Journal of Industrial Microbiology & Biotechnology (1998) 20, 55–60

Thermostable β-xylosidase from *Thermomonospora curvata*

F Stutzenberger[1] and AB Bodine[2]

[1]*Department of Microbiology;* [2]*Department of Animal, Dairy and Veterinary Sciences, Clemson University, Clemson, SC 29634, USA*

Thermomonospora curvata **produced a thermostable β-xylosidase during growth on birch xylan. The enzyme, extracted by sonication of early stationary phase mycelia, was purified by isoelectric focusing and size exclusion HPLC. The isoelectric point was pH 4.8. The molecular weight was estimated to be 102 000 by size exclusion HPLC and 112 000 by SDS-PAGE. Maximal activity occurred at pH 6–7 and 60–68°C. K_m values for xylobiose and *p*-nitrophenyl-β-D-xylopyranoside were 4.0 M and 0.6 M respectively. The enzyme was sensitive to low levels of Hg^{2+} (50% inhibition at 0.2 μM), but was stimulated by Co^{2+} and Pb^{2+}. Addition of the xylosidase to a xylanase reaction mixture increased the liberation of xylose equivalents from xylan and decreased the proportion of xylobiose in the hydrolysate.**

Keywords: actinomycete; *Thermomonospora*; xylan; xylosidase

Introduction

Xylan, the most abundant component of hemicellulose, constitutes up to 35% of the total dry wt of higher plants and ranks second only to cellulose as an abundant renewable polysaccharide [17]. The major components of microbial xylanolytic systems are endoxylanase (1,4-β-d-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidase (1,4,β-d-xylan xylohydrolase, EC 3.2.1.37) which catalyze the initial and secondary depolymerizations respectively; the action of these enzymes is facilitated by a variety of synergistic enzymes which remove substituents from the xylan backbone [25].

In nature, actinomycetes play an important role in the decay of hemicellulose in soil and composts [13]. Most of the information on xylan-degrading enzymes from actinomycetes has been obtained from studies on mesophilic streptomycetes [30]. However, the potential for use of thermostable enzymes in industrial bioconversion processes [19,20] has stimulated recent interest in the purification and characteristics of xylanases from thermophilic sources [8,11,15]. Thermophily and enzyme thermostability confer valuable advantages in industrial scale processes [29]. Of the thermophilic actinomycetes, the *Thermomonospora* species have been found to be the most xylanolytic [2]. *Thermomonospora curvata* establishes itself as the numerically dominant cellulolytic population in composting biomass [7,21]; it produces three extracellular endoxylanases which are excellent candidates for application in paper pulp pre-bleaching [27] since they are thermostable and active over a wide pH range [23]. However, xylanases are competitively inhibited by xylobiose [16]. Therefore, supplementation with a β-xylosidase will be required for maximal activity [18]. Here we describe the production and purification of a thermostable β-xylosidase from *T. curvata*.

Correspondence: F Stutzenberger, Dept Microbiology, Clemson University, Clemson, SC 29634-1909, USA
Received 14 April 1997; accepted 21 October 1997

Materials and methods

Chemicals

Buffers, vitamins and sugars were purchased from Sigma (St Louis, MO, USA). Reagents and inorganic chemicals were from Fisher Scientific (Norcross, GA, USA).

Cultures and media

Washed cell stocks of *Thermomonospora curvata* CUB993 [14], initially isolated from municipal solid waste compost [21], were maintained in 50% glycerol–mineral salts–vitamin minimal medium at –86°C. Cultures were routinely grown in 100 ml of minimal medium [22] to which carbon sources and vitamins, autoclaved separately, were added after cooling. Production of β-xylosidase during growth on a variety of soluble carbon sources (glucose, cellobiose, maltose, pectin or starch at concentrations of 0.2–0.5%, w/v) was compared to that on comparable concentrations of insoluble birch xylan (Sigma type X0502), consisting of >90% xylose residues. Cultures were grown at 55°C in 250-ml baffled Erlenmeyer flasks shaken (140 orbits min^{-1}) at an angle of 30° from vertical to provide maximal aeration. Cells were transferred twice (1% v/v) before assays were performed, to preclude interference by glycerol carryover from the stock culture inoculum.

Growth on soluble carbon sources was spectrophotometrically estimated at 610 nm (1-cm light path) and recorded as 1 mg dry cell wt per 1.7 OD units. In the presence of insoluble carbon sources, growth was estimated as insoluble nitrogen [9] in culture solids twice washed with 10 volumes of distilled water and centrifuged (13 300 × *g*, 10 min, room temp). Mycelial mass was recorded as 1.0 mg dry cell wt per 0.113 mg nitrogen.

Enzyme assays

Samples of culture fluid clarified by centrifugation were assayed for enzyme activity and protein content. Xylanase was measured in 2.5-ml (final volume) reaction mixtures containing 25 mg xylan (Sigma), 0.1 M HEPES buffer (pH 8.1) and appropriate volumes (10–100 μl) of clarified

culture fluid. Samples taken at zero time and after 20 min at 75°C were clarified by centrifugation (2 min, 13 300 × *g*, 4°C in pre-chilled tubes). The increase in soluble reducing sugars in the 20-min sample, spectrophotometrically compared at 540 nm to the zero time sample, was estimated by the dinitrosalicyclic acid method of Bernfeld [3] using a xylose standard. A xylanase unit (U) was defined as the activity required to liberate one μmole of xylose equivalents min^{-1}.

Activity of β-xylosidase was measured by addition of 20 μl of cell extract or culture fluid to 1 ml (final volume) reaction mixtures containing 2 mM *p*-nitrophenyl-β-d-xylopyranoside (Sigma) buffered with 0.1 M MES to pH 6.1. After incubation for 10 min at 62°C, 2 ml of 1.0 M Na_2CO_3 were added to stop the reaction and enhance the color of the released *p*-nitrophenol measured spectrophotometrically at 400 nm (E_m of 2×10^4/M cm^{-1}) against reagent blanks receiving water instead of enzyme. U were expressed as μmoles of *p*-nitrophenol released min^{-1}.

Cellulase-associated activities (endoglucanase and β-glucosidase) were measured in selected samples as previously described [4]. Endoglucanase U were expressed as μmoles of glucose equivalents released from carboxymethylcellulose (Hercules type 7L, Charlotte, NC, USA) per min. The β-glucosidase U were recorded as μmoles of nitrophenol released per min from *p*-nitrophenyl-β-d-glucopyranoside (Sigma).

Other assays

Protein concentrations were estimated by the dye-binding method of Bradford [6] as described in the Bio-Rad technical bulletin 89-0301, using an albumin/globulin protein standard (Sigma 540-10). Specific soluble sugar concentrations in culture fluid were measured by HPLC after centrifugation (13 300 × *g*, 5 min, room temp) and filtration (0.45-μm pore size, Micron Separations, Wormath, MA, USA) to remove residual particulates. The 20-μl samples were moved in degassed distilled water (0.67 ml min^{-1}) by a model 314 high pressure syringe pump (Isco, Lincoln, NE, USA) through an RCM-Resex 7.8 × 300-mm column (Phenomenex, Torrance, CA, USA) heated to 85°C. Sugar retention times were compared to those of known standards (Sigma) in a model ERC-7510 refractive index detector (Erma Optical Works, Tokyo). Sugar concentrations were quantitated by peak area integration in a model CDS-402 data collection system (Varian, Palo Alto, CA, USA).

Xylosidase purification

Late exponential phase xylan-grown mycelia (1.1 mg dry cell wt ml^{-1}) were collected by centrifugation (10 000 × *g*, 10 min, 4°C). Mycelial pellets were washed twice with 10 volumes of chilled 0.05 M phosphate, pH 7.0, containing 10^{-4} M phenylmethylsulfonyl fluoride (Sigma) to inhibit serine protease activity. Mycelia were resuspended in chilled buffer and either sonicated (maxi-probe of a Fisher Demembranator at 60% power) for 1–2 min in an ice bath or disrupted in a chilled French pressure cell (Aminco) at 20 000 p.s.i. Extracts were clarified (15 000 × *g*, 30 min, 4°C) and concentrated about 6-fold via ultrafiltration in a Centricon-30 (30 000 nominal mol wt exclusion limit, Amicon, Lexington, MA, USA). Retained proteins were washed with three volumes of 0.01 M Tris buffer, pH 8.0.

One milliliter of washed ultrafiltration retentate was applied to a flat bed of Pevikon C-870 copolymer in an LKB 2117 preparative multiphor system. Isoelectric focusing (IEF) was performed in a pH 4–7 gradient (Bio-lyte ampholytes, Bio-Rad, Richmond, CA, USA) during 18 h at 7°C under 8 W constant power. Pevikon bed segments were washed with 0.15 M NaCl (final fraction volumes of 3.1 ml). After pH determination, fractions were assayed for protein and β-xylosidase activity. Active fractions were pooled, reconcentrated by ultrafiltration in a Centricon-50 (mol wt exclusion of 50 000) and buffered to pH 7.0 in 0.1 M phosphate. The ultrafiltration retentate was applied (50 μl per run) to a Biosep SEC-2000 size exclusion analytical HPLC column (7.8 × 300 mm, Phenomenex) calibrated for mol wt determinations (Sigma MW-GF-200 protein marker kit) in a Perkin Elmer system consisting of a Series 4 pump (flow rate of 0.67 ml of a mixture of 0.1 M phosphate, pH 7.0, and 0.1 M NaCl per min), LC-85 UV detector (280 nm) and B-100 plotter integrator. Fractions having peak activity were pooled from multiple runs and reconcentrated on Centricon-50 units.

SDS-PAGE

The purified β-xylosidase and Bio-Rad *Kaleidoscope* prestained molecular weight markers (myosin [202 kDa], β-galactosidase [133 kDa], bovine serum albumin [71 kDa], carbonic anhydrase [42 kDa], soybean trypsin inhibitor [31 kDa], lysozyme [18 kDa] and aprotinin [7 kDa], Bio-Rad, Hercules, CA, USA) were electrophoresed by the method of Laemmli [10] through a 4–20% acrylamide gradient gel (pre-cast by ESA Inc, Chelmsford, MA, USA) in a Bio-Rad Mini-Protean II Cell and stained with Coomassie Blue R250.

Statistical analysis

Coefficients of variation (CV) in growth estimations were calculated in samples taken from three replicate cultures. CV values for all other assays were obtained by running six replicate determinations on representative samples; CV for growth, protein, reducing sugar, xylanase and xylosidase were 17.4%, 3.7%, 1.3%, 3.2% and 5.6% respectively. In the HPLC analysis of sugar concentrations, the CV values, based on peak heights, were 7.6%. Presented results are the averages of at least two separate experiments unless noted otherwise.

Results

Production and extraction of β-xylosidase

In a comparison of β-xylosidase extraction methods, the French pressure cell released more soluble protein (average of 195 mg g^{-1} dry cell wt), but less β-xylosidase (average of 45.4 U g^{-1} dry cell wt) than extraction by sonication (averages of 129 U and 144 mg protein g^{-1}). Therefore, sonication was routinely employed for β-xylosidase extraction. The rapid release of β-xylosidase relative to that of total soluble protein suggested that the enzyme might be located on the cell surface; however, there was little activity

detected using intact mycelia in the routine β-xylosidase assay (data not shown).

The β-xylosidase specific activity of extracts from sonicated *T. curvata* mycelium grown on a variety of soluble carbon sources (glucose, cellobiose and maltose, pectin and soluble starch) were relatively low (<0.1 U mg^{-1} protein) compared to extracts from mycelia grown on birch xylan. The β-xylosidase specific activity of soluble extracts from xylan-grown *T. curvata* increased rapidly during exponential growth and peaked (0.82–1.02 U mg^{-1} protein) in early stationary phase (Figure 1a). During that time, β-xylosidase activity in clarified culture fluid remained very low (< 0.01 U ml^{-1}) compared to that of endoxylanase activity (Figure 1b). This intracellular retention of β-xylosidase prevented the extracellular hydrolysis of xylanase inducers which were liberated from xylan by xylanases. Xylo-oligosaccharides and cello-oligosaccharides were previously found to be effective inducers of xylanase biosynthesis [23]; however, when 1 mM xylose was introduced into a *T. curvata* culture, it caused a transient repression of xylanase biosynthesis. The ability of extracellular β-xylosidase activity to abolish the xylanase-inductive capacity of xylo-oligosaccharides was confirmed by an experiment in which an enzymatic hydrolysate of xylan was introduced into a mid-exponential phase culture at a final concentration of 1 mM xylose equivalents. Within 1 h, all sugars detectable by HPLC in the series xylopentaose-xylobiose were removed from culture fluid and extracellular xylanase activity tripled compared to the control culture receiving no hydrolysate. However, in a culture receiving xylan hydrolysate incubated for 1 h with purified β-xylosidase (0.08 U ml^{-1} final concentration), then added to the culture, xylanase was not induced.

Purification

Peak β-xylosidase activity was found at pH 4.8 after IEF (Figure 2). On size exclusion HPLC (Figure 3), the mol wt of the β-xylosidase, calculated from the column calibration plot, was 102 kDa. The reconcentrated pool of fractions containing the β-xylosidase peaks from multiple HPLC runs had a specific activity of 12.3 U mg^{-1} protein with a 13.4-fold purification.

Characterization

The purified β-xylosidase had no detectable activity in the xylanase endoglucanase or β-glucosidase assays. The mol wt was estimated to be 112 kDa on SDS-PAGE (Figure 4). The enzyme was stable for at least 6 h at 60°C, but was inactivated at higher temperatures (half-lives at 62°C and 70°C of 409 min and 3.1 min respectively). The optimal pH values for stability and activity were 8.0 and 6.5 respectively. At pH 6.5, the energy of activation (E_a), calculated from an Arrhenius plot, was 11.44 kC $mole^{-1}$. The temperature coefficient (Q_{10}) was 1.82 over the range of 24–68°C.

The effect of a variety of metals on β-xylosidase reaction rates was tested. The enzyme had no apparent metal requirements for activity; addition of 1 mM EDTA had a slight stimulatory effect. This effect may have been due to removal of trace amounts of Hg to which the enzyme was very sensitive (50% inhibition at 0.2 μM). Low concen-

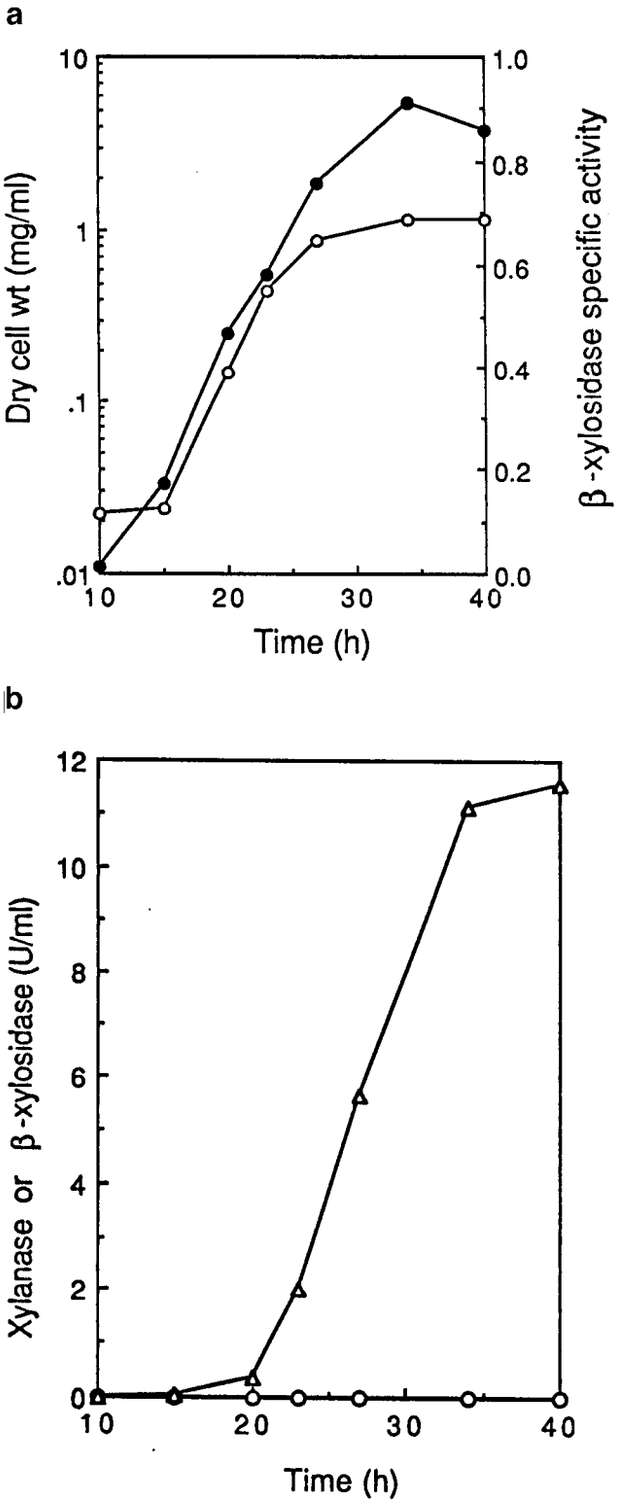

Figure 1 (a) Increase in intracellular β-xylosidase specific activity (●) during growth (○) of *Thermomonospora curvata* in xylan-minimal medium; (b) accumulation of endoxylanase (△) and β-xylosidase (○) in culture fluid.

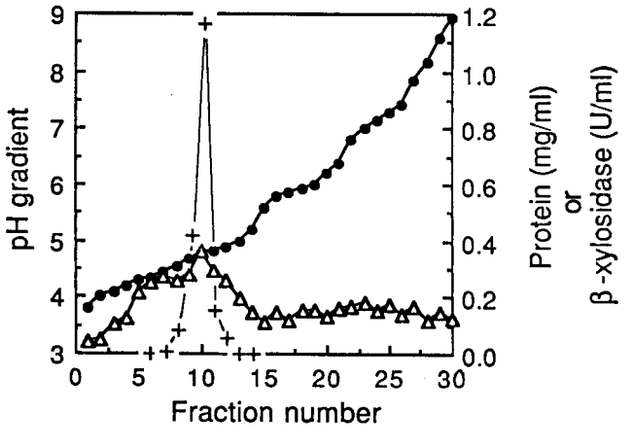

Figure 2 Profile of β-xylosidase (+) and total protein (△) over a pH gradient (●) formed during an IEF fractionation of a soluble extract from sonicated *Thermomonospora curvata* cells grown on xylan.

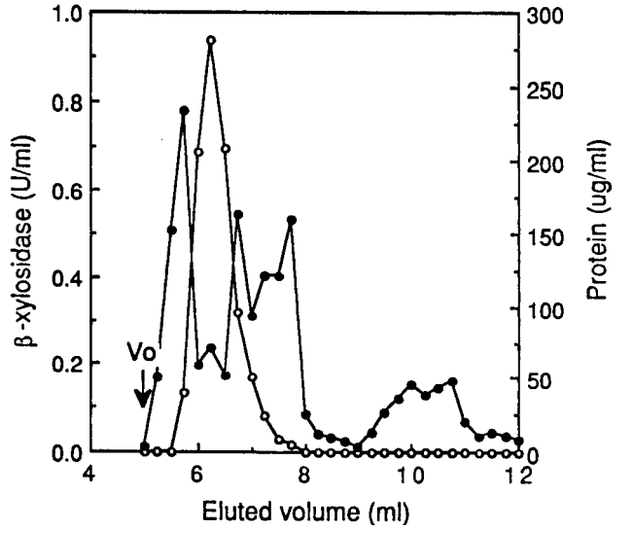

Figure 3 Size exclusion chromatography elution profile of β-xylosidase (○) and soluble protein (●) from pooled active IEF fractions as shown in Figure 2. Column void volume (V_o) indicated by arrow.

trations of other heavy metals caused both stimulation and inhibition, depending on their concentrations (Figure 5).

Xylobiose and PNPX were compared as substrates for the β-xylosidase. K_m values, calculated from double-reciprocal plots (Figure 6), were 4.0 mM and 0.6 mM respectively. The K_i values for xylose and xylobiose, determined with 1 mM PNPX as substrate, were 42.5 mM and 3.4 mM respectively.

Effect of β-xylosidase on the xylanase reaction rate

When purified xylosidase was added in a ratio of one unit per six xylanase units to a xylanase reaction mixture, the effect was apparent during prolonged incubation (Figure 7). During the latter half of the 400-min incubation, the average rate of reducing sugar release (as xylose equivalents) in the presence of the added β-xylosidase was about 2.5 times that of the reaction employing the xylanase alone. The xylooligoside concentrations in mixtures with and without added β-xylosidase were compared (Table 1). In

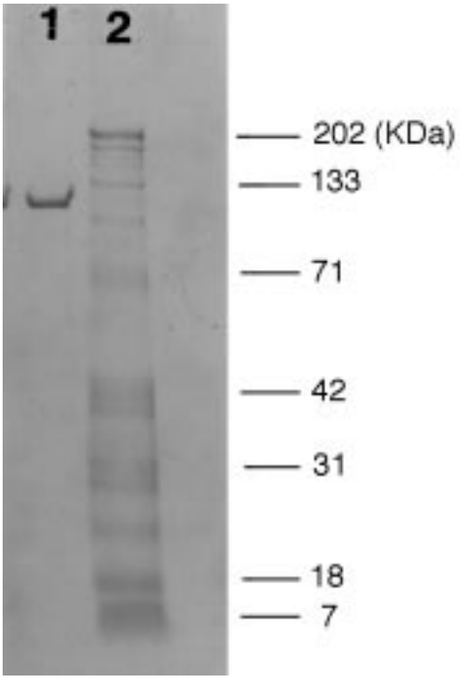

Figure 4 SDS-PAGE of purified β-xylosidase in lane 1, and molecular weight standards (Bio-Rad Kaleidoscope prestained kit) in lane 2.

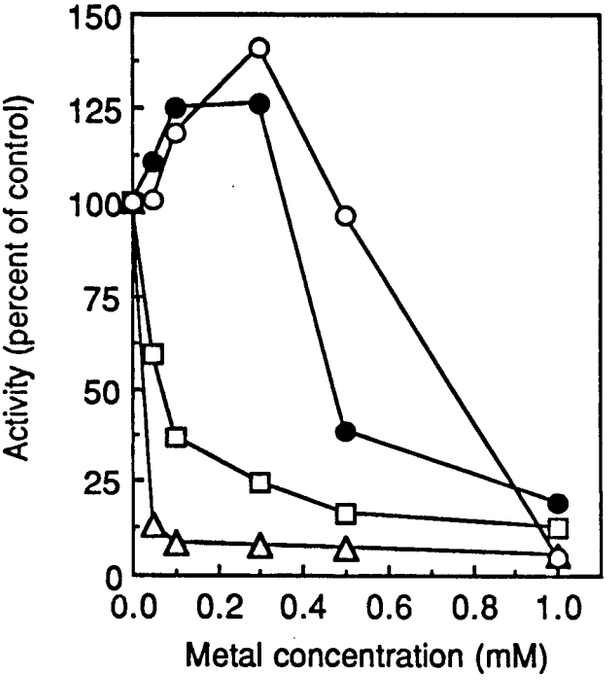

Figure 5 Stimulation and inhibition of β-xylosidase activity by heavy metals. Symbols: Cd, △, Co, ●, Ni, □, Pb, ○.

the xylosidase-supplemented reaction mixture, xylotetraose and xylopentaose were absent from the product mixture, and the xylose/xylobiose ratio was increased about 8.7-fold.

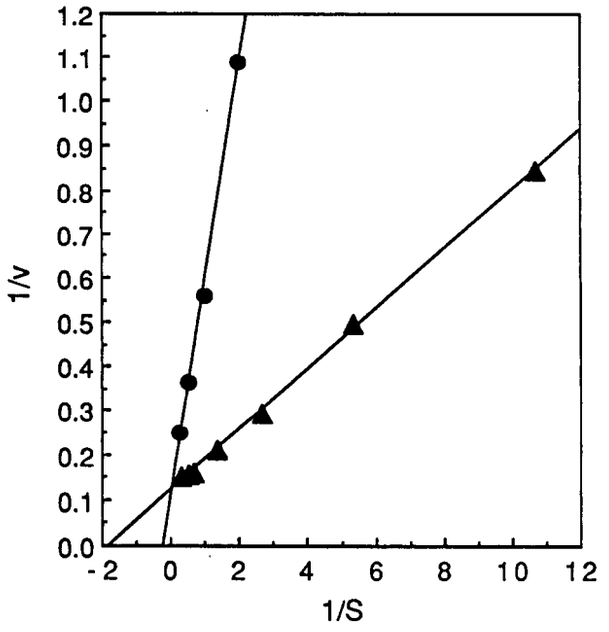

Figure 6 Double reciprocal plots in the determination of K_m values for xylobiose (●) and *p*-nitrophenyl xylopyranoside (▲) as substrates in the β-xylosidase reaction.

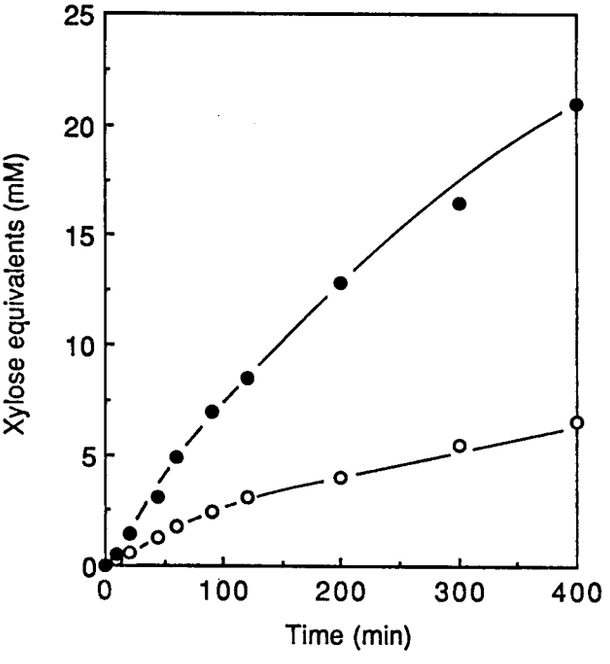

Figure 7 Accumulation of soluble reducing sugars (as xylose equivalents) liberated from xylan by the *Thermomonospora curvata* extracellular xylanase in the presence (●) and absence (○) of added β-xylosidase.

Discussion

The intracellular localization of β-xylosidase in *T. curvata* appears to be in common with a variety of other thermophilic bacteria [1,24,26,28]. Retention of the enzyme in a soluble cytoplasmic form facilitates a membrane-bound signaling system sensitive to specific xylo-oligosaccharides [5] which confers several advantages in nature. Since xylose is more readily utilized by a wide range of microbes than are xylo-oligosaccharides, cleavage of xylan hydrolysis products to xylose inside the cell would make its xylose source less accessible to competing microflora. Moreover, the intracellular localization would keep the xylosidase in close contact with stabilizing factors not available in the harsh extracellular environment. However, for the effective large-scale combination of xylanase and β-xylosidase activities in applications requiring high xylose yields, the intracellular localization of β-xylosidase poses difficulties, since little xylosidase activity would be found in the extracellular culture fluid where the xylanases abound. Another problem in the applied synergism of the two enzymes is the lower thermostability of the xylosidase compared to that of xylanase [5]. Although the thermostability of the *T. curvata* β-xylosidase is comparable to that of other thermophilic bacteria, it could not be employed at the optimal reaction temperature (75°C) of the *T. curvata* extracellular xylanases over a prolonged incubation, although it is most thermostable at pH 8.0 (the optimum for activity of the extracellular xylanase [23]). Lowering the reaction temperature to 62°C would greatly extend the half-life of the xylosidase while reducing the initial reaction rate of the extracellular xylanases only by about 20%. Even with this compromise, the β-xylosidase of *T. curvata* would be better suited to a wide range of high-temperature applications than its fungal counterparts [12].

Table 1 Effect of β-xylosidase supplementation on xylo-oligosaccharide concentrations in xylan hydrolysate

Xylo-oligosaccharide	Xylanase without β-xylosidase (mM)	Xylanase plus β-xylosidase (mM)
Xylose	0.16	3.56
Xylobiose	2.62	6.72
Xylotriose	1.54	3.26
Xylotetraose	0.81	ND*
Xylopentaose	0.44	ND

*ND, not detected.

Acknowledgements

We thank Rebecca Rogers for excellent technical assistance. This study was supported by the US Army Research Office and by the Department of Energy through the South Carolina University Research and Education Foundation.

References

1 Bachman SL and AJ McCarthy. 1989. Purification and characterization of a thermostable β-xylosidase from *Thermomonospora fusca*. J Gen Microbiol 135: 293–299.
2 Ball AS and AJ McCarthy. 1989. Production and properties of xylanases from actinomycetes. J Appl Bacteriol 66: 439–444.
3 Bernfeld P. 1955. Amylases, alpha and beta. Meth Enzymol 1: 149–154.
4 Bernier R and F Stutzenberger. 1987. Preferential utilization of cellobiose by *Thermomonospora curvata*. Appl Environ Microbiol 53: 1743–1747.

5 Biely P. 1985. Microbial xylanolytic systems. Trends Biotechnol 3: 286–290.
6 Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.
7 Fergus CL. 1964. Thermophilic and thermotolerant molds and actinomycetes on mushroom compost during peak heating. Mycologia 56: 267–284.
8 Fontes CMGA, GP Hazelwood, E Morag, J Hall, BH Hirst and HJ Gilbert. 1995. Evidence for a general role for non-catalytic thermostabilizing domains in xylanases from thermophilic bacteria. Biochem J 307: 151–158.
9 Johnson MJ. 1941. Isolation and properties of a pure yeast polypeptidase. J Biol Chem 137: 575–586.
10 Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680–684.
11 Lapidot A, A Mechaly and Y Shoham. 1996. Overexpression and single-step purification of a thermostable xylanase from *Bacillus stearothermophilus* T-6. J Biotechnol 51: 259–264.
12 Matsuo M and T Yasui. 1988. β-Xylosidases of several fungi. Meth Enzymol 160: 684–700.
13 McCarthy AJ, AS Ball and SL Bachmann. 1988. Ecological and biotechnological implications of lignocellulose degradation by actinomycetes. In: Biology of Actinomycetes '88 (Okami Y, Beppu T and Ogawara H, eds), pp 283–287, Japan Scientific Societies Press, Tokyo.
14 McCarthy AJ and T Cross. 1984. A taxonomic study of *Thermomonospora* and other monosporic actinomycetes. J Gen Microbiol 130: 5–25.
15 Nanmori T, T Watanabe, R Shinke, A Kohno and Y Kawamura. 1990. Purification and properties of thermostable xylanase and b-xylosidase produced by a newly isolated *Bacillus stearothermophilus* strain. J Bacteriol 172: 6669–6672.
16 Poutanen K, M Ratto, J Puls and L Viikari. 1987. Evaluation of different microbial xylanolytic systems. J Biotechnol 6: 49–60.
17 Puls J and K Poutanen. 1989. Mechanisms of enzymatic hydrolysis of hemicelluloses (xylans) and procedures for determination of the enzyme activities involved. In: Enzyme Systems for Lignocellulose Degradation (Coughlan M, ed), pp 151–165, Elsevier, London.
18 Reilly PJ. 1981. Xylanases: structure and function. In: Trends in the Biology of Fermentation for Fuels and Chemicals (Hollaender AE, ed), pp 111–129, Plenum Press, New York.
19 Saddler JN. 1992. Biotechnology for the conversion of lignocellulosics. Biomass Bioenergy 2: 229–238.
20 Senior DJ and J Hamilton. 1992. Biobleaching with xylanases brings biotechnology to reality. Pulp Paper 66: 111–114.
21 Stutzenberger FJ, AJ Kaufman and RD Lossin. 1970. Cellulolytic activity in municipal solid waste compost. Can J Microbiol 16: 553–560.
22 Stutzenberger F. 1972. Cellulolytic activity of *Thermomonospora curvata*: nutritional requirements for cellulase production. Appl Microbiol 22: 147–152.
23 Stutzenberger F and AB Bodine. 1992. Xylanase production by *Thermomonospora curvata*. J Appl Bacteriol 72: 504–511.
24 Sunna A and G Antranikian. 1996. Growth and production of xylanolytic enzymes by the extreme thermophilic anaerobic bacterium *Thermotoga thermarum*. Appl Microbiol Biotechnol 45: 671–676.
25 Thomson JA. 1993. Molecular biology of xylan degradation. FEMS Microbiol Rev 104: 65–82.
26 Uchino F and T Nakane. 1981. A thermostable xylanase from a thermophilic acidophilic *Bacillus* sp. Agric Biol Chem 45: 1121–1127.
27 Viikari L, A Kantelinen, J Sundquist and M Linko. 1994. Xylanases in bleaching: from an idea to the industry. FEMS Microbiol Lett 13: 335–350.
28 Weimer PJ. 1985. Thermophilic anaerobic fermentation of hemicellulose and hemicellulose-derived aldose sugars by *Thermoanaerobacter* strain B6A. Arch Microbiol 143: 130–136.
29 Zamost BL, HK Nielsen and RL Starnes. 1991. Thermostable enzymes for industrial applications. J Ind Microbiol 8: 71–82.
30 Zimmermann W. 1989. Hemicellulolytic enzyme systems from actinomycetes. In: Enzyme Systems for Lignocellulose Degradation (Coughlan M, ed), pp 167–182, Elsevier, London.