buckeridge *et al*., 2000). The polymeric

backbone comprises D-glucose and D-mannose residues bonded together by β-1,4-linkages (Fig 3). These polysaccharides are made up of approximately 60% D-mannose and 40% D-glucose residues (Li *et al*., 2005). D-Glucose and D-mannose residues may be acetylated at *O*-2 (Ratcliffe *et al.,* 2005), while in some plant species short chains consisting of 11-16 hexose molecules may form branches that are linked to the main chain through β-(1-6) or β-(1-3) linkages (Hua *et al*., 2004).

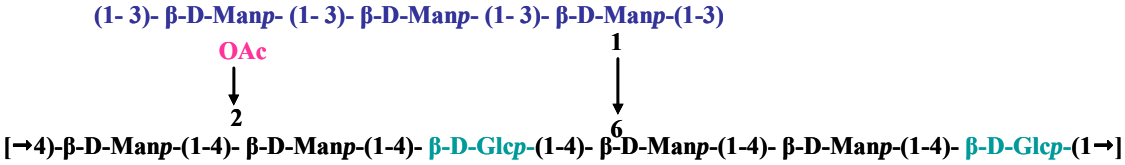

Figure 3. Glucomannan showing repeating units of mannose residues substituted by glucose residues in the main chain and containing some branches of acetyl groups.

In some plants e.g. *Dendrobium officinale* (*Herba dendrobii*) glucomannan may have branches containing an acetyl group at carbon 2 or small mannooligosaccharide chains (Hua *et al*., 2004). Although the molar ratio of mannose to glucose varies from plant species to species, 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). Glucomannan products are widely used in countries like Japan and China as general health aids, topically, for skin care and as a thickening agent for foods (Li *et al*., 2005; Katsuraya *et al.,* 2003).

1.3.2.4 Galacto-glucomannans

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

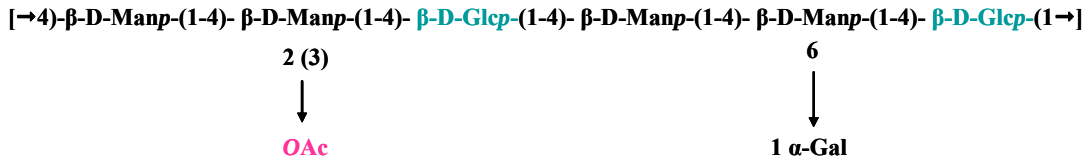

Figure 4. The structure of softwood *O*-acetyl galacto-glucomannan, *O*Ac: *O*-linked acetyl group, Man*p*: Mannopyrannoside residues, Gal: Galactose residues, Glc*p*: Glucopyrannoside residues

The Gal:Glc:Man ratio varies, depending on the plant source and developmental stage of the tissue (Capek *et al*., 2002). Galacto-glucomannans isolated from primary cell walls, or those secreted into extracellular space by suspension cells, contain approximately equal amounts of galactose, glucose and mannose residues. In contrast, the water insoluble galacto-glucomannan extracted from the secondary cell walls of gymnosperms and angiosperms has 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).

1.3.3 Galactomannan degrading enzyme

Mannan-based polysaccharides are relatively complex and their complete hydrolysis often requires a concerted action of several enzymes (de Vries, 2003; Ratto and Poutanen, 1988). These include: endo-β-1,4-mannanase (EC 3.2.1.78) which cleaves the β-1,4 linkages of the mannan backbone releasing manno-oligosaccharides; α-galactosidase (EC 3.2.1.22) which hydrolyses the α-1,6 linkages in the side chains of mannan backbones and β-mannosidases (EC 3.2.1.25) which release mannose from the non-reducing end of manno-oligosaccharides (Fig 5). Fungi, particularly moulds, produce a cocktail of these enzymes when growing on various mannan polysaccharides (Stålbrand *et al*., 1993; Ademark *et al*., 1998).

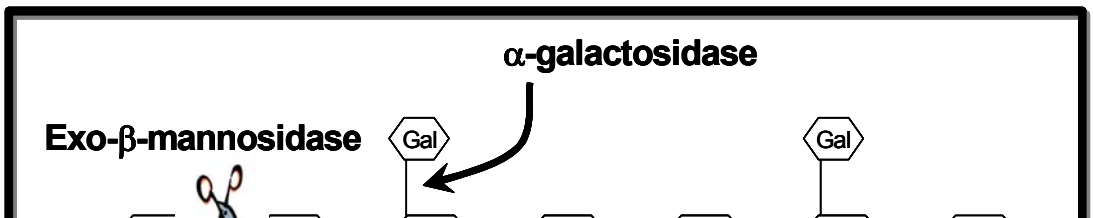

Figure 5. A schematic representation of galactomannan, showing positions of enzyme hydrolysis.

1.3.3.1. β-1,4-Mannanases

Endo-β-1,4-mannanase (EC 3.2.1.78) is a crucial enzyme for the depolymerization of unsubstituted mannans, galactomannans and galacto-glucomannans. This enzyme catalyses the random hydrolysis of β-1,4-mannosidic linkages in the main chain of mannan polymers (Stålbrand *et al*., 1993; de Vries and Visser, 2001). Its action causes rapid decrease in the viscosity of polysaccharide solutions, thus increasing accessibility of the polymer to other enzymes. β-1,4-Mannanase releases linear and branched manno-oligosaccharides of various lengths. These are then further hydrolysed into monomers by β-mannosidase (EC 3.2.1.25) and α-galactosidase (3.2.1.22) (Kremnický and Biely, 1997).

1.3.3.1.1 Structural organisation of β-1,4-mannanases

The mode of action of β-1,4-mannanase on a substrate often depends upon the source of the enzyme as well as the type of mannan (de Vries and Visser, 2001). Hydrolysis of substituted or branched polysaccharides by β-1,4-mannanases is hindered by the degree and pattern of galactose substitution on the mannan backbone and the distribution of glucose within the main chain (de Vries, 2003). In most cases an uninterrupted sequence of 3 to 5 unsubtituted mannose residues is required for cleavage to occur (Stålbrand *et al.,* 1993). Apart from their ability to hydrolyse different mannans, some β-1,4-mannanases display a transglycosylation activity (McCleary, 1983; Harjunpää *et al.,* 1995; Gübitz *et al.,* 1996b).

Several bacterial and fungal mannanases posses non-catalytic carbohydrate binding modules (which can either be a cellulose-binding or mannan-binding module) that are linked to the catalytic domain through flexible linkers (Sunna *et al*., 2001; Hägglund *et al*., 2003). These non-catalytic modules are thought to enhance enzyme activity by anchoring and localizing the catalytic domain onto the surface of the substrate thus improving enzyme-substrate association and local enzyme concentration (Bolam *et al*., 2004; Boraston *et al.,* 2004). Hägglund and co-workers demonstrated that removal of the cellulose binding module from *T. reesei* Man5A mannanase, decreases the rate of degradation of mannose/cellulose complexes (Hägglund *et al.,* 2003).

1.3.3.1.2 Biochemical properties of β-1,4-mannanases

Increasing interest in the potential application of β-1,4-mannanases in various industrial processes over the years, stimulated research into the biochemical properties of these enzymes. The fungal β-mannanases have been purified and characterized and reported to exhibit acidic to neutral pH optima, molecular weights ranging from 33 - 80 kDa and mesophilic to moderately thermophilic temperature optima (Table 3).

Table 3. A summary of 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

Fungal mannanases are often secreted into the culture liquid as multiple enzyme forms such as those from *Schlerotium rolfsii* (Gübitz *et al.,* 1996a), *Aspergillus fumigatus* (Puchart *et al.,* 2004) and *Trichoderma reesei* (Stålbrand *et al*., 1993). The multiplicity of these mannanases is thought to be due to their ability to bind and degrade different substrates (Johnson *et al*., 1990). These β-1,4-mannanase isoforms may be secreted as products of the same gene differing only in their post-translational modification, such as with *A. fumigatus* (Puchart *et al*., 2004) or their production may be regulated differently such as *S. rolfii* isoforms that exhibited different functions on substrates of varying sizes (Großwindhager *et al*., 1999).

1.3.3.1.3 Industrial applications of β-1,4-mannanase

β-1,4-Mannanases produced through biotechnology have become ubiquitous in industrial applications. They are widely applied in the food, instant coffee processing, paper and pulp together with xylanases and poultry feed industries (Wong and Saddler, 1993; Montiel *et al*., 1999; Sachslehner *et al.*, 2000; Ferreira and Filho, 2004; Gübitz *et al*., 1997). Recently β-1,4-mannanases have shown to be effective in laundry detergents (McCoy, 2001; Schäfer *et al.,* 2002).

Laundry detergents

Mannan-based polysaccharides (e.g. guar gum) are used as food thickeners, in fruit juices and dairy products such as ice cream (Maier *et al.,* 1993; Wong and Saddler., 1993). When the food or juices spill on fabrics, they form tough stains that are not easily removed with normal laundry detergents. In 2000, a washing detergent called Procter and Gamble's Tide Deep Clean® liquid formula containing β-1,4-mannanase, was developed. This enzyme breaks down the mannosidic linkages in the mannan polymers that form tough stains, making them easy to wash out and never reappear (Schäfer *et al.,* 2002; McCoy, 2001).

Poultry feed industries

Meals such as guar soybean, sesame and corn soybean diets are used for poultry feed. Mannan in these meals act as an anti-nutritional factor resulting in decrease in growth, feed efficiency, egg production and egg weight and an increase in viscosity of the digesta (Lee *et al*., 2003a,b; Lee *et al*., 2005). Addition of β-mannanases into these feeds has been shown to alleviate these problems because they break down the mannan to oligossacharides and reduce intestinal viscosity, which then improves feed efficiency and increases growth (Lee *et al*., 2003a; Wu *et al.,* 2005).

1.3.3.2 α-Galactosidase

The enzyme α-galactosidase (α-galactoside galactohydrolase [EC 3.2.1.22]) releases α-1,6-linked galactoside residues from a variety of substrates including galacto-oligosaccharides, galacto-glucomannans, and galactolipids (Luonteri *et al.,* 1998 ; de Vries and Visser, 2001). α-Galactosidases can be divided into two groups based on their substrate specificities. The first group comprises α-galactosidases that exhibit activity only on oligosaccharides e.g. melibiose, raffinose, stachyose and short fragments of galacto-glucomannans (Puchart *et al*., 2000; Kirk *et al*., 2002). The second group of α-galactosidases consists of enzymes that are mainly active on polymeric substrates. However, some of them attack short oligosaccharides, mainly fragments of degraded polymers, as well as artificial α-galactosides (Puchart *et al.,* 2000; Ademark *et al*., 2000a,b). Several fungi have been shown to produce both groups of enzymes depending on the carbon source on which they grow (Ademark *et al.,* 2000b; Lounteri *et al.,* 1998; Margolles-Clark *et al*., 1996a; Kaneko *et al.,* 1990).

α-Galactosidases have not gained as much interest as β-mannanases and only a few of them have been purified and characterized. Most of the fungal α-galactosidases act optimally at pH ranging from 4 to 5, their molecular weights are between 50 - 95 kDa and they also exhibit moderate to thermophillic temperature optima (60 - 70) (Table 4). These enzymes are generally secreted to the extracellular environment, although some fungi e.g. *Humicola* sp. may produce both intracellular and extracellular α-galactosidase (Kotwal *et al*., 1999).

Table 4. Biochemical properties of α-galactosidases produced by filamentous fungi

Organisms	**MW (kDa)**	**Temperature optima (°C)**	**pH optima**	**pI**	**Reference**
Thermomyces lanuginosus	57	65-70	4.5-5	5.2	Puchart *et al*., 2000
Humicola sp.	87.1	65	5	4	Kotwal *et al*., 1999
Trichoderma reesei	50	60	4	5.2	Zeigler *et al*., 1993
Aspergillus niger (α-galI - α-galIV)	64-94	60	4.5	4.15-4.8	Ademark *et al.,* 2000b

1.3.3.2.1 Industrial applications of α-galactosidase

The most important industrial application of α-galactosidases is in the sugar-making industry where they are used to eliminate small amounts of raffinose or stachyose which are known to negatively affect crystallization of sucrose in beet sugar syrups (Kirk *et al*., 2002). α-Galactosidases are also used to remove the side groups and improve the gelling properties of galactomannans which are used as food thickeners (Bulpin *et al.*, 1990).

1.3.3.3 β-Mannosidases

β-Mannosidases (EC 3.2.1.25) are exo-acting enzymes that catalyse the hydrolysis of terminal β-D-mannopyranosyl residues from the non-reducing end of various β-1,4-linked manno-oligosaccharides or mannose-containing glycopeptides (Ademark *et al.,* 1998; Samonte, 2002). The activity of β-mannosidases is essential for the complete hydrolysis of plant polysaccharides such as galactomannan and mannan, and readily converts the manno-oligosaccharides produced by β-mannanase to mannose (Ademark *et al.,* 1998; Reese and Shibata, 1965). These enzymes are generally thought to depend upon prior endo-β-mannanase and α-galactosidase activity to provide its oligomeric mannan substrate (Ademark *et al*., 1998, 2000a)

1.3.3.3.1 Biochemical properties of β-mannosidases

Relatively few microbial β-mannosidases have been purified and characterized from extracellular medium. They exhibit an acidic pH optimum ranging from pH 2 to 5.0 and are generally more active at temperatures between 40°C and 55°C (Table 5). The *A. niger* β-mannosidase is currently the only one that was reported to retain activity at 70°C (Ademark *et al*., 1998).

Table 5. A summary of the biochemical properties of purified β-mannosidases from fungi.

Organisms	MW	Tempereture	pH	pI	Reference

	(kDA)	optima (°C)	optima		
Aspergillus niger	135	70	2.4-5.0	5.0	Ademark *et al.,* 1998
Trichoderma reesei	150	NR	3.5	4.8	Kulminskaya *et al*., 1999
Schlerotium rolfsii	57.5	55	2.5	4.5	Gübitz *et al.,* 1996a
Aspergillus awamori	NR	60-70	5	NR	Kurakake and Komaki, 2001

NR=Not reported

1.3.2.3.2 Industrial applications of β-mannosidases

There is great interest in using β-mannosidase and related enzymes in the synthesis of oligosaccharides for medical and other purposes (Kobata, 1993). Apart from the synthetic field, there are two new fields of industrial interest in the study of β-mannosidases:

i. Hydrolysis of agricultural wastes for the release of reducing sugars that will serve as raw material for the production of alcohol (Itoh and Kamiyama, 1995).
ii. Genetic manipulation of β-mannosidase for the production of efficient synthetic catalysts (Ennis and Osborn, 2003).

Although β-mannosidases are less studied compared to other glycosidases, there is emerging interest in research of these enzymes in various industrial applications.

1.3.4 Reaction-mechanism of mannan-degrading enzymes

Mannan-degrading enzymes have been grouped and classified in families on the basis of their amino acid sequence similarities (Henrissat *et al*., 1998). β-Mannanases belong to glycosidase hydrolase family 5 and 26, α-galactosidases fall under family 27 and while β-mannosidases are grouped into sub-family 2A of family 2 glycosidases (Stoll *et al*., 2000; Hogg *et al*., 2001; Golubev *et al*., 2004).

The enzymes in these families hydrolyse their substrates by a retaining mechanism which occurs via double-displacement reaction (Fig 6) (Claeyssens and Henrissat., 1992; Stoll *et al*., 2000). The key amino acids involved in the retaining mechanism reaction are two carboxylic acid

residues, one of which functions as the acid-base and the other as the nucleophile (Henrissat *et al.*, 1995).

In the first reaction, the nucleophile attacks the anomeric centre of the substrate while the acid catalyst protonates the departing aglycone (glycosylation step), leading to the formation of a covalent glycosyl-enzyme intermediate of inverted stereo-chemistry at the anomeric centre relative to the substrate (Fig 6).

In the second reaction, the base catalyst promotes the attack of a water molecule on the opposite face of the anomeric centre, displacing the nucleophile and releasing the glycone with the same anomeric configuration as the substrate (deglycosylation step) (Fig 6). The enzymes can also form glycosides by transglycosylation of the glycone moiety of the covalent glycosyl-enzyme intermediate to an acceptor aglycone rather than to water. The active site residues responsible for this acid/base catalysis have been proven to be a pair of carboxylic acids (Glu or Asp) in most cases (Davies *et al*., 2005; Viladot *et al*., 2001; Mclntosh *et al*., 1996; Stoll *et al*., 2000).

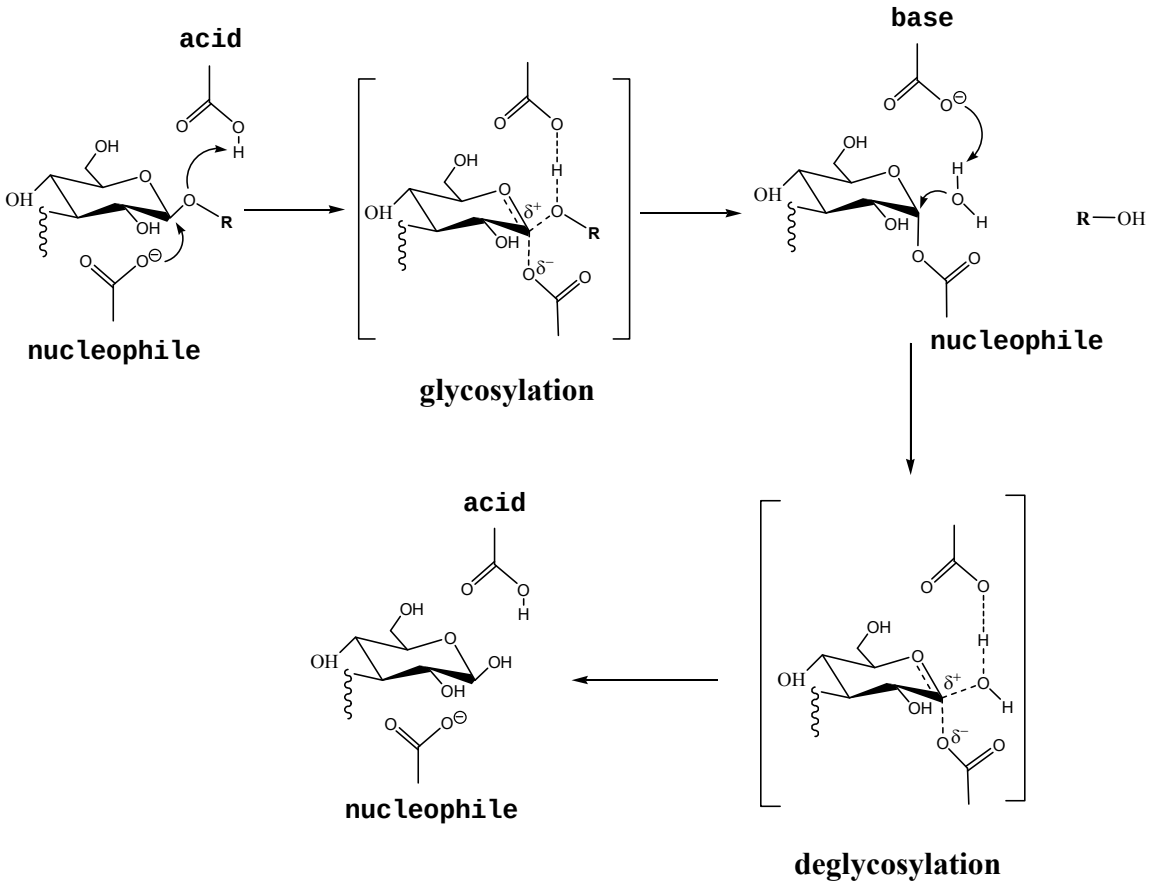

Figure 6. Mechanism of a retaining β-glucosidase by a double–displacement reaction through a covalent glycosyl-enzyme intermediate.

In family 5 enzymes of *Thermonospora .fusca* Glu128, Glu225, Asn127, His196 and Tyr198 are some of the strictly conserved amino acids (Hilge *et al*., 1998). In this organism Glu128 and Glu225 perform the roles of catalytic proton donor and nucleophile respectively, and Asn127, His196 and Tyr198 stabilize the active-site environment and are likely to influence the protonation state of the two glutamate residues (Sabini *et al*., 1999). Aspartate and glutamate residues are strictly conserved in family 26 (Bolam *et al*., 1996), while family 27 α-galactosidases employ two Asp residues as the catalytic acid-base molecules. In mannosidase sub-family 2 the Glu519 is entirely conserved not only for this sub-family but also within the whole family 2. This is a residue identified as an active-site nucleophile in a β-mannosidase (Stoll *et al*., 2000)

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Chapter Two

Production of galactomannan degrading enzymes by halotolerant *Scopulariopsis candida* strains isolated from the salt pan

(Some of the results in this chapter have been published, Appendix A)

2.1 Abstract

The current study reports on *Scopulariopsis candida* strains LMK004 and LMK008 isolated from a salt pan. These strains were used in the production of β-mannanase, β-mannosidase and α-galactosidase. The effect of medium composition and carbon source on growth and enzyme production was determined using salt water medium and Vogel's medium supplemented with varying concentrations of NaCl, and locust bean gum and α-cellulose as carbon sources. A combination of 1X Vogel's medium, 10% NaCl, 1% LBG and 1% yeast extract was found to be the best for high β-mannanase production. Strain LMK004 produced 7800 nkat/g biomass whereas LMK008 produced 13300 nkat/g biomass under these conditions. When α-cellulose was used as carbon source LMK004 produced seven times less β-mannanase while LMK008 produced five times less β-mannanase compared to the 126 nkat/ml and 116 nkat/ml produced on locust bean gum. Both strains secreted negligible amounts (less than 1 nkat/ml) of β-mannosidase and α-galactosidase resulting in β-mannanase: α-galactosidase: β-mannosidase ratio of about 700:1:2 in strain LMK004 and 1933:1:6.7 in strain LMK008.

2.2 Introduction

Mannan and mannan-based polysaccharides mainly occur in the hemicellulose fraction of plant cell walls (de Vries, 2003). The backbone of these polysaccharides generally contains β-1,4-linked mannose residues, and depending on their source they may be substituted with galactose, and glucose residues. The backbone of mannose and glucose residues may be acetylated at C-2 or C-3 (Buckeridge *et al*., 2000). The complete degradation of these polymers requires a synergistic action of enzymes including β-mannanase (EC 3.2.1.78), α-galactosidase (EC 3.2.1.22), β-mannosidase (EC 3.2.1.25), β-glucosidase (EC 3.2.1.21) and acetyl-mannan esterase (EC 3.1.1.6). These enzymes are usually produced by microorganisms, plants and animals (Franco *et al.,* 2004). Endo-β-1,4-mannanase breaks down β-1,4-linkages within the mannopyranosyl backbone releasing mannooligosaccharides of various sizes, while exo-β-1,4-mannosidase releases mannose residues from the reducing ends of mannooligosaccharides. The galactose, glucose and acetate residues are removed from the mannan backbone by α-

galactosidase, β-glucosidase and acetyl-mannan esterase, respectively (Stålbrand *et al*., 1993; Ademark *et al.,* 1998).

Over the years, there has been a growing interest, in the industrial applications of mannan-degrading enzymes. β-Mannanase is widely applied in poultry feed to reduce the anti-nutritional factor of mannan polymers found in corn-soy based feeds (Wu *et al*., 2005 ; Lee *et al*., 2005), and also plays a significant role in the detergent industry (Schäfer *et al.*, 2002; McCoy, 2001). β-Mannosidase is used in the synthesis of oligosaccharides which are then used in pharmaceutical industries (Taubken *et al*., 1993) while α-galactosidase is used in the sugar making-industry for removal of raffinose as well as in food products to improve the gelling properties of mannans (Kirk *et al.,* 2002; Bulphin *et al*., 1990)

Hypersaline environments including salt lakes, desiccating salt marshes, the Dead Sea and other environments where salt concentration exceed 3 M, represent habitats occupied by a wide variety of extremophillic microorganisms including filamentous fungi (van den Burg, 2003; Litchfield and Gillevet, 2002). These halophillic organisms accumulate inorganic ions or polyols within the cell walls, at concentrations equivalent to or greater than those of the environment in order to survive and adapt to the halophilic environment (Demirijian *et al*., 2001). The hypersaline environment may serve as a potential source of industrially important microorganisms.

Scopulariopsis is an anamorphic genus comprising mainly soil species (Aguilar *et al*., 1999), some are isolated from food, paper and other materials. Some species of *Scopulariopsis* including *S. brevicaulis* and *S. brumptii* have previously been isolated from hypersaline environments (Grishkan *et al*., 2004; Steiman *et al*., 1997). Research on species of *Scopulariopsis* including *S. candida* has generally focused on their potential ability to cause onychomycosis and invasive sinonasal infection in immunocompromised hosts (Kriesel *et al*., 1994). However, little work has been done on the hydrolytic properties of these organisms and there are no reports on mannan-degrading enzymes from any of these species.

In the current study, we report on the production of mannan-degrading enzymes by *Scopulariopsis candida* strains LMK004 and LMK008 isolated from a salt pan, with focus on the effect of NaCl concentration, mineral composition and carbon sources on β-mannanase production.

2.3 Materials and methods

2.3.1 Fungal isolates

Scopulariopsis candida (Guéguen) Vuillemin strains LMK004 and LMK008 were 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, 2006) and maintained on Potato dextrose agar (PDA) slants.

2.3.2 Effect of NaCl and media composition

Stock cultures were streaked on agar plates containing 1X Vogel's medium or 2.5% salt water medium, 0.5% locust bean gum (LBG) (Sigma, St. Louis, MO, USA) as carbon source and 1% yeast extract (Merck Chemicals, Darmstadt, Germany) as nitrogen source and incubated at 30 ^{o}C for 5 days. Vogel's medium (1X) was prepared from a 50X stock solution with the following mineral composition [(g/l) 130 Na_3citrate.$2H_2O$, 126 KNO_3, 144 $(NH_4)H_2PO_4$, 80 KH_2PO_4, 10 $MgSO_4.7H_2O$]. Five grams of $CaCl_2.2H_2O$ was dissolved in 20 ml distilled water and added drop-wise into the solution, 2.5 ml of a 0.1 mg/ml biotin solution dissolved in 50% ethanol was also added. Trace elements were prepared [(g/l) 5 Citric acid.H_2O, 5 $ZnSO_4.7H_2O$, 1 $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.25 $CuSO_4.5H_2O$, 0.05 $MnSO_4.H_2O$, H_3BO_3 and $Na_2MoO_4.2H_2O$] (Metzenberg, 2003). Five millilitres of trace elements and 3 ml chloroform were added to the mineral solution and the mixture was made up to 1 litre by distilled water. Salt Water (SW) medium (30% w/v) was prepared by dissolving the salts in warm distilled water [(g/l) 240 NaCl, 30 $MgCl_2.6H_2O$, 35 $MgSO_4.7H_2O$, 7 KCl and 0.2 $NaHCO_3$] (Dyall-Smith, 2000). Five millilitres of 1 M $CaCl_2.2H_2O$ as well as 2 ml of 1 M Tris-HCl buffer pH 7.5 was added and the volume of the solution was adjusted to a litre with distilled water. Agar pieces (cm^2) were cut out from the plates and used to inoculate 100 ml liquid media containing 1X Vogel's medium with 0% to 20% NaCl or 2.5%, 5% and 10% SW medium, 1% LBG and 1% yeast extract. The inoculated flasks were incubated at 25 ^{o}C while shaking at 150 rpm. Samples were collected daily and β-mannanase production and biomass was determined using the culture filtrate. Biomass determinations were carried out by drying the mycelium on pre-weighed Ø 47 mm glass

fibre filters (Laboratory and Scientific equipment company, (Pty, Ltd) at 100 ºC. The filtered mycelia were washed three times with distilled water prior to drying to remove insoluble medium constituents.

2.3 Effect of carbon sources on growth and enzyme production

Agar plates prepared with 1X Vogel's medium as above were used to streak *Scopulariopsis candida* LMK004 and LMK008 and the agar pieces (cm^2) were used to inoculate 100 ml liquid media containing 1% yeast extract, 10% NaCl and 1X Vogel's medium and either 1% LBG, 1% and 2% α-cellulose (Sigma) as a carbon source. The inoculated flasks were incubated at 25 ºC while shaking at 150 rpm. Samples were collected daily and β-mannanase and biomass production was determined as described above.

2.4 β-Mannanase assays

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 boiled. The mixture was cooled and left overnight with continuous stirring. The insoluble materials were removed by centrifugation at 3 840 X *g* for 5 min (Stålbrand *et al*., 1993). The assay mixture contained 900 µl of the substrate and 100 µl of suitably diluted supernatant. Reactions were performed at 50 ^{o}C for 10 min, followed by determination of reducing sugars using modified dinitrosalicylic acid (DNS) method (Miller, 1959). Dinitrosalicylic reagent (1 litre) was prepared without light exposure by first dissolving 10 g 3,5-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 slowly added and the solution was 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 1µmol.min^{-1}).

2.5 α-Galactosidase and β-mannosidase assays

The β-Mannosidase activity was determined using 1 mM 4-nitrophenyl-β-D-mannopyranoside (Sigma, St. Louis, MO, USA) in 50 mM citrate buffer, pH 5 as a substrate. Hundred microlitres of the diluted enzyme sample was incubated with 900 µl of the substrate at 50 °C for 10 min. The reaction was stopped by adding 1 ml of 1 M sodium bicarbonate and the liberated 4-nitrophenol was measured spectrophotometrically at 400 nm. α-Galactosidase was determined using 1 mM 4-nitrophenyl-α-D-galactopyranoside (Sigma) in 50 mM citrate buffer, pH 5 as a substrate using a procedure similar to the β-mannosidase assay. The standard used was (spectrophotometric grade) 4-nitrophenol (Sigma), β-mannosidase and α-galactosidase activities were expressed in nkat as previously defined.

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2.4. Results

2.4.1 Effect of medium composition on growth and enzyme production

Vogel's medium and SW medium were used to determine the effect of mineral composition on growth and β-mannanase production in *Scopulariopsis candida* strains LMK004 and LMK008. The production of β-mannanase by both strains increased proportionally with salt concentrations and similar amounts of biomass were formed in both media. However, increased levels of β-mannanase were obtained on Vogel's medium. *S. candida* LMK008 generally produced more β-mannanase than LMK004 on both media reaching levels of up to 13 000 nkat/g biomass and 8600 nkat/g biomass on Vogel's medium and SW medium, respectively. In contrast, LMK004 produced 7800 nkat/g biomass when cultivated on Vogel's medium while poor production levels were observed on SW medium (Fig 1). High NaCl concentrations (15 - 20%) resulted in reduced β-mannanase production levels (data not shown).

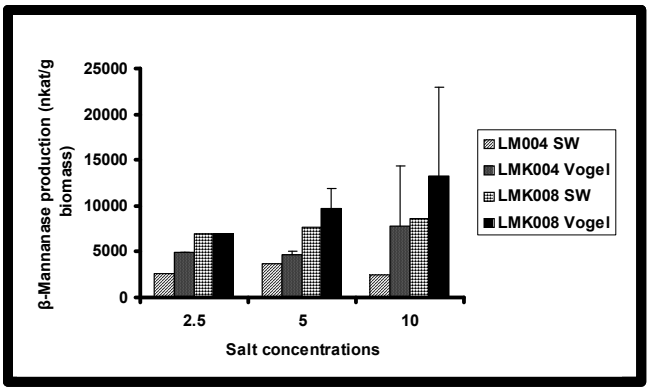

Figure 1. The effect of SW (2.5% to 10%) and Vogel's media with 2.5% to 10% NaCl on growth and β-mannanase production in *S. candida* LMK004 and LMK008. Average values of independent duplicate experiments were used.

2.4.2 Effect of carbon source on growth and enzyme production

The effect of carbon source was determined under the conditions of highest β-mannanase production using the medium containing 1X Vogel's medium with 10% NaCl, 1% yeast extract, and either LBG or α-cellulose as carbon sources. The cultures were cultivated for 6 days and the

assays were performed daily to determine β-mannanase activity. There was generally poor growth and β-mannanase production when α-cellulose was used as a carbon source. Strain LMK004 produced only 18 nkat/ml when cultivated on 1% and 2% α-cellulose whereas LMK008 produced up to 16.4 nkat/ml and 25.16 nkat/ml, respectively. In contrast, locust bean gum (LBG) stimulated increased β-mannanase production, LMK004 produced up to 126 nkat/ml and LMK008 produced 116 nkat/ml which were seven times higher than when both strains were cultivated on α-cellulose (Fig 3).

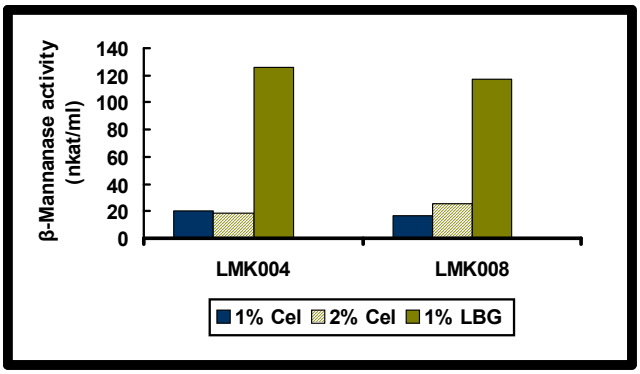

Figure 3. The influence of carbon source, 1% and 2% α-cellulose and 1% LBG galactomannan on β-mannanase production. Average values of independent duplicate experiments were used.

Both strains secreted trace amounts of β-mannosidase and α-galactosidase when LBG was used as a carbon source. None of these enzymes were detected when the strains were grown on α-cellulose as carbon source (Table 1).

Table 1. Production of β-mannanase, α-galactosidase and β-mannosidase by *Scopulariopsis candida* strain LMK004 and LMK008

Enzymes	**LMK004**		**LMK008**	
	LBG	α-Cellulose	LBG	α-Cellulose
β-mannanase (nkat/ml)	126	18	116	25
α-galactosidase (nkat/ml)	0.18	-	0.06	-
β-mannosidase (nkat/ml)	0.36	-	0.4	-

2.5 Discussion and conclusion

Hypersaline environments including the Dead Sea, salt lakes and solar salterns have been shown to harbour a wide variety of cosmopolitan lignicolous (wood) and cellulolytic moulds. However, microbial degradation of plant cell wall polysaccharides (particularly mannans) in such environments has not been investigated.

Mannan degrading enzymes are usually secreted into the extracellular environment by the producing microorganisms and are therefore affected by the prevailing environmental conditions under which the organism is growing (de Vries, 2003; Stålbrand *et al*., 1993). In the current study, we investigated the production of β-mannanase, α-galactosidase and β-mannosidase by two *Scopulariopsis candida* strains isolated from a salt pan.

Two salt media (Salt water media and Vogel's medium) were compared to determine the effect of mineral composition and NaCl content on growth and enzyme production. Although SW medium is commonly used for isolation and cultivation of halobacteria (Dyall-Smith, 2000), it was employed in this study as its mineral composition closely resembles that of the salt pan from which the organisms were isolated (Mudau and Setati, 2006). Vogel's medium is commonly used for cultivation of various moulds (Metzenberg, 2003). Both *S. candida* LMK004 and LMK008 proliferated well on both media and could produce and secrete functional β-mannanases into the extracellular environment. However, it was evident that Vogel's medium was more suitable for cultivation of these organisms. The low β-mannanase levels in SW medium could be due to direct enzyme deactivation by elevated Mg^{2+} in the medium rather than negative influence of the medium on growth and enzyme production. Similar observations were reported by Wainø and Ingvorsen (2003) where they showed that the activity of a β-xylanase from the halophilic archaeon *Halorhabdus utahensis* decreased linearly with increasing $MgSO_4$ concentrations in the presence of 3% - 21% NaCl. The effect of carbon sources on β-mannanase production investigated using locust bean galactomannan because is generally known to produce high amount of β-mannanase and α-cellulose was used because it was reported to trigger higher β-mannanase production as compared to LBG in *Sclerotium rolfsii* (Gübitz *et al*., 1996), however the opposite was observed in the current study.

Scopulariopsis candida LMK004 and LMK008 secreted trace amounts of β-mannosidase and α-galactosidase simultaneously with the β-mannanase. The auxiliary enzymes are generally secreted in low amounts relative to the β-mannanase in various moulds (Table 2). However, the levels recorded in the current study are very low and could indicate that the β-mannosidase and α-galactosidase in *S. candida* are localized intracellularly as has been observed in *Aureobasidium pullulans* (Kremnický and Biely, 1997) and *Humicola* species (Kotwal *et al*., 1999)

Table 2. The ratio of secreted β-mannanase, α-galactosidase and β-mannosidase in filamentous fungi

Organism and carbon source	**β-Mannanase (nkat/ml)**	**α-Galactosidase (nkat/ml)**	**β-Mannosidase (nkat/ml)**	**References**
Aspergillus niger (LBG)	11	5.8	1	Ademark *et al*., 1998
Sclerotium rolfsii				Gübitz *et al*., 1996
LBG	65.3	1.35	1	
(Cellulose)	141.8	1.5	1	
Thermoascus aurantiacus				Gomes *et al*., 2000
(Cellulose)	5	6	1	
Pleurotus ostreatus	32	NR	1	Valášková and Baldrian, 2006
Trametes versicolor	1.2	NR	1	
Piptoporus betulinus	0.5	NR	1	
(Cellulose)				
Scopulariopsis candida (LMK004)				This study
(LBG)	700	1	2	
Scopulariopsis candida (LMK008)				This study
(LBG)	1933	1	6.7	

NR: Not Reported

There are currently few reports on the production of polysaccharide degrading enzymes by the genus *Scopulariopsis*. Amongst those reported was a *Scopulariopsis* species that produced two isoenzymes of endo-1,4-β-xylanase (Afzal *et al.,* 2005) and *Scopulariopsis brevicaulis* TOF-1212 which produces endoglucanase (Nakatani *et al*., 2000). *Scopulariopsis candida* strains (in this study) were found to produce β-mannanase that exhibit activity on locust bean galactomannan. This report shows that *Scopulariopsis* species may be useful in the production of enzymes that are industrially important. Production of mannan-degrading enzymes from fungi isolated from hypersaline environment has not been extensively studied. The ability of

various fungi to survive in such environments has triggered an interest for the isolation of fungi that may be useful in the production of these enzymes for industrial applications such as in poultry feeds, laundry and in instant coffee processing.

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Chapter Three

Purification and characterization of endo-β-1,4-mannanase from two *Scopulariopsis candida* strains isolated from a hypersaline environment

3.1 Abstract

Extracellular β-mannanases were produced from two *Scopulariopsis candida* strains designated LMK004 and LMK008 during growth using locust bean gum (galactomannan) as a carbon source. The highest concentration of β-mannanase produced by LMK004 and LMK008 was 105 nkat/ml and 173 nkat/ml, respectively. Both enzymes were partially purified by ammonium sulphate precipitation and anion-exchange chromatography. The molecular weight of LMK004 β-mannanase was estimated to be ≈41 kDa whereas that of LMK008 β-mannanase could not be determined due to excessive loss of protein material during dialysis. The β-mannanase from LMK004 was most active at pH 5 and 50 °C, and retained ≥ 80% of its activity at pH 5 – 6.5 after 24 hrs of incubation at 4 °C. In contrast, the LMK008 β-mannanase retained ≥ 60% activity between pH 6 – 7. Both enzymes remained stable for 3 hrs between 30 °C and 40 °C, and showed loss of activity at higher temperatures. The LMK008 β-mannanase tolerated high NaCl concentrations with 60% activity remaining after incubation for 2 hrs at 20% NaCl, whereas the LMK004 β-mannanase was only active between 0% - 10% NaCl.

3.2 Introduction

Mannan and mannan-based polysaccharides consist of a backbone of β-1,4-linked mannose residues (de Vries and Visser, 2001). The complete degradation of these polymers requires an array of enzymes including 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). β-Mannanase is an important enzyme for the depolymerization of these polymers. It hydrolyses the β-1,4-linkages within the mannan backbone releasing the mannooligosaccharides of various lengths (Franco *et al.,* 2004). β-Mannosidase and α-galactosidase remove mannose residues and galactose residues from the mannan backbone, respectively (Stålbrand *et al.,* 1993).

Mannan-degrading enzymes have been grouped and classified into different families of glycosyl hydrolases families on the basis of their amino acid sequence similarities (Henrissat *et al*., 1998). β-Mannanase belongs to family 5 and 26 (Hogg *et al.,* 2001). These enzymes hydrolyze their substrates via a double-displacement mechanism that involves two catalytic amino acids, one of

which functions as the acid-base catalyst and the other as the nucleophile (Henrissat *et al.*, 1995). These have been identified as two glutamates in family 5 (Hilge *et al*., 1998) and a glutamate and aspartate in family 26 (Bolam *et al*., 1996).

Over the years, there has been an increasing interest in the potential application of β-1,4-mannanases in various industrial processes. This triggered research interest into the biochemical properties of these enzymes. As a result β-mannanases have been purified from both bacterial and fungal sources (Zakaria *et al*., 1998; Jiang *et al*., 2006; Ferreira and Filho, 2004; Ademark *et al*., 1998). The β-mannanases reported so far exhibit acidic to neutral pH optima, molecular weights ranging from 33 kDa to 90 kDa and mesophillic to moderately thermophillic temperature optima (Araujo and Ward, 1990; Stålbrand *et al*., 1993; Puchart *et al*., 2004). β-Mannanases are currently used in various industries such as in laundry detergents, poultry feeds, coffee processing and in paper and pulp industries together with xylanases (Schäfer *et al*., 2002; Sachslehner *et al*., 2000; Wu *et al*., 2005; Wong and Saddler, 1993). In the current study we report the purification and characterization of endo-1,4-β-mannanase from two *Scopulariopsis candida* strains isolated from a hypersaline environment.

3.3 Materials and methods

3.3.1 Growth experiments

Scopulariopsis candida (Guéguen) Vuillemin strains LMK004 and LMK008 were maintained on Potato dextrose agar (PDA) slants. Stock cultures were streaked on agar plates containing 1X Vogel's medium, 0.5% locust bean gum (LBG) (Sigma, St. Louis, MO, USA) as carbon source and 1% yeast extract (Merck Chemicals, Darmstadt, Germany) as nitrogen source and incubated at 30 ^{o}C for 5 days. A fifty times concentrated stock solution of Vogel's medium was prepared with the following mineral composition [(g/l) 130 Na_3citrate.$2H_2O$, 126 KNO_3, 144 $(NH_4)H_2PO_4$, 80 KH_2PO_4, 10 $MgSO_4.7H_2O$] 5g $CaCl_2.2H_2O$ was dissolved in 20 ml distilled water and added drop-wise into the solution, 2.5 ml of 0.1 mg/ml biotin dissolved in 50% ethanol was also added. Trace elements were prepared [(g/l) 5 citric acid.H_2O, 5 $ZnSO_4.7H_2O$, 1 $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.25 $CuSO_4.5H_2O$, 0.05 $MnSO_4.H_2O$, 0.05 H_3BO_3, 0.05 $Na_2MoO_4.2H_2O$] (Metzenberg, 2003). Five milli-litres of trace elements and 3 ml chloroform were added to the mineral solution and the mixture was made up to 1 litre with distilled water. Agar pieces (cm^2) were cut out from the plates and used to inoculate 100 ml liquid media composed of 1% LBG, 1% yeast extract, 10% NaCl and 1X Vogel's medium. The inoculated flasks were incubated at 25 ^{o}C with shaking at 150 rpm. Samples were collected daily and β-mannanase production was determined using the culture filtrate.

3.3.2 β-Mannanase assays

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 boiled. The mixture was cooled and left overnight with continuous stirring. The insoluble materials were removed by centrifugation at 3 840 X *g* for 5 min (Stålbrand *et al*., 1993). The assay mixture contained 900 µl of the substrate and 100 µl of suitably diluted supernatant. The reaction mixture was incubated at 50 ^{o}C for 10 min. Reducing sugars produced due to enzyme activity were determined as mannose reducing equivalents using modified dinitrosalicylic acid (DNS) method (Miller, 1959). 3,5-Dinitrosalicylic reagent (1 litre) 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 hundreds grams of Rochelle salt (Potassium-Sodium-Tartrate) was slowly added and the solution was 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 1μmol.min^{-1}).

3.3.3 β-Mannanase purification

Scopulariopsis candida LMK004 and LMK008 were cultivated until peak β-mannanase production. The culture filtrates were collected by passing fungal cultures through a Mira cloth (Calbiochem, USA) to remove mycelia and then precipitated with ammonium sulphate. The precipitates were collected by centrifugation at 153 X *g* for 15 min and resuspended in 50 mM-Tris-HCl buffer pH 7.5 followed by dialysis against the same buffer using Snakeskin® pleated dialysis tubing with molecular weight cut-off of 10 KDa (Pierce, Rockford, USA). The dialysates were filtered through Cameo 0.45 μm nylon syringe filters (Micron Separations Incorporated, USA) and loaded onto an Econo-pac® high Q anion exchange column (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. The fractions were assayed for β-mannanase activity using the DNS method for the determination of reducing sugars. Protein concentrations in the samples before and after purification were determined using Micro BCA™ protein assay reagent kit (Pierce).

3.3.4 Gel electrophoresis and zymogram analysis

One millilitre of purified sample was freeze-dried, resuspended in 100 μl citrate-buffer pH 5 and dialysed for 2 hrs. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and native poly-acrylamide gel electrophoresis (Native-PAGE) in conjunction with zymogram were employed to assess the purity of the fractions and to determine the molecular weight of the proteins. Thirty microlitres of the concentrates were mixed with 30 μl Laemmli sample buffer from Bio-Rad laboratories (prepared according to manufacturer's instructions) and denatured by boiling for 10 min. These samples were separated on 10% SDS-PAGE gels and visualized by

staining with Coomassie brilliant blue. The Pageruler™ prestained protein ladder (Fermentas, Hanover, Maryland, USA) was used to estimate the molecular weight of the proteins. Undenatured protein samples were separated on a 10% Native-PAGE. A substrate gel was prepared by adding 2% agarose to a 0.5% locust bean gum solution (prepared as in the β-mannanase assay, section 2.2) and heating until the agarose dissolved. The mixture was cast into a glass petri-dish and allowed to solidify. After electrophoresis the native gel was placed on the substrate gel. The two gels were then incubated at 50 °C for 1 hour followed by staining of the substrate gel using 0.1% (w/v) Congo red solution for 2 hrs. The gel was destained using 1 M NaCl and then transferred to 0.5% acetic acid to enhance clarity of hydrolysis zones that indicated β-mannanase activity.

3.3.5 Determination of temperature and pH optimum and stability

The β-mannanase activity of the purified samples was assayed at different temperatures ranging from 30 °C – 70 °C at pH 5 in 50 mM citrate-buffer. Temperature stability was determined by incubating the enzyme samples at the same temperatures in 50 mM citrate buffer pH 5 for 3 hrs, followed by standard β-mannanase assay at 50 °C. The pH optimum was determined using buffers ranging from pH 3 to 7. Citrate buffer (50 mM) was used in the pH range of 3 to 6 and 50 mM citrate-phosphate buffer was used for pH 6 - 7. To study the stability of purified β-mannanase samples under different pH conditions, appropriate dilutions of β-mannanase were made in the buffers in the range of pH 3 - 7 and incubated at 4 °C for 24 hrs. The residual activities were estimated under standard conditions of the β-mannanase assay.

3.3.6 Effect of salt concentration on enzyme stability

The effect of NaCl on the two β-mannanases was determined by incubating the enzyme samples at 4 °C for 2 hrs in 50 mM citrate buffer (pH 5) containing 0% - 20% (w/v) NaCl. Hundred microlitres were used to determine the residual activity under standard conditions for β-mannanase assay.

3.4 Results

3.4.1 Production and purification of β-mannanases

Scopulariopsis candida strains LMK004 and LMK008 were cultivated under conditions of highest β-mannanase produced and monitored daily for β-mannanase production. The conditions were liquid medium supplemented with 10% locust bean gum galactomannan, 1X Vogel's medium with 10% NaCl as well as 1% yeast extract (chapter 2). LMK004 produced up to 105 nkat/ml within four days of cultivation followed by an unexplained decline on day five. In contrast LMK008 produced a maximum of 173 nkat/ml of β-mannanase on day 5 and these levels were maintained for at least another 24 hrs (Fig 1).

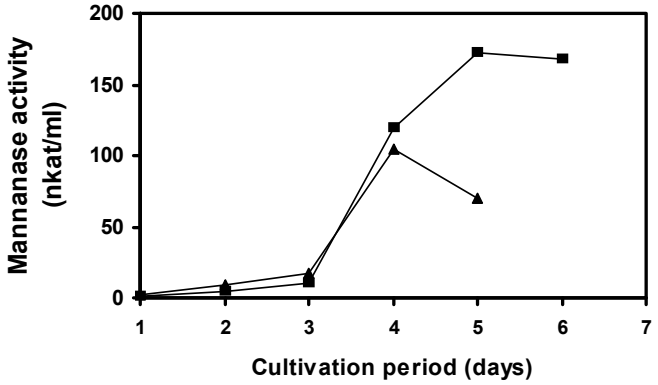

Figure 1. A time course of β-mannanase production by *S. candida* LMK004 (▲) and LMK008 (■). Average values of independent duplicate experiments were used.

The culture filtrates of LMK004 and LMK008 were collected after day 4 and 5 of cultivation, respectively. β-Mannanase was purified from these filtrates using ammonium sulphate precipitation and anion exchange chromatography. A 65 fold purity level was attained during purification of the LMK004 β-mannanase resulting in an increase in specific activity from 431 nkat/mg to 27865 nkat/mg (Table 1)

Table 1. Purification of β-mannanase from *Scopulariopsis candida* strain LMK004

Purification step	Volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Protein yield (%)	Degree of purification
Culture filtrate	250	51900	120.6	430.5	100	1
$(NH_4)_2SO_4$ Precipitation	7	4654.72	1.93	2414.9	8.97	5.61
Anion-exchange chromatography	4	3107.6	0.1115	27864.6	5.99	64.73

In contrast, LMK008 β-mannanase purification was hampered by excessive loss of protein material following dialysis resulting in the formation of insoluble precipitate in the dialysate. Consequently, only 5.7 purification fold was achieved resulting in a specific activity of 3321 nkat/mg (Table 2). Although this fraction could not be visualized on SDS-PAGE using either silver-staining and coomasie staining techniques, it was used for determination of biochemical properties.

Table 2. Purificaton of β-mannanase from *Scopulariopsis candida* strain LMK008

Purification step	Volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Protein yield (%)	Degree of purification
Culture filtrate	500	136315.8	272.16	500.86	100	1
$(NH_4)_2SO_4$ Precipitation	7	1153.7	1.52	763	0.85	1.53
Anion-exchange chromatography	9	418.5	0.126	3321	3.07	5.67

3.4.2 Biochemical properties

SDS-PAGE analysis of the purified LMK004 β-mannanase revealed an intense band of approximately 41 kDa and two faint bands of 27 kDa and 17.15 kDa (Fig 2A). The 41 kDa band was confirmed to be a β-mannanase through Native-PAGE in conjunction with zymogram and activity staining (Fig 2B)

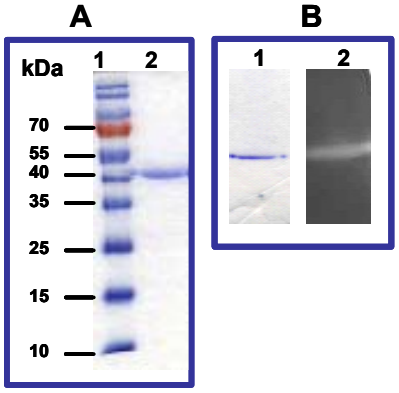

Figure 2. An SDS-PAGE analysis of the purified β-mannanase from LMK004 (A), showing molecular weight marker (lane 1) and LMK004 β-mannanase (lane 2). Native-PAGE and zymogram (B) used to confirm β-mannanase activity. Protein band resolved on native gel (lane 1) and β-mannanase activity on the substrate gel (lane 2).

The effect of pH, temperature and NaCl concentration on the activity and stability of the β-mannanase from LMK004 and LMK008 was determined. The β-mannanase from LMK004 showed optimal activity at pH 5 whereas that of LMK008 was most active at pH 6 (Fig 3A). The LMK004 β-mannanase was stable at pH 5 - 6.5 while LMK008 was stable at pH 6 - 7 (Fig 3B).

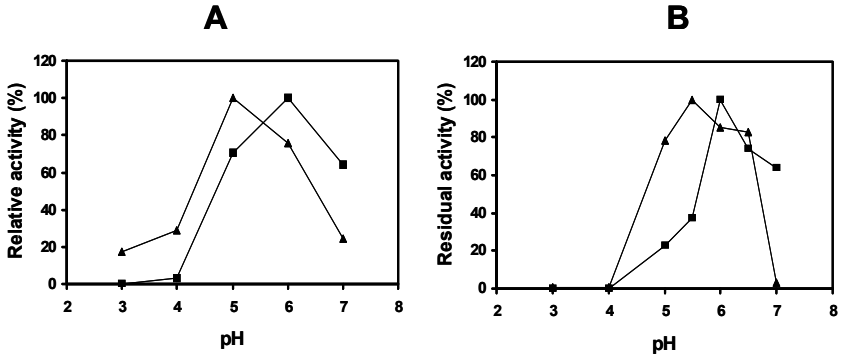

Figure 3. **A**ctivities (A) and stabilities (B) of the purified β-mannanases form *S. candida* LMK004 (▲) and LMK008 (■) at different pH.

The β-mannanase from LMK004 and LMK008 displayed optimal activity at 50 °C and 40 °C, respectively (Fig 4A). Both enzymes remained stable for 3 hrs at temperatures between 30 °C and 40 °C, and showed rapid loss of activity at higher temperatures (Fig 4B)

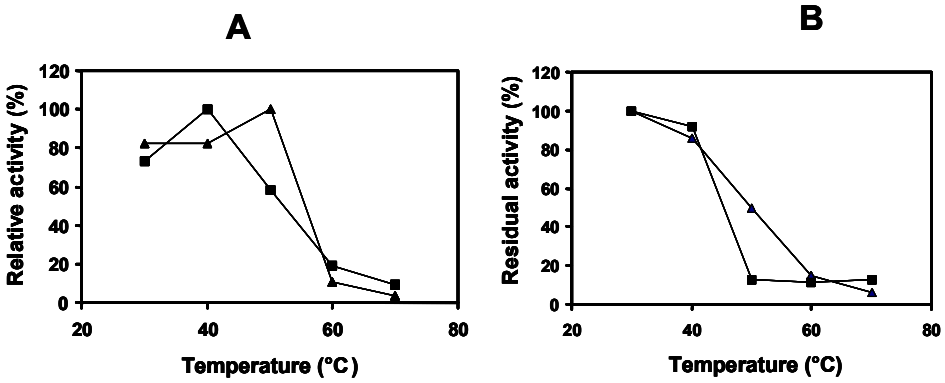

Figure 4. Temperature optimum (A) and stabilities (B) of the purified β-mannanases from LMK004 (▲) and LMK008 (■).

The effect of NaCl on β-mannanases was determined by incubating the enzyme samples at 4 °C for 2 hrs at various NaCl concentrations. Increase in NaCl concentration between 10% - 20% (w/v) led to loss of activity of the β-mannanase from LMK004. In contrast, the LMK008 β-mannanase remained stable between 0% and 20% NaCl with only 25% - 30% of activity lost (Fig 5).

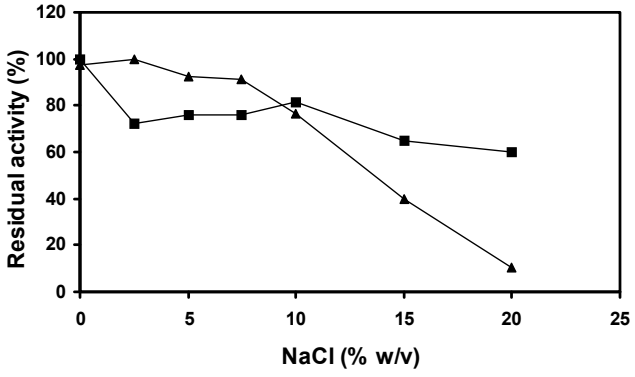

Figure 5. The effect of NaCl on the stability of β-mannanases from LMK004 (▲) and LMK008 (■).

3.5 Discussion and conclusion

Growing interest in the potential application of β-1,4-mannanases in various industries, has triggered increasing research towards biochemical characterization of these enzymes. Consequently, β-mannanases have been purified and characterized from both fungal and bacterial sources (Ferreira and Filho, 2004; Takeda *et al*., 2004). Extreme environments are becoming an important source of organisms that produce enzymes of industrial importance including β-mannanases.

Scopulariopsis candida strains LMK004 and LMK008 investigated in the current study were isolated from a hypersaline environment. They both secrete significant amounts of β-mannanase into the extracellular medium. The purified β-mannanases from these strains exhibited acidic pH optima and remained stable at acid to neutral pH although the LMK008 β-mannanase was stable within a pH range of 6 - 7. In contrast, the β-mannanase from LMK004 retained ≥ 80% of its activity between pH 5 – 6.5. The temperature optimum and stability of both enzymes is the lowest amongst fungal β-mannanases (Ademark *et al*., 1998; Sachslesner *et al*., 2000). The molecular weight of LMK004 β-mannanase was found to be amongst the range reported for most fungal β-mannanases including those purified from *Sclerotium rolfsii* (Gübitz *et al.,* 1996), *Aspergillus niger* (Ademark *et al.,* 1998), *Polyporus versicolor* (Johnson *et al.,* 1990), *Aspergillus aculeatus* (Setati *et al*., 2001).

β-Mannanases are extracellular enzymes and are consequently affected by the medium into which they are secreted. It was therefore important to investigate the effect of NaCl on the activity of the β-mannanases purified from *S. candida* LMK004 and LMK008. The two enzymes displayed different degrees of halotolerance with generally higher activities at low salinity. The LMK008 β-mannanase tolerated high NaCl concentrations with 60% activity remaining after 2 hour incubation at 20% NaCl. In contrast, the LMK004 β-mannanase was only stable up to 7.5% NaCl. The level of halotolerance observed with both enzymes is similar to other polysaccharide hydrolysing enzymes from halophilic archaea, bacteria and halotolerant fungi. The include β-xylanases and β-xylosidases from *Halorhabdus utahensis* (Wainø and Ingvorsen, 2003), *Aspergillus oryzae* (Hashimoto and Nakata, 2003) and a novel halophilic bacterium strain CL8

(Wejse *et al.,* 2003). The differences in the properties of β-mannanases in the current study could mean that the two strains used in the current study are completely different, further identification of the strains might be necessary to confirm the speculations.

β-Mannanases are currently used in low water activity industrial applications such as processing and manufacture of coffee (Sachslehner *et al*., 2000) as well as in poultry feeds (Lee *et al*., 2005. Halotolerant β-mannanase may be suited for such applications as they are normally produced and secreted into hypersaline environments which have low water potential. It will therefore be of scientific interest if the structure and function of such enzymes is understood. It is clear from the current study the two strains of *S. candida* produce distinct β-mannanases which make them potential candidates for comparative studies. Therefore, future research will investigate sequence similarities, amino acid composition and structure prediction.

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Chapter Four

General discussion and conclusions

Hypersaline environments are habitats usually occupied by a wide variety of halophilic microorganisms including archaea, fungi and bacteria (van den Burg, 2003). In these environments low water availability and high salt concentrations (exceeding 3 M) greatly influence the growth, development and adaptation of the organisms to extreme conditions (Grishkan *et al*., 2004). Salterns and hypersaline lakes, such as the Great Salt Lake, the Dead Sea and solar lakes are examples of hypersaline environments (Gomes and Steiner, 2004; Steiman *et al*., 1997). Biodiversity investigations of these environments have demonstrated that a variety of filamentous fungi do proliferate under hypersaline conditions. The prevailing mycobiota comprises halotolerant ubiquitous terrestrial species of *Aspergillus* and *Penicillium*, and a wide variety of lignocolous phytopathogens many of which have been endowed with the potential to hydrolyse plant cell wall polysaccharides.

Plant cell wall degrading enzymes such as cellulases, xylanases and mannanases are usually secreted into the extracellular environment by the producing organisms. Therefore, it may be expected that the enzymes produced and secreted by filamentous fungi into hypersaline environments have evolved to function optimally under the prevailing environmental conditions. However, degradation of plant polysaccharides in such ecosystems remain poorly characterized.
In the current study, we investigated the degradation of mannan polysaccharides by halotolerant *Scopulariopsis candida* strains that were isolated from a salt pan using enrichment techniques. The strains secreted a cocktail of active galactomannan degrading enzymes: endo-1,4-β-mannanase, exo-β-mannosidase and α-galactosidase that remained functional at NaCl concentrations between 10% – 15%, thus showing that these organisms can depolymerise plant polysaccharides in hypersaline environments. In this study the purified β-mannanase from *S. candida* LMK004 and LMK008 exhibited varying degrees of halotolerance. It became apparent that the β-mannanase from LMK008 could tolerate higher NaCl concentrations with ≥ 60% of its activity remaining even at 20% (w/v) NaCl. In addition, removal of $MgSO_4$ by dialysis resulted in loss of activity which could signify inactivation of this enzyme at lower salt concentrations, a behaviour common amongst halophillic enzymes (Madern *et al*., 2000). We can therefore, infer that the LMK008 β-mannanase would be more suitable for reactions performed at low water activity than the LMK004 β-mannanase.

Other polysaccharides degrading enzymes have been isolated from halophillic organisms such as starch-degrading α-amylase from *Halothermothrix orenii* (Mijts and Patel, 2002) and two halotolerant xylanases (Xyl 1 and Xyl 2) purified from a novel halobacterium, strain CL8 (Weije *et al*., 2003). β-Mannanases from non-halophilic sources are currently used in laundry detergents, manufacture of coffee and poultry feed. Some of these environments, particularly animal feed are generally low water activity environments. However, there are currently no known applications of halotolerant β-mannanases in these or other related industries.

Future research will therefore be to:

i. Develop a purification method for LMK008 β-mannanase
ii. Further characterize the β-mannanases from *S. candida* strains LMK004 and LMK008
iii. Study their structure, function and amino acid composition in order to identify their halophilic nature and to evaluate their potential applications in the industries that requires low water activity.

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Chapter Five

Summary/Opsommig

5.1 Summary

Mannan polysaccharides occur in hemicellulose fraction of the plant cell walls. The hydrolysis of these polymers involves the action of enzymes such as β-mannanase, β-mannosidase and α-galactosidase which are produced by both fungi and bacteria. The current study reports on the production of β-mannanase, β-mannosidase and α-galactosidase by newly isolated *Scopulariopsis candida* strains LMK004 and LMK008. The effect of medium composition and carbon source on growth and enzyme production was evaluated in a liquid culture. A combination of Vogel's medium and locust bean gum was found to stimulate growth and increase β-mannanase production. Optimal β-mannanase production of 7800 nkat/g biomass for LMK004 and 13300 nkat/g biomass for LMK008 was achieved in media containing 10% NaCl, 1X Vogel's medium, 1% yeast extract and 1% locust bean gum. Both strains secreted trace amounts (less than 1 nkat/ml) of β-mannosidase and α-galactosidase indicating that these enzymes may be retained intracellularly. Native-PAGE and SDS-PAGE were used together with the zymogram to assess purity and to estimate the molecular weight of the proteins. The molecular weight of LMK004 β-mannanase was estimated to be ≈41 kDa whereas that of LMK008 β-mannanase could not be determined due to excessive loss of protein material during dialysis. The β-mannanase from LMK004 was most active at pH 5 and 50 °C, and retained ≥ 80% of its activity at pH 5 – 6.5 after 24 hrs of incubation at 4 °C. In contrast, the LMK008 β-mannanase retained ≥ 60% activity between pH 6 – 7. Both enzymes remained stable for 3 hrs at temperature between 30 °C and 40 °C, and showed loss of activity at higher temperatures. The two enzymes displayed different degrees of halotolerance. The LMK008 β-mannanase tolerated high NaCl concentrations with 60% activity remaining after incubation for 2 hrs at 20% NaCl, whereas the LMK004 β-mannanase was only active between 0% - 10% NaCl. It is clear from the current study that the two strains of *S. candida* produce distinct β-mannanases which may be useful candidates in low water activity reactions.

Keywords: *Scopulariopsis candida*, salt pan, production, purification, characterization, β-mannanase

7.2 Opsomming

Mannaanpolisakkariede kom voor in die hemisellulose fraksie van plantselwande. Ensieme soos β-mannanase, β-mannosidase en α-galaktosidase, geproduseer deur beide fungi en bakterië, is betrokke by die hidrolise van hierdie polimere. Hierdie studie handel oor die produksie van β-mannanase, β-mannosidase en α-galaktosidase deur nuwe isolate van *Scopulariopsis candida*, stamme LMK004 en LMK008. Die invloed van mediumsamestelling en koolstofbron op groei en ensiemproduksie is geëvalueer in n vloeibare kultuur. Daar is gevind dat 'n kombinasie van Vogel se medium en locust bean gum groei stimuleer en produksie van β-mannanase verhoog. Optimale b-mannanase produksie van 7800 nkat/g biomassa vir LMK004 en 13300 nkat/g biomassa vir LMK008 is verkry in media bestaande uit 10% NaCl, 1X Vogel se medium, 1% gisekstrak en 1% locust bean gum. Beide stamme het spoorhoeveelhede (minder as 1 nkat/ml) β-mannosidase en α-galaktosidase uitgeskei, wat daarop mag dui dat hierdie ensieme intrasellulêr behou word. "Native"-PAGE en SDS-PAGE is saam met zymogram gebruik om die suiwerheid en molekulêre massas van die proteïene te bepaal. Die molekulêre massa van die LMK004 β-mannanase is ≈41 kDa terwyl dié van die LMK008 β-mannanase nie bepaal kon word nie van weë n uitermatige verlies van proteïen gedurende dialise. Die LMK004 β-mannanase was mees aktief by pH 5 en 50 ^{o}C en het ≥ 80% aktiwiteit behou by pH 5 – 6.5 na 24 uur inkubasie by 4 ^{o}C. Inteenstelling daarmee, het die LMK008 β-mannanase ≥ 60% aktiwiteit behou by pH 6 - 7. Beide ensieme was stabiel vir 3 ure by temperature tussen 30 ^{o}C en 40 ^{o}C en het n verlies aan aktiwiteit getoon by hoer temperature. Die twee ensieme het verskilende grade van halotoleransie getoon. Die LMK008 β-mannanase kon hoer NaCl konsentrasies verduur met 60% aktiwiteit na inkubasie vir 2 ure by 20% NaCl, waa die LMK004 β-mannanase slegs aktief was tussen 0% - 10% NaCl. Uit hierdie studie blyk dit duidelik dat die twee stamme van *S. candida* erskillende β-mannanases produseer wat nuttig mag wees in reaksies by lae wateraktiwiteite.

Appendix A

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Current Microbiology
An International Journal

Screening and Identification of Endomannanase-Producing Microfungi from Hypersaline Environments

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Abstract. A culture-dependent enrichment technique was used to isolate endo-1,4-β-mannanase–producing fungi from a hypersaline environment. Galactomannan was used as carbon source and resulted in isolation of strains of *Scopulariopsis brevicaulis*, *S. candida*, and *Verticillium dahliae*. The *Scopulariopsis* isolates were found to be more dominant and could be isolated from consecutive evaporation ponds, whereas *Verticillium* was only isolated from one pond. The *Scopulariopsis* strains exhibited only endomannanase activity, whereas *Verticillium* displayed broad-activity spectrum by secreting endoxylanases and cellulases in addition to endomannanases. *S. candida* LMK004 and LMK008 produced 7420 and 14750 nkat g^{-1} biomass, respectively. Endomannanase production in these strains increased with an increase in NaCl concentration up to 10% (w/v), after which both growth and enzyme production was decreased. *V. dahliae* LMK006 grew and produced up to 5000 nkat g^{-1} biomass endomannanase in the absence of NaCl. Increased NaCl concentration had a negative effect on this strain. *S. brevicaulis* LMK002 showed poor endomannanase production but a similar growth trend as the other *Scopulariopsis* strains. In general, the *Scopulariopsis* strains exhibited better halotolerance than *V. dahliae* and could grow in the presence of 20% NaCl on solid medium.

Mannan and heteromannans are widely distributed in nature as part of the hemicellulose fraction in plant cell walls. (Galacto)glucomannans are significant structural components in angiosperms and gymnosperms [1] whereas galactomannans are largely present as storage carbohydrates in the endosperms of leguminous plants [2]. Complete hydrolysis of heteromannans mainly entails a cooperative action between endo-1,4-β-mannanases and the auxiliary enzymes, which are exo-β-mannosidases, 1,6-α-galactosidases and in some cases acetyl mannan esterases and β-glucosidases [3, 4]. Endomannanases are retaining glycoside hydrolases that randomly cleave the main chain β-1,4-mannosidic linkages to release mannooligosaccharides of various lengths. Compared with the auxiliary enzymes, these enzymes are often produced in larger amounts by most fungi and are widely used in several industrial applications, including animal feed industries, which use corn-soybean meal and guar meal–based poultry diets [5, 6], and more recently the detergent industry [7]. Although molds are regarded as efficient producers of extracellular enzymes, only a few fungal endomannanases have been purified and characterized.

Hypersaline environments—including solar salterns, natural inland lakes, and the Dead Sea—have been shown to house a wide variety of molds [8–10]. The prevailing mycobiota comprises halotolerant terrestrial species, which are generally widely distributed in various soil types. Amongst them are several lignicolous and cellulolytic phytopathogens of the genera *Alternaria, Ulocladium, Humicola, Phoma*, and *Cladosporium*, for which soil is only a temporary habitat [8–10]. This pool of mycobiota could contain an untapped source of potential endomannanase-producing fungi. Therefore, the purpose of the current study was to use routine enrichment culture techniques to isolate and identify endomannanase-producing molds from a hypersaline environment and to investigate the effect of NaCl on growth and enzyme production.

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Materials and Methods

Description of the salt pan. The current study investigated the cultivable mycobiota of a small-scale salt work in the Free State province of South Africa. The solar salterns form one of the small localities around the Florisbad salt pan, northwest of the city of Bloemfontein, where farmers produce salt through solar evaporation of brines. The high salt season occurs during October and November. The salterns investigated consist of four consecutive evaporator ponds and five crystallizer ponds. Salt is produced through four phases. Phases one, two, and three are evaporation phases where brine is drawn from underground with boreholes into the first pond. The brine is allowed to evaporate during phases two and three, after which it is pumped into the crystallizer ponds for phase 4, where it is allowed to evaporate completely, leaving salt crystals on the floor of the ponds. At the time of sampling, the pH of the brine varied between 6.9 and 8.7 throughout the different phases, whereas the temperature was between 21°C and 26°C. The Institute on Groundwater studies (University of the Free State, South Africa) analyzed the mineral composition of the brine samples. The brine in the ponds contained Na^+ and Cl^- as the major ions followed by Ca^{2+}, Mg^{2+}, SO_4^{2-}, and K^+ (Table 1). The redox potential of the inlet brine was +200 mV at the time of sampling.

Sample collection and enrichment. Mud and brine samples were collected from three evaporator ponds and two crystallizer ponds in sterile 50-ml Falcon centrifuge tubes. Brine samples, including the inlet brine at the first evaporator pond, and mud samples from three evaporator ponds were enriched by mixing 10 ml sample with 10 ml media containing 1% yeast extract and 1% locust bean gum (Sigma Chemical, St. Louis, MO) in 250 ml Erlenmeyer flasks. Soil samples from crystallizer ponds and one from the last evaporator pond were enriched with media containing only 1% yeast extract. The flasks were incubated at 26°C with shaking. Samples were collected after 1 week of incubation, and 200 µl were plated out on the following media: malt extract agar plates supplemented with 100 µg/ml chloramphenicol (MEA-C) and 2.5%, 5%, and 10% (w/v) NaCl, respectively. Ten milliliters of media containing 1% yeast extract and 1% glucose were added to the flasks, and incubation was continued for 2 more weeks with samples collected weekly and plated out as described above. Plates were assessed on a daily basis to monitor growth. Fungal isolates obtained from MEA-C plates were purified through subsequent streaking on malt extract agar (MEA) plates supplemented with 2.5% to 10% NaCl.

Identification of fungal isolates. Microscopic observation was performed to determine the morphological characteristics of the fungal isolates. The isolates were also sent to Centraalbureau voor Schimmelcultures (CBS) for further phenotypic characterization. In addition, the isolates were cultivated in 10 ml medium containing 1% yeast extract, 2% bacteriological peptone, and 2% glucose), and genomic DNA was isolated according to Raeder and Broda [11]. Universal primers internal transcribe spacer (ITS) 5 and ITS 4 [12] were used to amplify the gene encoding the ITS regions (1 and 2) together with the 5.8S rRNA by polymerase chain reaction (PCR). The resulting amplicons were purified using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences UK Limited), ligated to pGEM®-T Easy Vector System (Promega Corporation, Madison, WI) and sequenced using the T7 and SP6 primers. The sequencing reactions were performed using the ABI PRISM® BigDye™ Terminator v3.0 Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA) and analyzed using the ABI PRISM™ 377 DNA Sequencer. Identification of some of the isolates was confirmed by amplification and sequencing of the D1/D2 domain of the large subunit (26S) ribosomal DNA using NL-1 and NL-4 primers [13].

Table 1. Mineral composition of brine before it enters into the first evaporation pond and the brine during the crystallization period

Minerals (g L^{-1})	Inlet brine	Phase 4
Calcium (Ca^{2+})	0.86	0.26
Magnesium (Mg^{2+})	0.80	10.40
Sodium (Na^+)	40.00	114.00
Potassium (K^+)	0.10	1.47
Chlorine (Cl^-)	63.30	197.50
Suphate (SO_4^{2-})	0.64	31.70

Screening for enzyme production and NaCl tolerance. The axenic isolates were streaked on MEA plates, and growth was monitored at different salt concentrations ranging from 2.5% to 20% (w/v) NaCl. Hydrolytic properties were determined by point-inoculating fungal isolates in parallel on agar plates containing 2.5% NaCl, 1% yeast extract, and 1× Vogel's medium prepared from a 50× concentrated solution with the following composition per liter of distilled water (130 g Na_3citrate $2H_2O$; 126 g KNO_3; 144 g $(NH_4)H_2PO_4$; 80 g KH_2PO_4; 10 g $MgSO_4$ $7H_2O$; 5 g in 20 ml water $CaCl_2$ $2H_2O$ added dropwise; 5 mL trace elements; and 2.5 mL 0.1 mg/mL biotin solution) [14]. This medium was then supplemented with either 0.5% (w/v) locust bean gum for determination of endo-1,4-β-mannanase activity, 0.1% (w/v) Remazol Brilliant Blue D-xylan (Sigma) for endo-1,4-β-xylanase activity, or 1% (w/v) carbomethyl cellulose for cellulase activity. Endo-1,4-β-mannanase and cellulase activities were observed after hydrolysis zones around the colonies were resolved by staining plates with 0.1% (w/v) Congo red solution and destaining with 1 M NaCl.

The effect of NaCl on growth and endo-1,4-β-mannanase. Endo-1,4-β-mannanase–producing isolates were maintained on potato dextrose agar (PDA) slants. The isolates were streaked out on agar medium containing 1× Vogel's medium with 0.5% locust bean gum and incubated at 30°C for 7 days. Agar pieces (1 cm^2) were cut out and used to inoculate 100 ml liquid medium containing 1% locust bean gum and (0% to 20%) NaCl. The cultures were incubated at 26°C with shaking at 120 rpm, and samples were collected daily to determine biomass (mycelial dry weight) formation and enzyme production. Endomannanase activity was determined using 0.5% locust bean gum prepared in citrate buffer pH 5.3 according to the method described by Stålbrand et al. [15]. The enzyme (0.1 mL) was added to 0.9 ml of the substrate pre-equilibrated to 50°C. The reaction was carried out for 10 minutes at 50°C, and reducing sugars were then determined by adding 1.5 mL DNS reagent, boiling for 10 minutes followed by measuring absorbance at 540 nm. Mannose was used to prepare a standard curve. The DNS reagent was prepared by sequentially dissolving 10 g dinitrosalicylic acid (Sigma), 16 g NaOH, and 300 g potassium sodium tartrate in 600 ml distilled water while slowly warming the mixture to 45°C. The solution was then made up to 1000 mL with distilled water. Enzyme activities are expressed in nkat (1 nkat = 0.06 IU).

Results and Discussion

Isolation and screening. Enrichment culture technique was used to isolate endo-1,4-β-mannanase–producing fungi in mud samples from a salt pan. After 3 weeks of enrichment fungal isolates belonging to the genera *Scopulariopsis* and *Verticillium* were obtained.

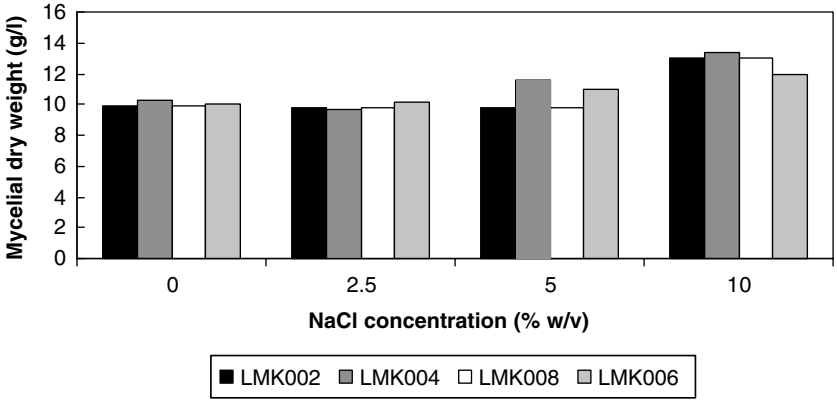

Fig. 1. The effect of NaCl on growth and biomass production in *S. brevicaulis* LMK002, *S. candida* LMK004 and LMK008, and *V. dahliae* LMK006.

Scopulariopsis species were more dominant and could be isolated from three consecutive evaporation ponds. After phenotypic characterization the isolates were confirmed as strains of *S. brevicaulis* Bainier, *S. candida* (Guéguen) Vuillemin, and *V. dahliae* Klebahn, and were therefore designated *S. brevicaulis* LMK002, *S. candida* LMK004, *S. candida* LMK008, and *V. dahliae* LMK006. *S. candida* LMK004 was added to the CBS culture collection with accession number CBS 118736. The *Scopulariopsis* isolates displayed halotolerance and the ability to grow in the presence of NaCl up to 20% (w/v) on solid medium. The isolates only secreted endomannanases into the external medium, and no endoxylanase or cellulase activity could be detected. In contrast, the growth of *V. dahliae* was decreased at NaCl concentrations above 10% (w/v). This organism had endomannanase, endoxylanase, and cellulase activities. *Scopulariopsis* spp. are common soil saprophytes, and a number of the species including *S. brevicaulis, S. brumptii*, and *S. chartarum* have previously been isolated from other hypersaline environments such as the Dead Sea [8, 10]. *V. dahliae* is a plant pathogen known to cause verticillium wilt in a wide variety of plants. This organism has never been isolated from hypersaline environments before and only one species of *Verticillium*, *V. luteoalbum*, has been recovered from the Dead Sea [8]. The sequence for the ITS region of *V. dahliae* LMK006 was submitted to GenBank and has been allocated accession number DQ282123. The isolates obtained in this study support the suggestion that the mycobiota of most hypersaline environments consists of halotolerant fungal species, which are distributed worldwide in various soil types rather than true halophilic species.

Growth and endomannanase production. Four isolates designated *S. brevicaulis* LMK002, *S. candida* LMK004, *S. candida* LMK008, and *V. dahliae* LMK006

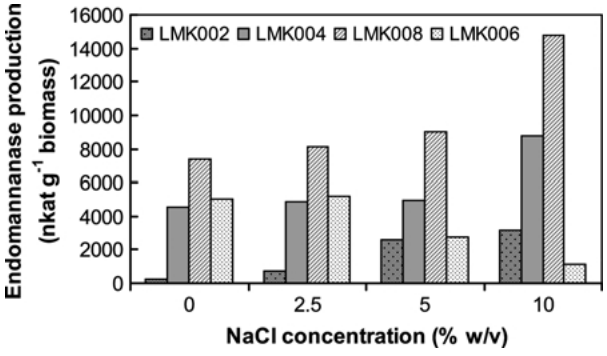

Fig. 2. Shows the amount of endomannanase that was produced by *S. brevicaulis* LMK002, *S. candida* LMK004 and LMK008, and *V. dahliae* LMK006 when cultivated on locust bean galactomannan in the presence of different concentrations of NaCl.

were cultivated in liquid media, and the relationship between NaCl content, growth, and endomannanase production was assessed. The isolates were not affected by the increase in NaCl and produced similar amounts of dry biomass (12 to 14 g L^{-1}) when growing at 0% to 10% NaCl, above which growth became severely decreased, and no biomass could be observed or measured within 7 days of cultivation (Fig. 1). Endomannanase production in *Scopulariopsis* isolates increased with increase in NaCl content reaching the highest levels at 10% NaCl (Fig. 2). *S. brevicaulis* LMK002 produced low amounts of endomannanases reaching a high of only 320 nkat g^{-1} biomass when grown on 10% NaCl, whereas *S. candida* LMK 004 and LMK008 produced 7420 and 14750 nkat g^{-1} biomass, respectively. In contrast, *V. dahliae* produced more endomannanase in the absence of NaCl and showed a decrease in enzyme production as NaCl content increased. Endomannanase production levels in this strain decreased five-fold from 5000 nkat g^{-1} biomass in

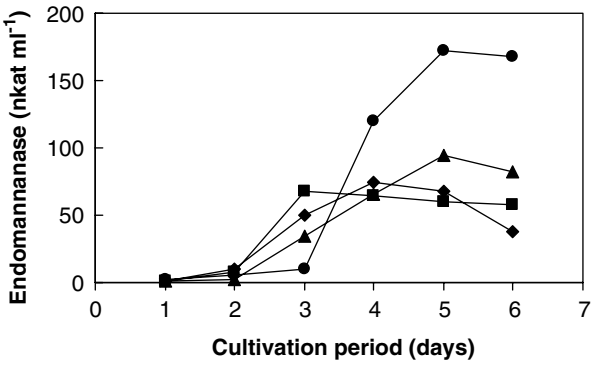

Fig. 3. Time course of endomannanase production from *S. candida* LMK008 at 0% NaCl (closed triangle), 2.5% NaCl (closed square), 5% NaCl (closed diamond), and 10% NaCl (closed circle) in the presence of 1% locust bean gum galactomannan.

Table 2. A comparison of endomannanase production in some molds

Organism	Mannanase levels (nkat ml^{-})	Reference
Sclerotium rolfsii	2591	[16]
S. rolfsii CBS 191.62	55	[17]
Aspergillus niger ATCC 46890	56	[18]
A. niger NCH-189	131	[19]
A. fumigatus IMI 385708	668	[20]
Thermomyces lanuginosus CBS 395.62	247	[21]
S. Candida LMK004 (CBS 118736)	104	This study
S. Candida LMK008	172	This study
V. dahliae LMK006	56	This study

the absence of NaCl to 1000 nkat g^{-1} biomass at 10% NaCl. Volumetric endomannanase production in *S. candida* LMK008 increased steadily for 4 to 5 days reaching a maximum level, after which a decrease was observed (Fig. 3). The amount of endomannanase produced increased from 75 nkat mL^{-1} at 0% NaCl to 172 nkat mL^{-1}. Similar trends were observed with *S. candida* LMK004 (data not shown). The activity levels obtained in the current study using *Scopulariopsis* isolates is comparable with some of the best known endomannanase producers previously reported (Table 2), indicating that extreme environments such as salt pans could provide an untapped resource of fungal isolates that may produce enzymes of interesting industrial potential. Endomannanases from both *Scopulariopsis* and *Verticillium* species have never been characterized previously, thus our future research will focus on optimization of cultivation conditions to achieve better production levels and purification of the enzymes.

ACKNOWLEDGMENTS

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