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Full Length Research Paper

Partial purification and characterization of endo-β-1,4-mannanases from *Scopulariopsis candida* strains isolated from solar salterns

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Scopulariopsis candida **strains LMK004 and LMK008 previously isolated from a solar saltern were cultivated in Vogel's medium supplemented with NaCl and locust bean gum galactomannan as carbon source and inducer for β-mannannase production.** ***S. candida*** **LMK004 produced up to 180 nkat/ml whereas LMK008 produced 116 nkat/ml. These levels dropped significantly when α-cellulose was used as carbon source. Both enzymes were partially purified by ammonium sulphate precipitation and anion-exchange chromatography. The molecular mass of LMK004 and LMK008 β-mannanases were estimated to be 41 and 28 kDa, respectively. 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 h of incubation at 4°C. In contrast, the LMK008 β-mannanase retained ≥ 60% activity between pH 6 – 7. Both enzymes remained stable for 3 h between 30 and 40°C, and showed loss of activity at higher temperatures. The LMK008 β-mannanase tolerated high NaCl concentrations with 70% activity remaining after incubation for 2 h at 20% NaCl, whereas the LMK004 β-mannanase was only active between 0 - 10% NaCl. The current study shows that fungi that inhabit hypersaline environments produce plant cell wall degrading enzymes that display similar properties to other fungi from low-salt environments.**

Key words: Endo-β-1,4-mannanase, halophiles, mannan, *Scopulariopsis candida*, solar salterns.

INTRODUCTION

Fungal degradation of plant biomass in hypersaline environments has not yet been investigated especially since fungi are not generally regarded as common inhabitants of such environments. However, several researchers have shown that fungi are present in solar salterns and evaporator ponds either as transient populations or as normal microbiota (Cantrell et al., 2006, Mudau and Setati, 2006, Grishkan, 2004). These and other studies have confirmed that fungi are common inhabitants of a wide variety of hypersaline environments. It is therefore necessary to understand the role of these fungi in decomposition of plant biomass and to explore the biotechnological potential of the enzymes they produce.

Mannan-based polysaccharides are complex heteropolymers that occur in the hemicellulose fraction of plant cell walls mostly in the form of glucomannan and galactoglucomannan (De Vries, 2003), and also as storage polymers in the form of galactomannan in the seeds of leguminous plants (Lee et al., 2005). The complete degradation of these polymers requires a cocktail of enzymes including endo-β-1,4-mannanases (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), α-galactosidase (EC 3.2.1.22), β-glucosidase (EC 3.2.1.21) and acetylmannan esterase (EC 3.1.1.6). β- Mannanase initiates depolymerization by randomly hydrolyzing the β-1,4-linkages within the mannan backbone releasing manno-oligosaccharides of various lengths (Franco et al., 2004), while β-mannosidase and α-galactosidase remove termi-

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nal mannose and galactose residues from the mannan backbone, respectively (Stålbrand et al., 1993).

Over the years, there has been an increasing interest in the potential application of β-1,4-mannanases in industrial processes such as production of animal feed (Wu et al., 2005; Lee et al., 2005) and laundry detergents (Schäfer et al., 2002; McCoy, 2001). These enzymes have been purified from bacteria (Kataoka and Tokiwa, 1998; Jiang et al., 2006; Tamaru et al., 1995; Zakaria et al., 1998; Takeda et al. 2004), fungi (Johnson et al., 1990; Ferreira and Filho, 2004; Puchart et al., 2004; Ademark et al., 1998; Stålbrand et al., 1993) and animals (Xu et al., 2002).

In the current study we report on the purification and characterization of endo-1,4-β-mannanase from two *S. candida* strains isolated from a hypersaline environment. *Scopulariopsis* is an anamorphic genus comprising mainly soil species. 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; Mudau and Setati, 2006; Cantrell et al., 2006). There are currently no reports on mannan degrading enzymes from this genus. The current study aimed to investigate the effect of NaCl concentration and carbon source on β-mannanase production as well as determining the biochemical characteristics of purified β-mannanases from two *S. candida* strains.

MATERIALS AND METHODS

Fungal isolates

S. 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.

Effect of NaCl and media composition

Stock cultures were streaked on agar plates containing 1× Vogel's medium or 2.5% salt water medium, 0.5% (w/v) locust bean gum galactomannan (LBG) (Sigma, St. Louis, MO, USA) as carbon source and 1% (w/v) yeast extract (Merck Chemicals, Darmstadt, Germany) as nitrogen source and incubated at 30°C for 5 days. Vogel's medium (1×) was prepared from a 50 × stock solution which was prepared by dissolving in (g/l) 130 $Na_3C_6H_5O_7.2H_2O$, 126 KNO_3, 144 $(NH_4)H_2PO_4$, 80 KH_2PO_4, 10 $MgSO_4.7H_2O$]. 5 g of $CaCl_2.2H_2O$ was dissolved in 20 ml distilled water and added dropwise into the solution; 2.5 ml of a 0.1 mg/ml biotin solution dissolved in 50% (v/v) ethanol was also added. Trace elements were prepared by dissolving in (g/l) 5 $H_3C_6H_5O_7.H2O$, 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). 5 ml of trace elements and 3 ml chloroform were added to the mineral solution and the mixture was made up to 1 L with distilled water.

Agar pieces (1 cm^2) were cut out from the plates and used to inoculate 100 ml liquid media containing 1× Vogel's medium with 0 to 20% NaCl, 1% LBG and 1% yeast extract. The inoculated flasks were incubated at 25°C while shaking at 150 rpm. Samples were collected daily and β-mannanase production was determined using the culture filtrate. To determine the effect of carbon source on β-mannase production, LMK004 and LMK008 cultures were used to inoculate 100 ml liquid media containing 1% yeast extract, 10% NaCl and 1× Vogel's medium and either 1% LBG, 1% or 2% α-cellulose (Sigma) as a carbon source.

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 × *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°C for 10 min, followed by determination of reducing sugars using modified dinitrosalicylic acid (DNS) method (Miller, 1959). Mannose was used as a standard and β-mannanase activity was expressed in nkat (1 nkat = 0.06 IU) where 1 nkat is defined as the amount of enzyme required to release 1 nmol of reducing sugars per second per milliliter at 50°C.

β-Mannanase purification

S. 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 1500 × *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^{-1}. The fractions were assayed for β-mannanase activity. Protein concentrations in the samples before and after purification were determined using Micro BCA™ protein assay reagent kit (Pierce).

Gel electrophoresis and zymogram analysis

One milligram per millilitre of culture filtrates from LMK004 and LMK008 were loaded onto isoelectric focusing (IEF) precast gels (Bio-Rad) to determine the pI of the proteins and to identify which ion exchange column would be suitable for purification purposes. The samples were run for 1 h: 30 at 200V, followed by 1 h 30 min at 400 V. A substrate gel was prepared by adding 2% agarose to a 0.5% locust bean gum solution and heating until the agarose dissolved. The mixture was cast into a glass Petri-dish and allowed to solidify. After IEF the gel was placed on the substrate gel and incubated at 50°C for 1 h followed by staining of the substrate gel using 0.1% (w/v) Congo red solution for 2 h. The substrate gel was then destained using 1 M NaCl and then transferred to 0.5% acetic acid to enhance clarity of hydrolysis zones that indicated β-mannanase activity (Stålbrand et al., 1993).

One millilitre of purified samples was freeze-dried, resuspended in 100 µl citrate-buffer pH 5 and dialysed for 2 h. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and native poly-acrylamide gel electrophoresis (Native-PAGE) in conjunction with zymogram were employed to asses the purity of the fractions and to determine the molecular masses of the proteins.

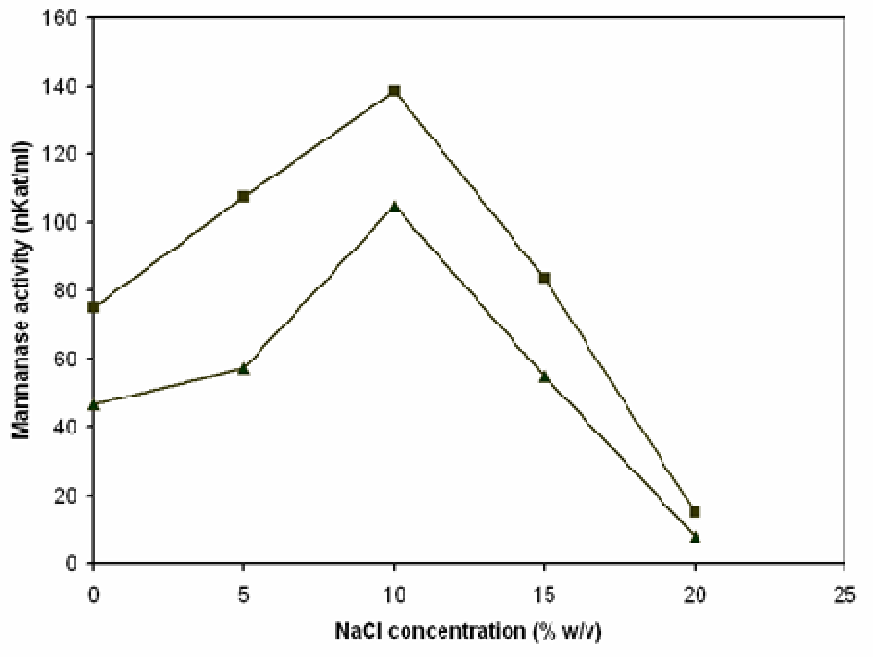

Figure 1. The effect of NaCl on the production of β-mannanase in *S. canadida* LMK004 (▲) and LMK008 (■).

30 µl 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. 20 µl of each sample was 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 mass of the proteins. To confirm the activity and molecular mass of the purified proteins, undenatured protein samples were separated on a 10% Native-PAGE followed by Zymogram. The native gel was placed on a substrate gel which was prepared as described in the previous paragraph for the IEF gel. The procedure for staining and destaining was followed.

Determination of temperature and pH optimum and stability

The temperature optima of the purified β-mannanases were determined by incubating the enzymes with 0.5% locust bean gum galactomannan substrate for 10 min at different temperatures ranging from 30 – 70°C at pH 5, followed by determination of reducing sugars. Temperature stability was determined by incubating the enzyme samples at the same temperatures in 50 mM citrate buffer pH 5 for 3 h without the substrate, 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 - 6 and 50 mM citrate-phosphate buffer was used for pH 6 - 7. To study the stability of purified β-mannanase samples at different pH, appropriate dilutions of β-mannanase were made in the buffers in the range of pH 3 – 7 and incubated at 4°C for 24 h. The residual activities were estimated under standard β-mannanase assay conditions.

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 h 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.

RESULTS AND DISCUSSION

β-Mannanase production on different media compositions

The production of β-mannanase by *S. candida* LMK004 and LMK008 in media containing various NaCl concentrations and the effect of carbon source on enzyme production was evaluated. In both strains, β-mannanase production increased proportionally with increasing salt concentration (Figure 1). However, NaCl concentrations above 10% (w/v) resulted in reduced β-mannanase production levels. The effect of carbon source was determined using the medium containing 1× Vogel's medium (Metzenberg, 2003) with 10% NaCl, 1% yeast extract, and either LBG or α-cellulose as carbon sources. Both strains produced more mannanase while growing on locust bean gum, reaching levels of 180 nkat/ml for LMK004 and 116 nkat/ml for LMK008 (Figure 2). Both strains produced more mannanase while growing on locust bean gum, reaching levels of 180 nkat/ml for LMK004 and 116 nkat/ml for LMK008. Although α-cellulose was previously reported to be the best inducer for the β-mannanase production in *Sclerotium rolfsii* (Sachslehner et al., 1998), it did not have the same effect of *S. candida*. The production of β-

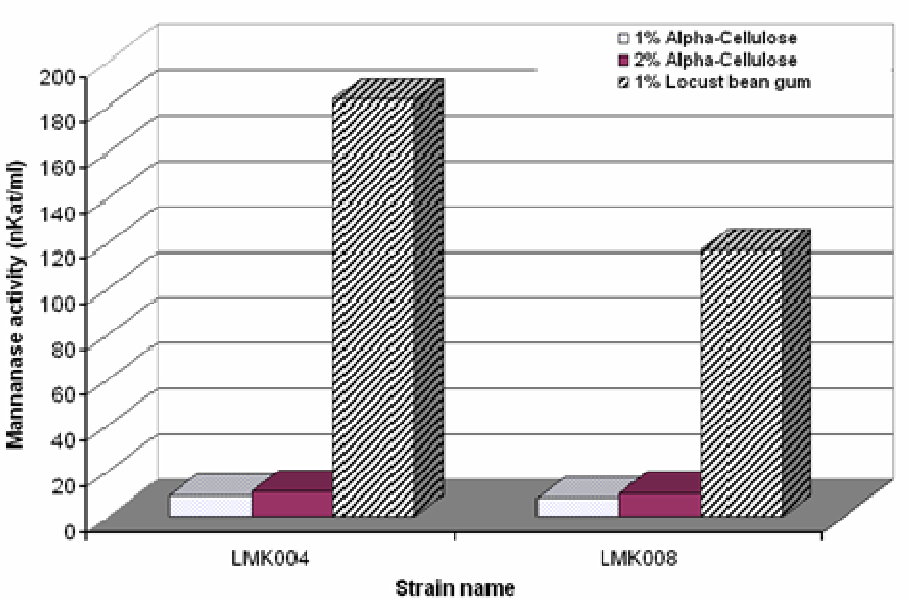

Figure 2. The production of β-mannanase by *S. candida* LMK004 and LMK008 when cultivated on either α-cellulose or locust bean gum galactomannan as carbon source.

mannanases in both LMK004 and LMK008 was significantly reduced when α-cellulose was used as carbon source. For instance, LMK004 produced only 9 and 11 nkat/ml when cultivated on 1 and 2% α-cellulose whereas LMK008 produced up to 8 and 10 nkat/ml, respectively (Figure 2). These levels are almost eight times less than when both strains were cultivated on locust bean gum.

Purification and characterization of β-mannanases

The β-mannanases from *S. candida* LMK004 and LMK008 both displayed acidic plus and were therefore purified from culture 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 to 27 865 nkat/mg while only 5.7 purification fold was achieved for LMK008 resulting in a specific activity of 3321 nkat/mg (Table 1). SDS-PAGE analysis of the purified LMK004 β-mannanase revealed an intense band of approximately 41 kDa and two faint bands of 27 and 17.15 kDa (Figure 3). The 41 kDa band was confirmed to be a β-mannanase through native-PAGE in conjunction with zymogram and activity staining (data not shown). In contrast, the β-mannanase from LMK008 was found to have a molecular mass of 28 kDa (Figure 3). 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).

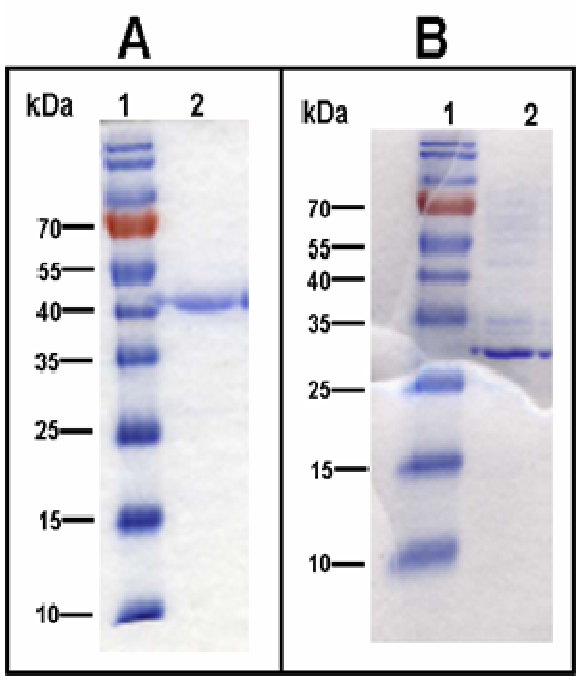

Figure 3. SDS-PAGE analysis of partially purified β-mannanase from LMK004 (A) and LMK008 (B). Lane 1 shows the molecular weight markers and lane 2 is the protein sample.

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-manna-

Table 1. Summary of the purification of β-mannanases from *S. candida* LMK004 and LMK008.

Purification step	Volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (nkat mg^{-1})	Protein yield (%)	Degree of purification
***S. candida* LMK004**						
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
***S. candida* LMK008**						
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

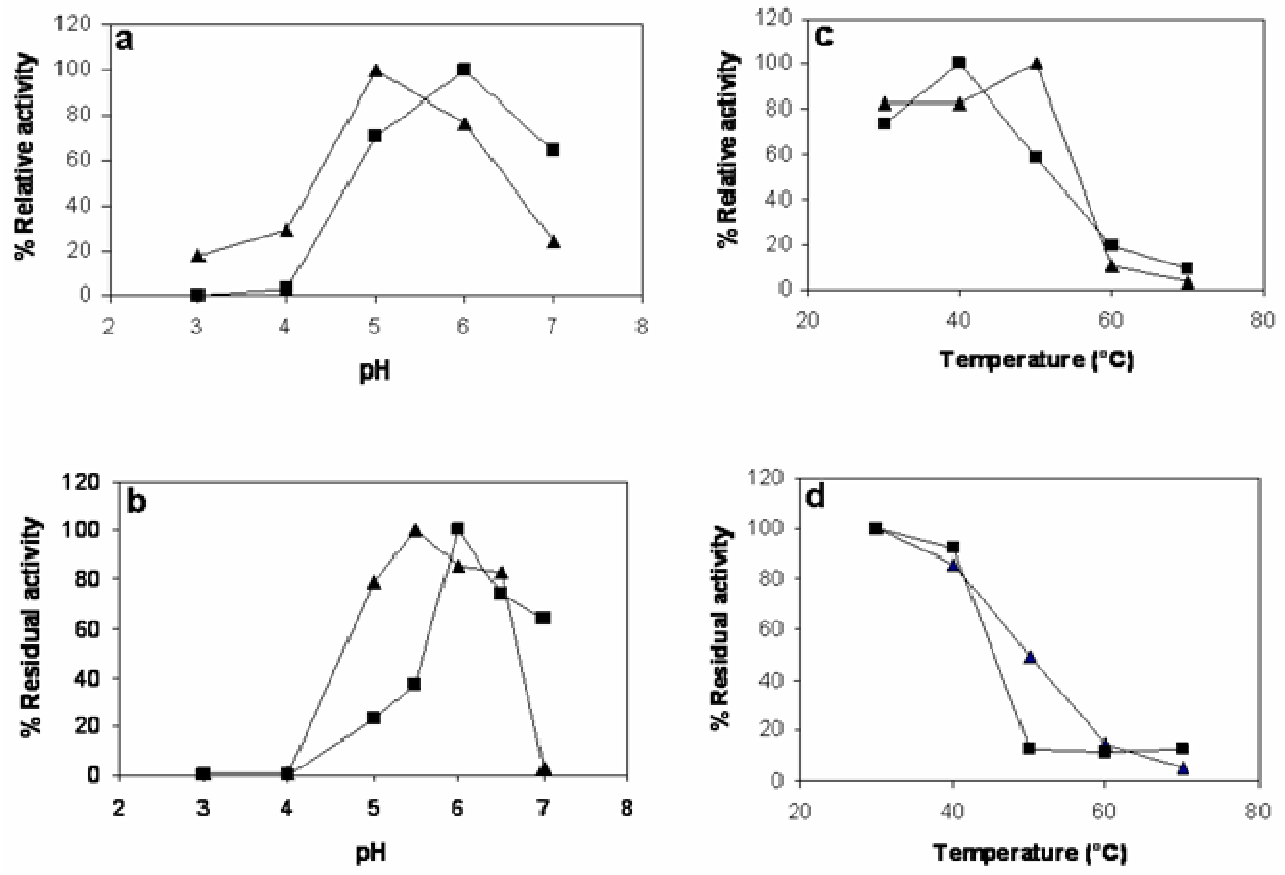

Figure 4. Activity profile of the partially purified β-mannanases from *S. candida* LMK004 (▲) and LMK008 (■) showing the influence of pH on the activity (a) and stability (b) as well as the effect of temperature on the activity (c) and stability (d) of the two enzymes.

nase from LMK004 showed optimal activity at pH 5 whereas that of LMK008 was most active at pH 6 (Figure 4a). The LMK004 β-mannanase was stable at pH 5 - 6.5 while LMK008 was stable at pH 6 - 7 (Figure 4b). The β-mannanase from LMK004 and LMK008 displayed optimal activity at 50 and 40°C, respectively (Figure 4c). Both en-

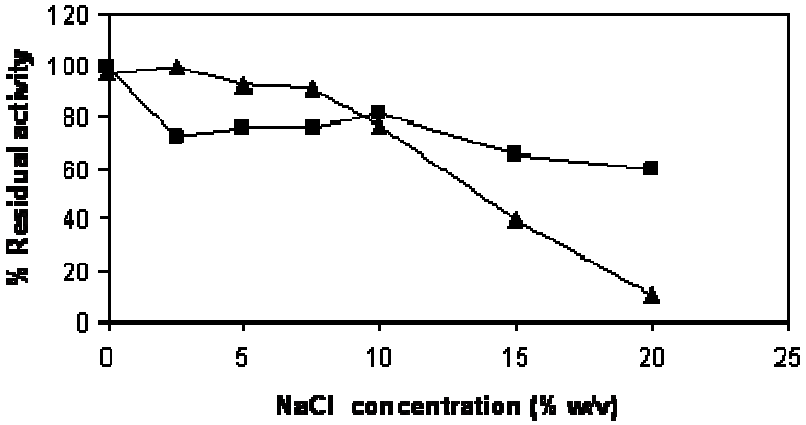

Figure 5. The effect of NaCl on the stability of β-mannanases from *S. candida* strains LMK004 (▲) and LMK008 (■) at pH 5 and 4°C.

zymes remained stable for 3 h at temperatures between 30 and 40°C, and showed rapid loss of activity at higher temperatures (Figure 4d). The β-mannanases reported so far exhibit acidic to neutral pH optima, molecular masses ranging from 33 to 90 kDa and temperature optima between 40 and 70°C with a few exception of thermophilic β-mannanase (Araujo and Ward, 1990; Stålbrand et al., 1993; Puchart et al., 2004). The application of these enzymes in various industrial processes such as those within the pulp and paper industries is often hampered by the fact that they turn to be less stable under the prevailing conditions. Therefore, this necessitates a quest for new enzyme sources especially from extreme environments.

The effect of NaCl on β-mannanases was determined by incubating the enzyme samples at 4°C for 2 h 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 (Figure 5). The level of halotolerance observed with both enzymes is similar to other polysaccharide hydrolysing enzymes from halophilic archaea, bacteria and halotolerant fungi. They 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).

Conclusions

β-Mannanases are currently used in low water activity industrial applications such as processing and manufacture of instant coffee (Sachslehner et al., 2000) as well as in poultry feeds (Lee et al., 2005). The current study shows that the fungi in hypersaline environments produce and secrete plant cell wall degrading enzymes (specifically mannanases) with similar biochemical properties to other fungi that prevail in low-salt environments. 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 that the two strains of *S. candida* produce distinct β-mannanases which can possibly be attributed to the fact that these strains were isolated from separate evaporator ponds and therefore could produce enzymes which might be active under slightly different conditions. Therefore, future research will investigate sequence similarities, amino acid composition and structure prediction.

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