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A xylan-degrading strain of *Sulfolobus solfataricus*: isolation and characterization of the xylanase activity

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Abstract Two strains (O_α and X_2) of the hyperthermophilic crenarchaeon *Sulfolobus solfataricus* strain MT4 were selected and isolated for their ability to grow on xylan. O_α and X_2, grown on media containing oat spelt xylan and birchwood xylan as the sole nutrient source, respectively, produced the same thermostable xylanase that was demonstrated to be inducible in xylan cultures. In an oat spelt medium, *S. solfataricus* O_α underwent interesting morphological changes in the cell envelope, exhibiting mobile appendages not present in the typical coccal shape. The enzyme was prevalently membrane associated and showed a molecular mass of approximately 57.0 kDa. It was also highly thermostable, with a half-life of 47 min at 100°C, and exhibited an optimal temperature and pH of 90°C and 7.0, respectively. Xylo-oligosaccharides were the enzymatic products of xylan hydrolysis, and the smallest degradation product was xylobiose, thus indicating that the enzyme was an endoxylanase. The enzyme was able to bind weakly to crystalline cellulose (Avicel) and more strongly to insoluble xylan in a substrate amount-and temperature-dependent manner.

Keywords Archaea · *Sulfolobus solfataricus* · Xylan · Xylanase

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Introduction

Cellulose, hemicellulose, and lignin, the main components of the wood and plant cell walls, are the major reservoirs of energy and nutrients in nature (Taiz and Zeiger 1991). Being the most abundant hemicellulose, xylan is the main food source of farm animals and is also a major component of raw materials for many industrial processes (Thomson 1993). Xylan is a heterogeneous polysaccharide consisting of a backbone of β-1,4-linked xylopyranosyl units, half of which are linked to acetyl, α-methylglucuronyl, or L-arabinofuranosyl residues (Biely 1985). Although the total breakdown of xylan requires the cooperative action of many enzymes (endo-β-1,4-xylanase, β-D-xylopyranosidase, α-L-arabinofuranosidase, acetyl xylan esterase, α-D-glucuronidase), the key enzyme is the endo-β-1,4-xylanase because it cleaves the internal glycosidic bond of the polysaccharide.

Consequently, this enzyme has acquired major biotechnological interest, and some applications have already begun, ranging from the biobleaching of paper pulp (Viikari et al. 1994) and improvements to the digestibility of animal feeds (Bedford 1995) to applications in the baking industry, e.g., as a flour additive (Maat et al. 1992). Xylanases from hyperthermophilic bacteria are attracting increasing interest at the industrial level because of their possible exploitation for xylan digestion processes at high temperatures. Their hydrolysis products can be converted into fuel and non-cariogenic sweeteners (xylitol) or can be used in the food industry as thickeners or fat substitutes (Wong and Saddler 1993; Hayes 2001). Moreover, xylo-oligosaccharides, particularly xylobiose, exhibit prebiotic properties that have been demonstrated by a stimulatory effect on the growth of the intestinal bacterium *Bifidobacterium* (Okazaki et al. 1990; Hopkins et al. 1998).

Most hyperthermophilic xylanases described so far belong to family 10 of glycosyl hydrolases, and only three are included in family 11 (Henrissat and Coutinho 2001). To date, only a few cases of archaeal xylanases

have been reported in the literature and none of them is included in the two families. The first reports of hemicellulases from Archaea indicate the presence of xylanolytic activities in two *Thermofilum* strains and in *Pyrococcus furiosus* (Bragger et al. 1989; Uhl and Daniel 1999).

The most recent report of a xylanase from Archaea describes activity from the deep-sea hyperthermophile *Pyrodictium abyssi*. Although this is the first study on fermentation strategies to improve the production and secretion of xylanases in Archaea, little information is provided on the main features of the enzyme (Carvalho Andrade et al. 2001). The only archaeal xylanase purified and partially characterized is the xylanase from the Euryarchaeon *Thermococcus zilligii* strain AN1 (Uhl and Daniel 1999), which is mainly detected in the culture supernatant. This enzyme shows a unique N-terminal sequence that has no significant homology with any xylanase. Recently, however, Rolland et al. (2002) demonstrated that the amino acid sequence of the enzyme shows significant similarities with a maltodextrin phosphorylase. Among hyperthermophilic Archaea, the genus *Sulfolobus* (Brock et al. 1972) has been especially studied with regard to its physiological requirements. Many species of *Sulfolobus* have been shown to grow on different sugars, namely, monosaccharides or α-linked polysaccharides, but no information is available on their ability to grow on β-linked polysaccharides.

This paper describes the detection and the partial purification of a xylanase from the *Sulfolobus solfataricus* strain MT4 adapted to grow on xylan. The strain O_α, selected from a medium containing oat spelt xylan as the sole nutrient source, shows interesting morphological changes in the cell envelope when compared to the usual coccal shape present in more common media.

This is the first paper dealing with the isolation and characterization of a xylanase from a crenarchaeon. It also reports the first evidence of the ability of *S. solfataricus* to metabolize a β-linked polysaccharide as a growth substrate.

Materials and methods

Materials

Gelrite, oat spelt xylan, beechwood xylan, birchwood xylan, D-xylose, Remazol Brilliant Blue R-D-xylan, and Congo Red were obtained from Sigma. Reagents for electrophoresis analyses and SDS-Broad Range standard protein mixture were purchased from BioRad. Low- and high-molecular-weight gel filtration calibration kits were obtained from Amersham Pharmacia Biotech.

Isolation of O_α and X_2 strains and cultivation

Sulfolobus solfataricus strain MT4 (DSM 5833) was supplied by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). *S. solfataricus* was grown aerobically at 80°C in Brock's salt basal medium (Brock et al. 1972) containing 0.3% (w/v) glucose buffered at pH 3.7 and was gradually adapted to grow on xylan. A 500-ml Erlenmeyer flask containing 80 ml Brock's salt basal medium supplemented with 0.2% (w/v) glucose and 0.1% (w/v) oat spelt xylan or birchwood xylan was inoculated with 20 ml of the previous culture. Subsequently, a 500-ml Erlenmeyer flask containing 80 ml Brock's salt basal medium supplemented with 0.2% (w/v) oat spelt xylan or birchwood xylan was inoculated with 20 ml of the precedent culture. For solid plates, the Brock's medium was supplemented with 0.8% (w/v) gelrite (Gellan gum; Sigma) and 0.1% (w/v) oat spelt xylan or birchwood xylan. Twenty-microliter aliquots of the liquid cultures grown on xylan were spotted onto the gelrite plates and incubated at 80°C. Four colonies were isolated from each plate and were streaked onto fresh gelrite plates containing 0.2% of the appropriate xylan. After 10 days' growth at 80°C, colonies of *S. solfataricus* were isolated from oat spelt xylan and indicated as O_α, while the isolated colonies from birchwood xylan were indicated as X_2.

One-liter cultures in liquid medium were performed by inoculating the Brock's salt basal medium containing 0.1% or 0.2% oat spelt xylan or birchwood xylan with the corresponding xylan-adapted culture. Parallel experiments to determine the inducible expression of the xylanase were performed by growing strain O_α in rich medium (Brock's basal medium supplemented with 0.1% w/v yeast extract and 0.1% w/v casamino acids) or in Brock's basal medium supplemented with 0.2% D-xylose as the sole nutrient.

Characterization of *S. solfataricus* O_α

The morphological properties and taxonomic characteristics of *S. solfataricus* O_α were studied. Light and electron microscopy were used in order to investigate the morphological changes of the cells when grown in Brock's basal medium supplemented with oat spelt xylan as the sole carbon source. The micrographs were produced by C.I.S.M.E. (Centro Interdipartimentale di Servizio per la Microscopia Elettronica, University of Naples Federico II, Italy) according to the following protocols. Light microscopy: cells were inspected with a Zeiss microscope equipped with an oil immersion objective of 100/1.6; negative staining: a drop of 1% (w/v) uranyl acetate was dropped on the cells and left for 1 min. The sample was dried with filter paper and after 1 h was observed under the microscope; scanning electron microscopy: the cells were fixed with 0.02% OsO_4 in 1% glutaraldehyde. Then the fixed cells were dehydrated with 30%, 50%, 80%, and 100% ethanol and 100% acetone, mounted with carbon paint on stubs, coated with gold, and observed under the microscope.

The nucleotide sequences of 16S rDNA were isolated by PCR amplification of the corresponding genes on the *S. solfataricus* MT4 and O_α chromosomal DNAs according to the basic protocol by Sambrook and Russell (2001). The oligonucleotide primers were designed against the already known *S. solfataricus* P2 16S rDNA and mapped at the positions 119–141 and 1370–1392, respectively. The sequences obtained were compared by the Phylip Interface Program available on the Internet at the Ribosomal Database Project II site (http://rdp.cme.msu.edu/html).

Enzyme isolation

Wet cells (25 g) from 5 l Brock's basal medium supplemented with 0.2% oat spelt xylan were harvested in the stationary phase, suspended in 10 ml 50 mM Tris-HCl pH 7.0, and ground in a mortar with sand (25 g, 50–150 mesh) for 1 h. After centrifugation at 2,000 *g* for 10 min in order to remove sand and unbroken cells, the supernatant was ultracentrifuged at 55,000 *g* for 30 min. The clear crude extract was stored at 4°C, while the pellet, containing membrane fragments, was suspended in 25 ml 50 mM Tris-HCl pH 7.0/0.5% Triton X-100 and incubated overnight at 70°C.

After incubation, the suspension was ultracentrifuged as described above. The pellet was discarded and the supernatant (30 ml) was extensively dialyzed against 25 mM Tris-HCl pH 7.0. After dialysis, the supernatant, exhibiting xylanase activity, was indicated as TX extract.

The TX extract was 20-fold concentrated by ultrafiltration with YM 10 membrane (Millipore). The resulting sample was dialyzed against 25 mM Tris-HCl, 200 mM NaCl, pH 8.4, and fractionated, using the same buffer at a flow rate of 0.5 ml/min, by the AKTA Fast Protein Liquid Chromatography system (Amersham Pharmacia Biotech) equipped with a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech). Fractions with xylanase activity were pooled, dialyzed against 25 mM Tris-HCl pH 7.0, and concentrated by ultrafiltration with YM 10 membrane. The concentrated sample was applied to a Mono Q HR 5/5 column (Amersham Pharmacia Biotech) equilibrated with 25 mM Tris-HCl pH 7.0 buffer, and the enzyme was eluted with a continuous NaCl gradient (0.0–0.5 M). Active fractions were pooled, dialyzed against 25 mM Tris-HCl pH 7.0, and used for subsequent studies.

Xylanase activity in the fractions was detected by spotting 10 µl of each fraction onto 1.5% agarose plates supplemented with 0.1% oat spelt xylan in 50 mM Tris-HCl pH 7.0, followed by incubation at 80°C for 1 h. Subsequently, the plates were stained with 0.1% (w/v) Congo Red for 30 min at room temperature and destained with 1 M NaCl. Xylanase activity was revealed as a white halo on a blue background after addition of 0.5% acetic acid.

Enzyme assays and protein determination

For enzyme activity estimation, an assay based on the use of a soluble chromogenic xylan was used (Biely et al. 1985). The activity was measured by adding 250 µl 0.2% Remazol Brilliant Blue R-D-xylan (RBB-xylan) to 25 mM Tris-HCl pH 7.0 to 100 µl of enzyme solution and incubating at 80°C for 10–30 min. The reaction was stopped by addition of 1 ml 96% ethanol to the mixture, followed by incubation at room temperature for 15 min and centrifugation at maximum speed for 5 min. The absorbance of the supernatant was measured at 590 nm. One unit of xylanase activity (RBB-unit) was defined as the amount of enzyme required to increase the absorbance at 590 nm of 1 Δ/min under standard conditions.

Alternatively, enzyme activity was measured by determining the amount of reducing sugars released from the oat spelt xylan. The standard reaction mixture consisted of 50 µl 1% xylan in 25 mM Tris-HCl pH 7.0 and 50 µl of enzyme solution. After 1 h incubation at 80°C, the reaction was stopped on ice and the amount of reducing sugars released was measured at 520 nm by the Somogyi-Nelson method (Nelson 1944). One unit of xylanase activity (SN-unit) was defined as the micromoles of xylose released per minute per milliliter, and it corresponds to 1.19 RBB-units.

Protein concentration was determined as described by Bradford (1976) using the BioRad protein staining assay and BSA as standard.

Electrophoretic analyses

SDS-PAGE was performed at room temperature in 10% polyacrylamide gel by the method of Laemmli (1970) using the BioRad Mini Protean II cell unit. Proteins were revealed by staining the gel with Coomassie Brilliant Blue R250 (BioRad). Specific xylanase staining was carried out as described by Schwarz et al. (1987). A solution of oat spelt xylan (0.1% w/v final concentration) was added to the separating gel before polymerization. After the run, the gel was treated with two 15-min washes in 25 mM Tris-HCl pH 7.0/isopropyl alcohol (4:1 v/v) to remove SDS and then rinsed with the buffer to remove the isopropyl alcohol. The gel was incubated in 25 mM Tris-HCl pH 7.0 at 80°C for 30–60 min, stained with 0.1% (w/v) Congo Red solution for 30 min at room temperature, and subsequently destained with 1 M NaCl. Xylanase activity could be detected as a white band on a dark blue background after submerging the gel in 0.5% acetic acid.

Molecular mass estimation

The molecular mass of the xylanase under denaturing conditions was estimated by 10% SDS-PAGE using a SDS Broad Range Standard Protein Mixture (BioRad). The determination of the native molecular mass was performed by size-exclusion chromatography using a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) connected to the AKTA Fast Protein Liquid Chromatography system (Amersham Pharmacia Biotech). Fractions were eluted with 25 mM Tris-HCl, 200 mM NaCl, pH 8.4 at a flow rate of 0.5 ml/min. Native molecular mass was estimated by comparing the specific retention time of the enzyme with a calibration run performed with aldolase from rabbit muscle (158.0 kDa), bovine serum albumin (67.0 kDa), and ovalbumin from hen egg (43.0 kDa) as molecular weight standards.

Influence of pH and temperature

The dependence of the activity on the pH was monitored at 80°C in universal buffer over the range 3.0–9.0 by the Somogyi-Nelson assay. The influence of temperature on xylanase activity was studied over the range 60–100°C in 25 mM Tris-HCl pH 7.0 for 1 h by the Somogyi-Nelson assay. For assays at 90°C, 95°C, and 100°C, the reaction mixture was incubated in Eppendorf tubes with mineral oil overlaid in order to avoid water evaporation.

The thermal stability was studied at 90°C and 100°C. Enzyme samples (170 µg/ml in 25 mM Tris-HCl, pH 7.0) were incubated in sealed Eppendorf tubes with mineral oil overlaid. Aliquots were withdrawn at the requested times and assayed at 80°C by the Somogyi-Nelson assay.

Substrate specificity

The substrate specificity of xylanase was determined using the following xylans: oat spelt, birchwood, and beechwood. The activity was measured by the Somogyi -Nelson assay, estimating the amount of reducing sugars released after 1 h from 1% xylans in 25 mM Tris-HCl pH 7.0 at the optimal temperature for xylanase activity (90°C).

Xylan degradation products were qualitatively determined by thin-layer chromatography (TLC) on pre-coated silica gel plates (60 F254, Merck) by using acetone-isopropyl alcohol-water (6:3:1.5 by volume) as eluent. The enzyme (10 RBB-mU) was added to 175 µl oat spelt xylan (1% in 25 mM Tris-HCl pH 7.0) and the mixture was incubated in sealed Eppendorf tubes at 80°C. Samples for the analysis were withdrawn at different times and centrifuged in an Eppendorf centrifuge at maximum speed for 5 min. The clarified supernatants were loaded onto the silica gel plate, and the hydrolysis products were detected, after separation, by spraying the plate with α-naphtol (3.5% w/v in 83% ethanol and 10% sulfuric acid) followed by heating at 150°C for 10 min.

Binding assay

The preparation of insoluble oat spelt xylan was performed by an alkali treatment method as previously reported (Irwin et al. 1994). Binding experiments were run by adding the enzyme (90 RBB-mU) to Avicel or insoluble xylan (2, 6, and 10% w/v) in 25 mM Tris-HCl pH 7.0. Samples were stirred for 1 h at 25°C, 50°C, and 70°C and then centrifuged. The amount of residual enzyme in the supernatant was determined by the RBB xylan assay.

Results

Identification of xylanolytic activity in *Sulfolobus solfataricus*

Aliquots of 10^6 cells of *Sulfolobus solfataricus* MT4, preliminarily adapted in liquid Brock's basal medium supplemented with oat spelt xylan or birchwood xylan as carbon source, were seeded onto gelrite plates containing the corresponding xylan. After 10 days' growth at 80°C, 10–20 colonies (named O_α from growth on oat spelt and X_2 from growth on birchwood) could be observed. Specific staining with Congo Red confirmed the presence of xylanase activity due to the white halo around the colonies, which indicated the ability of the adapted *S. solfataricus* strains to grow on xylan (Fig. 1). However, the wild type was not able to grow in either solid or liquid media containing Brock's basal medium plus xylan as the sole carbon source.

In order to verify the inducible expression of the enzyme, *S. solfataricus* O_α was also grown in Brock's basal medium supplemented with xylose, as well as in a rich medium. O_α was chosen for these growths, since this strain exhibited a threefold higher level of enzyme production with respect to X_2. In addition, the xylanase activity was 8.7-fold higher with respect to the basal level (rich medium) and was completely undetectable in the medium containing xylose, indicating the necessary presence of the polysaccharide for a high level of enzyme expression (Table 1).

In all conditions examined, the activity was found to be almost exclusively cell associated, and zymographic analysis revealed that *S. solfataricus* produced the same xylanolytic activity independent of the xylan source used in the culture media (Fig. 2).

Since Brock's basal medium supplemented with 0.2% oat spelt xylan was demonstrated to be the best medium for xylanase production among those tested, the enzyme was isolated from the *S. solfataricus* O_α grown in these conditions. Moreover, it was observed that O_α cells exhibited morphological changes on the surface, showing mobile extensions of variable length depending on the distance of the cell from xylan granules. It is interesting to underline that this phenomenon occurred only in the presence of oat spelt xylan. Negative staining of *S. solfataricus* O_α showed that the adhesion of xylan particles was specific, namely, located only around the induced appendages (Fig. 3).

Table 1 Distribution of xylanase activity from *Sulfolobus solfataricus* after growth in different carbon sources

Carbon source	Strain	Culture broth (U/l)	Cells[a] (U/l)
Rich medium	O_α	Undetectable	0.57
BBM[b] + 0.2% xylose	O_α	Undetectable	Undetectable
BBM + 0.1% birchwood	X_2	0.06	1.01
BBM + 0.2% birchwood	X_2	0.08	1.46
BBM + 0.1% oat spelt	O_α	0.33	2.30
BBM + 0.2% oat spelt	O_α	0.88	4.96

[a]Xylanase activity in the cells was the sum of the activity present in the cell extract and in the membrane fragments measured by the RBB xylan assay
[b]BBM: Brock's basal medium

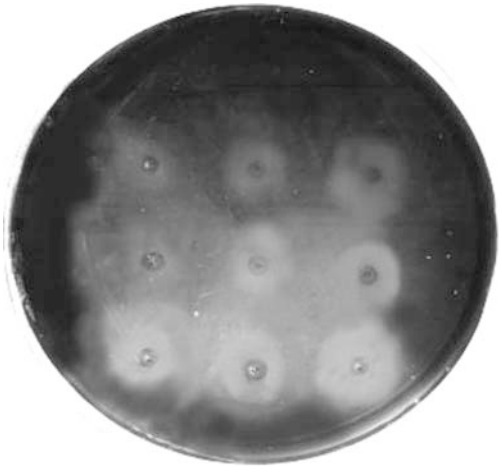

Fig. 1 Detection of xylanase activity in *Sulfolobus solfataricus* O_α grown on gelrite plate containing Brock's basal medium supplemented with 0.2% (w/v) oat spelt xylan. The plate was stained with 0.1% (w/v) Congo Red, destained with 1 M NaCl, and rinsed with 0.5% acetic acid. After this treatment, xylanase activity was evidenced as a white halo around the colonies on a dark blue background

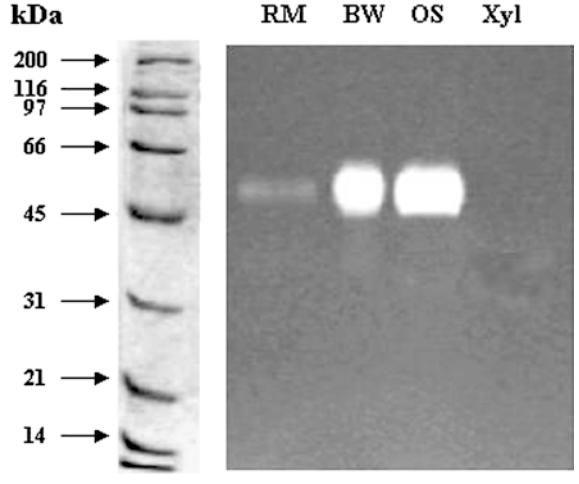

Fig. 2 Detection of xylanase activity in *S. solfataricus* grown in different carbon sources. The Triton X-100 extracts of membrane proteins (30 μg total proteins for each sample) were analyzed by zymogram on 10% SDS-PAGE containing oat spelt xylan (0.1% w/v final concentration). Lanes: *RM*, O_α grown in rich medium; *BW*, X_2 grown in Brock's basal medium supplemented with 0.2% (w/v) birchwood xylan; *OS*, O_α grown in Brock's basal medium supplemented with 0.2% (w/v) oat spelt xylan; *Xyl*, O_α grown in Brock's basal medium supplemented with 0.2% (w/v) xylose

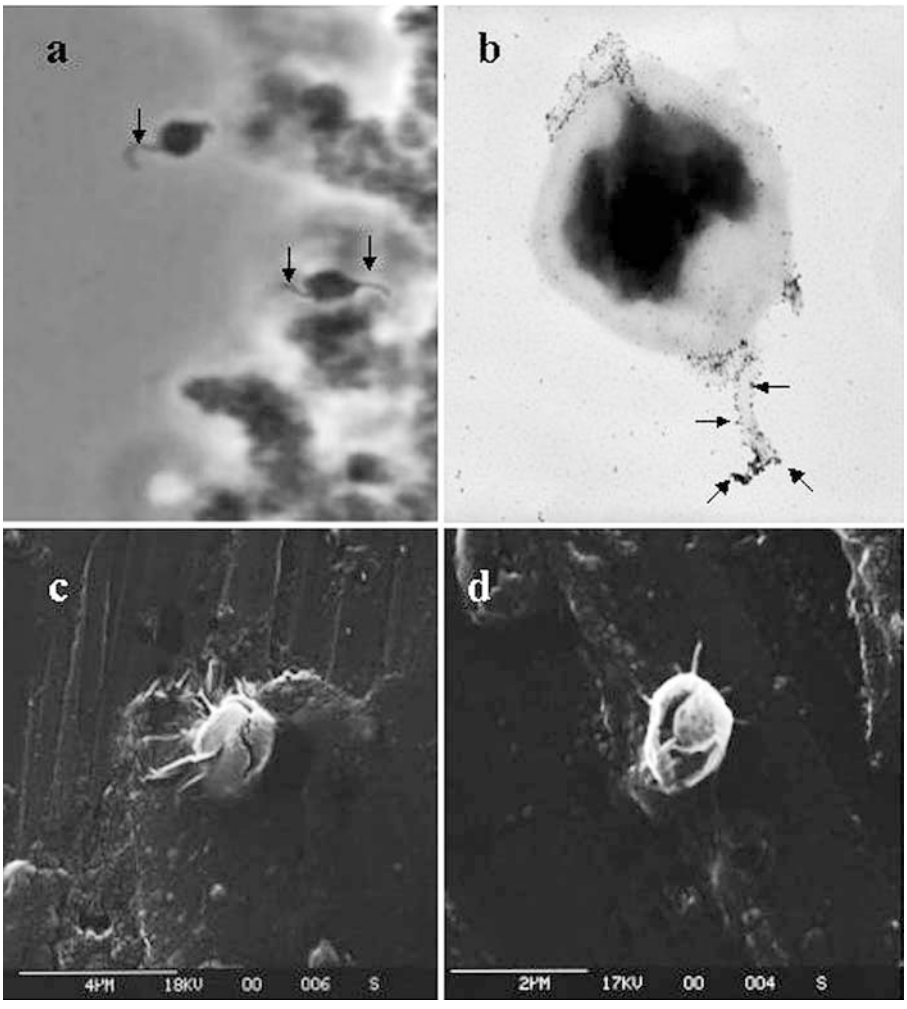

Fig. 3a–d Micrographs of *S. solfataricus* O_α grown in Brock's basal medium supplemented with 0.2% (w/v) oat spelt xylan. **a** Light microscopy: *arrows* indicate the mobile appendages. **b** Negative staining: *arrows* indicate the xylan particles around the appendage. **c, d** Scanning electron microscopy: cells showing several appendages

Table 2 Purification of xylanase from *S. solfataricus*. Purification was performed starting from 25 g (wet mass) of *S. solfataricus* O_α harvested from 5 l Brock's basal medium supplemented with 0.2% oat spelt xylan O_α

Purification step	Total activity[a] (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (*n*-fold)
TX extract	22	19.8	1.1	100	1
Superdex 200	10.1	5.6	1.8	46	1.6
Mono Q	4.0	0.35	11.4	18	10.4

[a]Xylanase activity was measured by the RBB xylan assay

Fractionation and purification of xylanase from *Sulfolobus solfataricus* O_α

In order to determine the distribution of xylanase at the cell level, the activity was measured in the crude extract and in the membrane fragments. Almost all the activity was localized in the membrane fragments, and 92% of its activity could be released by treatment with Triton X-100. This procedure led to the recovery of 4.41 RBB-units per liter of culture, with a specific activity of 1.1 U/mg of enzyme per liter of culture. The partial purification of the xylanase was performed starting from 25 g (wet mass) of *S. solfataricus* O_α grown in Brock's basal medium supplemented with 0.2% oat spelt xylan (Table 2). The TX extract containing the xylanase was then subjected to size-exclusion chromatography. This purification step was necessary for the separation of xylanase from other glycosyl-hydrolytic activities. Fractions containing the xylanase were pooled and further purified by anion-exchange chromatography. The enzyme was eluted at between 0.15 and 0.18 M NaCl and proved to be purified 10.4-fold with a specific activity of 11.4 U/mg.

Characterization of xylanase

The native molecular mass of the enzyme, which was determined by size-exclusion chromatography on a Superdex 200 column, was 58.8 kDa. Since the molecular mass, estimated by SDS-PAGE, was calculated to be 55.5 kDa, a monomeric structure was suggested for the enzyme. The optimal pH for activity at 80°C was 7.0, an unexpected value because the enzyme was localized in an external environment characterized by pH 3.7. In each case, the enzyme showed 46% maximal activity at pH 4.0. There was also considerable xylanase activity in an alkaline pH, retaining 63% activity at pH 8.0 and 40% at pH 9.0. The dependence of the activity on temperature over the range 60–100°C was determined at pH 7.0. After 1 h incubation, xylanase exhibited optimal temperature at 90°C and retained almost 30% maximal activity at 100°C. The resistance to heating was investigated at 90°C and 100°C at pH 7.0. Fifty percent maximal activity was measured after 85 min at 90°C, and 23% residual activity could still be measured after 3 h. The half-life at 100°C was reached after 47 min. The substrate specificity of the xylanase was investigated at the optimal temperature and pH (90°C, pH 7.0) with different xylans. Oat spelt was chosen because it is a cereal xylan, while beechwood and birchwood were chosen because they derive from hardwood.

The enzyme was active towards all the substrates tested, exhibiting the highest activity in the presence of beechwood xylan, followed by oat spelt xylan (87%) and birchwood xylan (67%).

The degradation products from oat spelt xylan were analyzed by TLC. The smallest end product obtained after 96 h incubation was xylobiose. Xylotriose and medium-sized oligomers were also produced, indicating that the enzyme was an endoxylanase. When oat spelt xylan was incubated with TX extract, which contained several glycosyl-hydrolytic activities, the polysaccharide degradation was increased and the main end product of hydrolysis was xylose.

Xylanase binding to Avicel and insoluble xylan

To evaluate the ability of the xylanase to bind to insoluble substances, the enzyme was incubated with Avicel or insoluble xylan at different temperatures. The soluble fraction was assayed to determine the unbound activity. The amount of bound enzyme rose as the temperature increased, and 63% of the enzyme bound at 70°C to insoluble xylan (100 mg/ml) (Fig. 4a). In contrast, the binding of the enzyme to Avicel was weaker, with a maximum of 35% of the total activity bound to 100 mg/ml Avicel (Fig. 4b).

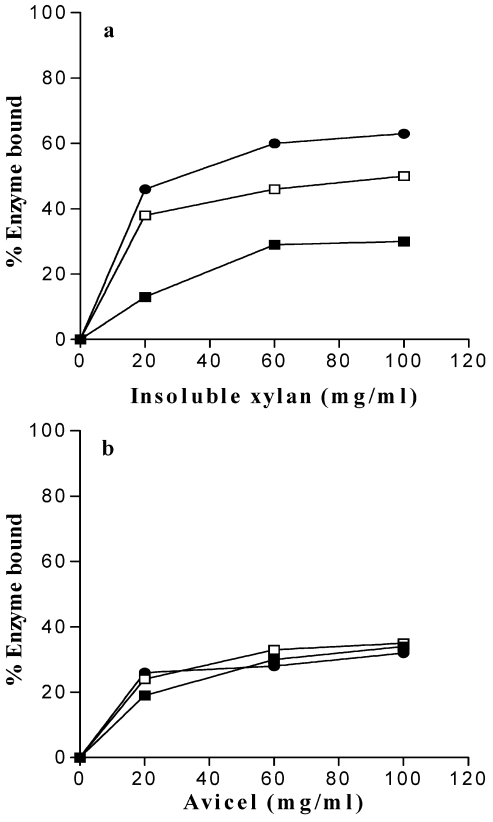

Fig. 4 Xylanase binding to insoluble xylan (**a**) and Avicel (**b**). The capacity of a constant amount of xylanase from *S. solfataricus* O_α (90 RBB-mU) to bind to different amounts of insoluble xylan or Avicel was investigated. The binding test was carried out for 1 h at 25°C (■), 50°C (□), and 70°C (●). Unbound xylanase was estimated by the RBB xylan assay

Discussion

This paper reports the first evidence of the ability of the crenarchaeon *Sulfolobus solfataricus* to grow using a β-linked polysaccharide as the carbon source. The *S. solfataricus* strain MT4 was gradually adapted to grow on minimal media containing birchwood xylan or oat spelt xylan as the sole carbon source. Two strains showing xylanolytic activity were subsequently isolated from the adapted cultures containing birchwood xylan or oat spelt xylan and were named X_2 and O_α, respectively. The identity score obtained from the alignment of the16S rRNA of the two isolated strains with the 16S rRNA of the MT4 strain was 100%, making it possible to establish that the isolated strains were not contaminants of the MT4 strain but actual derivatives. After the initial adaptation on xylan, O_α and X_2 were able to grow and produce xylanase when inoculated in a minimal medium supplemented with xylan for an indefinite time, thus confirming that the capacity acquired to grow and metabolize xylan was a stable characteristic. However, no growth was observed with the MT4 strain when it was directly inoculated in the same medium.

Since the capacity to grow on xylan as the sole carbon source following pre-adaptation could be explained as either an adaptation of the wild type or a mutation, it is difficult to establish at the moment whether the isolated strains were mutants or whether the parental MT4 was simply adapted to the new growth conditions.

Oat spelt xylan and birchwood xylan acted as inducers for the xylanase, which was almost exclusively membrane associated. Although O_α and X_2 expressed the same xylanase, strain O_α showed an interesting morphological change that was not observed in the cells grown on birchwood xylan. Oat spelt is an insoluble xylan, and the presence of mobile extensions at the membrane level could prove advantageous for the binding of the cell to the polysaccharide particles. This hypothesis is confirmed by the adhesion of the xylan particles to the cell appendages, which was observed by negative staining, suggesting the necessity of a "physical" contact to start the degradation of the polysaccharide by xylanase.

Besides this peculiar feature, *S. solfataricus* O_α produced a greater amount of xylanase than *S. solfataricus* X_2. Therefore, the isolation and the characterization of the enzyme were performed using this strain. The enzyme was active between 60°C and 100°C, demonstrating the highest level of activity at 90°C. Among the xylanases characterized from extremophilic microorganisms, only several endoxylanases from *Thermotoga* showed higher optimal temperatures (Simpson et al. 1991; Winterhalter and Liebl 1995; Sunna et al. 1996). However, xylanase from *S. solfataricus* appears to be the most thermostable at 100°C compared to the xylanases from the *Thermotoga* sp. strain FjSS3-B.1 and *Thermococcus zilligii*, ($t_{1/2}$: 47, 20, and 8 min, respectively) (Simpson et al. 1991; Uhl and Daniel 1999).

Xylanase from *S. solfataricus* O_α was highly active towards all three xylans tested, showing preference for beechwood and oat spelt. The lowest level of activity, which was still considerable, was shown towards birchwood xylan. In terms of substrate preference, it is difficult to rationalize the different behavior of the enzyme towards beechwood and birchwood since they are both 4-O-methylglucuronyl-xylans. The only reasonable explanation is that beechwood xylan and birchwood xylan can differ in purity grade in different preparations, especially because of their lignin content, which affects the water-solubility and the accessibility of the enzyme to the substrate.

The enzyme isolated is an endoxylanase, as demonstrated by the products from xylan hydrolysis, which proved to be a mixture of xylo-oligosaccharides. The pattern of hydrolysis obtained by TX extract, which increased the xylan degradation and produced xylose in addition to the oligomers, demonstrated the necessity of a cooperative action among the xylanase and other xylanolytic enzymes to achieve a more efficient breakdown of the polysaccharide.

Since the cellulose-binding domain (CBD) is a component of many xylanases (Black et al. 1997), we attempted to verify the presence of a CBD in the xylanase from *S. solfataricus*. The enzyme bound weakly to crystalline cellulose (up to 35%), while 63% total activity bound to insoluble oat spelt xylan at 70°C. Other xylanases possessing both a CBD and a xylan-binding domain have been described, and the xylanase STX-II from *Streptomyces thermoviolaceus* was seen to display the same behavior, namely, a stronger affinity for the insoluble xylan (Tsujibo et al. 1997). The presence of a substrate-binding domain can play an important role in targeting the enzyme toward the substrate, increasing its local concentration and facilitating the polysaccharide hydrolysis, especially in the case of barely soluble substrates.

The analysis of the fully sequenced *S. solfataricus* P2 genome published on the Internet at http://www-archbac.u-psud.fr/projects/sulfolobus/ (She et al. 2001) did not point out any sequence significantly matching with xylanases from other microorganisms. Moreover, the low yield of xylanase obtained did not make it possible to produce enough material in order to find significant homologies with xylanases and/or other glycosyl hydrolases. Therefore, large-scale fermentation of *S. solfataricus* O_α as well as strategies aimed at the direct cloning of the gene involved in the xylan degradation are currently being developed.

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