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Purification and characterization of a thermophilic alkaline xylanase from thermoalkaliphilic *Bacillus* sp. strain TAR-1

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Abstract

Thermoalkaliphilic *Bacillus* sp. strain TAR-1 isolated from soil produced an extracellular xylanase. The enzyme (xylanase R) was purified to homogeneity by ammonium sulfate fractionation and anion-exchange chromatography. The molecular mass of xylanase R was 40 kDa and the isoelectric point was 4.1. The enzyme was most active over the range of pH 5.0 to 10.0 at 50°C. The optimum temperatures for activity were 75°C at pH 7.0 and 70°C at pH 9.0. Xylanase R was stable up to 65°C at pH 9.0 for 30 min in the presence of xylan. Mercury(II) ion at 1 mM concentration abolished all the xylanase activity. The predominant products of xylan-hydrolysate were xylobiose, xylotriose, and higher oligosaccharides, indicating that xylanase R was an endo-acting enzyme. Xylanase R had a K_m of 0.82 mg/ml and a V_{max} of 280 μmol min^{-1} mg^{-1} for xylan at 50°C and pH 9.0.

Keywords: Xylanase; *Bacillus* sp.; Alkaliphiles; Thermophiles; Xylan; Biobleaching

1. Introduction

Hemicellulose is the second most abundant polysaccharide in plant cell walls, accounting for 10 to 30% of the dry weight of wood. Xylans, the major hemicellulose component, contain β-1,4-linked D-xylose backbones with arabinose, 4-*O*-methyl-D-glucuronic acid and acetic acid substituents [1]. The xylan-degrading enzymes include xylanase (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). Xylanases catalyze the hydrolysis of xylan to xylo-oligosaccharides and xylose, while β-xylosidases release xylose residues from the nonreducing ends of xylo-oligosaccharides.

Various applications for xylanases in bioconversion, food and feed industries have been suggested [2–4]. One of the major potential applications of xylanases involves the pulp and paper industry [5]. In the process of making kraft pulp, lignin is completely removed from cellulose fibres traditionally with chlorine-based chemicals (bleaching). Although this chlorine bleaching

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process is highly effective, it results in the release of polluting organic chlorine compounds such as dioxins. Viikari et al. [6] initially demonstrated that xylanases can be used in the prebleaching of kraft pulp in order to reduce the amount of chlorine required to achieve a target pulp brightness and consequently reduce the chlorinated organics released in the effluent. The pulp is normally processed at high temperature under high alkaline condition in a kraft mill. Therefore, xylanases that are active at higher temperatures and pH values are needed in order to make enzymatic process technically and economically more feasible.

Xylanases are produced by various microorganisms [2–4]. Several xylanases from thermophilic microorganisms have been shown to have high temperature optima (ca. 80°C) in neutral or acidic pH conditions [7–13]. The xylanase from extremely thermophilic *Thermotoga* sp. has a temperature optimum of 105°C at pH 5.5 [14] and is probably the most thermo-active xylanase reported previously. On the other hand, alkaline-active xylanases from alkaliphilic [15–25] and alkali-tolerant [26] microorganisms have been studied to date. However, they were not so active and stable at high temperatures. Xylanases active at high temperatures and pH values have not previously been described.

Recently, we have isolated a thermoalkaliphilic *Bacillus* sp. strain TAR-1 from a soil sample [27]. Strain TAR-1 produced a novel thermophilic alkaline xylanase extracellularly. Production of the xylanase was induced by xylan and xylose, but was repressed by glucose. In this paper, we report on the purification and characterizations of the xylanase from strain TAR-1.

2. Materials and methods

2.1. Bacterial strain and medium

Thermoalkaliphilic *Bacillus* sp. strain TAR-1 was used in this investigation. The strain was isolated from the Japanese forest soil as described previously [27]. The medium contained 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.5% xylose. The initial pH was adjusted to 10.5 by adding 1% (w/v) Na_2CO_3.

2.2. Purification of xylanase

All purification steps were carried out at 4°C.

Step 1. The organism was inoculated into the medium (400 ml) in 2-l baffled Erlenmeyer flasks (5 flasks) and then cultivated aerobically at 50°C on a rotary shaker for 12 h. The culture supernatant was obtained by centrifugation at $8000 \times g$ for 10 min.

Step 2. Solid ammonium sulfate was added to the culture supernatant (1850 ml) until 50% saturation was obtained. After standing overnight, the precipitate was discarded by centrifugation ($7200 \times g$, 60 min). To the resultant supernatant, ammonium sulfate was added to give 90% saturation. The precipitate was collected by centrifugation, resuspended in 60 ml of 10 mM citrate buffer (pH 5.0), and dialyzed five times against 10 l of the same buffer for 3 to 10 h.

Step 3. The dialyzed solution (final volume, 90 ml) was applied to a DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan) column (2.5 × 17 cm) which had been equilibrated with 10 mM citrate buffer (pH 5.0). The column was washed with 400 ml of the buffer and then eluted with a linear gradient of 0 to 300 mM NaCl in 1.0 l of the buffer. Fractions (10 ml each) were automatically collected, scanned for their A_{280}, and assayed for xylanase activity. The active fractions that eluted at around 200 mM NaCl were pooled and stored at 4°C.

2.3. Xylanase assay

Xylanase activity was assayed by measuring the amount of reducing sugars liberated from xylan by the 3,5-dinitrosalicylic acid method [28]. Briefly, 40 μl of the enzyme preparation was added to 160 μl of a 2.0% (wt/vol) birchwood xylan (Sigma, St. Louis, MO) suspension in a 100 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0). The

reaction was carried out at 50°C for 10 min. The enzyme was inactivated by adding 400 μl of the 3,5-dinitrosalicylic acid reagent and boiling for 5 min. Absorbance at 500 nm was measured immediately after 2.4 ml of deionized water was added to the mixture. One unit was defined as the amount of enzyme which produced reducing sugars equivalent to 1 μmol of xylose per min under the conditions described above.

2.4. Estimation of protein concentration

Protein concentration was measured by the method of Bradford [29] using bovine serum albumin (fraction V, Sigma) as a standard.

2.5. Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [30] using 12.5% gels. Samples were dissolved in a solution containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 15 mM Tris–HCl (pH 6.8) and heated in a boiling water bath for 3 min. After electrophoresis, the gels were stained with Coomassie brilliant blue (CBB) R-250. The molecular mass markers used were the SDS-PAGE standards (low range, Bio-Rad Labs., Richmond, CA).

Isoelectric focusing (IEF) was done using Servalyt Precotes (pH 3 to 10) (Serva, Heidelberg, Germany). The gels were stained with CBB G-250. IEF calibration kit (pH 3 to 10) (Pharmacia, Uppsala, Sweden) was used as isoelectric point (p*I*) markers.

2.6. Zymogram analysis

Zymogram analysis was performed by the method of Morag et al. [31]. Samples were electrophoresed on an SDS-12.5% polyacrylamide gel containing 0.1% birchwood xylan as described above. Then, the gel was washed four times for 30 min at 4°C in a 100 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0) (the first two washes contained 25% (v/v) isopropyl alcohol) to remove SDS and renature proteins in the gel and further incubated in the buffer for 10 min at 50°C. The gel was soaked in 0.1% Congo red solution for 15 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. A zymogram was prepared after an introduction of the gel into 0.2% acetic acid. The background turned dark blue and clear zones were observed in areas exposed to xylanase activity.

A zymogram was also prepared after IEF as described previously [32]. The polyacrylamide gel with separated proteins was layered on an 1% agarose gel with 1 mm of thickness containing 0.1% birchwood xylan in a 100 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0). After incubation at 50°C for 10 min, the agar layer was separated from the polyacrylamide gel and then active bands were visualized by Congo red staining as described above.

2.7. Hydrolysis of xylan and xylo-oligosaccharides

Xylan hydrolysis reaction was carried out with 3.2 mg of birchwood xylan and 0.75 U of purified xylanase in 200 μl of an 80 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0) at 50°C. The aliquots were periodically withdrawn and used for chromatographic analysis. Enzymatic hydrolysis of xylose and xylo-oligosaccharides (xylobiose, xylotriose, xylotetraose, and xylopentaose: gifts of T. Yasui of the Seitoku Junior College of Nutrition) was performed at 50°C for 30 min with 80 μg of each substrate and 75 mU of purified enzyme in 20 μl of an 80 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0).

A 0.5-μl portion of each sample was spotted onto thin-layer chromatography silica gel plate 60 F_{254} (Merck, Darmstadt, Germany) and chromatographed in a solvent system containing acetonitrile–water (3:1, v/v) at room temperature. Saccharides were located by the orcinol–sulfuric acid reaction [33]. Xylose and xylo-oligosaccharides were used as standards.

2.8. Determination of kinetic parameters

The enzyme (47 mU) was incubated with different amounts of birchwood xylan (0 to 3.2 mg) in 200 μl of an 80 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0) at 50°C. Initial reaction rate was measured for each condition. The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined from Lineweaver-Burk plots [34].

2.9. Amino acid analysis

Amino acid analysis was carried out by the ninhydrin method with a Seiko (Chiba, Japan) SAC4800 automatic amino acid analyzer.

2.10. Sequencing of N-terminal amino acids

The *N*-terminal amino acid sequencing was performed by automated Edman degradation of the native enzyme using a Shimadzu (Kyoto, Japan) PPSQ-10 protein sequencer and an on-line PTH-10 PTH analyzer.

3. Results and discussion

3.1. Purification of xylanase

Thermoalkaliphilic *Bacillus* sp. strain TAR-1 was grown in xylose-containing alkaline medium (pH 10.5) at 50°C for 12 h. The zymogram of the culture supernatant of the organism showed a single active band corresponding to a molecular mass of 40 kDa (Fig. 1A, lane 1). A major protein band was detected at the position of 40 kDa by CBB staining, suggesting that the 40-kDa xylanase (hereafter designated as xylanase R) seemed to be the major extracellular product of strain TAR-1.

The xylanase in the culture supernatant was concentrated by ammonium sulfate fractionation with 50 to 90% saturation. The concentrated xylanase R was further purified by anion-exchange chromatography with DEAE-Toyopearl 650M. A zymogram prepared after IEF of the culture supernatant from strain TAR-1 revealed that the p*I* of xylanase R is about 4.1 [27]. Therefore, pH 5.0 was chosen for the chromatographic conditions. Most of the enzyme activity was adsorbed on the resin and eluted from the column

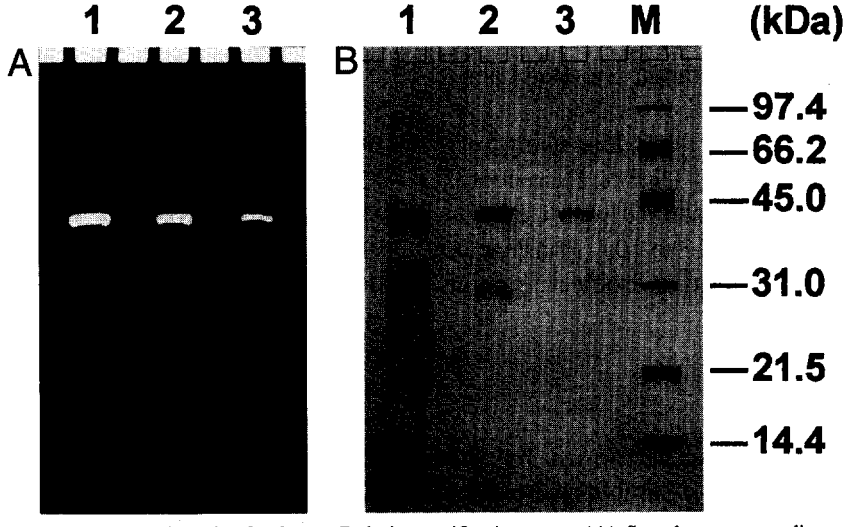

Fig. 1. SDS-polyacrylamide gel electrophoresis of xylanase R during purification steps. (A) Samples corresponding to 80 μl of the original culture were electrophoresed on an SDS-12.5% polyacrylamide gel containing 0.1% xylan; the gel was examined for xylanase activity at pH 9.0. (B) Samples corresponding to 300 μl of culture were also subjected to SDS-12.5% PAGE; the gel was stained with CBB. Lanes 1, culture supernatant (2.6 μg of protein for panel A and 9.6 μg for panel B); lanes 2, precipitate with 50 to 90% saturated ammonium sulfate (1.3 μg of protein for panel A and 4.8 μg for panel B); lanes 3, fraction from the DEAE-Toyopearl 650M column (final preparation in purification step, 0.35 μg of protein for panel A and 1.3 μg for panel B). M, molecular mass markers.

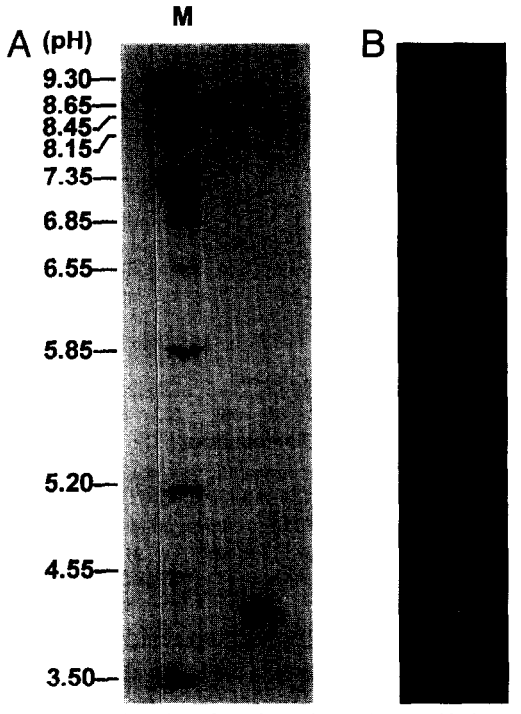

Fig. 2. Isoelectric focusing of purified xylanase R. A purified enzyme preparation was electrophoresed on a polyacrylamide gel with a pH gradient from 3 to 10. (A) CBB staining of the gel; (B) overlay with an agarose gel (pH 9.0) containing 0.1% xylan for detecting xylanase activity. The amounts of the purified enzyme loaded were 0.67 μg for CBB staining and 0.067 μg for the zymogram, respectively. M, p*I* markers.

at 180 to 220 mM NaCl. The purified xylanase R appeared as a single band in SDS-PAGE with a molecular mass of 40 kDa under the reducing condition (Fig. 1B, lane 3). The enzyme activity was found to comigrate with the protein band in SDS-PAGE (Fig. 1A, lane 3). Furthermore, both protein and activity were focused around pH 4.1 (Fig. 2), suggesting that the p*I* of the purified xylanase R is 4.1. These results are in agreement with those predicted previously [27]. The homogeneity of the purified xylanase R thus obtained was confirmed by SDS-PAGE followed by silver-staining (data not shown), as well as by IEF (Fig. 2A).

Purification steps were monitored by SDS-PAGE and the following zymogram analysis (Fig. 1). Table 1 summarizes the procedure. The purified xylanase R preparation with the specific activity of 217 U/mg-protein was used in the experiments described below.

3.2. Effect of reaction pH on activity

The activities of xylanase R at various pH values were measured by using birchwood xylan as the substrate. The reaction pH values were adjusted to 4.0 to 11.0 with either 80 mM citrate buffer (pH 4.0 to 6.0), 80 mM phosphate buffer (pH 6.0 to 8.0), 80 mM $Na_4P_2O_7$–HCl buffer (pH 8.0 to 9.0), or 80 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0 to 11.0); the other experimental conditions were the same as those for the standard assay. Xylanase R showed a broad pH activity profile and was most active over a range of pH 5.0 to 10.0 (data not shown). This result was almost consistent with that of culture supernatant of strain TAR-1 [27]. Some xylanases from alkaliphiles have been described to have a broad range pH optima [16,18–23,25].

3.3. Effect of temperature on activity and stability

The enzyme activity was assayed at various temperatures. As shown in Fig. 3, the optimum temperatures of xylanase R were 75°C at pH 7.0 and 70°C at pH 9.0. The maximum activity was about 2–3 times higher than the activity at 50°C in both neutral and alkaline conditions, indicating the thermophily of this enzyme. The level of maximum activity at pH 7.0 was 1.7-fold as high as

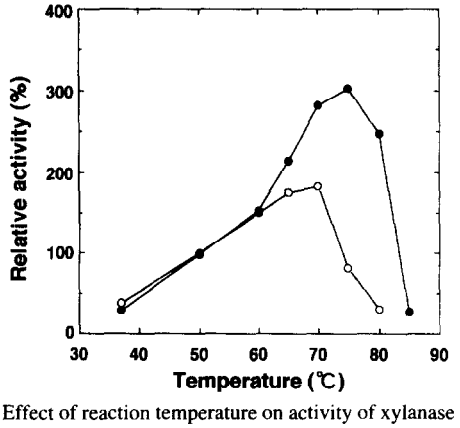

Fig. 3. Effect of reaction temperature on activity of xylanase R. The enzyme reaction was carried out for 10 min at pH 7.0 (●) using an 80 mM phosphate buffer, or pH 9.0 (○) using an 80 mM Na_2CO_3–$NaHCO_3$ buffer. The activity at 50°C and pH 9.0 (standard assay conditions) was taken as 100%.

that at pH 9.0. To the best of our knowledge, xylanase R is more thermo-active at a high alkaline condition (pH 9.0) than all the xylanases reported so far.

The thermostability of xylanase activity was assessed by incubating the enzyme sample in an 80 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0) at various temperatures between 4 and 75°C for 30 min in the presence or absence of 1.6% (w/v) birchwood xylan. The samples were cooled on ice, diluted 40–100 times into the same buffer, and then measured for residual activity at 50°C and pH 9.0 using standard assay. In the absence of xylan, the enzyme activity declined sharply at temperatures above 55°C (Fig. 4). Xylanase R, however, was stable at temperatures of up to 65°C in the presence of xylan. It is possible that xylan somehow protected and conserved the enzyme activity at higher temperatures.

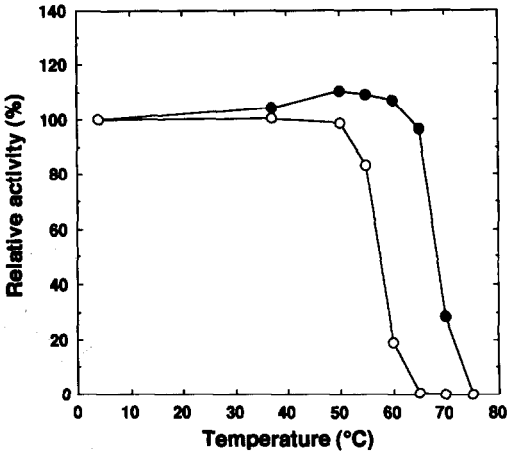

Fig. 4. Effect of temperatures on stability of xylanase R. The purified enzyme (0.13 U), in 50 μl of an 80 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0), was incubated at various temperatures for 30 min in the presence (●) or absence (○) of 1.6% (w/v) xylan. Then, the residual activity was measured under the standard assay conditions.

3.4. Influence of various reagents on activity

The activity of xylanase R was measured under the standard assay conditions in the presence of metal ions and other agents. One millimolar Ca^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, or Zn^{2+} did not influence the enzyme activity. On the other hand, Hg^{2+} (1 mM) completely inhibited the xylanase activity; Cu^{2+} (1 mM) resulted in a loss of activity of about 60%. *N*-Bromosuccinimide, *p*-chloromercuricbenzoate, EDTA, and monoiodoacetic acid had no remarkable effects on xylanase activity at 1 mM. Some xylanases from different microbial strains have been reported to be inactivated by *N*-bromosuccinimide, a potent oxidizing agent with specificity for tryptophan residues [35], indicating the existence of essential tryptophan residues in their active sites [16,32,36,37]. However, it is suggested here that tryptophan residues may have no relationship to the activity of xylanase R.

3.5. Mode of action

A silica gel thin-layer chromatogram of the hydrolysate of birchwood xylan by purified xylanase R is shown in Fig. 5. The enzyme degraded xylan at random, and the end products released were xylobiose, xylotriose, and higher oligosaccharides. Under prolonged incubation, a trace amount of xylose was detected. Thus, xylanase R was suggested to be an endoxylanase that randomly cleaves xylan as a substrate. The spot between those of xylotriose and xylotetraose was not identified but is presumed to be that of a xylo-oligosaccharide with side chains.

The action of xylanase R on xylose and xylo-oligosaccharides was also investigated. The enzyme had no activity toward xylose, xylobiose and xylotriose, but hydrolyzed xylotetraose and

Table 1
Purification of xylanase R from thermoalkaliphilic *Bacillus* sp. strain TAR-1

Purification step	Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)
Culture supernatant	5530	59.1	93.5	100
Ammonium sulfate fractionation	3150	29.8	106	57.1
DEAE chromatography	1740	8.01	217	31.5

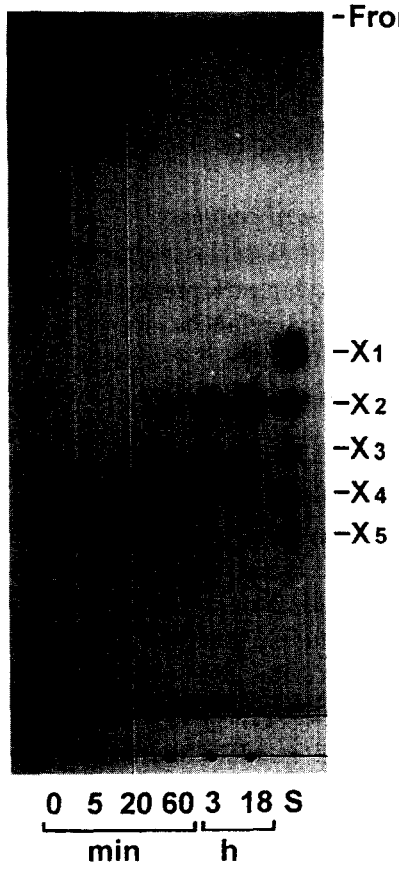

Fig. 5. Thin-layer chromatogram of the products of xylan degradation by xylanase R. The purified enzyme preparation (0.75 U) was mixed with 200 μl of 1.6% (w/v) xylan in an 80 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0) and incubated at 50°C for the times indicated. The samples (0.5 μl each) for each time interval were chromatographed on a silica gel plate in acetonitrile–water (3:1, v/v) and developed with an orcinol–sulfuric acid spray. The standards (S) used were xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), and xylopentaose (X_5).

xylopentaose to lower xylo-oligosaccharides (Fig. 6). Thus, xylanase R required at least four xylose residues for catalytic activity. It should be noted that small amounts of xylopentaose and higher oligosaccharides might be formed when xylotetraose was used as the substrate. This result suggests that xylanase R has transxylosidase activity as found with several xylanases from other *Bacillus* spp. [18,38,39].

3.6. Kinetic parameters

The kinetic parameters of xylanase R were determined at 50°C and pH 9.0 by using birchwood xylan as the substrate. The apparent K_m value of purified xylanase R was 0.82 mg/ml. The V_{max} value was calculated to be 280 μmol of xylose min^{-1} mg of $protein^{-1}$.

3.7. Amino acid composition and N-terminal sequence

The amino acid composition of xylanase R (Table 2) revealed that the protein contains high amounts of aspartic acid/asparagine and glutamic acid/glutamine, and low amounts of histidine and methionine. It has been suggested that methionine content is usually low in microbial xylanases

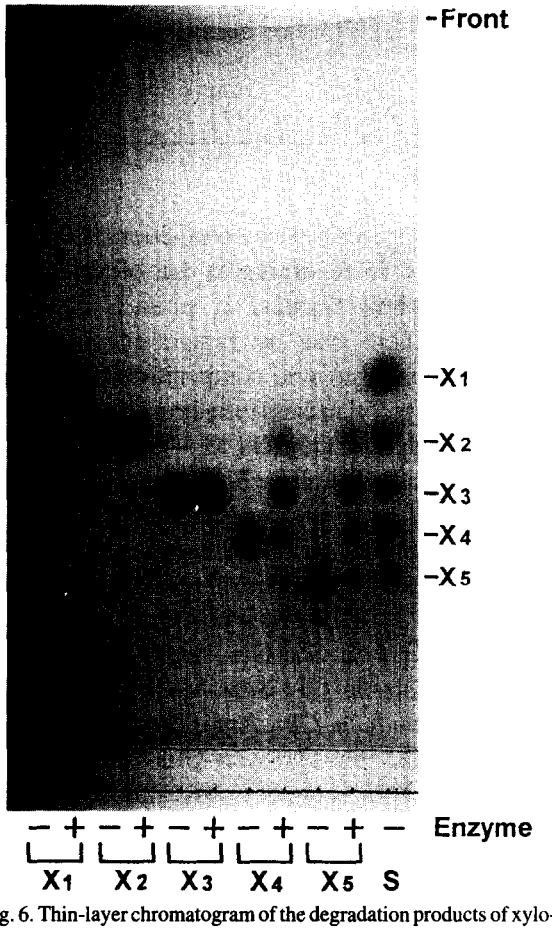

Fig. 6. Thin-layer chromatogram of the degradation products of xylo-oligosaccharides by xylanase R. The purified enzyme (75 mU) and substrate (80 μg of each: X_1, X_2, X_3, X_4, or X_5) was mixed in 20 μl of an 80 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0), and then incubated at 50°C for 30 min. The samples (0.5 μl each) were chromatographed as described in the legend to Fig. 6. The standards (S) used were X_1, X_2, X_3, X_4, and X_5.

Table 2
Amino acid composition of xylanase R from thermoalkaliphilic *Bacillus* sp. strain TAR-1

Amino acid	Composition (mol%)
Asx	14.8
Thr	4.3
Ser	4.3
Glx	15.4
Pro	4.3
Gly	7.0
Ala	7.3
Cys	0
Val	6.2
Met	1.5
Ile	5.2
Leu	8.1
Tyr	4.9
Phe	4.0
Lys	4.6
His	2.9
Trp	N.D. [a]
Arg	5.2

[a] Not determined.

[40,41]. The amino acid composition of xylanase R was found to be similar to that of xylanase A from alkaliphilic *Bacillus* sp. strain C-125 [42]. However, there was no remarkable similarity between the amino acid composition of xylanase R and those of xylanases from three closely related bacteria (*Bacillus subtilis* [43,44], *Bacillus pumilus* [39], and alkaliphilic *Bacillus* sp. strain 41M-1 [32]) and other microorganisms [2].

The *N*-terminal amino acid sequence of xylanase R was determined up to 49 residues as follows: H_2N–Asn–Asp–Gln–Pro–Phe–Ala–Trp–Gln–Val–Ala–Ser–Leu–Ser–Glu–Arg–Tyr–Gln–Glu–Gln–Phe–Asp–Ile–Gly–Ala–Ala–Val–Glu–Pro–Tyr–Gln–Leu–Glu–Gly–Arg–Gln–Ala–Gln–Ile–Leu–Lys–His–His–Tyr–Asn–Glu–Leu–Glu–Ala–Asp–. This sequence was almost identical with that of the near *N*-terminal region of xylanase A, corresponding to the sequence between Asn-18 and Glu-66, from alkaliphilic *Bacillus* sp. strain C-125 [42]. Xylanase R from strain TAR-1 and xylanase A from strain C-125 exhibit some important differences. First, xylanase R in this study is more thermo-active than xylanase A. The optimum temperature for activity at pH 7.0 is 75°C for xylanase R, while 70°C for xylanase A as described by Honda et al. [18]. Second, the molecular mass of xylanase R (40 kDa) is different from that of xylanase A (43 kDa) [18].

3.8. Activity toward cellulose

The purified xylanase R preparation hydrolyzed neither crystalline cellulose nor carboxymethyl cellulose. Some microbial xylanases act not only on xylan but also on cellulose [4]. Cellulase activity is, however, unwanted, especially for pulp and paper application, because it degrades cellulose fibers and destroys pulp properties. There was no cellulase activity in the culture filtrate of strain TAR-1 grown on xylan, xylose and cellulose, suggesting the strain does not produce cellulolytic enzymes by nature. Therefore, crude culture filtrates of strain TAR-1 can be used for pulp treatment without further purification.

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