Isolation and characterization of highly thermophilic xylanolytic *Thermus thermophilus* strains from hot composts

Pierre-François Lyon, Trello Beffa, Michel Blanc, Georg Auling, and Michel Aragno

Abstract: This is the first detailed report of xylanolytic activity in *Thermus* strains. Two highly thermophilic xylanolytic bacteria, very closely related to non-xylanolytic *T. thermophilus* strains, have been isolated from the hottest zones of compost piles. Strain X6 was investigated in more detail. The growth rate (optical density monitoring) on xylan was $0.404 \cdot h^{-1}$ at 75°C. Maximal growth temperature was 81°C. Xylanase activity was mainly cell-bound, but was solubilized into the medium by sonication. It was induced by xylan or xylose in the culture medium. The temperature and pH optima of the xylanases were determined to be around 100°C and pH 6, respectively. Xylanase activity was fairly thermostable; only 39% of activity was lost after an incubation period of 48 h at 90°C in the absence of substrate. Xylanolytic *T. thermophilus* strains could contribute to the degradation of hemicellulose during the thermogenic phase of industrial composting.

Key words: *Thermus*, thermophilic aerobic bacteria, xylanase, thermostable enzyme, compost.

Résumé : Il s'agit de la première étude détaillée menée sur des souches de *Thermus* xylanolytiques. Deux nouvelles souches hautement thermophiles, isolées dans la zone la plus chaude de tas de compost, sont affines aux souches de *T. thermophilus* non xylanolytiques déjà décrites. La souche X6 a été étudiée plus en détail. Son taux de croissance (suivi de la densité optique) sur xylane est de $0.404 \cdot h^{-1}$ à 75°C. Sa température maximale de croissance est de 81°C. L'activité xylanase est principalement liée à la cellule bactérienne, mais un traitement aux ultrasons la solubilise dans le milieu. Elle est induite par la présence de xylane ou de xylose dans le milieu. La température et le pH optimum des xylanases sont proches de 100°C et de pH 6, respectivement. L'activité xylanase est très thermostable, puisque l'on observe que 40 % de pertes d'activité après une incubation de 48 h à 90°C en absence de substrat. Les *T. thermophilus* xylanolytiques pourraient être responsables de la dégradation d'une partie de l'hémicellulose, durant la phase thermogène du compostage industriel.

Mots clés : *Thermus*, bactérie thermophile aérobie, xylanase, enzyme thermostable, compost.

Introduction

Industrial composting is a microbial, aerobic, self-heating, solid-phase controlled process bringing about the mineralization and the stabilization of the organic fraction of household waste (De Bertoldi et al. 1983; Finstein and Morris 1975; Miller 1996). In windrows, the large upper central zone may remain at temperatures higher than 70°C for several weeks (Beffa et al. 1996*b*; Lott Fischer et al. 1998). This hot zone harbors a high number of thermophilic bacteria forming a well diversified community (Beffa et al. 1996*a*; Blanc et al. 1997, 1999; Strom 1985*a*, 1985*b*), including the species *Thermus thermophilus* (Beffa et al. 1996*b*).

Several authors have shown the presence of extracellular lignocellulose-degrading enzymes during industrial and laboratory composting at temperatures up to 65°C (Ball and Jackson 1995; Bono et al. 1992; Godden et al. 1983; Herrmann and Shann 1993; Stutzenberger et al. 1970). The degradation of lignocellulose at higher temperatures has been little studied. Nevertheless, xylanase activity has been detected in hot composts up to 80°C (Lyon et al. 2000), though actinomycetes are the only thermophilic xylanolytic microorganisms that have so far been isolated from compost (Ethier et al. 1994; Holtz et al. 1991).

Xylanolytic microorganisms were repeatedly isolated from various environments and studied for their potential applications in industrial processes (Coughlan and Hazlewood 1993; Wong et al. 1988), especially for the bleaching of kraft pulp in the paper industry (Shoham et al. 1993; Viikari et al. 1994). Many studies focussed on thermostable xylanases, due to the operational conditions of the industrial processes (often high temperatures and (or) extreme pH

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Table 1. Media composition.

Medium name[a]	Substrate[b](g/L)			
	Yeast extract	Xylan	Xylose	Nutrient broth
Liquid				
Y	0.2	—	—	—
YX	0.2	—	0.5	—
YN	2.0	—	—	8.0
YXBW	0.2	2.0 (birchwood)	—	—
YXOS	0.2	2.0 (oat spelt)	—	—
Solid				
YXOS	0.5	5.0 (oat spelt)	—	—

[a]All media contained the basal mineral solution (BMM) of Beffa et al. (1996*a*) and the solid medium YXOS contained 15 g/L agar (Merck).
[b]Birchwood xylan was purchased from Sigma and oat spelt xylan from Fluka.

Table 2. DNA–DNA homology values (%) between xylanolytic strains (X6 and CS) and non xylanolytic *T. thermophilus* strains ($HB8^{TS}$ and CT1).

Strains	$HB8^{TS}$	X6	CT1
X6	103[a]		
CT1	78	81	
CS	69	67	86

[a]Similarity is given as percentage degree of homology according to De Ley et al. (1970).

values), and the interest in protein thermostability and gene transfer (Bergquist et al. 1999; Kulkarni et al. 1999). Until now, isolation of highly thermophilic xylanolytic microorganisms (optimum growth temperature >65°C) has been limited to geothermal sites and to anaerobic microorganisms (Lüthi et al. 1990; Nielsen et al. 1993*a*, 1993*b*; Saul et al. 1995; Shao and Wiegel 1995; Winterhalter and Liebl 1995). To our knowledge, a single report mentioned the presence of xylanolytic *Thermus* sp. in hot springs (Perttula et al. 1993), but the properties of their xylanases were not discussed.

This paper describes the isolation, identification, morphological characteristics, and growth of two highly thermophilic xylanolytic *T. thermophilus* strains from hot composts. The characteristics of the xylanases from strain X6 have been determined in cell free-extract without purification steps. The role of *Thermus* spp. in the degradation of organic matter during the thermogenic phase of composting is also discussed.

Materials and methods

Media

The composition of each medium is indicated in Table 1. Xylan powders (birchwood xylan was purchased from Sigma and oat spelt xylan from Fluka) were sterilized by UV treatment for 30 min before use, and the absence of thermophilic contaminants was checked by one-week incubation at 75°C and 60°C of non-inoculated xylan media YXBW and YXOS.

Sampling and isolation

The studied site was a classic open-air windrow-composting facility previously described by Lott Fischer et al. (1998). The substrate was constituted of separately collected kitchen and garden waste (50%), leaves and twigs (40%), mixed with rejects from the sieving (wood pieces from mature windrows, 10%). Windrows were turned every working day. Samples were taken from the hottest upper central part of the windrows (50 cm deep) as previously described (Beffa et al. 1996*b*; Blanc et al. 1999).

Thirty grams of compost (fresh wt) were placed in 270 mL of sterile water, homogenized at room temperature on a shaker (150 rpm) for 30 min, and serially diluted (10^{-2} to 10^{-10}) in the xylan medium YXOS, or in the non-elective rich and complex medium YN. Prior to incubation at 75°C, the serial dilutions were pre-incubated for 5 min in a water bath at 70°C. Screening for pure cultures of xylanolytic bacteria was carried out by successive plating, at 75°C, on the xylan medium YXOS. After one week of incubation, xylanolytic bacteria produced characteristic xylan hydrolysis zones that appeared on YXOS agar plates as clearing zones around the colonies.

DNA–DNA hybridization

Isolation of genomic DNA and hybridization were carried out by using the initial renaturation rate method of De Ley et al. (1970) according to Auling et al. (1986).

16S rDNA sequencing

Strains were cultivated in the rich and complex medium YN. Extraction and purification of the total cellular DNA from approximately 0.2 g (wet weight) washed cells from fresh cultures were carried out according to Beffa et al. (1996*b*). Cell lysis was increased by two extended heat-shock cycles (20 min at –80°C; 20 min at 65°C).

Using universal primers GM3f and GM4r, 16S rRNA genes were selectively amplified by PCR from purified genomic DNA (Muyzer et al. 1995). 16S rDNA products were inserted into a pGEM-T vector (Promega) which was used for the transformation of competent *E. coli* XL1 cells (Promega). Primers RNA1, RNA3, and RNA6 (Saul et al. 1993), as well as primers SP6 and T7 were used by Microsynth (Balgach, Switzerland) for the sequencing of the 16S rRNA genes (1478 pb for strain X6 and 1477 pb for strains and CT1 and CS).

Sequence analysis

Sequences of 16S rDNA were compared with those of other *Thermus* strains available in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk). Percentage identity of the 16S rDNA sequences and UPGMA-clustering were calculated using the GeneBase software (v. 1.0, Applied Maths BVBA, Kortijk, Belgium).

Nucleotide sequence accession numbers

The 16S rDNA sequences of the two xylanolytic strains and that of the *T. thermophilus* strain CT1, isolated from a hot compost by Beffa et al. (1996*b*), were deposited in the EMBL database under

Fig. 1. Phylogenetic UPGMA-dendrogram showing the position of the xylanolytic strains X6 and CS, as well as the non-xylanolytic strain CT1 also isolated from hot compost (Beffa et al. 1996*b*). The scale bar indicates the percentage of sequence homology between the 16S rRNA gene sequences of the different strains.

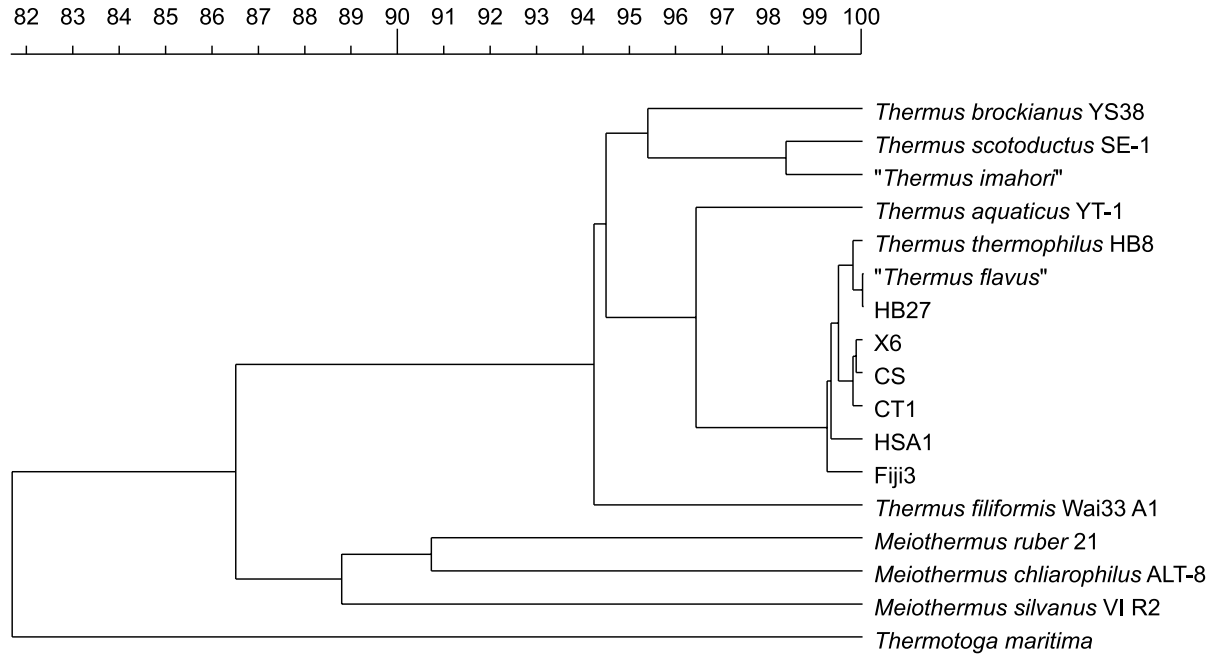


Table 3. Growth rates, and respiratory and xylanase activities of the strain X6 in function of the growth medium.

Growth[a]			Respiratory activity[b]		Xylanase activity[c]
			Yeast extract[f]	Birchwood xylan[f]	
Medium[d]	Specific growth rate ($\cdot h^{-1}$)	Final optical density[e] (A_{436nm})	(nmol O_2 · mg (dry wt)$^{-1}$ · min^{-1})		(U × mL^{-1})
Y	0.494	0.3	348	<5	$<0.3 \times 10^{-2}$
YX	0.436	1.1	280	193	20.5×10^{-2}
YXBW	0.404	1.6	267	182	15.1×10^{-2}

[a]Cultures were carried out at 75°C in fermentor.
[b]Measured for samples taken in the middle of the exponential growth phase and measured at 75°C with washed cells resuspended in sterile BMM.
[c]Measured for samples taken during the stationary growth phase and measured in the CFE fraction, at 70°C and pH 6.
[d]See Table 1.
[e]Measured at the end of the stationary phase.
[f]Respiratory substrate.

the following accession numbers: AJ251938 for strain CS, AJ251939 for strain X6, and AJ251940 for strain CT1.

Growth characteristics

The temperature and pH range, as well as substrate tests, were carried out in a water rotatory shaker (Aquatron, Infors AG, Basel, Switzerland), at 150 rpm. The temperature growth range was determined in the rich and complex medium YN, by incubation between 30°C and 85°C. The range of pH for growth was determined, at 75°C, in YN at pH values of 6, 7, and 8. For the following pHs, the different buffering salts replaced phosphate in YN: For pH 9, 84 mM boric acid–NaOH; for pH 8, 55 mM boric acid–NaOH; and for pH values of 6, 5, and 4, 50 mM citric acid–Na_2HPO_4. The pH of each medium was adjusted at 75°C after autoclaving.

The ability to grow in saline conditions was assessed in YN medium supplemented with 30 g/L of NaCl.

To evaluate the capacity of strain X6 to use organic substrates, the low concentration complex medium Y was supplemented with different substrates (acetate, cellobiose, cellulose (Sigmacell), D-arabinose, D-galactose, D-glucose, D-lactose, D-mannose, glutamate, nutrient broth, pyruvate and xylose) each at a concentration of 2 g/L.

Determinations of batch growth kinetics and monitoring of xylanase activity were carried out in a fermentor as previously described (Beffa et al. 1996*b*), using the xylan medium YXBW, the xylose medium YX, and the low concentration complex medium Y. Growth was followed turbidimetrically at 436 nm using 1-cm cuvettes in a Perkin-Elmer Lambda 6 spectrophotometer.

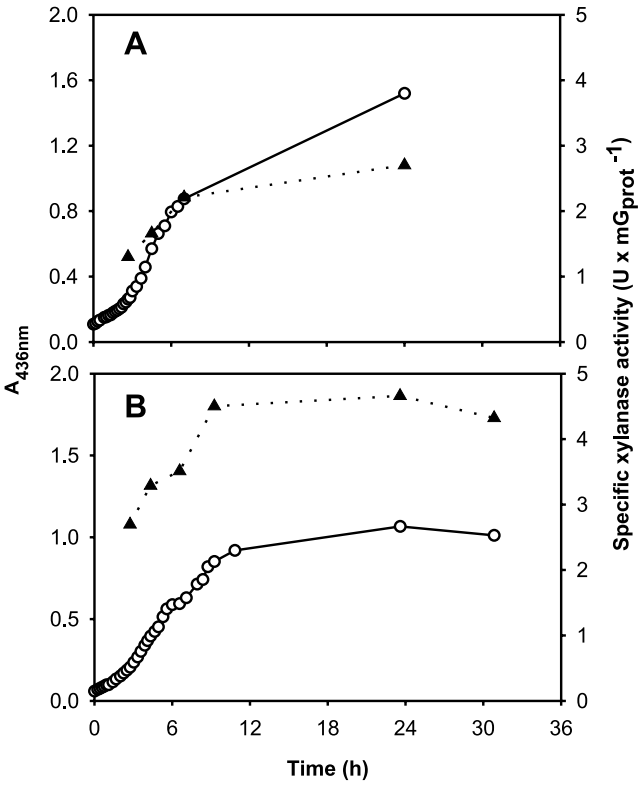
Oxygen uptake measurements

The respiratory activity was measured polarographically at 75°C with an oxygen electrode system as previously described (Beffa et al. 1996*a*). Respiratory activity was expressed as nmol O_2 consumed per mG (dry wt) and per min.

Xylanase assays

Xylanase activity (XA) was determined by the dinitrosalicylic acid method (Miller 1959) according to Lyon et al. (2000). One unit (U) of XA was defined as the release of 1 µmol of xylose equivalent per min. Protein concentration was determined by the

Fig. 2. Specific xylanase activity during growth of strain X6 at 75°C in fermentor. (A) Growth in the xylan medium YXBW; (B) growth in the xylose medium XY. (○) A_{436nm}, (▲) XA. Xylanase activity was measured in CFE fractions at 70°C and at pH 6.



method of Bradford (1976) with bovine serum albumin as the standard.

Strain X6 was cultivated overnight in 50 mL of the xylan medium YXBW (250 mL conical flasks). Unless otherwise indicated, XA was tested with freshly obtained cell-free extract fractions (CFE) in 50 mM potassium hydrogen phthalate–NaOH buffer (pH 6.0), as indicated below, and the reaction mixture was incubated for 1 h at 70°C.

Six different culture fractions were tested for the localization of the XA. The *supernatant* (SN) was obtained by centrifugation of a culture sample at 6000 × *g* during 20 min at 4°C. The cell pellet was washed twice by centrifugation (6000 × *g*; 2 min; 4°C) in sterile physiological water, then the washed cells (WC) were obtained by resuspension of the cells to the initial volume in the sterile basal mineral solution (BMM) used for the media (Table 1). The crude extract (CE) was obtained after sonication (Branson Sonifier 450; 5 × 30 s; power 20 W; duty cycle 50%; with pauses of 30 s) of fraction WC. The CE was centrifuged (12 000 × *g*; 1 h; 4°C) and thus divided into the cell-free extract (CFE) and cell-wall fragments (CWF). The cell-wall fragments were resuspended to the initial volume in the sterile BMM. Finally, the soluble cell-free extract (SCFE) was obtained by ultra-centrifugation of the CFE (100 000 × *g*; 1 h; 4°C).

The temperature optimum of xylanases was determined between 27 and 100°C. The pH optimum was ascertained between pH 4 and 9 with the previously described buffers. The thermal stability was determined by pre-incubation of CFE without substrate during 1, 3, 6, 24, and 48 h at 70°C, 80°C, 90°C, and 100°C, prior to the assay.

Table 4. Xylanase activity measured in the different fractions of the a culture of strain X6. Growth in the xylose medium YX, at 75°C.

Fraction tested	Xylanase activity[a] ($U \cdot mL^{-1}$)
Culture supernatant	5.0×10^{-2}
Washed cells	10.6×10^{-2}
Crude extract	19.0×10^{-2}
Cell-free extract	16.1×10^{-2}
Soluble fraction of the cell free extract	16.1×10^{-2}
Cell-wall fragments	1.9×10^{-2}

[a]Measured at 70°C in mineral solution used for the medium (pH 7.2).

Results

Sampling and isolation

The xylanolytic strain X6 was isolated from a compost sample collected after 14 days of composting (beginning of the thermogenic phase). The temperature at the sampling site was 75°C. The oxygen and carbon dioxide concentrations were 5% and 16% (v/v), respectively. The values for pH and moisture content of the sample were 6.7% and 56%, respectively. Bacterial growth occurred up to a dilution of 10^{-6} of the elective xylan medium YXOS, and strain X6 was isolated from this dilution.

The second xylanolytic strain, called CS, was isolated in the non-elective rich and complex medium YN, at 75°C, from the serial dilution enrichment 10^{-8} of a compost sample collected after 25 days of windrow composting. Temperature at the sampling site was 75°C. The values for pH and moisture content of the sample were 8.4% and 51%, respectively. Strain CS was the only xylanolytic strain among 26 highly thermophilic strains isolated in non-elective conditions from hot composts.

Morphological characteristics

Strains X6 and CS are non-spore-forming, Gram-negative, non-motile rods. The morphology of cells for both strains varied in function of the growth medium: long rods (2 to 5 × 0.5 μM) in the rich and complex medium YN, short rods (1 × 0.5 μM) in the xylan medium YXBW.

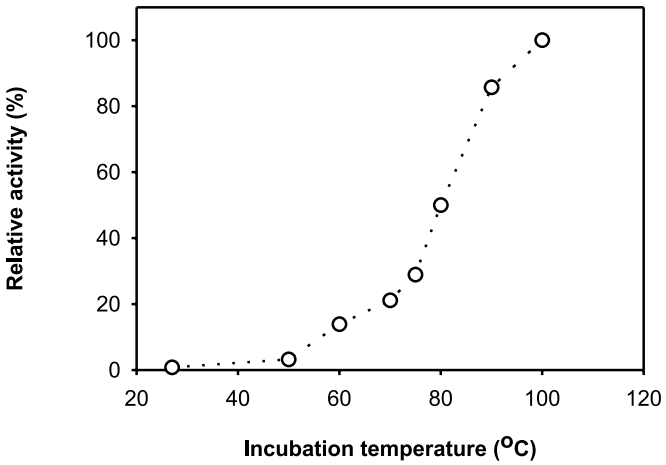
DNA–DNA hybridization

The xylanolytic strains X6 and CS were compared to the non-xylanolytic *T. thermophilus* strain CT1, also isolated from hot compost (Beffa et al. 1996*b*) and to *T. thermophilus* $HB8^{TS}$ isolated from a Japanese hot spa (Oshima and Imahori 1974) (Table 2). Strain X6 is more closely related to the type strain HB8 than to the strains CS and CT1, but DNA–DNA homology values exceeded 66% in all instances.

16S rDNA gene sequence

16SrDNA of the strains X6, CS, and CT1 were sequenced (1478 pb for strain X6 and 1477 pb for strains and CT1 and CS). Sequence homologies ranging from 99.1% to 99.9% confirm the three strains isolated from compost samples to be very closely related to other *T. thermophilus* strains (Fig. 1). Conversely, sequence homologies ranging from

Fig. 3. Xylanase activity of strain X6 as a function of temperature. Xylanase activity was measured in the CFE fraction, at pH 6.



94.2% to 96.4% clearly indicate that these three compost strains differed from the other species of *Thermus*.

Growth characteristics

Temperature growth range for strains X6 and CS was 40°C to 81°C, with a maximal growth rate at 70°C. Two strains were able to grow at pH values comprised between pH 6 and 8. No growth was detected at pH 5.5 and 9.0. Growth was also detected in medium YN supplemented with 30 g/L of NaCl.

Strains X6 and CS required a low concentration of yeast extract for growth. Good growth was obtained on cellobiose, D-galactose, D-glucose, D-mannose, nutrient broth, starch, xylan, xylose (0.5 g/L), and yeast extract. Weak growth was observed on acetate, D-arabinose, glutamate, and pyruvate, whereas no growth occurred on cellulose (Sigmacell) and D-lactose. Xylose concentrations over 1 g/L in culture medium were totally growth inhibitory.

Growth rate, final optical density, respiratory activity, and xylanase activity for strain X6 are shown in Table 3. Growth rate was slightly higher in the low concentration complex medium Y than in the xylose medium YX and the xylan medium YXBW. However, due to the low concentration of yeast extract (0.2 g/L), the medium Y allowed only a very low final optical density. Xylan-dependent respiratory activity and XA were only induced in the presence of xylan or xylose in the culture medium.

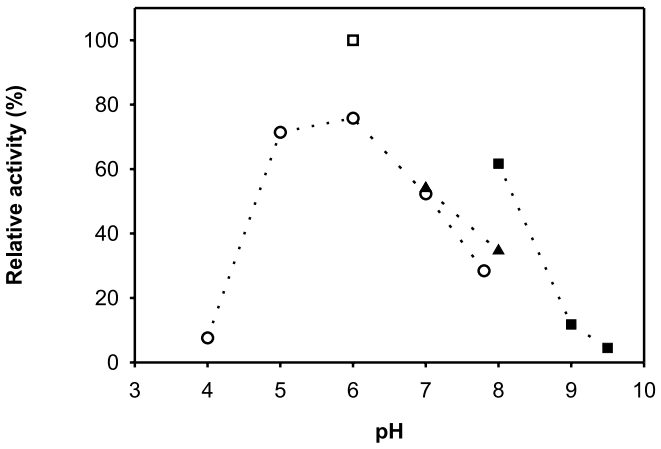
Characterization of xylanase activity of strain X6

During growth in the xylan medium YXBW and in the xylose medium YX, XA was detectable since the beginning of the culture. Then, the specific activity increased during the active growth phase, whereas it remained constant during the stationary phase (Fig. 2).

Table 4 shows that XA was mainly bound to intact cells, but sonicating the cells caused its solubilization.

Within the range of temperature tested, the temperature optimum of XA was close to 100°C at pH 6.0 (Fig. 3). At temperatures below 60°C, XA was close to the detection threshold. The pH optimum value, at 70°C, was around pH 6.0, but XA appeared to be also buffer dependent (Fig. 4).

Fig. 4. Xylanase activity of strain X6 as a function of pH. The following buffers were used: (○), citric acid-Na_2HPO_4; (□), potassium hydrogen phthalate-NaOH; (▲), potassium phosphate; (■), boric acid-NaOH. Xylanase activity was measured in the CFE fraction, at 70°C.



Xylanase activity was very stable between 70°C and 90°C, but decreased strongly at 100°C, the temperature optimum of XA (Fig. 5).

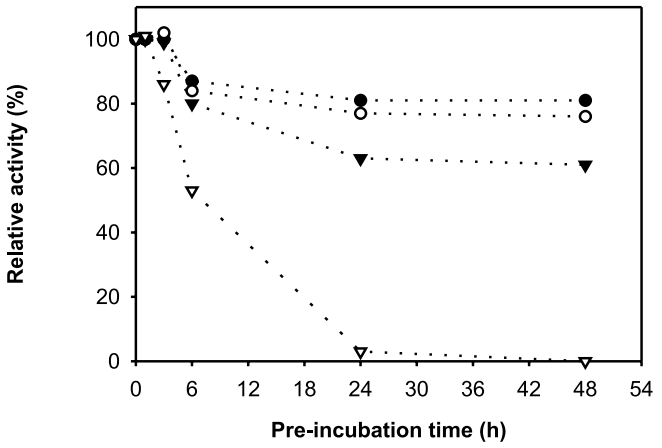
Discussion

This paper reports the first characterization of highly thermophilic aerobic xylanolytic bacteria belonging to the genus *Thermus*. The 16S rDNA sequences of the xylanolytic strains X6 and CS, as well as that of the non-xylanolytic strain CT1 (also isolated from hot composts), shared more than 99% sequence homology within the *T. thermophilus* strains and less than 97% with other valid *Thermus* species. Moreover, DNA–DNA homologies of the three strains isolated from compost piles and the *T. thermophilus* $HB8^{TS}$ show a clustering above 73%. DNA–DNA homology values between the different *Thermus* spp. type strains were reported to range from 35% to 43% (Williams et al. 1995, 1996). It is generally admitted that a species includes strains with an approximately 70% or greater DNA–DNA relatedness and with a ΔT_m = 5°C or less (Wayne et al. 1987). Therefore, both xylanolytic strains should be considered members of the species *T. thermophilus*, while accounting for the comments of Stackebrandt and Goebel (1994).

The presence of xylanolytic activity (XA) linked to a subpopulation of a highly thermophilic species belonging to the same phylum as *Thermus*/*Deinococcus*, in which no other xylanolytic member has been described so far, poses the question of its origin and its evolution. Indeed, a similarly thermophilic XA was also observed in *Thermotoga* (Simpson et al. 1991; Sunna et al. 1996; Winterhalter and Liebl 1995), a genus altogether more thermophilic and branching at a deeper position in the Bacteria domain than *Thermus*.

Xylanolytic and non-xylanolytic *T. thermophilus* metabolize a wide variety of low-molecular-weight organic substrates. In the highest temperature zones (72°C–82°C), they constitute the dominant bacterial population during the thermogenic phase of composting (Beffa et al. 1996*b*; Blanc

Fig. 5. Thermostability of xylanases from the strain X6 determined by pre-incubation, in absence of substrate, at the following temperatures: (●), 70°C; (○), 80°C; (▼), 90°C; (▽), 100°C. Xylanase activity was measured in the CFE, at 70°C and pH 6.



et al. 1999). Therefore, they probably contribute to the biological activity during composting to a great extent. However, XA measured in cell-free extracts from the hottest compost samples were low compared with those measured from samples taken in the cooler peripheral compost zones, and the optimal temperature for XA never exceeded 80°C in compost samples (Lyon et al. 2000). Due to the cell-bound location of their xylanases, it is therefore difficult to evaluate the actual importance of xylanolytic *T. thermophilus* in the degradation of hemicellulose at high temperatures. Previous enzymatic assays in compost samples were also carried out without sonication (Ball and Jackson 1995; Bono et al.1992; Godden et al. 1983; Herrmann and Shann; 1993 Stutzenberger et al. 1970). Moreover, they have only considered the extracellular and easily solubilized enzymes.

Kulkarni et al. (1999) indicated in a recent review that the highest thermostable xylanases synthesized by aerobic bacteria, actinomycetes, and fungi, had their optimal activity close to 80°C, with half lives never exceeding one hour at these temperatures. Therefore, *T. thermophilus* strain X6 appears to possess the most thermophilic and thermostable XA observed in an aerobic microorganism. This opens new perspectives in biotechnological applications, and further investigations should be directed towards the in-depth characterization of the individual enzymes associated with this XA.

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