Extremophiles (2003) 7:423–431
DOI 10.1007/s00792-003-0342-7

ORIGINAL PAPER

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Purification and characterisation of two extremely halotolerant xylanases from a novel halophilic bacterium

Received: 25 February 2003 / Accepted: 10 June 2003 / Published online: 18 July 2003

Abstract The present work reports for the first time the purification and characterisation of two extremely halotolerant endo-xylanases from a novel halophilic bacterium, strain CL8. Purification of the two xylanases, Xyl 1 and 2, was achieved by anion exchange and hydrophobic interaction chromatography. The enzymes had relative molecular masses of 43 kDa and 62 kDa and pI of 5.0 and 3.4 respectively. Stimulation of activity by Ca^{2+}, Mn^{2+}, Mg^{2+}, Ba^{2+}, Li^{2+}, NaN_3 and isopropanol was observed. The K_m and V_{max} values determined for Xyl 1 with 4-*O*-methyl-D-glucuronoxylan are 5 mg/ml and 125,000 nkat/mg respectively. The corresponding values for Xyl 2 were 1 mg/ml and 143,000 nkat/mg protein. Xylobiose and xylotriose were the major end products for both endoxylanases. The xylanases were stable at pH 4–11 showing pH optima around pH 6. Xyl 1 shows maximal activity at 60°C, Xyl 2 at 65°C (at 4 M NaCl). The xylanases showed high temperature stability with half-lives at 60°C of 97 min and 192 min respectively. Both xylanases showed optimal activity at 1 M NaCl, but substantial activity remained for both enzymes at 5 M NaCl.

Keywords Endo-xylanase · Enzyme purification · Halophilic · Halotolerance · Transglycosidation

Communicated by W.D. Grant

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Introduction

The majority of enzymes utilised in biotechnology still derive from well-characterised non-extremophilic bacteria and fungi. Significant changes in the properties of such non-extremophilic enzymes, e.g. towards higher thermostability, have been made by protein engineering (Minagawa et al. 2002; Tisi et al. 2002). However, enzymes with such unique properties may already exist in cultured and un-cultured microbes from extreme environments. These environments include extremes in temperature, pH and salt content, and the organisms that live in these ecosystems remain poorly characterised, yet represent a vastly under-utilised resource for new metabolites and biocatalysts. It may be expected that enzymes produced by these organisms have evolved to function optimally under harsh conditions. From a practical standpoint, it may be advantageous and efficient to utilise (and, if necessary, genetically engineer) enzymes obtained from extreme ecosystems to function under artificially harsh conditions in chemical and biochemical processing (Ryu et al. 1994). In the search for xylanases from extremophilic microorganisms, acidophiles, alkaliphiles and thermophiles have been studied (Fushinobu et al. 1998; Lopez et al. 1998; Bataillon et al. 2000). However, previous research on xylanases has only dealt with halophilic enzymes to a very limited extent (Johnson et al. 1986). Until recently, the known halophilic bacterial and archaeal isolates had not been reported to degrade xylan, and thus nothing was known about the microbial degradation of this substrate in hypersaline environments. Recently, a few xylanolytic halophiles have been isolated and described (Wainø and Ingvorsen 1999; Wainø et al. 1999; Wainø and Ingvorsen 2003), but no xylanases of halophilic origin have been purified and characterised until now.

Xylanases degrade the xylan part of hemicelluloses, which represent an enormous reserve of utilisable biomass as hardwoods consist of about 25–32% hemicelluloses, whereas softwoods contain 15–25% hemicelluloses. In

monocotyls, hemicelluloses may amount to 40% and exceed the cellulose portion (Wong et al. 1988; Saake et al. 2001). Undoubtedly, a commercial potential exists for xylanases, as hemicelluloses are the second most abundant renewable resource, only exceeded by cellulose. Therefore, xylanolytic enzymes derived from fungi and bacteria have been extensively studied in recent years (Coughlan and Hazlewood 1993). Most of the xylanases characterised show optimal activity at slightly acidic pH values (5.0–6.0) and at temperatures between 40°C and 70°C. The last decade has seen a rise in studies aiming at the isolation of thermophilic and alkali tolerant xylanases applicable to pulp and paper processes. Apart from their use in the pulp and paper industry, xylanases have several uses in the food and feed industries (Biely 1985; Bedford and Classen 1992; Maat et al. 1992; Wong and Saddler 1992; Kuhad and Singh 1993). In the present paper we report for the first time on the purification and properties of two new, extremely halotolerant xylanases exhibiting a hitherto unseen stability and activity in NaCl concentrations from 0 to 5 M. Further investigation of these special enzymes might lead to enhanced understanding of the halostability of proteins.

Materials and methods

Source of micro-organisms and enzyme production

Strain CL8, isolated from Great Salt Lake (USA) by K. Ingvorsen, was from the laboratory collection of our department. Strain CL8 is a moderately halophilic, gram-negative, aerobic bacterium which is able to grow at salt concentrations ranging from 0.2% to 20% (w/v) NaCl. On the basis of 16S rRNA gene sequence data, strain CL8 belongs to the γ-group of the Proteobacteria, showing highest sequence similarity values to *Oceanospirillum linum* (91.6%) and *Marinobacter* sp. str. CAB (91.6%). The sequence data thus suggest that strain CL8 could represents a new taxon (Trinderup et al., in preparation). The strain is deposited in Deutsche Sammlung von Mikroorganismen with accession number DSM-12619.

For enzyme production the following medium, developed by simplex optimisation (Shavers et al. 1979; Greasham and Inamine 1986), was used (quantities per litre): xylan (Lenzing, Austria) 1.66 g; natural sea salt from the Red Sea 73.29 g; NH_4Cl 0.73 g; Trisma base 4.0 g; trace metal solution 2 ml. The pH was adjusted to 8.3 with NaOH before autoclaving (121°C for 20 min). After cooling of the medium the following sterile solutions were added (per litre): KH_2PO_4 solution (at 50 g/l) 1.22 ml; MC solution 6.56 ml; vitamin mix 2.55 ml. The final pH of the medium was 8.3. The trace metal solution contained the following (per litre): HCl (32%) 10 ml; $FeCl_2{\cdot}4H_2O$ 2 g; $CoCl_2{\cdot}6H_2O$ 250 mg; $MnCl_2{\cdot}4H_2O$ 100 mg; $ZnCl_2$ 70 mg; H_3BO_3 6 mg; $Na_2MoO_4{\cdot}2H_2O$ 40 mg; $NiCl_2{\cdot}6H_2O$ 70 mg; $CuCl_2{\cdot}2H_2O$ 2 mg; $AlCl_3{\cdot}6H_2O$ 60 mg; $Na_2WO_4{\cdot}2H_2O$ 6 mg (Ingvorsen and Jørgensen 1984). The MC solution contained the following (per 100 ml 10 mM HCl): $CaCl_2{\cdot}2H_2O$ 10 g; $FeCl_2{\cdot}4H_2O$ 0.8 g; $MnCl_2{\cdot}4H_2O$ 0.8 g. The vitamin mix contained the following (mg/l): cyanocobalamine 17; thiamin chloride 33; 4-aminobenzoic acid 13; D-biotin 3.3; nicotinic acid 33; Ca-pantothenate 17; pyridoxine dihydrochloride 50; folate 10.

Cells were aerobically cultivated in 500-ml Erlenmeyer flasks containing 300 ml medium at 30°C in an orbital shaker (240 rpm). Cultures were inoculated with a volume of 2.5% from cultures grown under similar conditions. Cell free supernatant was obtained by centrifugation (30 min at 9,500 *g* and 5°C) after 24 h growth.

Assays for xylanase activity and protein

Xylanase activities were determined spectrophotometrically by measuring the amount of water-soluble dyed compounds released from the hydrolysis of finely ground insoluble azurine cross-linked birchwood xylan (AZCL-xylan) (Megazyme, Ireland). During xylanase purification all assays were conducted at 10% (w/v) NaCl using a substrate solution containing the following (in 100 ml MilliQ water): NaCl 10 g; $MgSO_4{\cdot}7H_2O$ 1 g; finely ground AZCL-xylan 0.2 g; $NaH_2PO_4{\cdot}2H_2O$ 0.3 g. The pH of the substrate solution was adjusted to 7.0 with NaOH. Enzyme solution (0.5–20 µl depending on activity) was added to 0.9 ml substrate solution and incubated at 30°C in an Eppendorf thermomixer (1,300 rpm) for 10–30 min. After incubation, samples were centrifuged and the amount of dye present in the supernatant was determined spectrophotometrically by measuring the absorbance at 590 nm (A_{590}). Using this assay protocol, one xylanase unit is defined as the amount of enzyme yielding an A_{590} of 1 in 1 h. For the characterisation of the pure enzymes, the assay was performed with different buffers, and 0.2 mg/ml bovine serum albumin (BSA) was included to protect the enzymes against adsorption.

Alternatively, xylanase activity was measured by monitoring the production of reducing sugars using the dinitrosalicylic acid (DNS) method of Miller (Miller 1959). This method was applied when determining the halotolerance, as unpublished data have shown a great effect of ionic strength on the AZCL-xylan based assay, due to swelling phenomena (Wejse et al. in preparation) The DNS method was also used for determining K_m and V_{max}, as these experiments require a soluble substrate. The substrate used was birchwood 4-*O*-methyl glucuronoxylan (product 7500, Carl Roth) prepared by a procedure similar to the one devised by Bailey et al. (1992), except for the buffer used. The citrate buffer used by Bailey was replaced by MilliQ water, and aliquots of the solution were pipetted into 2-ml Eppendorf tubes and freeze-dried in the tubes. The xylan could easily be re-solubilised in 200 µl buffer for the various experiments (10 mg/ml final substrate concentration). The assay was incubated at 50°C (halotolerance) or 60°C (kinetic determinations) for 5 min. Standardisation of the DNS assay was performed according to Bailey et al. (1992). Because of interference from NaCl and KCl, standard curves were prepared at each salinity tested. Protein content was monitored spectrophotometrically at 280 nm during purification. Protein concentrations of pure xylanase preparations were estimated by the method of Bradford (Bradford 1976) with BSA as a standard.

IEF, SDS-PAGE and zymograms

Isoelectric focusing (IEF) was performed using an XCell SureLock Mini-Cell (Invitrogen) with a Novex pre-cast vertical IEF focusing gel, pH 3–10, with 10 wells (Invitrogen). The anode, cathode and sample buffer were from Invitrogen. The pI marker was the Broad pI Kit from Amersham Biosciences. Denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gel was performed according to Laemmli (1970) using a Bio-Rad Mini-PROTEAN II system (Bio-Rad PROTEAN II system for zymograms) and standard molecular weight markers (LMW, Amersham Biosciences). Proteins were stained by Coomassie brilliant blue R250. For zymograms, un-boiled samples were run in parallel with regular (boiled) samples to analyse for xylanase activity in SDS-PAGE. The lane with un-boiled enzyme was sliced into 5-mm pieces and each was incubated in AZCL-xylan in buffer. Activity was clearly visualised by the blue colour developed after 24 h incubation. Slices with activity could be related to stained protein bands in the un-boiled and boiled form, as well as the molecular weight standard.

Purification of xylanases

All procedures were carried out at 4°C, unless otherwise stated.

The following buffers were used during purification:

- buffer A: 1.2 M $(NH_4)_2SO_4$, 50 mM NaH_2PO_4/NaOH, pH 7.0;
- buffer B: 10 mM acetic acid/NaOH, pH 5.0;
- buffer F: 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes)/KOH, pH 7.5.

Protease inhibitor (phenyl methyl sulfonyl fluoride) and ethylenediaminetetraacetic acid (EDTA) were added to the cell free supernatant (approximately 3 l) to final concentrations of 0.1 mM and 3 mM respectively. Solid ammonium sulphate was added to a final concentration of 1.2 M and the pH was adjusted to 7.0 by addition of HCl. Phe-Sepharose HP (Amersham Biosciences) was added (2 ml settled gel per litre of supernatant) and incubated for 20 min with mixing. The gel was recovered by filtration through a nylon filter (10 μm pore size) and packed in a column (16 mm×30 mm). The column was washed with 30 ml buffer A. Adsorbed proteins were eluted by a 120-ml linear gradient to 5 mM NaH_2PO_4/NaOH, pH 7.0, containing 10% isopropanol, at a flow rate of 0.5 ml min^{-1}. Fractions of 6 ml were collected and assayed for xylanase activity. Fractions containing xylanase activity were analysed by SDS-PAGE and those containing the first peak of activity were combined into pool 1. The least contaminated fractions containing the other peak of activity were combined into pool 2. Pool 1 was dialysed against buffer B and applied to a Mono Q HR 5/5 column (Amersham Biosciences) equilibrated with buffer B. The column was washed with 2 ml buffer B. Adsorbed proteins were eluted by a 20-ml linear NaCl gradient (0–300 mM) at a flow rate of 0.9 ml min^{-1}. Fractions of 1.0 ml were collected, assayed for xylanase activity and analysed for purity by SDS-PAGE. The apparently pure fractions were pooled, concentrated and changed to buffer C by ultrafiltration using a membrane with a 30-kDa molecular weight cut-off, and used as the pure Xyl 1 preparation for characterisation experiments. Solid ammonium sulphate was added to pool 2 (176 g l^{-1}) and the solution applied to a 1-ml column (50 mm×5 mm) packed with Phe-Sepharose HP (Amersham Biosciences) equilibrated with buffer A. The column was washed with 4 ml buffer A. Adsorbed proteins were eluted by stepping to 0.6 M $(NH_4)_2SO_4$, with 10% isopropanol, followed by a 40-ml linear gradient of 0.6–0 M $(NH_4)_2SO_4$, with 10%–20% isopropanol, at a flow rate of 0.7 ml min^{-1}. Fractions of 1.5 ml were collected, assayed for xylanase activity and checked for purity by SDS-PAGE. The apparently pure fractions were pooled, concentrated and changed to buffer C by ultrafiltration using a membrane with a 30-kDa molecular weight cut-off, and used as the pure Xyl 2 preparation for characterisation experiments. Impure fractions with high xylanase activity were combined and re-purified following the above procedures.

Analytical gel filtration

Analytical gel filtration of pure xylanases was performed with a TSK G2000 SW column (0.75 cm×60 cm) and pre-column. The buffer was 50 mM NaH_2PO_4, pH 7.0, 200 mM NaCl and the flow rate was 0.8 ml min^{-1}. Calibration was carried out with the following proteins: BSA 67 kDa; ovalbumin 43 kDa; chymotrypsinogen A 25 kDa; and ribonuclease A 13.7 kDa.

Characterisation of xylanases

Effects of metal ions and organic chemicals on enzyme activity were determined ($n = 3$) by incubating the enzymes with the respective compounds at concentrations of 1 mM and 10 mM in 50 mM Hepes/KOH, pH 7.5, with 0.1 mg/ml BSA for 1 h at room temperature. The residual activity was determined by the addition of AZCL-xylan followed by incubation at 30°C for 12–82 min. The pH stability was determined ($n = 3$) by incubating the enzymes for 1 h at 30°C at pH 2–12 in an organic polybuffer made up of glycine, acetic acid, succinic acid, 3-(*N*-morpholino) propanesulfonic acid and Tris-HCl, each at 50 mM in 2 M NaCl and 0.2 mg/ml BSA. The residual activity was determined by diluting the incubations 18-fold into 50 mM succinic acid, pH 6.0 containing 0.2 mg/ml BSA and AZCL-xylan followed by incubation for 20 min at 40°C. The effect of pH on enzyme activity was determined ($n = 3$) by incubating the enzymes for 20 min at 40°C at pH 3–11 in the above-mentioned organic polybuffer containing 0.2 mg/ml BSA and AZCL-xylan. The temperature stability was determined ($n = 3$) by incubating the enzymes at temperatures from 41–93°C in 100 mM Hepes/KOH, pH 7.50, with 10 mM $CaCl_2$, 0.2 mg/ml BSA and 0 M or 2 M NaCl. Samples were withdrawn and assayed for residual activity at different times, after dilution (45×) in NaCl-free organic polybuffer containing AZCL-xylan. The assays were incubated for 20 min at 40°C. The effect of temperature on enzyme activity was determined ($n = 3$) by incubating the enzymes in 50 mM succinic acid/NaOH, pH 6.0 (pH for optimal activity) containing 10 mm $CaCl_2$, 0.2 mg/ml BSA, 4 M NaCl and AZCL-xylan. The buffer was pre-heated at temperatures from 0–80°C before addition of enzymes. The absorbance was determined spectrophotometrically and related to incubation time when the absorption at 590 nm was 0.1–0.3 (5 min to 2 days). The effect of salinity on enzyme activity was determined ($n = 3$) by incubating the enzymes with soluble birchwood 4-*O*-methyl glucuronoxylan (prepared as described above) in 50 mM succinic acid/NaOH, pH 6.0 containing 10 mm $CaCl_2$, 0.2 mg/ml BSA and varying NaCl or KCl concentration at 50°C.

Substrate specificity

The substrate specificity of the xylanases was determined ($n = 3$) using the following substrates: azurine cross-linked hydroxyethyl cellulose (AZCL-HE-cellulose), AZCL-arabinoxylan (wheat), AZCL-xylan (birchwood), AZCL-galactomannan, AZCL-pullulan, AZCL-amylose (MegaZyme), chitin-azure (Sigma), xylobiose, birchwood xylan (Carl Roth, Germany), Avicel PH-101 (Fluka BioChimika), and ultra-low viscosity carboxymethylcellulose (CMC) with a degree of substitution in the range 0.60–0.95 (Fluka BioChimika). The incubations were performed in 50 mM succinic acid/NaOH, pH 6.0 containing 10 mm $CaCl_2$, 0.2 mg/ml BSA, 1 M NaCl and the substrate. Dyed substrates were used at 0.02% (w/v) and the colour release compared to that of AZCL-arabinoxylan (wheat). Xylobiose was analysed by high performance liquid chromatography using the Dionex system as described below. Avicel, 4-*O*-glucuronoxylan and CMC were used at 1% (w/v) and the release of reducing sugars was compared to that from 4-*O*-glucoronoxylan. The incubations were performed at 40°C for 5 min (xylans) to 18 h (other substrates).

Product analysis

The products from the enzymic hydrolysis of soluble birchwood xylan were separated by anion-exchange chromatography on a Diones CarboPak PAI column (4 mm×250 mm) (Dionex, Sunnyvale, Calif., USA). A pulsed amperometric detector (PAD-2, Dionex) with a gold electrode was used for oligosaccharide detection. Peaks were identified by reference sugars prepared according to Puls et al. (1988).

Results and discussion

Purification of xylanases

The results of the purification are summarised in Table 1. Initial zymogram analysis of concentrated (ultra filtration) cell free supernatant (data not shown) revealed the presence of two xylanases of

Table 1 Purification of two halotolerant endo-xylanases

Step	Total protein (mg)[a]	Total units (U_{Dye})[b]	Specific activity (U_{Dye}/mg)	Recovery (%)	Purification (fold)
Supernatant	3,021.0	380,640	126	100.0	1.0
Phe-Sepharose pool 1	56.4	12,857	228	3.4	1.8
Phe-Sepharose pool 2	39.0	46,903	1,203	12.3	10.1
Mono Q on pool 1	8.9	5,814	653	1.5	5.2
Phe-Sepharose on pool 2	4.6	6,471	1,407	1.7	11.8

[a]Based on A_{280}
[b]One xylanase unit (U_{Dye}) is defined as the amount of enzyme yielding an A_{590} of 1 in 1 h in the hydrolysis of azurine cross-linked birchwood xylan under assay conditions

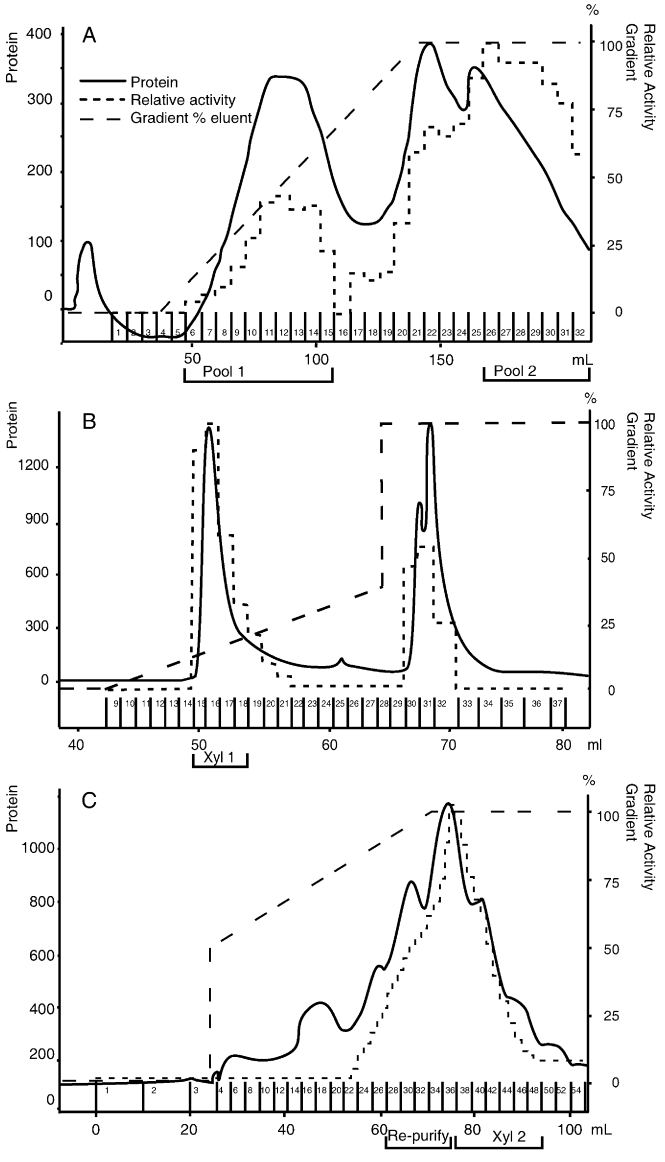

Fig. 1A–C Column chromatography of xylanases; protein axis shows absorbance (mAU) and the light path was 2 mm. **A** Initial chromatography on Phe-Sepharose. **B** Chromatography of pool 1 on Mono Q. **C** Chromatography of pool 2 on Phe-Sepharose

approximately 40 kDa and 60 kDa. In the first purification step, batch adsorption by hydrophobic interaction chromatography (HIC), the xylanase activity was concentrated and the two xylanases were separated from each other as they eluted at approximately 50% and 100% buffer B (Fig. 1A). The fractions were analysed for activity and for purity by SDS-PAGE. The first activity peak (fractions 6–15, 60 ml), were combined in pool 1. The second activity peak contained significant amounts of contaminants in the first fractions, therefore only the last fractions (numbers 26–32, 42 ml) were combined in pool 2. The xylanases were purified 1.8-fold and 10.1-fold respectively. Though the recovery was very low, this first step served as an efficient procedure for handling a large volume with low concentration of enzymes and high concentration of NaCl. Pool 1 was dialysed prior to anion exchange chromatography on Mono Q. Xyl 1 eluted in a narrow peak at approximately 200 mM NaCl (Fig. 1B). The fractions were analysed for activity and for purity by SDS-PAGE. The apparently pure fractions (fractions 15–18) were combined and concentrated by ultrafiltration; the buffer was also changed to 50 mM Hepes/KOH, pH 7,5 and the pure Xyl 1 preparation was stored at −20°C. A total purification of 5.2 fold was achieved. Solid ammonium sulphate (176 g/l) was added to pool 2 from the initial step and this pool was subjected to HIC again under slightly different conditions. Xyl 2 elutes in a broad peak at approximately 100% buffer C (Fig. 1C). The fractions were analysed for activity and for purity by SDS-PAGE. The apparently pure fractions, fractions 38–48, were pooled and concentrated by ultrafiltration; the buffer was changed to 50 mM Hepes/KOH, pH 7,5 and the pure Xyl 2 preparation was stored at −20°C. A total purification of 11.8 fold was achieved. Fractions 28–37 were subjected to repeated chromatography by the above procedure, yielding more pure Xyl 2. Though the recovery of both xylanases is, for unknown reasons, low, requiring large volumes (3 l) of cell free supernatant, pure and active xylanases are obtained by the procedure employed (Fig. 2). Furthermore zymogram analysis shows that the xylanases retained their initial size during the purification procedure (data not shown).

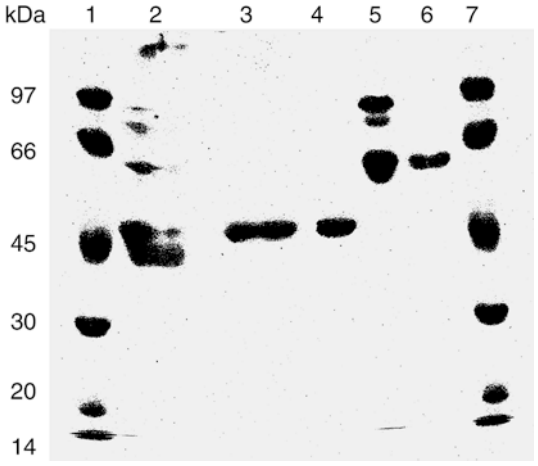

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the purification of halophilic xylanases. *Lanes 1 and 7* Molecular weight standards, *lane 2* concentrated supernatant, *lane 3* pool 1, *lane 4* purified Xyl 1, *lane 5* pool 2, *lane 6* purified Xyl 2

Characterisation

The relative molecular masses for Xyl 1 and 2, as determined by SDS-PAGE, were 43 kDa and 62 kDa respectively. By analytical gel filtration the relative masses of Xyl 1 and Xyl 2 were estimated to be 31 kDa and 76 kDa respectively, suggesting monomeric proteins. Xyl 2 also eluted with an apparent molecular weight of 16 kDa, due to interaction with the column. The pIs of Xyl 1 and 2 as determined from the IEF gel were 5.0 and 3.4 respectively. These data suggest that both xylanases belong to glycanase family 10 (formerly named F), characterised as acidic and of high molecular weight (> 30 kDa) (Henrissat and Bairoch 1993; Biely et al. 1997), but ongoing sequence analysis shows that they are in fact family 11 xylanases (Wejse et al., in preparation).

Effects of metals and organic chemicals

The effects of metal ions and various organic chemicals are summarised in Table 2. Some metal ions were found to inhibit the activity of both xylanases completely, even at 1 mM concentration. This is commonly observed for Hg^{2+} and Pb^{2+} (Gupta et al. 2000), but in this study Cu^{2+}, Al^{3+} and Fe^{3+} had similar effect. Aluminium has been shown to have inhibitory effect on other xylanases, whereas the cupric and ferric ions have been shown to have inhibitory or stimulatory effects (Raj and Chandra 1996; Gupta et al. 2000; Chivero et al. 2001). Ferrous iron and zinc exerted a strong inhibitory effect. Ferrous iron has been found to have a stimulating effect on some xylanases (Gupta et al. 2000), and the result may be influenced by chemical oxidation of Fe^{2+} to Fe^{3+} during the experiment. Ni^{2+} had a modest negative effect on the activity of both xylanases. Stimulating divalent cations were: $Ca^{2+} > Mn^{2+} > Mg^{2+} > Ba^{2+} > Li^{2+}$. EDTA has a positive effect on Xyl 1 whereas it affects Xyl 2 slightly negatively. As Xyl 2 displays the largest positive response to activating divalent cations, the negative effect of EDTA can probably be ascribed to the decreased availability of these ions in the presence of EDTA. Divalent cations are therefore not necessary for the activity of the xylanases, but they enhance the activity. Positive effects of these metal ions are not commonly observed among xylanases. Thus, the large effect observed in this study can probably be ascribed to the halophilic nature of the xylanases. Co^{2+} has an intermediate position, stimulating Xyl 2 at 1 mM, but inhibiting at 10 mM. Co^{2+} inhibited Xyl 1 at both concentrations. NaN_3 was found to stimulate the xylanases; this was also true for isopropanol. An activating effect of isopropanol has, to our knowledge, not been observed for xylanases before. The effect of dithiothreitol, β-mercaptoethanol and iodoacetamide indicated that cysteine residues are not a part of the catalytic site of these xylanases. Acetic anhydride affects the two xylanases very differently; the activity of Xyl 1 is strongly inhibited, whereas that of Xyl 2 is enhanced.

Table 2 The effect on enzyme activity of metal ions and organic chemicals as a percentage of control

Compound	Xyl 1 1 mM	Xyl 1 10 mM	Xyl 2 1 mM	Xyl 2 10 mM
Control	100	100	100	100
$HgCl_2$	0	0	0	0
$PbCl_2$	0	0	0	0
$CuCl_2$	0	0	0	0
$AlCl_3$	0	0	0	0
$FeCl_3$	0	0	0	0
$ZnCl_2$	57	0	39	0
$FeCl_2$	0	0	4	0
$NiCl_2$	51	19	91	75
$CoCl_2$	73	32	163	70
Li_2SO_4	101	160	148	254
$BaCl_2$	139	163	366	353
$MgCl_2$	113	217	226	654
$MnCl_2$	124	246	465	1,161
$CaCl_2$	140	237	773	1,135
NaN_3	92	121	123	206
NH_4Cl	98	134	140	217
EDTA[a]	102	185	64	94
L-cysteine	78	89	114	67
Iodoacetamide	87	88	124	114
β-mercaptoethanol	95	92	114	45
Dithiothreitol	75	76	43	33
Ascorbic acid	103	137	129	201
Urea	81	94	140	139
Guanidine-HCl	96	129	132	221
SDS[b]	60	6	127	112
PMSF[c]	99	76	104	121
Isopropanol[d]	112	134	160	188
Acetic anhydride	88	8	179	172

[a]Ethylenediaminetetraacetic acid
[b]Sodium dodecyl sulphate
[c]Phenylmethylsulfonyl fluoride
[d]Isopropanol tested at 1% and 10% (v/v)

Table 3 Substrate specificity

Substrate[a]	Xyl 1 (%)	Xyl 2 (%)
AZCL-arabinoxylan (wheat)	100	100
AZCL-xylan (birchwood)	29	41
AZCL-HE-cellulose[b]	< 0.1[c]	< 0.1
AZCL-galactomannan	< 0.1	< 0.1
AZCL-pullulan	< 0.1	< 0.1
AZCL-amylose	< 0.1	< 0.1
Chitin-azure (Sigma)	< 0.1	< 0.1
Xylan, birchwood	100	100
CMC[d]	0.25	0.34
Avicel	< 0.1	< 0.1
Xylobiose	< 0.1	< 0.1

[a] *AZCL* azurine cross-linked
[b] Azurine cross-linked hydroxyethyl cellulose
[c] Not detectable
[d] Carboxymethylcellulose

Substrate specificity and kinetic parameters

The substrate specificity is summarised in Table 3. Both purified xylanases were quite specific towards xylans, showing higher activity on wheat arabinoxylan than on birchwood xylan. This has been observed in other xylanases as well (Winterhalter and Liebl 1995; Karlsson et al. 1998). The different activities towards xylans of differing structure are under further study at the moment. The very low but significant activity against CMC suggests some degree of cross-specificity, but no activity is detected against avicel or HE-cellulose. Cross-specificity has been observed before in family 10 xylanases (Biely et al. 1997), and the finding may therefore support the xylanases under study belonging to this family. Since the activity against CMC is very low, comparable activities may have been ignored in other studies, as it might have been below the detection limit (Leathers 1989). With a shorter time of incubation the activity would not have been significant in this study either. No activity was observed against galactomannan, pullulan, amylose, chitin or xylobiose. This confirms that the xylanase preparations are of high purity. On the basis of Lineweaver-Burk plots, apparent K_m values calculated for Xyl 1 and 2 were 5 mg ml^{-1} and 1 mg ml^{-1} respectively, and V_{max} values were 125,000 nkat/mg and 143,000 nkat/mg protein respectively. The experiments were performed with a buffer containing 1 M NaCl and 10 mM $CaCl_2$. The K_m values found for Xyl 1 and Xyl 2 are very typical of xylanases, while the V_{max} values are among the highest reported in the literature (Beg et al. 2001). The high specific activity is in agreement with the observation of activity in zymograms without visible protein bands (data not shown). However, the enormous variation in kinetic parameters reported in the literature is beyond doubt partly due to differences in assay procedures; the substrate especially has a large effect on the values obtained (Bailey et al. 1992; Beg et al. 2001).

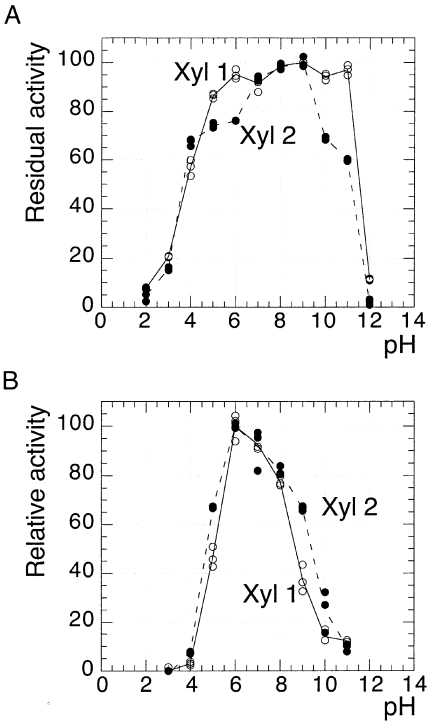

Fig. 3 A pH-stability of Xyl 1 and Xyl 2 at 2 M NaCl, 30°C. **B** The effect of pH on xylanase activity at 40°C in the presence of 2 M NaCl. The substrate used was azurine cross-linked birchwood xylan (AZCL-xylan)

Hydrolysis pattern

The two xylanases appear to exhibit very similar hydrolysis patterns. The products from the enzymic hydrolysis of soluble birchwood xylan were mainly xylobiose and xylotriose with only trace amounts of xylose. This observation is a proof of the endo activity of the xylanases. Xylotetraose and xylopentaose are observed as intermediate breakdown products, and their degradation without accumulation of xylose can only be explained by the presence of transglycosidation activity by the endoxylanases. This mode of action is similar to that observed with the xylanases from *Bacillus sp., Humicola grisea* var. *thermoidea*, *Aspergillus fumigatus* Fresenius and *Rhodothermus marinus* (Honda et al. 1985; Karlsson et al. 1998; Silva et al. 2000).

Effects of pH and temperature

Xyl 1 and Xyl 2 were very stable over a wide pH range from 4–11 (1 h, 30°C, 2 M NaCl); residual activities were even detected at pH 3 (Fig. 3). This is an extremely wide range compared with other known xylanases e.g. pH 3–8.5 for a xylanase from *Trichoderma reesei* or pH 6–11 for a xylanase from *Bacillus* sp. NG-27 (Beg et al. 2001). Both Xyl 1 and Xyl 2 had typical pH optima at pH 6 (20 min, 40°C, 2 M NaCl), and at pH 8 they retained 80% of optimal activity (Fig. 4). The lack of activity at pH below 4 probably reflects the ionisation of the

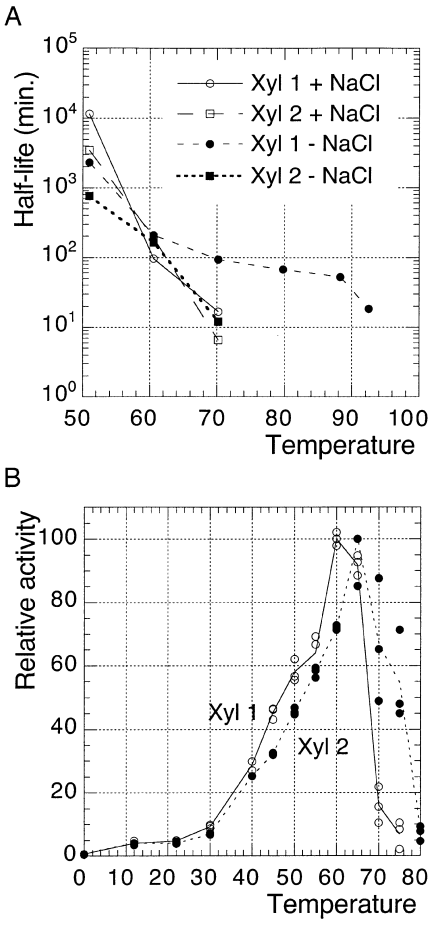

Fig. 4 **A** The effect of temperature on enzyme stability with and without 2 M NaCl, pH 6.0. **B** The effect of temperature on activity of the two xylanases in the presence of 4 M NaCl, pH 6.0. The substrate used was AZCL-xylan

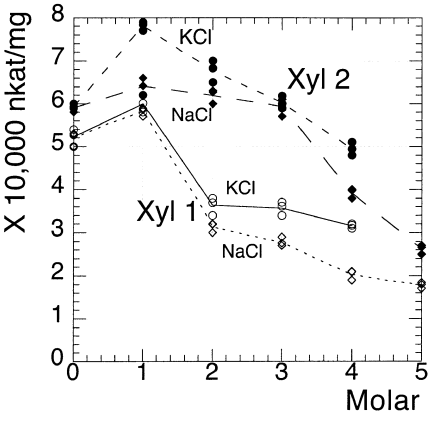

Fig. 5 The effect of NaCl and KCl on the activity of the two xylanases at 50°C, pH 6.0. The substrate used was soluble birchwood xylan

general base catalyst (Tull and Withers 1994). Temperature stability was quite high for both xylanases; the half-lives at 60°C were 97 min and 192 min respectively (pH 7.5, 2 M NaCl). At 70°C they decreased to 17 min and 7 min (Fig. 5). The high stability is comparable or superior to that found for xylanases from *Bacillus* (Beg et al. 2001). The linearity of the plot indicates first-order inactivation kinetics. Without salt, Xyl 1 deviates considerable form the other curves by having a much higher stability at high temperatures; the half-life at 88°C was 53 min decreasing to 18 min at 93°C. The temperature optima for Xyl 1 and Xyl 2 were 60°C and 65°C, respectively (pH 6.0, 4 M NaCl) (Fig. 4B). This is at the high end of what is typically reported for xylanases from mesophilic organisms.

Effect of NaCl and KCl

Maximal xylanase activities were detected at 1 M NaCl for both xylanases (pH 6.0, 50°C) (Fig. 5). They retained most of their activity even without NaCl added to the buffer, when the NaCl concentration was approximately 50 mM. The activity of Xyl 1 decreases rapidly between 1 M and 2 M NaCl (from 100% to 54%). From 2 M to 5 M it decreases slowly to 31%. Xyl 2 is even more halotolerant with near maximal activity throughout the salinity range from 0 M to 3 M NaCl. From 3 M to 5 M it only decreases to 42% of the maximal activity. For KCl almost the same pattern is observed, though Xyl 2 has a clear maximum at approximately 1 M KCl. The xylanases seem very well adapted to the environment, as they have maximal activity when glacial streams introduce new hemicellulosic substrates and dilutes Great Salt Lake in spring. These enzymes can still support the microbial population, though at a lower rate, when evaporation leads to higher salinity during summer. The observed response to NaCl is unique, as the xylanases are highly active at all possible salinities. In fact, the enzymes have a much wider range of activity than the bacterium producing them (R. Trinderup, unpublished results), which grew in the range 0.2–20% NaCl. No single xylanase has ever been reported to cover as broad a range of salinities. This finding therefore indicates that the purified xylanases of CL8 may differ considerably with respect to function compared to previously characterised xylanases. Usually, non-halophilic enzymes will precipitate and/or become inactivated at high salinity, whereas halophilic enzymes will inactivate, often irreversibly, at lower salt concentrations (Madern et al. 2000; Wright et al. 2002). This is observed in intracellular enzymes from the relatively constant cellular environment e.g. the well-studied malate dehydrogenase from *Haloarcula marismortui* (Mevarech et al. 2000; Richard et al. 2000) and in extracellular enzymes e.g. a protease from *Halobacterium halobium* (Ryu et al. 1994). The xylanases in this study are completely different, and they may be described as extremely halotolerant. These characteristics are in good agreement with the strongly fluctuating salinities occurring in the southern part of Great Salt Lake. The special characteristics of Xyl 1 and 2 unify stability and activity at conditions of low

salt, such as production and purification, as well as in applications at any concentration of salt. This positions Xyl 1 and 2 as ideal candidates for biotechnological applications demanding high halostability, flexibility and activity. This study raises a broader question as to what the underlying structural basis for the unprecedented halotolerance observed is. The xylanases might possess typical halophilic adaptations such as more acidic surface and less hydrophobic core (Ryu et al. 1994), but, more probably, new halo-stabilising mechanisms are at play. This hypothesis is supported by the absence of destabilisation of Xyl 1 and Xyl 2 at low salinity due to extensive repulsive electrostatic interactions (Elcock and McCammon 1998). In fact an increase in stability to temperature denaturation was observed for Xyl 1 (Fig. 5). Most probably, special adaptations are necessary to impose such special characteristics. A possible mechanism for stabilisation of halophilic proteins allowing them to function at low salinity has been proposed by Polosina et al. (2002), but as this stabilisation relies on quaternary structure, other mechanisms must be at play in these new monomeric xylanases. Ongoing structural investigations will hopefully shed light on to this question. Further studies are needed to determine whether this extreme halotolerance also correlates with a broad tolerance to low concentrations of water and inorganic ions in organic solvents.

Acknowledgments We thank Dr. Jürgen Puls for generous help with product analysis and Jens Frisbæk Sørensen at Danisco A/S for performing the isoelectric focusing analysis. Rene Trinderup is also acknowledged of his information on the characteristics of strain CL8.

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