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Alkaline-active xylanase produced by an alkaliphilic *Bacillus* sp isolated from kraft pulp

VW Yang[1], Z Zhuang[1,2], G Elegir[1,3] and TW Jeffries[1]

[1]*Institute of Microbial and Biochemical Technology, USDA Forest Service, Forest Product Laboratory, One Gifford Pinchot Drive, Madison, WI 53705, USA*

A *Bacillus* sp (V1–4) was isolated from hardwood kraft pulp. It was capable of growing in diluted kraft black liquor at pH 11.5 and produced 49 IU (μmol xylose min^{-1} ml^{-1}) of xylanase when cultivated in alkaline medium at pH 9. Maximal enzyme activity was obtained by cultivation in a defined alkaline medium with 2% birchwood xylan and 1% corn steep liquor at pH 9, but high enzyme production was also obtained on wheat bran. The apparent pH optimum of the enzyme varied with the pH used for cultivation and the buffer system employed for enzyme assay. With cultivation at pH 10 and assays performed in glycine buffer, maximal activity was observed at pH 8.5; with phosphate buffer, maximal activity was between pH 6 and 7. The xylanase temperature optimum (at pH 7.0) was 55° C. In the absence of substrate, at pH 9.0, the enzyme was stable at 50° C for at least 30 min. Elecrophoretic analysis of the crude preparation showed one predominant xylanase with an alkaline pI. Biobleaching studies showed that the enzyme would brighten both hardwood and softwood kraft pulp and release chromophores at pH 7 and 9. Because kraft pulps are alkaline, this enzyme could be used for prebleaching with minimal pH adjustment.

Keywords: enzymatic prebleaching; alkaliphilic *Bacillus*; xylanase; chromophore release

Introduction

Dioxin production as a consequence of kraft pulp chlorine bleaching has led researchers to seek alternative methods to obtain high levels of pulp brightness in printing and writing grades of paper. In 1986, Viikari *et al* [38] introduced the use of xylanases for prebleaching kraft pulp. This discovery led to extensive futher studies [5,18,19,27,39,40,43]. Successful mill trials using xylanase for prebleaching kraft pulp have been reported since 1989 [37]. In this process, a xylanase solution is mixed with washed pulp in a brown-stock holding tank prior to alkali extraction and chemical bleaching.

Although many bacteria and fungi have been studied for xylanase production [1,4,8,20,27,29,30] only a few are alkaliphiles [2,16,23,24]. Initial xylanase prebleaching studies focused on the use of known enzymes from fungi and yeasts. However, their pH optima are acidic. Kraft pulping is carried out under strong alkaline conditions, and even after multiple washings, alkali continues to leach from the fiber. Therefore, activity under alkaline conditions is an important characteristic for an enzyme to function in enzymatic prebleaching.

To our knowledge, no previous report has examined the characteristics of organisms found growing in the processing tanks of kraft pulp mills. In the present study, we report the characteristics of a *Bacillus* sp (V1–4) that we isolated from fresh hardwood pulp. The organism is capable of growing in diluted black liquor at pH 11.5 and producing high levels of xylanase when cultivated in alkaline medium (pH 9 and 10). Electrophoretic analysis of crude enzymes showed one predominant basic xylanase produced by the organism. Biobleaching studies showed that the enzyme would brighten both hardwood and softwood kraft pulp at pH 7 and 9. These results suggest that the application of this enzyme to the paper and pulp industry is very promising.

Correspondence: TW Jeffries, Forest Products Laboratory, One Gifford Pinchot Drive, Madison, WI 53705, USA
[2]Present address: Institute of Microbiology, Academia Sinica, Beijing, China
[3]Present address: Stazione Sperimentale Cellulosa carta e Fibre Tessili, Milano, Italy
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Materials and methods

Media

Defined xylan (DX) medium contained (per L): K_2HPO_4, 1.0 g; NaCl, 1.0 g; $(NH_4)_2SO_4$, 2.0 g; birchwood xylan (Sigma, St Louis, MO, USA) 10 g; $CaCO_3$, 2 g; for solid medium, 18 g agar. After autoclaving the medium, its pH was adjusted to 9.0 with sterile 1% Na_2CO_3 [24]. To avoid hydrolysis during autoclaving, birchwood xylan was steam-sterilized dry (in aluminum foil packets) and added to molten agar. For liquid inocula, cultures were primed in trypticase soy broth (TSB, Difco, Detroit, MI, USA) at pH 7 and inoculated into alkaline medium prepared as described by Nakamura *et al* [24]. Alkaline medium contained (per L) Bactopeptone, 5 g; yeast extract, 5 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; birchwood xylan, 5 g; and after autoclaving, 2–3 g of Na_2CO_3 were added to adjust pH to 9 or 10 according to experimental needs. Birchwood xylan was used for substrate throughout this study.

Xylan clearing assays

Colonies were evaluated for xylan hydrolysis by the production of clearing zones on DX plates. Cell-free broths were tested for xylanase activities by spotting 2 μl onto an

agarose gel containing 3% birchwood xylan plus 1% agarose in 50 mM glycine buffer, pH 9.0. After staining with Congo red dye (1% aqueous solution) for 30 min, the gel was destained with 1 M NaCl [42].

Strain isolation

Bacillus strain V1–4 was isolated from freshly obtained hardwood pulp (mainly aspen), provided by the Potlatch Corp, Cloquet, MN, USA. A 5-g sample of pulp was placed in a Petri dish, teased apart, soaked in 50 ml sterile distilled water in a 125-ml flask, and shaken at 180 rpm at 37° C for 3 h. The extract was collected and centrifuged at $3700 \times g$ for 15 min. The resulting pellets were plated on DX agar medium. After incubation at 37° C for 3–5 days, colonies showing clearing zones on DX agar were further purified. Cell suspensions were plated on DX agar medium using serial dilution with sterile water to obtain isolated colonies. Thirty isolates were screened for xylanase activities by cultivating each in 1 ml DX broth medium in 17 mm × 100 mm tubes for 2 days. Cultures were shaken at 180 rpm. The highest xylanase-producing strain (V1–4) was selected for further investigation.

Growth in black liquor

Blow pit hardwood and softwood pulp samples were obtained from the Potlatch Corp. Pulp samples were washed with three volumes of sterile water and filtered through Whatman no 1 filter paper. Black liquor was collected, sterilized, and its pH was measured as 11.5. The *Bacillus* sp V1–4 culture was primed by cultivation overnight in TSB at 37° C and shaken at 180 rpm. Four percent of inoculum was used to inoculate 30 ml of black liquor (diluted 1 : 1 with sterile water) in a 125-ml Erlenmeyer flask. Cell growth was monitored by direct cell counts using a hemocytometer. A separate cell growth experiment was performed by collecting black liquor cell suspension, diluting it with DX medium without xylan (pH 9) and plating it directly on alkaline medium agar plates. After the plates were incubated for 2 days, colonies were counted.

Fermentor cultivation

Fermentor cultivations were carried out in alkaline medium with 1% xylan using a 1.4-L MultiGen fermentor (New Brunswick Scientific Co, NJ, USA) with a working volume of 1.2 L. Aeration was set at 1.2 V/Vm. Temperature and pH were set at 32° C and initially adjusted to pH 9.

Medium optimization

Several carbon sources (fine wheat bran, corn stalk flour, malt extract, oat spelt xylan, and birchwood xylan) were studied at levels of 0.5 to 3% (w/v) with alkaline medium, pH 9, containing 0.5% yeast extract and 0.5% peptone as nitrogen sources. One percent solutions of xylose, glucose, or arabinose were also examined. The inoculum was as previously described. Xylanase activity was measured on 2- and 3-day cultures at pH 7. Effects on enzyme level of cultivation on 1 and 2% birchwood xylan were compared to enzyme levels that had previously been obtained with 0.5% xylan during initial screening studies. Five nitrogen sources (yeast extract, peptone, casein, soy bean meal, and corn steep liquor) were tested at concentrations equivalent to 0.5% yeast extract plus 0.5% peptone. For these studies, 0.5% xylan was used as the carbon source.

Enzyme detection

Xylanase activity was determined by measuring the release of reducing sugars from 1% (w/v) water-soluble birchwood xylan using the arsenomolybdate method [35]. Soluble xylan was prepared by suspending 4 g of birchwood xylan in 100 ml of 50 mM K_2HPO_4/KOH buffer, pH 7, and stirring the suspension for 4 h followed by centrifugation. The soluble fraction corresponded to 50% (w/v) of the total xylan. Crude enzyme preparations were appropriately diluted to obtain maximal activity consistent with a linear response. Buffers used for studying the effect of pH on enzyme activity were 50 mM of phosphate buffer (pH 5–8), glycine buffer (pH 8–10) [17], and AMPSO (3-[1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid) buffer (pH 9–11) (Sigma). Xylose was used as the standard for reducing sugar measurement. Xylanase activity was determined by incubating diluted enzyme solutions with substrate in buffer at 50° C for 10 min. Activity was expressed as IU (μmol xylose min^{-1}). All units were corrected for substrate background reducing groups in the pH region of the working buffer.

Cellulase activity was measured by two methods. Carboxymethyl cellulose activity (CMCase) was assayed at pH 7 by replacing 1% xylan with 1% of low viscosity carboxymethyl cellulose (Sigma) in the assay described previously. Filter paper activity (FPase) was determined by incubating Whatman no 1 filter paper with the culture filtrate in 50 mM phosphate buffer, pH 7 [11]. Enzyme activity was expressed as μmol glucose released min^{-1}.

Protease activity was measured according to Fukushima *et al* [9]. Two milliliters of 1% azocasein solution in 0.05 M phosphate buffer, pH 7, were mixed with 0.1 ml of enzyme sample, incubated at 30° C for 15 min, then 2 ml of 10% trichloroacetic acid was added to stop the reaction. The mixture was filtered through Whatman no. 1 filter paper, and the optical density at 410 nm of the filtrate was measured. Alkaline protease activity was measured by a similar method after the sample was brought to a final concentration of 15 mM EDTA at 30° C for 15 min [21]. Protease activity was calculated from a calibration curve correlated with protease units (PU) by a modified Hagiwara–Anson method using casein [22]. One unit of protease activity was defined as the amount that catalysed release of 1 μg tyrosine min^{-1}.

Electrophoretic analysis

Isoelectric focusing was performed on a Bromma 2117 Multiphor™ horizontal slab-gel system (LKB, Sweden) using Servalyt Precotes™ (pH 3–10, Serva, Heidelberg, Germany). Protein bands were revealed by staining with Serva Blue W™. Zymogram analysis of xylanase activity in isoelectric focusing gels (IEF) was performed as previously reported [7].

Enzyme treatment for chromophore release and prebleaching

Chromophore release was assayed with red oak pulp at 10% consistency. According to the enzyme activity at pH 7 or 9,

1 IU enzyme g^{-1} oven-dried pulp was diluted with 50 mM phosphate buffer, pH 7 or glycine buffer, pH 9, and then applied to 3 g pulp in a sealed plastic bag. After incubation for 3 h at 60° C, the pulp was dewatered on a Büchner funnel using Whatman no 1 filter paper. Chromophore content in the filtrate was measured by optical density at 465 nm.

Xylanase pretreatment was carried out on hardwood and softwood pulps at 10% consistency. Methods were the same as described for the chromophore release. Enzyme dosage was 4 IU (pH 7.0 activity) g^{-1} oven-dried pulp. Samples of pulp (30 g oven-dried) and appropriate volumes of buffer, pH 7 and 9, were equilibrated at 52° C or 60° C for 30 min. Samples were occasionally mixed by kneading the bags during the 3-h incubation. After dewatering them, pulps were subsequently treated with 1% NaOH for 1 h at 60° C and washed with water until pH decreased to 7.0. Pulp samples were then subjected to a bleaching sequence to determine the amount of chlorine required to attain a target brightness.

Chlorine bleaching

The control (buffer only) and xylanase-treated samples were bleached with a conventional bleaching sequence (C70/D30)EDED as described in the following section.

Hardwood pulp samples (20 g of oven-dried basis) were bleached in polyethylene bags for 30 min at 45° C and 3.5% consistency. The chlorine charge applied was a 0.18-multiple based on a Kappa number of 9, which was equivalent to 1.62% active chlorine on oven-dried pulp. A 30% chlorine dioxide substitution was used (C70/D30). Chlorine and chlorine dioxide were added to water and applied simultaneously to the pulp in dilute form. The chlorine solution was distributed manually throughout the pulp prior to placing the samples in a water bath. At the end of chlorination, bleaching chemicals were removed from the pulp samples on a Büchner funnel. The samples were washed thoroughly, dewatered, and extracted with 1% NaOH (w/w oven-dried pulp) for 90 min at 65° C and 10% consistency (E1 stage). The pulp was washed thoroughly after extraction, and 5 g pulp was removed to determine brightness and viscosity. The remaining pulp was bleached with 0.5% chlorine dioxide (calculated as active chlorine on an oven-dried pulp basis) for 3 h at 65° C and 12% consistency (D1 stage).

The same procedure was followed for softwood as described for hardwood with the exception of the chemical charge applied. The higher kappa number for the softwood pulp, 20, required more chlorine. Based on a 0.18-multiple, 5.2% chlorine was applied. Again, a 30% chlorine dioxide substitution was used. Extraction was carried out with 3% NaOH; 1% chlorine dioxide was applied in the D1 stage. Brightness was determined by measuring the reflectance at 457 nm on handsheets using a Technidyne brightness meter (Tappi standard method T452 OM92). Viscosity was determined by the capillary viscosity method according to Tappi standard T230 OM 89.

Results

Strain characterization

Bacillus strain V1–4 was isolated from hardwood pulp. It forms a distinct clearing zone on a xylan DX agar plate, pH 9.0. The organism is capable of growing over a wide pH range (from 5 to 12 in TSB). It grows at temperatures ranging from 25° C to 37° C, but it does not grow at 55° C. *Bacillus* V1–4 is an aerobic, Gram-positive, catalase and oxidase-positive, rod-shaped bacterium. Based on the morphology and physiological characteristics, the organism was keyed to the genus *Bacillus* and appeared closely related to *B. subtilis* using the criteria of Bergey's Manual of Systematic Bacteriology [14].

Effect of culture conditions on growth and xylanase production

Black liquor collected from hardwood and softwood was used to test the growth of *Bacillus* V1–4 at pH 11.5. The organism is capable of growing in 1/2 strength hardwood or softwood black liquor. Diluted black liquor provided a high background absorbency reading that interfered with growth measurements by optical density. Therefore, a direct cell count of growth rate was taken using a hemocytometer and results represent the average of triplicate determinations (Figure 1). Xylanase activity was undetectable by the Nelson Somogyi method. In a separate cell growth experiment, diluted cell suspensions were plated on alkaline medium agar plates and colony numbers were counted after 2 days. The growth profile was similar to that shown in Figure 1.

An alkaline medium containing birchwood xylan was used to grow *Bacillus* sp V1–4 at pH 9 and 10 at 37° C in shake flasks. Figure 2 shows the growth and xylanase activity using 1.0% xylan as the substrate at pH 9. Similar growth profiles were observed at pH 10. Maximal xylanase activity was observed after 2 days of cultivation. Xylanase activity was assayed at pH 7, and activities were measured as 30 and 25 IU ml^{-1} for pH 9 and 10 culture filtrates, respectively. When comparing various xylan concentrations (0.5, 1, and 2%) as substrates, the highest activity was detected with 1% xylan at pH 9 on day 2. Activities were similar with 2% xylan, but the cultivation time was longer (4–5 days; data not shown). Addition of 0.2% Tween 80 to 1% xylan culture at pH 9 did not affect enzyme production.

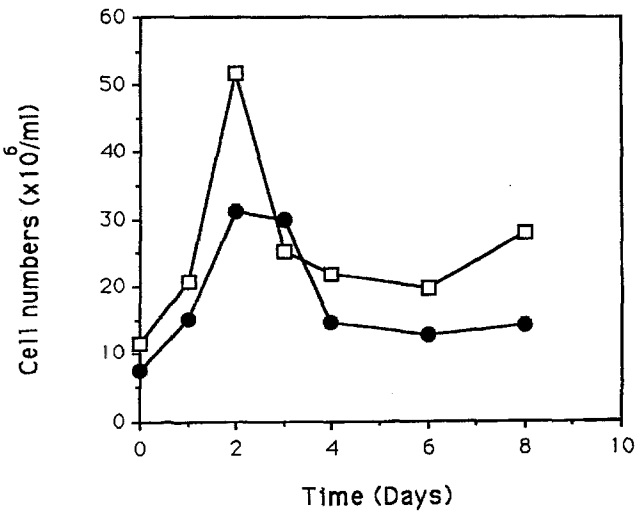

Figure 1 Growth curve of *Bacillus* V1–4 cultivated in half-strength diluted black liquor from hardwood and softwood pulp (pH 11.5). □ hardwood, ● softwood

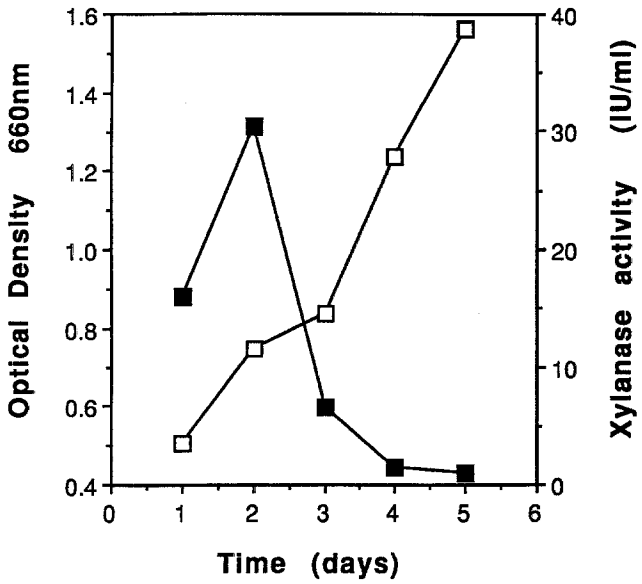

Figure 2 Cell growth and xylanase production by *Bacillus* V1–4. Enzyme activity was assayed in phosphate buffer at pH 7. The culture was cultivated with 1% xylan at pH 9.0. □ OD 660 nm, ■ IU ml^{-1}

Cultivation temperature did affect enzyme production. The highest enzyme production was observed at 32° C; 25° C was the next most suitable temperature for production (Figure 3). Activity decreased rapidly in cultures grown at 37° C.

Other enzyme activities, such as cellulase (CMCase and FPase) and protease, were examined. Minimal CMCase (0.1 IU ml^{-1}), FPase (0.5 IU ml^{-1}) and alkaline protease (34 PU) were detected in pH 9 culture broths.

Effect of pH and temperature on crude xylanase

The optimum reaction pH was determined with crude culture supernatant solution collected from day 2 cultures grown at pH 9 and 10. Xylanase activity was detected over a wide pH range from 5 to 10 (Figure 4); pH values of 6 to 8.5 were most suitable for xylanase activity. When glycine buffer was used, high activity was observed in the alkaline region; this was particularly true with cultures grown at pH 10.

The optimum temperature for crude xylanase activity from cells grown at pH 9 was 55° C (Figure 5) in the pH 7 assay. A similar temperature optimum was observed at pH 9. Crude enzyme from cells grown at pH 9 was stable at temperatures up to 55° C when heated from 30 min at pH 9.0 (Figure 6). Fifteen to 20% of residual activity remained after incubation at 60° C.

Effect of carbon and nitrogen sources

The effects of various carbon and nitrogen sources on production of xylanase by *Bacillus* V1–4 are given in Table 1. Both day 2 and 3 levels are presented. Birchwood xylan

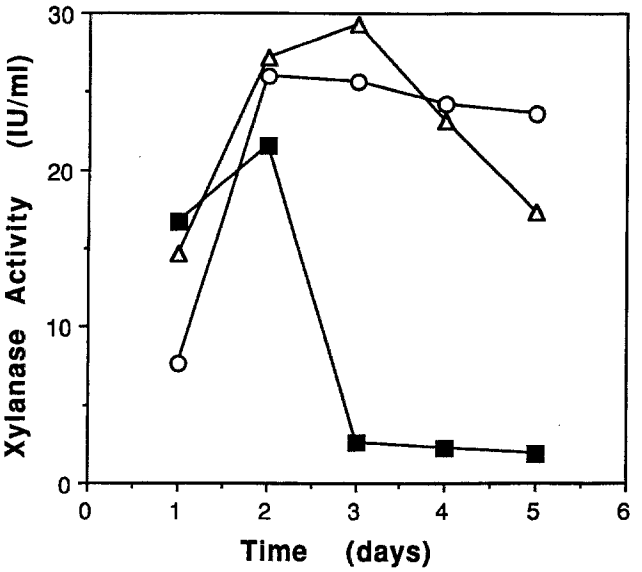

Figure 3 Effect of cultivation temperature on xylanase production by *Bacillus* V1–4. Enzyme activity was determined at pH 7. ○ 25° C, Δ 32° C, ■ 37° C

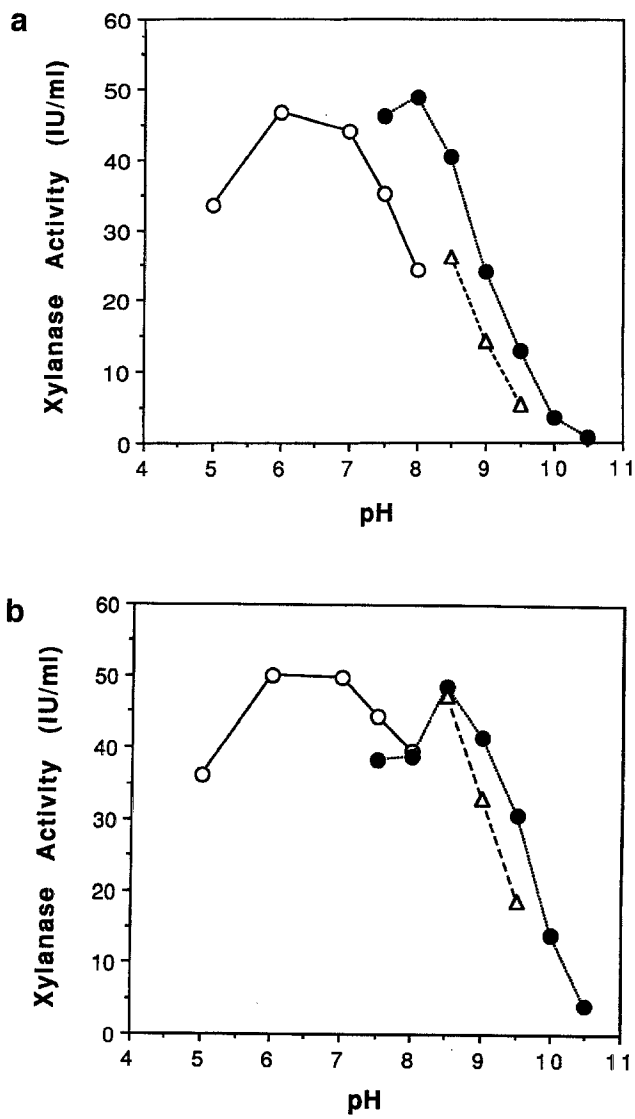

Figure 4 Effect of pH on the activity of *Bacillus* V1–4 xylanase produced on 1% birchwood xylan at (a) pH 9 and (b) pH 10 after 2 days growth. Buffer systems applied are described in text. ○ phosphate buffer, ● glycine buffer, Δ AMPSO buffer

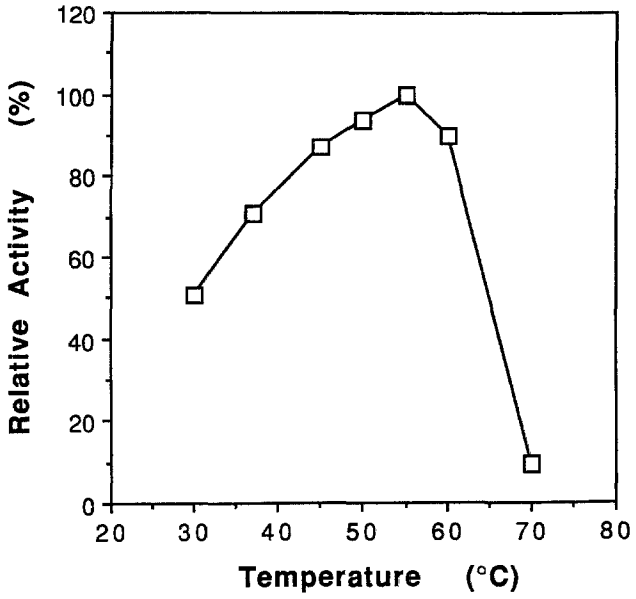

Figure 5 Effect of temperature on *Bacillus* V1–4 crude xylanase at pH 7. Relative activity is expressed as a percentage of the maximum

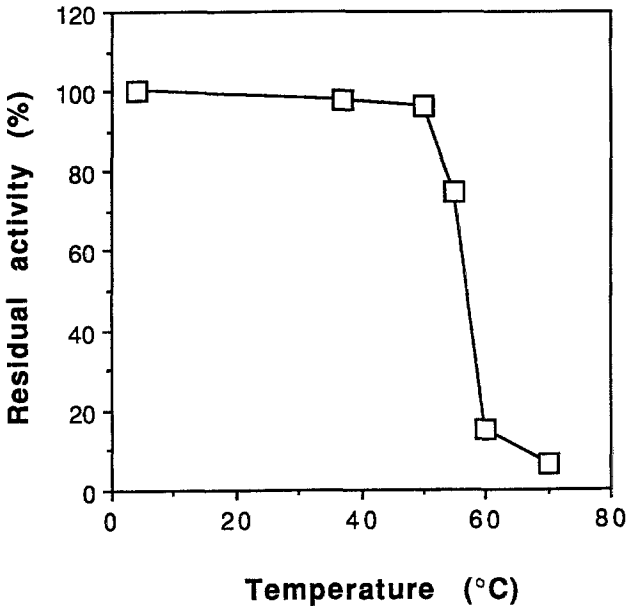

Figure 6 Thermostability of *Bacillus* V1–4 xylanase. Crude enzymes were preincubated at various temperatures for 30 min prior to xylanase determination at pH 9 assay condition. Residual activity is presented as a percentage of the original without heat treatment

(1%) and finely ground wheat bran (3%) were the best carbon-sources for enzyme production. Enzyme activity occurred more rapidly on birchwood xylan, but the crude insoluble substrates, such as wheat bran, are more economical. Birchwood xylan was a much better carbon source than that from oat spelt, and wheat bran was much better than corn stalk flour. Among the three sugars tested, only xylose resulted in significant activity.

Corn steep liquor was the best nitrogen source for enzyme production. The addition of corn steep liquor to 0.5% birchwood xylan substrate enhanced enzyme production. With 2% birchwood xylan medium, a 40–80% enhancement was observed and an activity as high as 49 IU ml^{-1} was obtained when measured by the arsenomolybdate method.

Electrophoretic analysis

Isoelectric focusing was performed on crude enzyme of *Bacillus* V1–4 grown in pH 9 alkaline medium. One major band was observed, and its isoelectric point was 9.1 (Figure 7). A zymogram of the native isoelectric focusing gel showed strong xylan clearing at that pI (Figure 7).

Effect of xylanase on chromophore release and pulp bleachability

Previous studies have shown that pulp bleachability correlates with chromophore release following treatment with *Streptomycetes* xylanase [28]. Therefore, it was of interest to determine how pH affected chromophore release by the alkaline active xylanase from *Bacillus* V1–4. As described in Materials and Methods, after pulps were treated with V1–4 enzyme, filtrates were collected and measured at 465 nm. The pH 9 filtrates (0.32 unit) had about 75% of the chromophore release activity of that observed at pH 7 (0.41 unit).

To evaluate the effects of enzyme on bleachability, enzymatic pretreatment of pulps combined with a bleaching sequence was studied. Both hardwood and softwood pulps were treated at pH 7 and 9 at 52° C and 60° C. Table 2 shows that high brightness and viscosity levels were attained with both hardwood and softwood pulps following xylanase pretreatment. Low incubation temperature (52° C) resulted in higher brightness and viscosity.

Discussion

Bacillus V1–4 grew over a wide pH range in TSB, and it grew especially well in the pH 9 alkaline medium. It grew slowly in black liquor at pH 11. The low level of cell growth observed under these conditions suggests that it might be capable of using xylan, saccharinic acids or other organic degradation products present in the pulp cooking liquors, and that it will survive under highly alkaline conditions. To our knowledge, this is the first published report of isolating an alkaliphilic microorganism from pulp.

Bacillus sp V1–4 produces one of the highest xylanase activities ever reported from bacteria or fungi. In screening for xylanase activity, the dinitrosiacylic acid (DNS) assay method is often used because it is rapid, convenient, and sensitive. However, the latter feature can also lead to misinterpretation, because the DNS assay gives higher values for xylanase activity than does the arsenomolybdate (Nelson–Somogyi) assay when compared under similar conditions. This difference is attributable to degradation of xylan oligosaccharides by the DNS reagent [10,32].

The xylanase activities produced by *Bacillus* V1–4 (as measured by the arsenomolybdate method) showed much higher activity than most other *Bacillus* strains, even when the latter were assayed by DNS [12,34]. Enzyme production was also greater than the activity produced by a previously reported alkaliphilic *Bacillus* sp [15,16,24,26]. The activity from *Bacillus* V1–4 is similar to levels

Table 1 The effects of various carbon and nitrogen sources on xylanase production by *Bacillus* V1–4

Source	(%)	Day 2 (IU ml^{-1})[a]	Day 3 (IU ml^{-1})
Carbon			
Oat spelt xylan	0.5	5.5	8.1
Birchwood xylan	0.5	15.1	15.0
Birchwood xylan	1.0	28.2	24.8
Corn stalk flour	2.0	8.2	11.0
Wheat bran	3.0	6.8	22.1
Malt extract	1.0	6.3	4.6
D-Glucose	1.0	2.6	1.8
D-Xylose	1.0	8.9	7.8
L-Arabinose	1.0	2.2	1.2
Nitrogen			
Yeast extract + peptone	0.5 + 0.5	17.1	20.3
Yeast extract	1.0	17.7	16.1
Peptone	1.0	16.9	14.9
Casein	1.0	22.2	20.9
Soybean meal	1.0	21.0	19.0
Corn steep liquor	2.8	10.6	25.0

[a]Enzyme activities are expressed in IU ml^{-1}

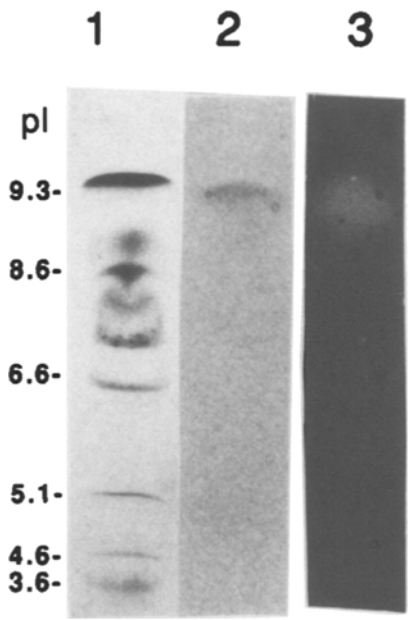

Figure 7 Isoelectric focusing and zymogram of crude enzymes of *Bacillus* V1–4. Lanes: 1. The position of pI marker proteins is indicated. 2. Isoelectric focusing gel (pH 3–10) stained with Serva Violet. 3. Xylanase zymogram corresponding to lane 2

reported for *Bacillus circulans* [31]. In comparison with the fungal enzymes [13], the production time for bacteria is much shorter, which is a crucial beneficial factor for large-scale production.

In the fermentor, there was a minimal pH decrease, from pH 9.0 to 8.7, during 3 days of cultivation. A similar observation was made with flask cultures. Therefore, it would not be necessary to readjust pH during production in a commercial application.

The pH optimum of the *Bacillus* V1–4 xylanase culture filtrate was 7. Of the maximal xylanase activity, 50–80% was present at pH 9.0 for cultures grown at either pH 9 or 10. This is a higher level than the alkaliphilic *Bacillus* species described previously. They have pH optima around 7.0, and their activities at pH 10 are 10–50% of the maximum activity [16,26]. A higher percentage of maximal activity was reported at pH 9 [2,24], but the overall xylanase activity was low.

Two commercially available xylanases, Ecozyme (Zeneca Bioproducts, Ontario, Canada) and Pulpzyme HC (Novo Nordisk Inc, Danbury, CT, USA), were tested for their optimal pH activity. Ecozyme exhibited a pH profile similar to that of *Bacillus* V1–4 xylanase; Pulpzyme HC exhibited higher activity in the alkaline region (pH 9–9.5) (data not shown). Both of these are cloned products, and their volumetric activities were considerably higher than what is reported here.

Enzyme stability was tested following storage at 4° C. Minimal degradation (< 10%) was found after 1 month. To reduce storage space for large scale production, lyophilization is recommended. The recovery of enzyme activity after reconstitution was about 90–95%.

Minor cellulosic activity was found in the crude filtrate. This was possibly due to xylan contamination of the CMC substrate [33]. Because the activity was very low, the culture filtrate can be used for treating pulp without further purification.

The enzyme exhibited 50% greater residual activity when 3% xylan was added to the temperature stability assays carried out at 60° C; the presence of substrate appeared to enhance thermostability of the enzyme. This effect was also observed by Bandivadekar *et al* [3].

Isoelectric focusing has shown that this organism produces one predominant xylanase enzyme under alkaline conditions. In comparison to other organisms which produced multiple xylanases [6,7,25,36,41], *Bacillus* V1–4 would be an attractive organism for cloning an alkaline xylanase gene.

Our laboratory bleaching trial showed improved brightness levels and viscosity values using both hardwood and

Table 2 Brightness and viscosity properties of hardwood and softwood pulps treated with *Bacillus* V1–4 xylanase prior to chlorine bleaching sequences

		pH 7						pH 9					
		52° C				60° C		52° C				60° C	
Pulp	Xylanase[b]	C/DE1	D1	Viscosity[c]	Kappa[d]	C/DE1	D1	C/DE1	D1	Viscosity[c]	Kappa[d]	C/DE1	D1
Hardwood	–	50	78	25	2	49[a]	61[a]	50	73	29	3	45[a]	53[a]
	+	56	78	30	2	50[a]	66[a]	53	76	32	2	48[a]	57[a]
Softwood	–	32	44	20	7	nd	nd	32	45	19	7	nd	nd
	+	35	52	21	5	nd	nd	35	48	20	6	nd	nd

[a]Indicates data from a separate experiment; nd is not determined; C/DE1 is chlorine/chlorine dioxide followed by NaOH extraction
[b]4 IU g^{-1} oven-dried pulp of xylanase was applied for pulp pretreatment
[c]Expressed as centipoise
[d]Original Kappa number is 9.0

softwood pulps. These results indicate that the effectiveness of *Bacillus* V1–4 xylanase could be useful in pulp and paper applications.

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