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Purification and Properties of Thermostable Xylanase and β-Xylosidase Produced by a Newly Isolated *Bacillus stearothermophilus* Strain

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We isolated a thermophilic bacterium that produces both xylanase and β-xylosidase. Based on taxonomical research, this bacterium was identified as *Bacillus stearothermophilus*. Each extracellular enzyme was separated by hydrophobic chromatography by using a Toyopearl HW-65 column, followed by gel filtration with a Sephacryl S-200 column. Each enzyme in the culture was further purified to homogeneity (62-fold for xylanase and 72-fold for β-xylosidase) by using a fast protein liquid chromatography system with a Mono Q HR 5/5 column. The optimum temperatures were 60°C for xylanase and 70°C for β-xylosidase. The isoelectric points and molecular masses were 5.1 and 39.5 kDa for xylanase and 4.2 and 150 kDa for β-xylosidase, respectively. Heat treatment at 60°C for 1 h did not cause inhibition of the activities of these enzymes. The action of the two enzymes on xylan gave only xylose.

Many microorganisms produce xylan-digesting enzymes. The enzymes involved, xylanase (endo-β-1,4-xylanase; EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37), have been purified and characterized from fungi and bacteria (3–5, 8–11, 13–15). However, these xylan-degrading enzymes are limited to those from microorganisms that grow at ordinary temperatures, and the enzymes do not show thermostability at higher temperatures. There have been very few reports about thermostable xylan-digesting enzymes. Moreover, they are limited to a few fungi and anaerobic bacteria (7, 17). It was reported that acidophilic *Bacillus* (16) or alkalophilic *Bacillus* (12) spp. could produce thermostable xylanase, but the investigators did not discuss whether or not the strain could produce a xylosidase. Gruninger and Fiechter have isolated thermostable bacteria which possess high xylanase activity that cleaves xylan to xylose. However, they did not purify the separate enzymes (6). The occurrence of a thermophilic *Bacillus* sp. that could produce both thermostable xylanase and β-xylosidase was not determined.

The purpose of this research was to isolate thermophilic bacteria capable of producing both xylan-digesting enzymes (xylanase and β-xylosidase) at higher temperatures (50 to 60°C) and to clarify the mechanism of xylan digestion. We describe the isolation and identification of a bacterium capable of producing thermostable xylanase and xylosidase and the purification of these enzymes. We then clarify the xylan digestion system in this bacterium.

MATERIALS AND METHODS

Culture medium. Agar plate A consisted of 1% soluble starch (Nakarai Tesque, Ltd.), 0.5% meat extract (Wako Pure Chemical Industry), 1% polypeptone (Wako Pure Chemical Industry), and 2% agar (Wako Pure Chemical Industry) at pH 7.0. This was used for selection of thermophilic bacteria.

Agar plate B contained 0.1% yeast extract (Wako Pure Chemical Industry), 1% xylan from oat-spelt (Nakarai Tesque), 2% agar, 0.4% KH_2PO_4, 0.2% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$), 0.005% $MnSO_4$, 0.005% $FeSo_4 \cdot 7H_2O$, 0.2% $CaCl_2 \cdot 2H_2O$, and 0.2% NH_4Cl at pH 7.0. This was used for screening bacteria capable of producing xylan-digesting enzymes.

The liquid medium contained 1% xylan from oat-spelt, 2% polypeptone, 0.25% yeast extract, 0.2% NH_4NO_3 and KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.005% $MnSO_4$ at pH 7.0.

Assay of xylanase and β-xylosidase and protein determination. (i) Xylanase. Enzyme solution (0.5 ml) was added to 2% xylan suspension (0.5 ml) in 0.1 M acetate buffer, pH 6.0, and the mixtures were incubated at 55°C for 30 min. After the mixtures were cooled rapidly on ice water, the insoluble xylan was removed by centrifugation (10,000 × *g*). To the resulting supernatant (0.5 ml), 1 ml of 3,5-dinitrosalicylate (0.5%) solution was added, and the mixture was cooked in boiling water. Color development was measured on a spectrometer (UVIKON 860; Contron Ltd.) at 535 nm. One unit of xylanase was defined as the activity releasing 1 μmol of xylose in 1 min.

(ii) β-Xylosidase. Enzyme solution (0.5 ml) was mixed with 0.5 ml of 20 mM phenyl-β-xyloside solution and incubated at 55°C for 30 min; 5 ml of 0.55 M Na_2CO_3 solution was added. Then 1 ml of Folin-Ciocalteu reagent was added. After 30 min at room temperature, color development was measured at 660 nm. One unit of β-xylosidase was defined as the activity releasing 1 μmol of phenol in 1 min.

Protein concentration was determined by the method of Bradford (1).

Purification of xylanase and β-xylosidase. Strain 21 was cultured in liquid medium at 55°C for 48 h, using a Bio-Shaker (Taiyo Co. Ltd.). Ammonium sulfate was added to the culture filtrate (500 ml) until 35% saturation was obtained, and the precipitates were removed.

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Clear supernatant was applied to a Toyopearl HW-65 column (2.5 by 33.5 cm) equilibrated with ammonium sulfate solution (35% saturation). Back-gradient elution (35 to 0% saturation of ammonium sulfate) was done. The resulting xylanase and β-xylosidase fractions were dialyzed against 50 mM acetate buffer, pH 6.0, and concentrated up to 10 ml by ultrafiltration with an ultrafilter (UK-10; Toyo Co. Ltd.). Both enzymes in the concentrated solution were then separated by gel filtration, using a Sephacryl S-200 column (2.7 by 120 cm) equilibrated with 50 mM acetate buffer, pH 6. Each enzyme (xylanase and β-xylosidase) was further purified to homogeneity by anion-exchange chromatography, using a fast protein liquid chromatography (FPLC) system equipped with a Mono Q HR 5/5 column (0.5 by 5 cm; Pharmacia-LKB). FPLC was performed at a flow rate of 1 ml/min and a pressure of 2 MPa. Elution of each enzyme was done by increasing NaCl concentration gradients up to 0.3 M for xylanase or 0.5 M for β-xylosidase. A_{280} was monitored, and the peak fractions were collected to assay enzyme activities.

Gel electrophoresis. Isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) were performed with an LKB2117 Multiphor II electrophoresis system. Ampholine gels (Ampholine PAGE plate; pH range, 3.5 to 9.5; Pharmacia-LKB) were run at 1,500 V for 1.5 h at 10°C. Exel gels (SDS gradient 8-18; Pharmacia-LKB) were run at 600 V and 50 mM for 75 min at 15°C.

Estimation of molecular mass of xylanase and β-xylosidase. The molecular mass of each enzyme was estimated by SDS-PAGE. Another estimation of β-xylosidase molecular weight was made by using gel filtration on a Superose 12 column (1.5 by 30 cm; Pharmacia-LKB) which was joined to the FPLC system.

Determination of sugars formed by xylanase and β-xylosidase action. Xylan suspension (2% oat-xylan, 1 ml) in 10 mM acetate buffer, pH 6.0, was added to 0.5 ml of purified (A) β-xylosidase solution (0.9 U/ml) or (B) xylanase solution (0/9 U/ml). Distilled water (0.5 ml) was added to each reaction mixture. Xylan suspension (1 ml) was added to the 1-ml solution (mixture of 0.5 ml of xylanase solution and 0.5 ml of β-xylosidase solution) (C). These reaction mixtures were incubated at 55°C for 1 or 2 h. The remaining xylan was removed by centrifugation (14,000 × *g*, 5 min). A 20-μl portion of each clear supernatant was spotted onto a silica plate (thin-layer chromatography aluminum sheets; Merck), and liberated xylose or xylo-oligosaccharides in each supernatant were separated in the gel by developing the solvent (*n*-butanol/water ratio, 85:15). After development, aniline-phthalate reagent was sprayed onto the gel so that each carbohydrate could be visualized.

Xylose and xylo-oligosaccharides (X_2–X_5) were separated in the gel. They were used as standards for identification of sugars.

RESULTS AND DISCUSSION

Isolation and determination of thermophilic bacterium producing xylan-digesting enzyme. Soil suspensions in sterilized water were poured and spread onto agar plates A. These plates were incubated at 70°C for 2 days. About 100 colonies were found on the plates. These colonies were transferred onto agar plates B. These plates were incubated at 70°C for 2 days. Of 100 bacterial colonies, only 1 showed a clear halo on the agar plate. This strain (tentatively named strain 21) showed an ability to digest xylan and was selected for further experiments.

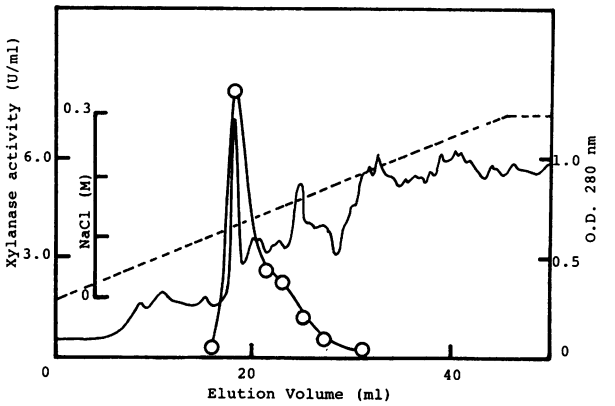

FIG. 1. FPLC-Mono Q column chromatogram of xylanase. O.D., Optical density.

The strain was gram positive, negative on the Voges-Proskauer test (at pH 7.2), and facultatively anaerobic and had a rod shape, 1.0 to 2.5 μm in diameter. Spore formation was observed at the terminal position at the swollen sporangium. The strain possessed the ability to hydrolyze both starch and gelatin. Strain 21 grew in nutrient broth at 54 to 70°C at neutral pH but could not grow at pH 5.5. There was acid formation from D-glucose but no gas formation from glucose. From these results, strain 21 was identified as *Bacillus stearothermophilus* by the criteria of *Bergey's Manual of Systematic Bacteriology* (2).

Purification of xylanase and β-xylosidase. Crude preparations of xylan-digesting enzymes were obtained from the culture filtrates (500 ml) after hydrophobic chromatography on a Toyopearl HW-65 column. These enzymes were collected in the eluents at a 25 to 17% ammonium sulfate saturation. Each enzyme (xylanase or β-xylosidase) was separated by gel filtration on a Sephacryl S-200 column.

The xylanase fraction obtained was dialyzed against 20 mM histidine-hydrochloric acid buffer, pH 6.0, and then led to adsorption on FPLC-Mono Q HR 5/5 gels, which were equilibrated with the same buffer. Elution was performed by increasing the concentration of NaCl in the histidine-hydrochloride buffer from 0 to 0.3 M. The enzyme was eluted at an NaCl concentration of approximately 0.12 M (Fig. 1).

The β-xylosidase fraction obtained after gel filtration was dialyzed against 20 mM histidine-hydrochloride buffer, pH 5.0, and applied to the FPLC-Mono Q HR 5/5 column,

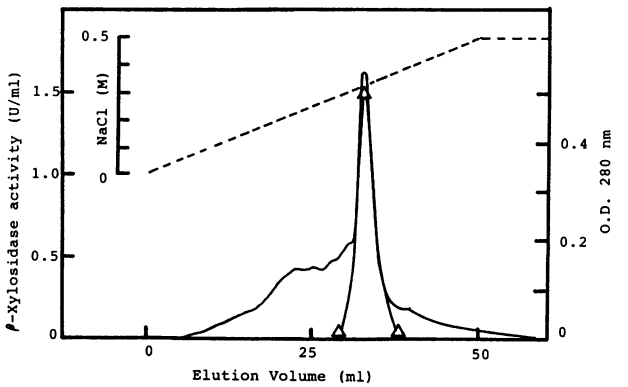

FIG. 2. FPLC-Mono Q column chromatogram of the β-xylosidase. O.D., Optical density.

TABLE 1. Purification of xylanase (A) and β-xylosidase (B)

Column	Total (U)		Sp act (U/ml)		Yield (%)		Purification (fold)	
	A	B	A	B	A	B	A	B
Crude	184	42.9	1.96	0.455	100	100	1	1
Toyopearl	100	10.3	7.59	0.775	54.3	24	3.87	1.7
Sephacryl	65	9.75	52.3	5.13	35.3	22	26.7	11.3
Mono Q	20.8	9.29	122	27.9	11.3	21	62.2	61.3
Mono Q		6.92		34.2		16.1		72.5

equilibrated with the buffer. Elution was performed by increasing the NaCl concentration in the histidine-hydrochloride buffer from 0 to 0.5 M. β-Xylosidase was eluted at an NaCl concentration of approximately 0.4 M (Fig. 2). Rechromatography with the FPLC-Mono Q column was performed to obtain well-purified β-xylosidase under the same conditions. The purification is summarized in Table 1.

Xylanase and β-xylosidase were purified up to 62.2- and 72.5-fold, respectively. The specific activity was 122 U/mg for xylanase and 34.2 U/mg for β-xylosidase. Isoelectrofocusing on a Ampholine PAGE plate gave a single band of each enzyme (xylanase or β-xylosidase), which assured the homogeneity of each (Fig. 3).

General properties of xylanase and β-xylosidase. Isoelectric points of xylanase and β-xylosidase were 4.83 and 4.13, respectively. SDS-PAGE gave a single band of each enzyme. The molecular mass of the xylanase was determined to be 39.5 kDa (Fig. 3). Another estimation of molecular mass, using gel filtration, revealed that the molecular mass of the β-xylosidase in the native state was 150 kDa, which indicates that the enzyme is a dimer (the molecular mass of each subunit was 75 kDa). The optimum pH and temperature were 7.0 and 60°C for xylanase and 6.0 and 70°C for β-xylosidase, respectively. Xylanase was stable in the pH range of 5 to 11, but β-xylosidase was stable in the range of pH 6 to 8. Heat treatment at 60°C for 1 h did not cause inhibition of the activities of either enzyme (Fig. 4). To date, a thermostable β-D-xylosidase has not been reported (9). The K_m of the xylanase for xylan or the β-xylosidase for *p*-nitrophenyl-β-D-xyloside was calculated to be 3.8 mg/ml or 1.2×10^{-3} M. The ability of the xylanase to degrade cellulose or carboxymethyl cellulose was tested, but activities were not detected, which is not in accordance with the results with other xylanases produced by acidophilic *Bacillus* spp. (16).

Xylan-digesting system in strain 21. Purified β-xylosidase could not digest xylan (Fig. 5, lanes B_0, B_1, and B_2), but

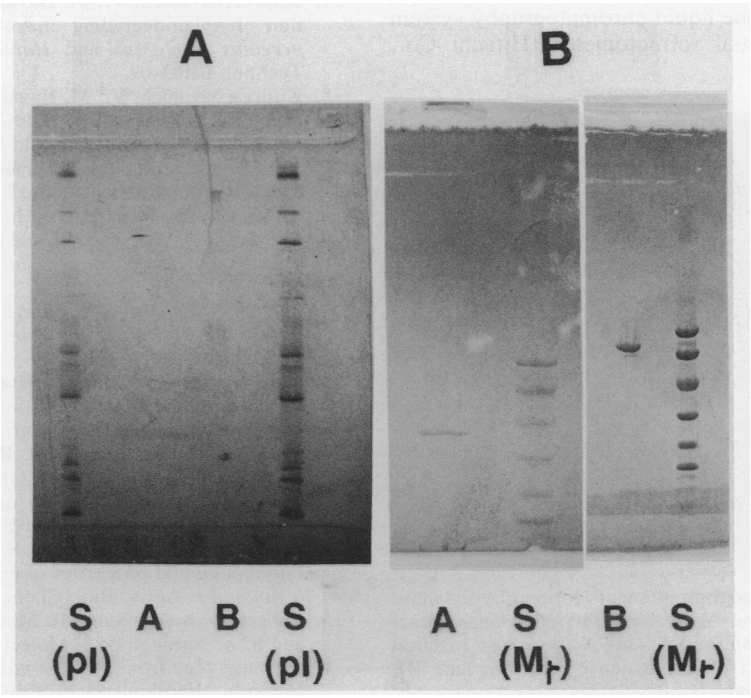

FIG. 3. Isoelectrofocusing and SDS-PAGE of xylanase and β-xylosidase. (A) Ampholine gel (pH range, 3.0 to 9.5) was used for isoelectrofocusing. (B) Excel gel (Pharmacia-LKB) was used for SDS-PAGE. The following were used as protein markers (isoelectric focusing calibration kit; pH range, 3 to 10; Pharmacia-LKB): amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin (6.85), horse myoglobin (7.35), lentil lectin (8.15), lentil lectin (8.45), lentil lectin (8.65), and trypsinogen (9.30). The following were used as molecular calibration markers (electrophoresis kit; Pharmacia LKB): phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Lane S, Each calibration marker; lane A, xylanase; lane B, β-xylosidase.

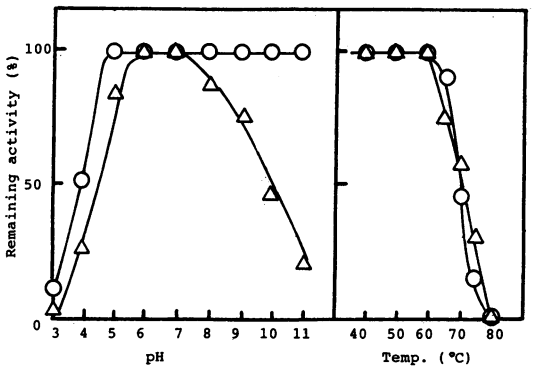

FIG. 4. Thermostability and pH stability of xylanase and β-xylosidase. Purified xylanase (2 U) and β-xylosidase (1 U) solutions, adjusted at each pH, were left at room temperature for 20 h. The remaining activity of each enzyme was measured. Purified xylanase solution (2 U) at pH 6.0 and β-xylosidase solution at pH 6.0 (1 U) were incubated at each temperature for 1 h. The remaining activity of each enzyme was measured at 60°C.

purified xylanase could digest xylan to form mainly xylobiose and xylotriose (Fig. 5, lanes A_0, A_1, and A_2).

When the enzymes concurrently reacted to the substrate (oat-xylan), only xylose was found in the reaction mixture (Fig. 5, lanes M_0, M_1, and M_2). These results indicate that, at first, xylanase cleaved the substrate to liberate xylooligosaccharides and then the resulting oligosaccharides were cleaved to form xylose by the β-xylosidase action. Xylan hydrolysates formed by the enzymes were analyzed on a LiChrosorb-NH_2 (5-μm) packed column (Cica-Merck) joined to a high-performance liquid chromatography system equipped with a differential refractometer (Hitachi Co., Ltd.), which supported the results on thin-layer chromatography.

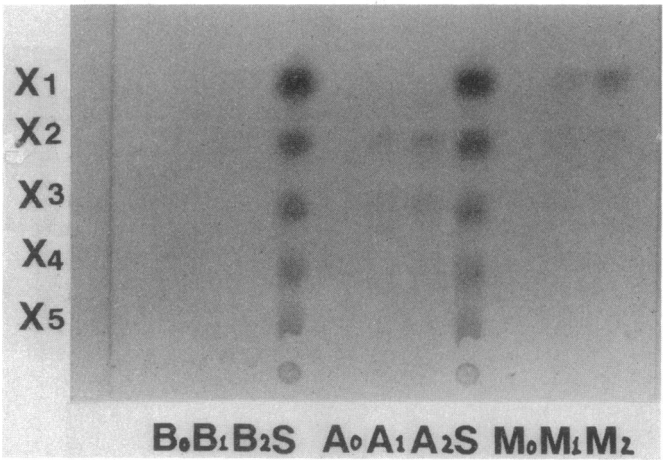

FIG. 5. Thin-layer chromatogram of sugars formed by xylanase and β-xylosidase actions. Lane A_0, Before start of xylanase reaction; lane A_1, xylanase reaction for 1 h; lane A_2, xylanase reaction for 2 h; lane B_0, before start of β-xylosidase reaction; lane B_1, β-xylosidase reaction for 1 h; lane B_2, β-xylosidase reaction for 2 h; lane M_0, before start of xylanase and β-xylosidase reaction; lane M_1, xylanase and β-xylosidase reaction for 1 h; lane M_2, xylanase and β-xylosidase reaction for 2 h; lane S, standard xylose (X_1) and xylo-oligosaccharides (X_2–X_5).

This strain, *B. stearothermophilus* 21, grows on xylan as the sole source of carbon and produces the two enzymes, although in smaller amounts when compared with those produced in the liquid medium containing polypeptone (components described in Materials and Methods). The strain has a characteristic xylan digestion system, i.e., secretion of both thermostable xylanase and β-xylosidase, and a two-step digestion of xylan that occurs in extracellular medium. This thermostable system of xylan digestion has not yet been reported in any other bacteria (9).

LITERATURE CITED

1. **Bradford, M. M.** 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.
2. **Claus, D., and R. C. W. Berkeley.** 1986. Genus *Bacillus* Cohn 1872, 174, p. 1105–1139. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
3. **Esteban, R., J. R. Villanueva, and T. G. Villa.** β-D-Xylanases of *Bacillus circulans* WL-12. Can. J. Microbiol. **28**:733–739.
4. **Frederick, M. M., J. R. Frederick, A. R. Fratzk, and P. J. Reilly.** 1981. Purification and characterization of a xylobiose- and xylose-producing endoxylanase from *Aspergillus niger*. Carbohydr. Res. **97**:87–103.
5. **Gorbacheva, I. V., and N. A. Rodionova.** 1977. Studies on xylan degrading enzymes. Biochim. Biophys. Acta **484**:79–93.
6. **Gruninger, H., and A. Fiechter.** 1986. A novel, highly thermostable D-xylanase. Enzyme Microb. Technol. **8**:309–314.
7. **Kitpreechavanich, V., M. Hayashi, and S. Nagai.** 1984. Production of xylan-degrading enzymes by thermophilic fungi, *Aspergillus fumigatus* and *Humicola lanuginosa*. J. Ferment. Technol. **62**:63–69.
8. **Kitpreechavanich, V., M. Hayashi, and S. Nagai.** 1984. Purification and properties of endo-1,4-β-xylanase from *Humicola lanuginosa*. J. Ferment. Technol. **62**:415–420.
9. **Lee, S. F., and C. W. Forsberg.** 1987. Isolation and some properties of a β-D-xylosidase from *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. **53**:651–654.
10. **Lee, S. F., C. W. Forsberg, and L. N. Gibbins.** 1985. Xylanolytic activity of *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **50**:1068–1076.
11. **Matsuo, M., A. Fujie, M. Win, and T. Yasui.** 1987. Four types of β-xylosidases from *Penicillum wortmanni* IFO 7237. Agric. Biol. Chem. **51**:2367–2379.
12. **Okazaki, W., T. Akiba, K. Horikoshi, and R. Akahoshi.** 1985. Purification and characterization of xylanases from alkalophilic thermophilic *Bacillus* spp. Agric. Biol. Chem. **49**:2033–2039.
13. **Paice, M. G., L. Jurasek, M. R. Carpenter, and L. B. Smillie.** 1978. Production, characterization, and partial amino acid sequence of xylanase A from *Schizophyllum commune*. Appl. Environ. Microbiol. **36**:802–808.
14. **Panbangred, W., A. Shinmyo, S. Kinoshita, and H. Okada.** 1983. Purification and properties of endoxylanase produced by *Bacillus pumilus*. Agric. Biol. Chem. **47**:957–963.
15. **Robert, C. A., C. Yang, R. Mackenzie, D. Bilous, V. L. Seligy, and S. A. Narang.** 1988. Molecular cloning and expression of a xylanase gene from *Bacillus polymyxa* in *Escherichi coli*. Appl. Environ. Microbiol. **54**:1023–1029.
16. **Uchino, F., and T. Nakane.** 1981. A thermostable xylanase from a thermophilic acidophilic *Bacillus* spp. Agric. Biol. Chem. **45**:1121–1127.
17. **Wiegel, J., C. P. Mothershed, and J. Puls.** 1985. Differences in xylan degradation by various noncellulolytic thermophilic anaerobes and *Clostridium thermocellum*. Appl. Environ. Microbiol. **49**:656–659.