World Journal of Microbiology and Biotechnology, 9, 221–224

Production of alkaline xylanase by a newly isolated alkaliphilic *Bacillus* sp. strain 41M-1

S. Nakamura,* K. Wakabayashi, R. Nakai, R. Aono and K. Horikoshi

Alkaliphilic *Bacillus* sp. strain 41M-1, isolated from soil, produced xylan-degrading enzymes extracellularly. Optimum pH for the crude xylanase preparation was about pH 9, confirming the production of novel alkaline xylanase(s) by the isolate. Xylanases were induced by xylan, but were not produced in the presence of xylose, arabinose or glucose. Xylanase productivity was influenced by culture pH, and production at pH 10.5 was higher than that at pH 8.0. Zymogram analaysis of the culture supernatant showed the alkaline xylanase with a molecular mass of 36 kDa.

Key words: Alkaline xylanase, alkaliphilic *Bacillus*.

There has been increasing interest in applying xylanases to kraft pulp bleaching in the paper industry (Viikari *et al.* 1986). As the pulp is normally at alkaline pH, alkaline xylanases are preferable for this purpose. Although xylanases from various microorganisms have been reported [for review, see Wong *et al.* (1988)], few of them are from alkaliphiles (Horikoshi & Atsukawa 1973; Okazaki *et al.* 1984; Honda *et al.* 1985; Ohkoshi *et al.* 1985; Tsujibo *et al.* 1990; Dey *et al.* 1992). In this study, we describe the isolation and characterization of a xylanolytic alkaliphile. Furthermore, the culture conditions necessary for optimal production of alkaline xylanase(s) by the isolate are also discussed.

Materials and Methods

Strain and Growth Media

Bacterial strain 41M-1 was isolated from a soil sample from Chiba, Japan, and used throughout this investigation.

The cells were grown at 37°C in an alkaline medium containing (w/v) 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.5% oat spelt xylan (Sigma, St. Louis, MO) and 1% Na_2CO_3, at pH 10.5. In some experiments some of the Na_2CO_3 in the medium was replaced with NaCl to give an initial pH of 8.0. Growth of the cells was monitored by optical density measurements at 660 nm.

Enzyme Assays

Xylanase activity was determined by measuring the amount of reducing saccharides liberated from 0.4% (w/v) larchwood xylan (Sigma) after 10 min at 37°C. The reducing saccharides formed were measured colorimetrically by the 3,5-dinitrosalicylic acid method (Sumner 1925). One unit (U) was defined as the amount of enzyme which released reducing saccharides equivalent to 1 μmol of xylose per min.

Culture supernatant was electrophoresed on a 12.5% (w/v) sodium dodecylsulphate (SDS)-polyacrylamide gel (Laemmli 1970) containing 0.1% (w/v) larchwood xylan. After electrophoresis, the gel was washed four times at 4°C for 30 min in buffer [the first two washes contained 25% (v/v) propanol] to remove SDS and to renature proteins in the gel. Zymograms were then prepared after incubation for 10 min at 37°C and staining the gel with Congo Red (Morag *et al.* 1990). Clear zones were observed in areas exposed to xylanase activity.

Results and Discussion

Characterization of the Isolate

A soil sample was suspended in sterilized water and spread on agar plates of the alkaline medium (pH 10.5) containing 0.02% (w/v) Congo Red. After incubation at 37°C for 2 to 3 days, colonies that formed clear zones were isolated and

S. Nakamura, K. Wakabayashi, R. Nakai, R. Aono and K. Horikoshi are with the Department of Bioengineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 227, Japan; fax: (+81) 45 923 0377. *Corresponding author.

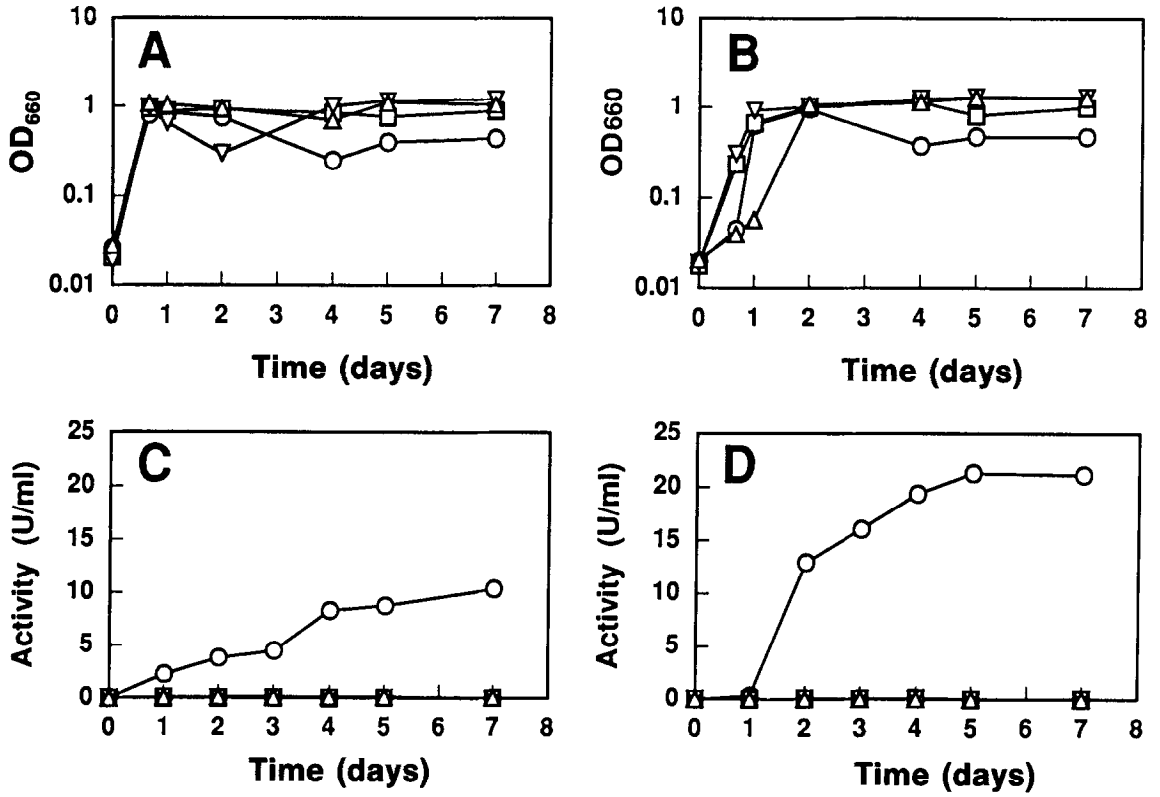

Figure 1. Time course of cell growth (A and B) and xylanase production (C and D) by alkaliphilic *Bacillus* sp. 41M-1. Cells were grown at pH 10.5 (A and C) or pH 8.0 (B and D) on 0.5% (w/v) xylan (○), xylose (△), arabinose (□) or glucose (▽). Growth was monitored by measuring optical density at 660 nm (OD_{660}). Xylanase activity in the culture supernatant was assessed at pH 9.0 using 80 mM $Na_2CO_3/NaHCO_3$ buffer.

screened further for xylanase activity. The isolated strain 41M-1 was capable of growing at pH 7 to 11 and was aerobic, spore-forming, Gram-positive, motile and rod-shaped (1.0 μm × 2.0 to 3.0 μm). From these results, strain 41M-1 should belong to the genus *Bacillus* by the criteria of Bergey's Manual of Systematic Bacteriology (Claus & Berkeley 1986).

Effect of Culture Conditions on Xylanase Production

In liquid medium at 37°C, *Bacillus* sp. 41M-1 grew rather faster at pH 8.0 than at pH 10.5, and there was no significant difference in cell growth with the carbon source used (Figure 1A and 1B). Xylanase activities in the culture supernatants were monitored at pH 9.0 (Figure 1C and 1D). High activities of extracellular xylanase(s) were produced with xylan as a carbon source, whereas very low activities were found on xylose, arabinose or glucose. The xylanase production with xylan was suppressed by the addition of xylose, arabinose or glucose to the medium (data not shown). The production was found to be dependent on culture pH: enzyme activity in the culture with xylan at pH 10.5 was higher than that at pH 8.0. The xylanase activity produced by *Bacillus* sp. 41M-1 in this study was higher than that of other alkaliphilic *Bacillus* spp. studied in this laboratory (Horikoshi & Atsukawa, 1973; Honda *et al.* 1985).

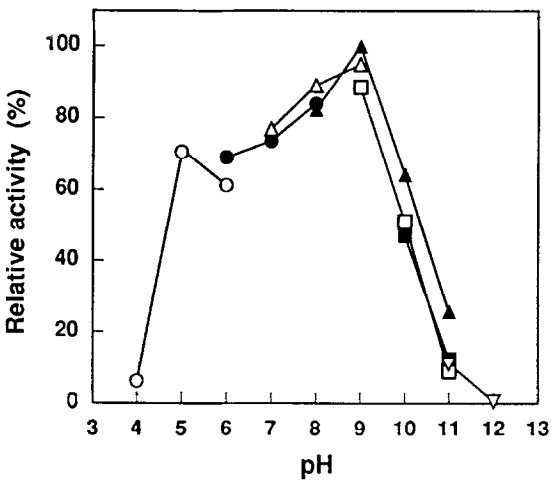

Figure 2. Effect of reaction pH on xylanase activity in various 80 mM buffer systems: ○—citrate buffer (pH 4.0 to 6.0); ●—KH_2PO_4/NaOH (pH 6.0 to 8.0); △—Tris/HCl (pH 7.0 to 9.0); ▲—NH_3/NH_4Cl (pH 8.0 to 11.0); □—$Na_2CO_3/NaHCO_3$ (pH 9.0 to 11.0); ■—Na_2HPO_4/NaOH (pH 10.0 to 11.0); ▽—KCl/NaOH (pH 11.0 to 12.0).

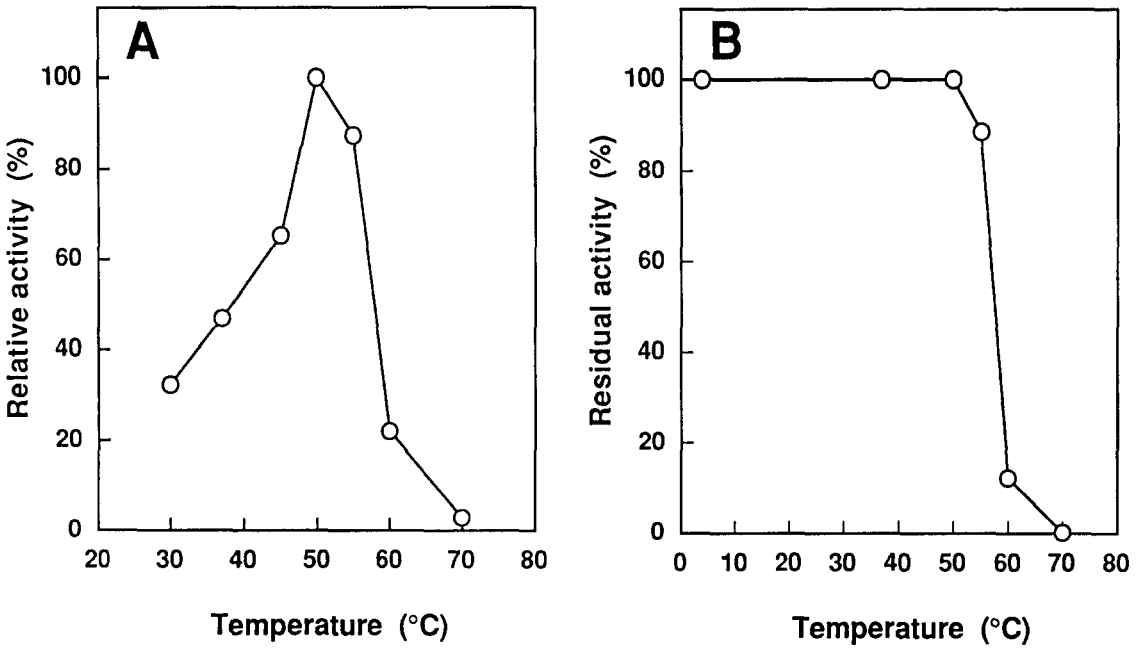

Figure 3. Effects of temperature on xylanase activity (A) and stability (B). For activity, the enzyme activity was assayed at the indicated temperatures, at pH 9.0, using 80 mM $Na_2CO_3/NaHCO_3$ buffer, and expressed here as a percentage of the highest activity. For stability, the enzyme, in 80 mM $Na_2CO_3/NaHCO_3$ buffer (pH 9.0), was exposed to the indicated temperatures for 30 min and the residual activity was measured at pH 9.0.

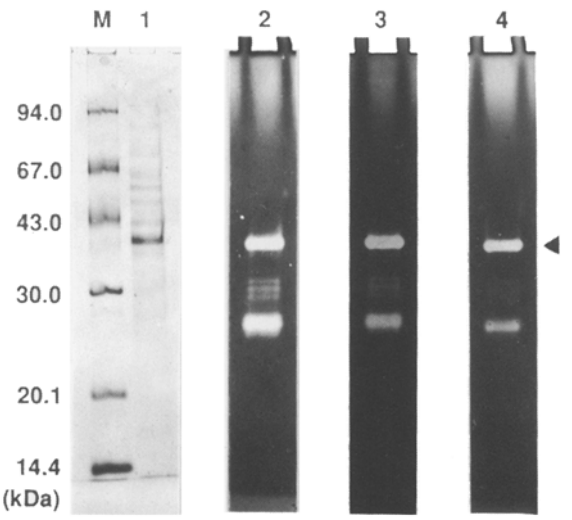

Figure 4. Activity patterns of extracellular xylanases of alkaliphilic *Bacillus* sp. 41M-1 in SDS-polyacrylamide gel containing 0.1% (w/v) xylan. Culture supernatant was applied to the gel after heating at 100°C for 3 min in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol, and then electrophoresed. Proteins in the gel were stained with Coomassie Brilliant Blue (lane 1) or renatured at 4°C in the buffer for xylan hydrolysis (see below). Zymograms were prepared after incubation at 37°C for 10 min (hydrolysis reaction) and staining the gel with Congo Red. For xylan hydrolysis reactions, 100 mM $KH_2PO_4/NaOH$ buffer (pH 7.0, lane 2), 100 mM $Na_2CO_3/NaHCO_3$ buffer (pH 9.0, lane 3) or 100 mM $Na_2HPO_4/NaOH$ buffer (pH 11.0, lane 4) were used. M—Molecular mass standards (Pharmacia, USA).

Properties of the Crude Xylanase

Culture supernatant obtained after 5 days' cultivation at 37°C in xylan-containing alkaline medium (pH 10.5) was used as a crude xylanase preparation. When the effect of reaction pH on xylanase activity was examined in this preparation (Figure 2), the optimum pH (at 37°C) for activity was found to be about pH 9. All the xylanases from alkaliphiles described earlier had pH optima in the near neutral range (Horikoshi & Atsukawa 1973; Okazaki *et al.* 1984; Honda *et al.* 1985; Ohkoshi *et al.* 1985; Tsujibo *et al.* 1990; Dey *et al.* 1992), although some of the them also had relatively high activity at alkaline pH. The optimum pH of the crude xylanase of strain 41M-1 lay in the alkaline region, indicating the existence of a novel enzyme with an alkaline pH optimum in the culture supernatant. Optimum temperature for activity at pH 9.0 was about 50°C (Figure 3A). The enzyme was stable at temperatures up to 55°C, during incubation at pH 9.0 for 30 min (Figure 3B). Predominant products of xylan hydrolysis were xylobiose, xylotriose and higher oligosaccharides (data not shown).

Zymograms of the culture supernatant are shown in Figure 4. Two major bands and several minor bands of xylanases were detected at pH 7.0 and 9.0. One of the major bands, with a molecular mass of 36 kDa, exhibited relatively high activity, even at pH 11.0. The major band detected, after Coomassie Brilliant Blue R-250 staining, was also of a protein of 36 kDa, suggesting that the 36-kDa alkaline xylanase was the major extracellular product of strain 41M-1.

The novel alkaline xylanase was produced efficiently by alkaliphilic *Bacillus* sp. 41M-1. A high pH optimum is especially important in the treatment of alkaline pulp. Furthermore, the feasibility of pulp bleaching by xylanases is also dependent on the absence of contaminating cellulolytic activity. The crude xylanase preparation of strain 41M-1 hydrolyzed neither native cellulose nor carboxymethyl cellulose and is therefore a good candidate for biological pulp bleaching. We are now investigating the purification of the 36-kDa alkaline xylanase from the culture supernatant of strain 41M-1.

Acknowledgements

We thank Professor T. Yasui of the University of Tsukuba for providing xylooligosaccharides and M. Tanimura for her secretarial assistance.

References

Claus, D. & Berkeley, R.C.W. 1986 Genus *Bacillus* Cohn 1872, 174. In *Bergey's Manual of Systematic Bacteriology*, Vol. 2, eds Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. pp. 1105–1139. Baltimore, MD: Williams and Wilkins.

Dey, D., Hinge, J., Shendye, A. & Rao, M. 1992 Purification and properties of extracellular endoxylanases from alkaliphilic thermophilic *Bacillus* sp. *Canadian Journal of Microbiology* **38**, 436–442.

Honda, H., Kudo, T., Ikura, Y. & Horikoshi, K. 1985 Two types of xylanases of alkalophilic *Bacillus* sp. No. C-125. *Canadian Journal of Microbiology* **31**, 538–542.

Horikoshi, K. & Atsukawa, Y. 1973 Xylanase produced by alkalophilic *Bacillus* No. C-59-2. *Agricultural and Biological Chemistry* **37**, 2097–2103.

Laemmli, U.K. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Morag, E., Bayer, E.A. & Lamed, R. 1990 Relationship of cellulosomal and noncellulosomal xylanases of *Clostridium thermocellum* to cellulose-degrading enzymes. *Journal of Bacteriology* **172**, 6098–6105.

Ohkoshi, A., Kudo, T., Mase, T. & Horikoshi, K. 1985 Purification of three types of xylanases from an alkalophilic *Aeromonas* sp. *Agricultural and Biological Chemistry* **49**, 3037–3038.

Okazaki, W., Akiba, T., Horikoshi, K. & Akahoshi, R. 1984 Production and properties of two types of xylanases from alkalophilic thermophilic *Bacillus* spp. *Applied Microbiology and Biotechnology* **19**, 335–340.

Sumner, J.B. 1925 A more specific reagent for the determination of sugar in urine. *Journal of Biological Chemistry* **47**, 393–395.

Tsujibo, H., Sakamoto, T., Nishino, N., Hasegawa, T. & Inamori, Y. 1990 Purification and properties of three types of xylanases produced by an alkalophilic actinomycete. *Journal of Applied Bacteriology* **69**, 398–405.

Viikari, L., Panua, M., Kantelinen, A., Sundquist, J. & Linko, M. 1986 Bleaching with enzymes. In *Proceedings of the Third International Conference on Biotechnology in the Pulp and Paper Industry*, Stockholm, pp. 67–69.

Wong, K.K.Y., Tan, L.U.L. & Saddler, J.N. 1988 Multiplicity of β-1, 4-xylanase in microorganisms: Functions and applications. *Microbiological Review* **52**, 305–317.

(Received 18 August 1992; accepted 8 October 1992)