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Full Length Research Paper

Isolation of hydrolase producing bacteria from Sua pan solar salterns and the production of endo-1, 4-β-xylanase from a newly isolated haloalkaliphilic *Nesterenkonia* sp.

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Eighty seven bacterial isolates were obtained from evaporator ponds using culture enrichment technique and screened for xylanase, mannanase and cellulase activity. Based on biochemical and phenotypic characteristics, the isolates were divided into 18 groups. Thirteen groups were *Bacillus* species, four were *Halomonas* species, while one group belonged to the genus *Nesterenkonia*. Four *Bacillus* isolates, Sua-BAC005, Sua-BAC012, Sua-BAC017 and Sua-BAC019, as well as *Nesterenkonia* sp. Sua-BAC020 were studied further. Isolate Sua-BAC005 affiliated with *Bacillus amyloliquefaciens* secreted 12.6 U/ml and 9.0 U/ml β-mannanase and β-xylanase, respectively, while isolates Sua-BAC012, Sua-BAC017 and Sua-BAC019 affiliated with *Bacillus licheniformis*, produced less than 2 U/ml of xylanase, cellulase and mannanase. *Nesterenkonia* sp. Sua-BAC020 grew at 0 – 20% NaCl with an optimum at 2.5% NaCl, and at pH 7 – 9.5 with an optimum at pH 9. This isolate produced 3.5 U/ml xylanase when cultivated at pH 8 in 10% NaCl. Five xylanase activity bands were detected on Native-PAGE coupled with zymogram.

Key words: *Halomonas*, xylanase, halophiles, alkaliphiles, xylanase multiplicity.

INTRODUCTION

Hypersaline environments such as solar salterns/salt pans are inhabited by a broad spectrum of halophilic and halotolerant bacteria which are classified into four groups depending on the NaCl concentration required for optimal growth. Halotolerant organisms are those that grow best in media containing < 0.2 M NaCl but can tolerate higher concentrations, while the slight and moderate halophiles grow best between 0.2 M – 0.5 M NaCl and 0.5 M - 2.5 M NaCl, respectively. The extreme halophiles on the other hand grow optimally in media containing 2.5 to 5.2 M (saturated) NaCl (Ventosa et al., 1998). Gram negative halophilic bacteria are mainly represented by members of the genera *Halomonas*, *Chromohalobacter* and *Salinivibrio* while gram positive bacteria are often species of *Bacillus*, *Halobacillus*, *Marinoccocus* and *Salinicoccus* (Ventosa et al., 1998; Sánchez-Porro et al., 2003). Several bacterial species from hypersaline environments have been shown to possess a wide spectrum of hydrolytic enzymes including xylanases (Wejse et al., 2003), α-amylases (Coronado et al., 2000) and proteases (Sanchez-Porro et al., 2003). Some of these enzymes such as xylanases do not only show increased activity in the presence of NaCl but are also stable within a broad pH and temperature range compared to their non-halophilic counterparts (Wejse et al., 2003).

Microbial xylanases in particular have vast industrial applications in the food industry (e.g. baking and processing of fruit juices), in the feed industry as well as technical industries such as paper and pulp, and textiles (Collins et al., 2005). Although the demand for enzymes continues to grow, most enzymes applied in industrial processes are mainly derived from mesophilic and/or neutrophilic origins with only a few extremophilic enzy-

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mes characterized. Recently, xylanases have been purified from halophilic microorganisms (Wainø and Ingorvsen, 2003; Wejse et al., 2003; Collins et al., 2005). The growing interest in halophilic enzymes is mainly due to their potential application in low water activity reactions such as the production of soy sauce (Hashimoto and Nakata, 2003).

The genus *Nesterenkonia* comprises Gram positive non-encapsulated, non-endospore forming, moderately halophilic or halotolerant species which may either be coccoid or short rods. Members of this genus may be either alkaliphilic or alkalitolerant, and are generally mesophilic with optimum growth at temperatures between 28–37°C (Li et al., 2005; Delgado et al., 2006; Li et al., 2008). *Nesterenkonia* spp. have been isolated from soda lakes, saline and non-saline alkaline soil, and commonly form part of the microbial population in hypersaline environments (Tiago et al., 2004). Some species such as *Nesterenkonia halobia* have been shown to produce industrially relevant enzymes such as amylases (Sanchez-Porro et al., 2003), while other species secrete unique serine proteases (Yang et al., 2008). However, no detailed studies have yet been performed on the production of hydrolytic enzymes from this genus. Therefore, the current study describes the isolation of hydrolase producing bacteria from evaporator ponds at Sua pan in Botswana, with more emphasis on the production of xylanase by a newly isolated *Nesterenkonia* species.

MATERIALS AND METHODS

Isolation and screening for hydrolases

A culture enrichment technique was used to isolate halophilic and halotolerant eubacteria from Sua pan evaporator ponds in Botswana. Fifty millilitres of brine samples were mixed with 50 ml of double strength broth containing 2% yeast extract. The samples were incubated at 37°C with shaking for 7 days. Eubacteria were subsequently isolated by spread-plating on 10% salt water (SW-10) medium containing in (g/l) 80 NaCl, 11.7 $MgSO_4.7H_2O$, 10 $MgCl_2.6H_2O$, 0.17 $CaCl_2.2H_2O$, 2.3 KCl and 20 bacteriological agar (Dyall-Smith, 2000). Pure isolates were screened for xylanase, cellulase and mannanase activity, on SW-10 agar supplemented with either, 0.5% carboxymethyl cellulose, 0.2% locust bean gum galactomannan or 0.2% birchwood xylan. The plates were incubated at 37°C for 48 h followed by staining with 0.1% (w/v) Congo red solution (Teather and Wood, 1982).

Phenotypic and biochemical characterization of bacterial isolates

Representative isolates were characterized and identified. Cellular morphology and spore formation were determined using standard Gram staining and endospore staining. Biochemical tests including nitrate reduction in liquid media, carbohydrate fermentation, catalase and oxidase production and Voges-Proskauer were performed using standard media amended with 3% (w/v) NaCl. Acid production from carbohydrates was determined in medium supplemented with 0.001% phenol red (Leifson, 1963). Cultures were incubated at 37°C for all tests.

16S rRNA sequence analysis

Genomic DNA was extracted from representative isolates according to Cheng and Jiang (2006). The 16S rRNA gene was PCR-amplified using the primer set E8F (5′-AGAGTTTGATCCTGGC TCAG-3′) and E1541R (5′-AAGGAGGTGATCCANCCRCA-3′) (Baker et al., 2003). PCR fragments were ligated into pGEM-T Easy (Promega) and cloned into *Escherichia coli* JM109 following routine procedures and sequenced using the ABI Big Dye Terminator Cycle sequencing kit 3.1. Separation of bases was carried out on a 3130XL genetic analyzer (Applied Biosystems). Sequence similarity searches were performed on the National Centre for Biotechnology Information (NCBI) database, using NCBI-BLASTN megablast.

Production of β-mannanase, β-xylanase and cellulase

Bacterial isolates (Sua-BAC005, Sua-BAC012, Sua-BAC017, Sua-BAC019, Sua-BAC020) that exhibited good production (hydrolysis zone greater than 8 mm) of β-xylanase, mannanase and cellulase during screening with plate assays were used for further studies. Pre-cultures for each of the four isolates were prepared by inoculating single colonies into 100 ml SW-10 broths and then incubated overnight at 37°C with shaking. The pre-cultures were used to inoculate 100 ml main cultures to an initial optical density of 0.01 absorbance units at 600 nm. The main cultures were prepared by inoculating SW broth, containing specific optimal salt concentrations for each strain, supplemented with the appropriate carbon source for each enzyme. For β-mannanase activity the medium was supplemented with 0.5% (w/v) locust bean gum, for β-xylanase activity the medium was supplemented with 1% (w/v) Birchwood xylan (Fluka), while for cellulase activity the medium was supplemented with 1% (w/v) carboxymethylcellulose. Samples were collected at specific time intervals and centrifuged at 12 000 × *g* for 10 min to remove the cells.

The supernatant was then assayed for β-mannanase, β-xylanase and cellulase activity. Enzyme activity was determined by measuring the release of reducing sugars from the above mentioned substrates using dinitrosalicylic reagent following the method described by Bailey et al. (1992). The substrate specific for each enzyme was prepared by boiling in 50 mM phosphate, pH 7.0 followed by continuous stirring overnight for birchwood xylan as well as locust bean gum. The assays were carried out by mixing 100 µl of culture supernatant with 900 µl of the substrate while incubating at 50°C for 10 min. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar (either, xylose, mannose or glucose equivalents) per minute per ml at 50°C.

The effect of NaCl and pH on growth of *Nesterenkonia* sp. Sua-BAC020

The pre-culture was prepared by inoculating a loop-full from 48 h old agar cultures into 100 ml LB broth containing 10% (w/v) NaCl in 250 ml Erlenmeyer flasks. The flasks were incubated at 37°C, with shaking at 160 rpm for 48 h. The pre-culture was used to inoculate 100 ml LB supplemented with different concentrations of NaCl to an initial optical density (OD_{600nm}) of 0.01 and incubated on a shaker at 37°C. Samples were collected at regular intervals over a cultivation period of 80 h for OD determination. Growth rates were determined from the slope of the exponential phase. Optimum pH for growth was determined in buffered LB using 50 mM phosphate buffer (pH

Table 1. Hydrolytic activities of bacterial isolates, collected from Botswana's Sua pan evaporator ponds.

Group	Number of isolates	Closest related species	Accession number	% Identity	XYL	CEL	MAN
BAC001	7	*Bacillus subtilis*	EU047884	99	++	–	+++
BAC002	3	*Bacillus licheniformis*	EU016215	99	++	+	++
BAC003	1	*Bacillus pumilus*	EU236743	100	+++	–	–
BAC004	6	*Bacillus pumilus*	EU239356	99	+++	–	–
BAC005	9	*Bacillus amyloliquefaciens*	EF433406	99	+++	+	+++
BAC006	4	*Bacillus licheniformis*	AY887129	99	+++	–	–
BAC008	2	*Bacillus licheniformis*	AY887129	99	–	–	–
BAC009	3	*Halomonas pacifica*	L42616	98	–	–	–
BAC010	6	*Halomonas pacifica*	L42616	98	–	–	–
BAC012	4	*Bacillus licheniformis*	CP000002	99	++	++	+++
BAC013	9	*Halomonas campaniensis*	AJ515365	99	–	–	–
BAC014	6	*Bacillus pumilus*	EU239356	99	–	–	+++
BAC015	3	*Halomonas pacifica*	L42616	98	–	–	–
BAC016	7	*Bacillus pumilus*	EU239356	99	++	++	+++
BAC017	5	*Bacillus licheniformis*	AY887129	99	+++	++	+++
BAC018	5	*Bacillus subtilis*	EU047884	99	+++	++	+++
BAC019	3	*Bacillus licheniformis*	AY887129	99	++	+++	+++
BAC020	4	*Nesterenkonia aethiopica*	AY574575	98	++	–	–

Diameter of hydrolysis zone (+ : 2 - 8 mm; +++ : 15 - 18 mm; ++ : 9 - 14 mm; – : No hydrolysis zone).

6-8) or 50 mM glycine-NaOH buffer (pH 8.6-9.5) while the NaCl concentration was maintained at 10% (w/v).

The effect of NaCl and pH on xylanase production

The production of xylanase was determined by incubating cultures in LB supplemented with 1% (w/v) birchwood xylan (Sigma-Aldrich), and 0-20% NaCl. Cultures were inoculated as described above. Samples were collected after 72 h and centrifuged for 10 min at 14550 × *g*, and the supernatant was assayed for xylanase activity. The effect of pH on xylanase production was monitored in buffered LB supplemented with 1% birchwood xylan. The NaCl concentration was maintained at 10% (w/v). Once the optimum pH and NaCl for growth and enzyme production were determined, they were combined and xylanase production was re-evaluated. *Nesterenkonia* sp. Sua-BAC020 was cultivated in LB medium supplemented with 1% birchwood xylan at 0% and 2.5% (w/v) NaCl, and pH 8. Samples were collected at 24 h intervals and assayed for xylanase activity.

Statistical analysis

Three flasks were inoculated for each experiment, triplicate samples were analysed per flask, and the experiments were performed three times. The results represent the means of three separate experiments.

Determination of xylanase multiplicity

The production of multiple xylanases was determined by cultivation of *Nesterenkonia* sp. Sua-BAC020 in LB broth (pH 7.5 and 10% [w/v] NaCl) containing 1% (w/v) oatspelt or birchwood xylan at 37°C, 160 rpm for 72 h. The supernatant was collected by centrifugation. Twenty millilitres of the samples were concentrated down to 500 µl using VIVASPIN centrifugal concentrators with a molecular weight cut-off of 10 kDa (Sigma-Aldrich). Twenty microlitre samples were loaded onto a 12% native-PAGE containing 0.13% birchwood xylan solution prepared in 50 mM phosphate buffer pH 7 (Ninawe et al., 2008). After electrophoresis, the gel was washed and stained 0.1% (w/v) Congo red.

Nucleotide sequence accession numbers

The 16S rRNA sequences of the representative isolates obtained in the current study were deposited in Gen Bank under the accession numbers EU870498 – EU870514 and FJ948172.

RESULTS

Hydrolytic potential and identification of bacterial isolates

In the current study, a total of 87 isolates were obtained through enrichment methods, and divided into 18 groups based on morphological characteristics and their ability to produce mannanases, cellulases and xylanases (Tables 1 and 2). All groups, except BAC008, BAC009, BAC010, BAC013 and BAC015, were capable of secreting one or more of the enzymes (Table 1). BLAST searches performed on the 16S rRNA gene fragments indicated that the isolates fall into three genera viz. *Bacillus*, *Halomonas*

Table 2. Differential phenotypic characteristics of the 18 bacterial isolates.

Group	Colony colour	Gram reaction	Morphology	Spore location	Nitrate reduction	Nitrite reduction	Oxidase	Catalase	Acid produced from			Voges Prokauer
									Sucrose	Glucose	Lactose	
BAC001	Cream	+	Short rods	Central	+	–	–	+	+	–	–	+
BAC002	White	+	Short rods		–	–	–	+	+	+	–	+
BAC003	White	+	Short rods	Endo-terminal	+	+	+	+	+	+	–	+
BAC004	White	+	Short rods	Central	+	+	–	+	+	–	–	+
BAC005	Cream	+	Short rods	Central	+	–	+	+	+	–	–	+
BAC006	White	+	Short rods	Central	+	+	+	+	+	+	–	+
BAC008	Cream	+	Short rods		+	+	+	+	–	+	–	–
BAC009	Cream	–	Short rods	Absent	+	–	+	+	–	–	–	–
BAC010	Cream	–	Short rods	Absent	+	_	+	+	–	–	–	–
BAC012	Cream	+	Rods	Central	–	–	+	+	+	+	–	+
BAC013	Beige	–	Rods	Absent	+	–	+	+	–	–	–	–
BAC014	White	+	Rods		+	–	+	+	+	–	–	–
BAC015	Cream	–	Short rods	Absent	+	–	–	+	+	–	–	–
BAC016	White	+	Short rods	Terminal	–	–	+	+	+	–	–	–
BAC017	Cream	+	Rods		–	–	+	+	+	–	–	+
BAC018	Cream	+	Rods	Central	+	–	+	+	+	–	–	+
BAC019	Cream	+	Rods	Central	–	–	+	+	+	–	–	+
BAC020	Yellow	+	Short rods - coccoid	Absent	–	–	–	–	+	–	–	–

and *Nesterenkonia*. Thirteen of the groups displayed 98-100% similarity to *Bacillus* species, four were found to be *Halomonas* species, while one displayed 99% similarity to *Nesterenkonia* species (Table 1). Isolates represented in group BAC001, BAC004, BAC006, BAC008, BAC009, BAC010, BAC014, BAC015 and BAC018 formed cream-white colonies that were smooth circular and raised, with entire margins, while BAC002, BAC0 03, BAC005, BAC012, BAC016, BAC017 and BAC019 formed colonies that were mucoid. Sua-BAC020 formed smooth, yellow convex colonies with entire margins after 48 h incubation, and the yellow colour intensified with extended incubation and storage, whereas Sua-BAC013 produced smooth, beige colonies (Table 2). All isolates were catalase positive and did not produce acid from lactose.

Production of β-mannanase, β-xylanase, and cellulase from selected *Bacillus* isolates

Quantitative enzyme production was evaluated by cultivating selected bacterial isolates: *Bacillus* sp.Sua-BAC005, Sua-BAC012, Sua-BAC017 and Sua-BAC019 in shake flasks using appropriate substrates for induction of β-mannanases, β-xylanases as well as cellulases. Isolate *B. amyloliquefaciens* Sua-BAC005 was evidently the best producer of β-mannanase and β-xylanase, with activities of 12.6 U/ml and 9.0 U/ml, respectively (Table 3), while the other three isolates Sua-BAC012, Sua-BAC017 and Sua-BAC019 produced less that 2 U/ml of these enzymes. Cellulase production was generally low in all the isolates with Sua-BAC019 exhibiting the highest cellulase activity of 1.4 U/ml.

The effect of NaCl, pH and carbon source on growth and xylanase production by *Nesterenkonia* sp. Sua-BAC020

Further studies were carried out *Nesterenkonia* sp. Sua-BAC020 as there are currently no reports on xylanase production from this genus. The isolate was able to grow in the presence and

Table 3. Comparison of enzyme production from selected *Bacillus* isolates.

Isolate name	Cellulase (U/ml)	Xylanase (U/ml)	Mannanase (U/ml)
Sua-BAC005	0.4 ± 0.12	8.9 ± 0.81	12.6 ± 1.1
Sua-BAC012	1.0 ± 0.06	0.9 ± 0.1	1.7 ± 0.21
Sua-BAC017	1.0 ± 0.09	1.4 ± 0.12	0.6 ± 0.04
Sua-BAC019	1.4 ± 0.08	0.7 ± 0.043	0.6 ± 0.05

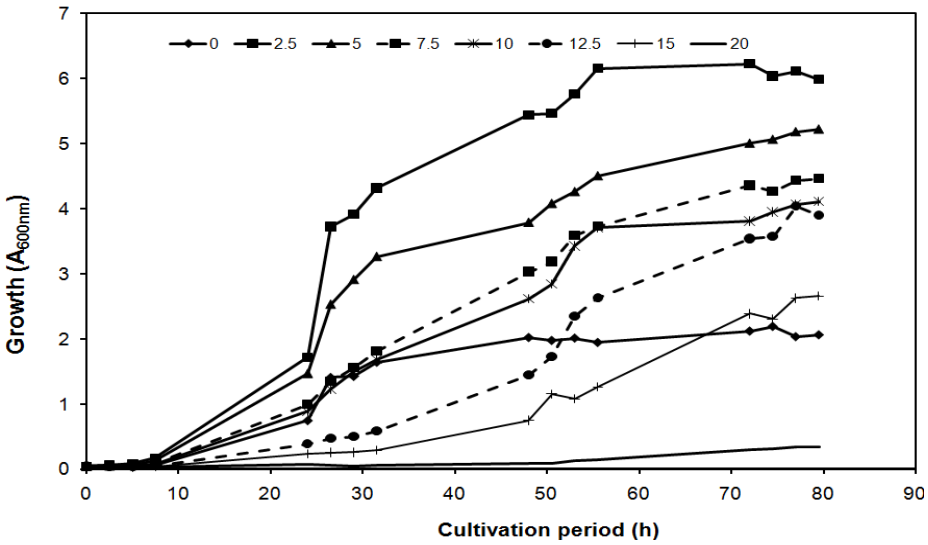

Figure 1. The effect of NaCl on growth of *Nesterenkonia* sp. BAC020 at pH 7.

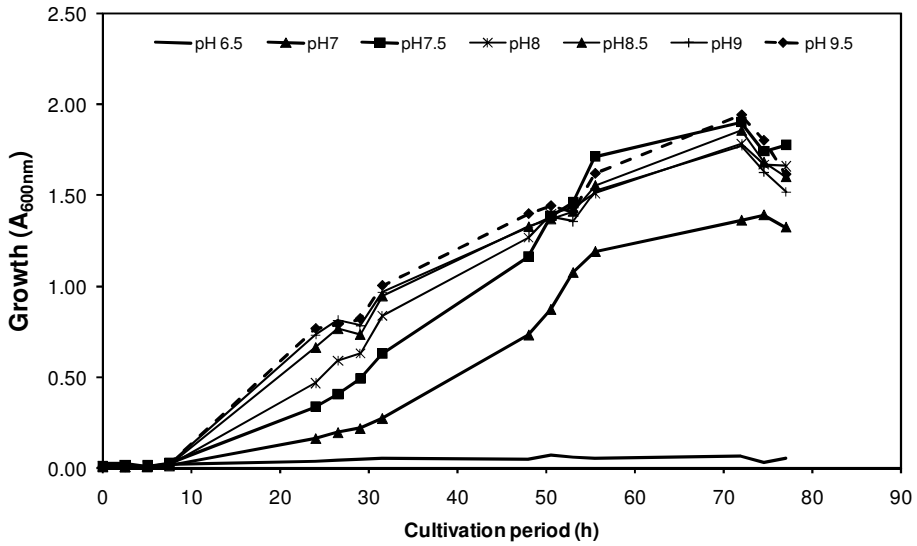

Figure 2. The effect of initial pH on the growth of *Nesterenkonia* sp. BAC020 in LB medium containing 10% (w/v) NaCl.

absence of NaCl at pH 7 in liquid cultures and could tolerate up to 20% NaCl. Optimal growth was observed at 2.5% NaCl (Figure 1). A lag phase up to 7.5 h was apparent at 20% NaCl. In addition, an increase in NaCl above 5% resulted in a decrease in growth rate. *Nesterenkonia* sp. Sua-BAC020 grew well from neutral to alkaline pH with an optimum at pH 9 -9.5 (Figure 2).

Isolate Sua-BAC020 produced xylanase at NaCl con-

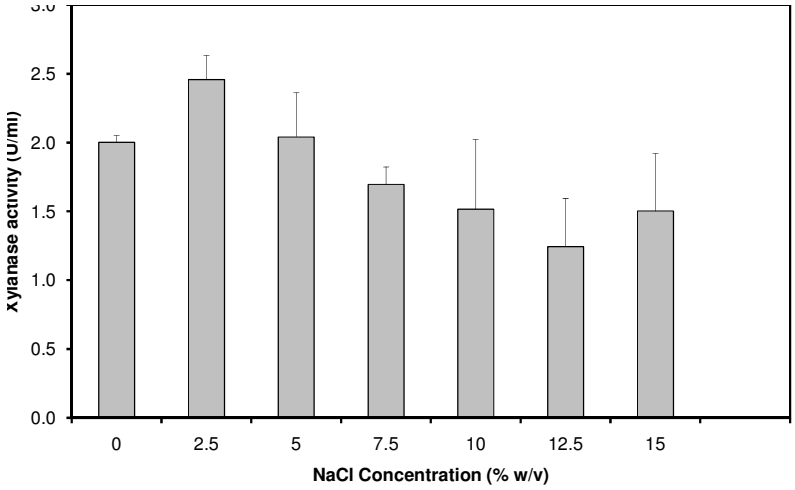

Figure 3. The production of xylanase by *Nesterenkonia* sp. BAC020 after 72 h of growth at different NaCl concentrations and an initial pH of 7.

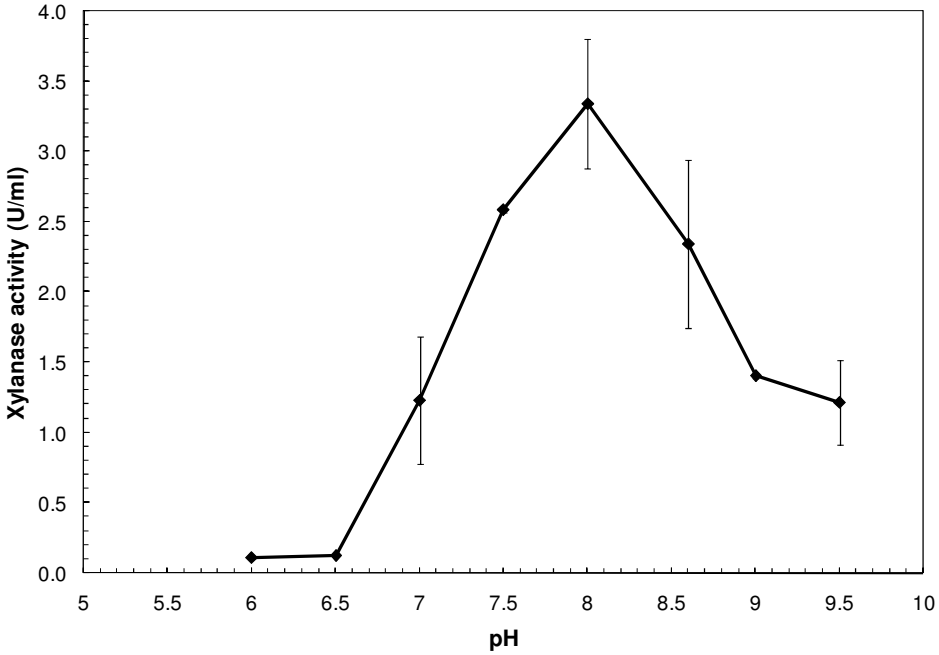

Figure 4. The effect of initial pH on the production of endoxylanase by *Nesterenkonia* sp. BAC020 after 96 h of cultivation in LB medium containing 10% NaCl.

centrations of 0-15% (w/v) at pH 7. Peak production levels of 2.5 U/ml were achieved at 2.5% NaCl followed by a decline (Figure 3). A gradual increase in xylanase production up to pH 8 was apparent (Figure 4). The xylanase production decreased at pH 8.6-9.5. Maximum xylanase production levels of 3.5 U/ml were achieved at pH 8, while minimal levels were produced at acidic pH in accordance with growth (Figure 4). When Sua-BAC020 was cultivated at pH 8 in the absence of NaCl or at 2.5% NaCl, similar amounts of xylanase were secreted into the extracellular environment. The xylanase production levels achieved at 0% NaCl and 2.5% NaCl were 3.1 U/ml and 3.4 U/ml, respectively.

However, peak xylanase production was attained at 72

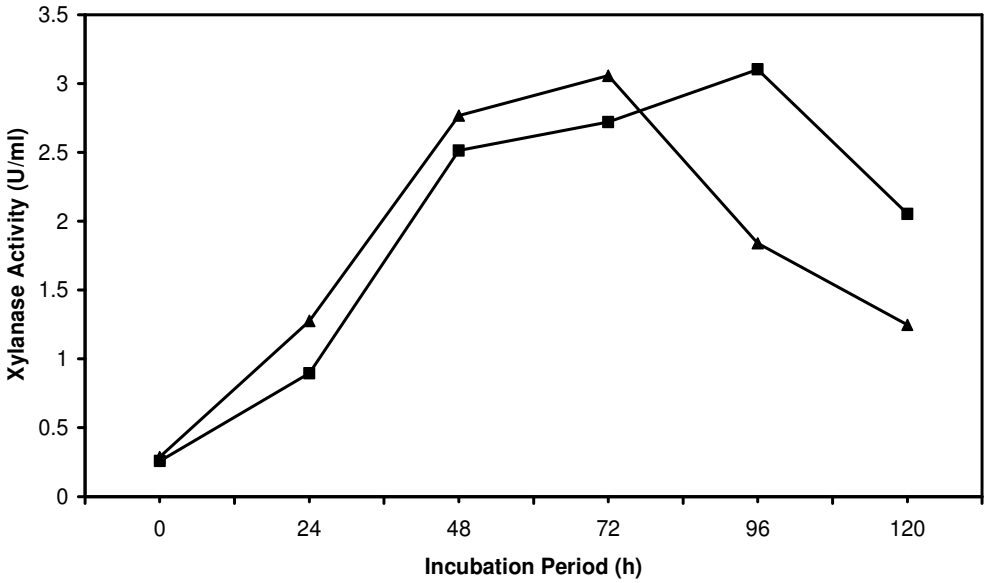

Figure 5. The time course of xylanase production at an initial pH of 8 and 0% (w/v) NaCl (▲) or 2.5% (w/v) NaCl (■).

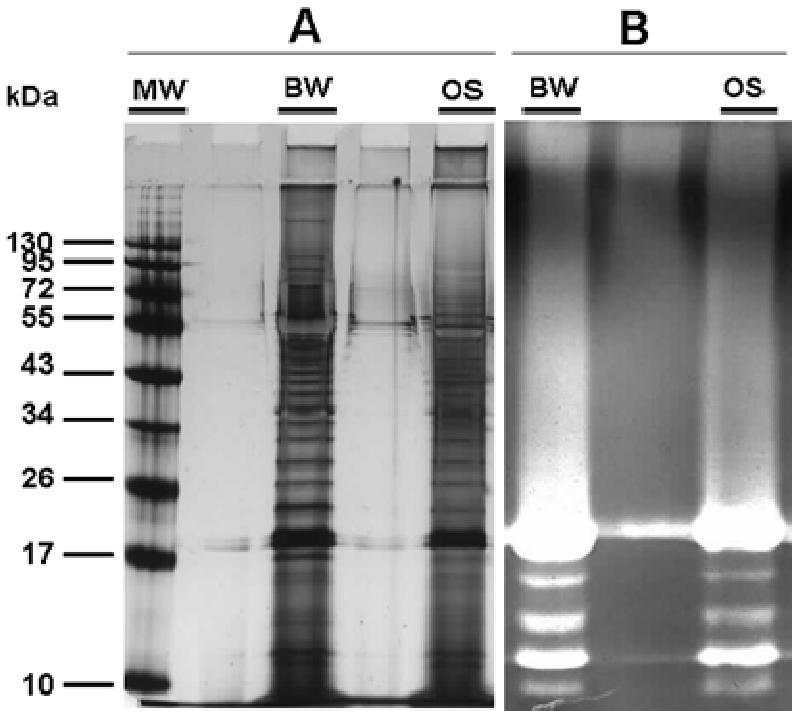

Figure 6. Xylanase production pattern during growth on birchwood (BW) and oatspelt (OS) xylan, resolved by native-PAGE (A) and zymogram (B).

h in the absence of NaCl and at 96 h in the presence of 2.5% NaCl (Figure 5). *Nesterenkonia* sp. Sua-BAC020 was found to produce five xylanases in the native form. Similar xylanase expression patterns were observed on birchwood and oatspelt xylan (Figure 6).

DISCUSSION

The hydrolytic potential of halophilic bacteria has not sparked as much interest in scientific research as thermophilic and alkaliphilic bacteria. Nevertheless,

several researchers have shown that halophiles do produce a wide variety of industrially relevant enzymes such as xylanases (Wejse et al., 2003), α-amylases (Coronado et al., 2002), proteases, lipases and pullulanases (Sanchez-Porro et al., 2003). The current study investigated the production of plant cell wall degrading enzymes such as xylanases, cellulases and mannanases from bacterial isolates obtained from evaporator ponds. All *Bacillus* spp. were found to display multiple hydrolytic activities whereas *Halomonas* spp. showed no hydrolytic activity. Previous studies in which halophilic bacteria were screened for production of hydrolytic enzymes, also found no xylanase activity amongst *Halomonas* species (Sanchez-Porro et al., 2003). *Bacillus* spp. BAC005, BAC012, BAC017 and BAC019 displayed similar enzyme production patterns to common bacilli such as *Bacillus amyloliquefaciens* (Breccia et al., 1998) Bacillus subtilis (Sá-Pereira et al., 2002) and Bacillus pumilus (Duarte et al., 1999). Generally, Gram positive bacteria have displayed broad hydrolase potential than Gram negative bacteria (Sanchez-Porro et al., 2003; Rhoban et al., 2009).

Nesterenkonia sp. Sua-BAC020 was studied in more detailed due to the potential for novelty in this organism's enzyme. The isolate grew optimally at 2.5% NaCl and produced higher amounts of xylanase at this concentration while poor growth and enzyme production was observed at 12.5-20% NaCl. This decrease may be due to high energetic cost for osmotic balance at high NaCl concentration (Oren, 1999). Similar growth responses have been previously reported for other halophilic bacteria such as *Halomonas meridiana* (Coronado et al., 2000), *Halobacillus* sp. (Amoozegar et al., 2003), and *Salinivibrio* sp. (Amoozegar et al., 2007).

Sua-BAC020 can be classified as moderately halotolerant as it does not show an absolute requirement for salt while its optimal growth at alkaline pH and mirrored by growth acidic pH, suggested that this isolate is also alkaliphilic. However, xylanase production at pH 8.6 – 9.5 decreased despite the high growth rates observed for this organism under those pH conditions. This may be attributed to early induction of proteases at high alkaline pH levels. When xylanase production was monitored at optimal growth conditions, it was observed that the amounts of xylanase produced at 0% and 2.5% NaCl at pH 8 were similar to those recorded at 10% NaCl at pH 8.

However, xylanase production in the presence of NaCl is slower, resulting in peak production after 96 h of growth while in the absence of NaCl peak production is attained after a 72 h growth period. Since Sua-BAC020 displayed optimal growth at 2.5% NaCl, it is possible that when the organism is cultivated in the absence of NaCl on xylan, it rapidly colonizes and utilizes this substrate to provide energy for growth. In the presence of NaCl, the utilization of xylan is delayed since this process is energy demanding but it is not necessary for growth of this organism, while on the other hand more energy is required to maintain osmotic balance. Therefore, energy is probably first channelled towards structural maintenance and generation of biomass from easily metabolizable nutrients such as yeast extract.

Sua-BAC020 produced five xylanases in the native form, which consist of two major and three minor xylanases based on the intensity of the bands. The multiple xylanases may be the result of genetic redundancy, different proteolytic processing or may be expressed as distinct gene products (Fernández-Abalos et al., 2003; Badhan et al., 2004; Collins et al., 2005). The molecular weights of the xylanases produced by Sua-BAC020 could not be determined due to complete loss of xylanase activity when SDS-PAGE was carried out and followed by SDS removal using 25% isopropanol.

Sua-BAC020 is the first *Nesterenkonia* species to be shown to produce xylanases. However, the isolate produced low amounts of xylanase. Therefore, it would be of great interest to optimize cultivation conditions for the production of xylanases in Sua-BAC020 and to purify and characterize the enzyme as this will provide insight about the properties of haloalkaliphilic enzymes.

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