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Production and Location of Xylanolytic Enzymes in Alkaliphilic *Bacillus* sp. K-1

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Abstract The production and location of xylanolytic enzymes in alkaliphilic *Bacillus* sp. K-1, isolated from the wastewater treatment plant of the pulp and paper industry, was studied. When grown in alkaline xylan medium, the bacteria produced xylanolytic enzymes such as xylanase, β-xylosidase, arabinofuranosidase, and acetyl esterase. Two types of xylanases (23 and 45 kDa) were found to be extracellular, but another type of xylanase (35 and/or 40 kDa) was detected as pellet-bound that was eluted with 2% triethylamine from the residual xylan of the culture. The xylanases were different in their molecular weight and xylan-binding ability. Arabinofuranosidase and β-xylosidase were found to be intracellular and extracellular, respectively, and acetyl esterase was found to be extracellular. The extracellular xylanolytic enzymes effectively hydrolyzed insoluble xylan, lignocellulosic materials, and xylans in kraft pulps.

Key words: Xylanolytic enzymes, alkaliphilic *Bacillus* sp. K-1, extracellular, pellet-bound and intracellular fractions

Xylan is one of the most abundant carbohydrates available in nature and composed of a backbone of 1,4-β-linked D-xylose units, which can be highly substituted. The substituents include acetic acid, L-arabinose, and 4-O-methyl D-glucuronic acid. It can be degraded by xylanolytic enzymes to sugars, which in turn can be converted to other valuable chemicals, energy, food, and feed [24]. There is a great interest in the use of xylanases in the pulp and paper industries to reduce the amount of chlorine required for the bleaching process and absorbable organic halogen in waste water [30].

Xylanolytic enzymes are inducible and produced by many prokaryotes and eukaryotes [6, 15, 17, 31]. These enzymes are secreted into the environment by microorganisms during their growth on xylan-containing substrate, but some enzymes are cell-bound [23]. Moreover, the multiplicity of hydrolases and the location of their components in cell fractions of several microorganisms have been reported: cellulases of *Pseudomonas fluorescens* var. *cellulose* [32] and *Trichoderma reesei* [5], β-glucanases and β-glucosidases of *Cellulomonas uda* [27], xylanases of *Cryptococcus albidus* [2], and protease of *Escherichia coli* [13]. In this study, the production and location of xylanolytic enzymes such as xylanase, β-xylosidase, arabinofuranosidase, and acetyl esterase in alkaliphilic *Bacillus* sp. K-1 was investigated when grown on xylan. This bacterium produces xylanolytic enzymes that are active and stable in alkaline condition without cellulase activity [22].

Materials and Methods

Microorganism and Culture Condition

Bacillus sp. K-1 was isolated from a wastewater treatment plant of the pulp and paper industry [21]. The bacterium was grown on Berg's mineral salts medium [1] containing $NaNO_3$ 0.2%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.02%, $MnSO_4 \cdot H_2O$ 0.002%, $FeSO_4 \cdot 7H_2O$ 0.002%, $CaCl_2 \cdot 2H_2O$ 0.002%, and xylan 0.5%. The pH of the medium was initially adjusted to 10.5 with 1% Na_2CO_3 after autoclaving

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and the culture was incubated in a rotary incubator at 200 rpm and 37°C for 3 days.

Enzyme Assays

Xylanase Assay. The assay mixture consisted of 0.1 ml of enzyme suspension and 0.5 ml of 1% oat spelt xylan in 0.1 M Tris-HCl buffer, pH 9.0 [14]. After incubation for 15 min at 50°C, the increase in reducing sugars was determined by the Somogyi-Nelson method [26] with xylose as a standard.

An enzyme unit (U) was defined as that amount of enzyme that catalyzed the release of 1 μmole of reducing sugar in 1 min.

Cellulase Assay. The cellulase activity was measured as described above using carboxymethyl cellulose (CMC) as a substrate.

β-Xylosidase Assay. The β-xylosidase assay mixture consisted of 0.9 mM *p*-nitrophenyl β-D-xylopyranoside in 50 mM phosphate buffer (pH 7.0), and enzyme to give a final volume of 1.1 ml. The reaction mixture was incubated for 30 min at 50°C, and 2.0 ml of 0.4 M sodium carbonate was then added to terminate the reaction. The *p*-nitrophenol released was measured at 405 nm [14].

β-Glucosidase and Arabinofuranosidase Assays. β-Glucosidase and arabinofuranosidase activities were measured under the same conditions as β-xylosidase activity, as mention above, except the substrates were changed: For the β-glucosidase assay, 1 mM *p*-nitrophenyl β-D-glucopyranoside was used, and 0.83 mM *p*-nitrophenyl α-L-arabinofuranoside was used for the arabinofuranosidase assay.

Acetyl Esterase Assay. The acetyl esterase activity was determined according to the method of Mackenzie *et al.* [19]. β-Xylosidase, β-glucosidase, arabinofuranosidase, and acetyl esterase activities were expressed as μmole of *p*-nitrophenol released per min per ml of enzyme suspension.

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* [18], using bovine serum albumin as a standard.

Isolation of Xylanolytic Enzymes

After the bacteria had been grown in alkaline xylan (0.5%) medium for 3 days at 37°C, the culture was centrifuged at 10,000 ×*g* for 10 min (4°C). The supernatant was assayed for the extracellular enzyme activities. The pellets (cells+ residual xylan) were washed with a large amount of phosphate-buffered saline (PBS) (0.15 M sodium chloride in 0.1 M potassium phosphate buffer, pH 7.0) by centrifugation. The pellets were resuspended in 2% triethylamine (TEA) for 30 min at 4°C with frequent stirring. After centrifugation, the supernatant was assayed for the activities of pellet-bound enzymes. The pellets were washed again with PBS under the same condition, resuspended in PBS, and incubated with lysozyme (10 mg/ml) at pH 8.0 (10 mM Tris-HCl buffer) for 20 min at 30°C [25]. After centrifugation, the supernatant was assayed for the activities of intracellular enzymes.

Preparation of Substrates

To prepare soluble and insoluble xylans, a suspension (0.5%) of oat spelt xylan (Sigma, St. Louis, MO, U.S.A.) in deionized water was stirred for 1 h at room temperature. The mixture was centrifuged at 3,000 ×*g* for 10 min and the supernatant, comprising the soluble fraction, was removed and freeze dried. The pellet, comprising the insoluble fraction, was washed twice with 20 volumes of deionized water and freeze dried. Lignocellulosic materials such as corn hull, sugarcane bagasse, eucalyptus wood, and rice straw were ground (40 mesh), washed several times in hot water to remove free reducing sugars remaining in these materials, and then dried. The washed kraft pulps were also ground and dried.

Hydrolysis of Xylan, Lignocellulosic Materials, and Unbleached Kraft Pulps

Birchwood and oat spelt xylans (Sigma, St. Louis, MO, U.S.A.) were hydrolyzed with crude enzyme (30 mg protein) at pH 7.0 and 50°C. At appropriate intervals, the reducing sugars released were determined by the Somogyi-Nelson method.

Xylanolytic enzymes produced by *Bacillus* sp. K-1 were analyzed in 3 fractions: intracellular, pellet-bound, and extracellular. The hydrolytic activaties of extracellular crude, unbound, and bound xylanase and pellet-bound xylanase on soluble and insoluble oat spelt xylans were analyzed. Soluble and insoluble oat spelt xylans (1% dry weight) were also assayed with the same amount of enzyme at pH 7.0 and 50°C for 20 min.

All of the lignocellulosic materials (1% dry weight) prepared were hydrolyzed with xylanolytic enzymes (1 U) at pH 7.0 and 50°C for 1 h, but unbleached kraft pulps (1% dry weight) were hydrolyzed at pH 9.0 and 40°C for 1 h. The reducing sugars released were determined.

RESULTS

Location of Xylanolytic Enzymes

The xylanolytic enzyme activities such as xylanase, β-xylosidase, arabinofuranosidase, and acetyl esterase were evaluated in three fractions: extracellular, intracellular, and pellet-bound (Table 1). Xylanase activity was detected in extracellular and pellet-bound fractions, whereas β-xylosidase and arabinofuranosidase were found in both extracellular and intracellular fractions. However, acetyl esterase was detected only in the extracellular fraction.

Production of Xylanolytic Enzymes

Time courses of production of extracellular xylanolytic enzymes, xylanase, β-xylosidase, arabinofuranosidase, and

Table 1. Location of xylanolytic enzymes in *Bacillus* sp. K-1.

Source of enzymes	Enzyme activity (U/mg protein)			
	Xylanase	β-Xylosidase	Arabino-furanosidase	Acetyl esterase
Extracellular	9.36	0.47	0.63	0.94
Pellet-bound	1.72	-	-	-
Intracellular	-	0.70	1.20	-

-, could not detect under the assay condition.

acetyl esterase, are shown in Fig. 1. Xylanase, β-xylosidase, arabinofuranosidase, and acetyl esterase were detected in the extracellular fraction, and reached to the highest in 3-days of incubation, when grown in alkaline xylan medium.

Only xylanase activity was detected in the pellet-bound fraction that was eluted with TEA (2%) from the pellet of the culture. The highest activity was found at 1 day of incubation and then decreased slightly, as shown in Fig. 2. Arabinofuranosidase and β-xylosidase were detected in the intracellular fraction and reached to the maximum in only 1 day of incubation. However, both enzymes were decreased in 2 days of incubation (Fig. 3).

Type and Size of Xylanase

We previously reported that the crude enzyme showed two major protein bands of xylanases on SDS-PAGE and zymography and their molecular masses were estimated to be about 23 and 45 kDa, and the low molecular size xylanase (23 kDa) had a xylan-binding region [22]. Figure 4 shows two protein bands of pellet-bound xylanase on SDS-PAGE and their molecular sizes were 35 and 40 kDa,

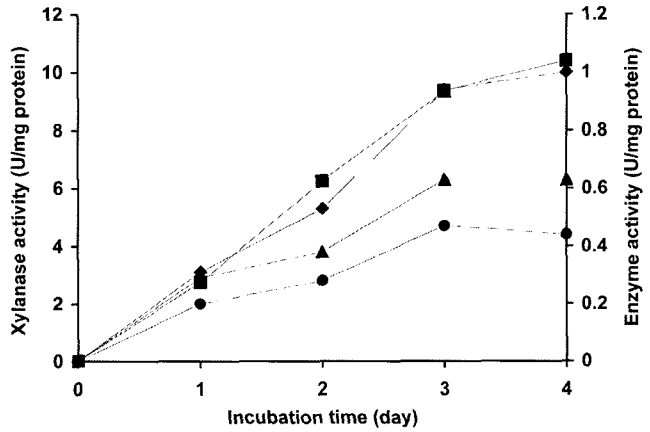

Fig. 1. Time course of production of extracellular enzymes in *Bacillus firmus* K-1.

Time-dependent xylanase activity. The enzyme activity of extracellular enzymes in *Bacillus firmus* K-1 was assayed in alkaline xylan medium. Left Y-axis and right Y-axis indicate the xylanase activity secreted and enzyme activity of extracellular enzymes as U/mg of protein, respectively. Filled circle, triangle, rhombus, and square indicate β-xylosidase, arabinofuranosidase, acetyl esterase, and xylanase, respectively. This graph represents data from similar results of three independent experiments.

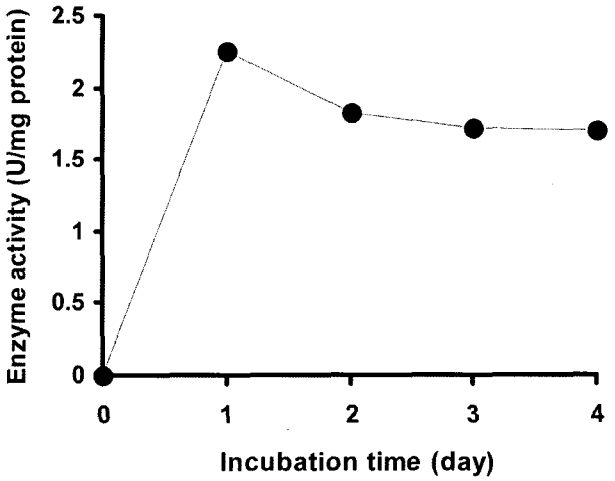

Fig. 2. Time course of production of pellet-bound xylanase in *Bacillus firmus* K-1.

Production of pellet-bound xylanase eluted from residual xylan of culture was assayed by growing in alkaline xylan medium. Filled circle indicates the production yield of the pellet-bound xylanase activity as U/mg of protein. This graph represents data from similar results of three independent experiments.

indicating that these pellet-bound xylanases are different from the extracellular xylanases. As the pellet-bound xylanases were eluted with 2% TEA from the pellet of the culture, they also had the xylan-binding region, which was stronger than the xylan-binding xylanase of extracellular enzyme: The latter was eluted with 1% TEA.

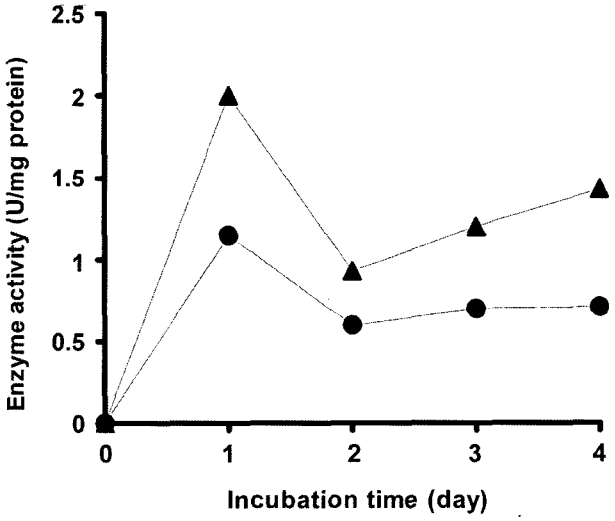

Fig. 3. Time course of production of intracellular enzymes by *Bacillus* sp. K-1.

Production of intracellular enzymes, β-xylosidase and arabinofuranosidase, in *Bacillus* sp. K-1 was assayed, when they were grown in alkaline xylan medium. Filled circle and triangle indicate the production yield of intracellular enzymes, β-xylosidase and arabinofuranosidase, respectively, as each enzyme activity (U/mg of protein). This graph represents data from similar results of three independent experiments.

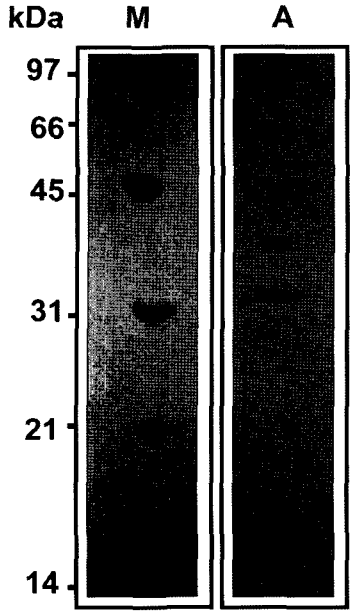

Fig. 4. Protein bands on SDS-PAGE of pellet-bound xylanase in *Bacillus* sp. K-1.
The pellet-bound xylanase was eluted with 2% TEA (triethylamine) from residual xylan of the culture. The prestained lower protein marker shows a wide range of molecular size (14 kDa–97 kDa).

Hydrolysis of Xylan

The hydrolysis of xylan (birchwood and oat spelt) by xylanolytic enzymes is shown in Fig. 5. The amount of reducing sugars released from birchwood was greater than that of oat spelt xylan.

The effect of extracellular crude, unbound, and bound xylanases and pellet-bound xylanase on the hydrolysis of soluble and insoluble oat spelt xylans are shown in Table 2. The bound extracellular enzyme and pellet-bound xylanase appeared to effectively hydrolyze insoluble xylan, because of its xylan-binding ability. The amount of reducing sugars jhydrolyzed by the bound enzyme was 83% of that of the crude enzyme. The unbound enzyme was more effective in hydrolyzing soluble xylan than the bound enzyme because of the presence of high molecular mass xylanase (45 kDa), together with xylan debranching enzymes. Thus, the xylan-binding region appears to be not important for hydrolysis of soluble xylan.

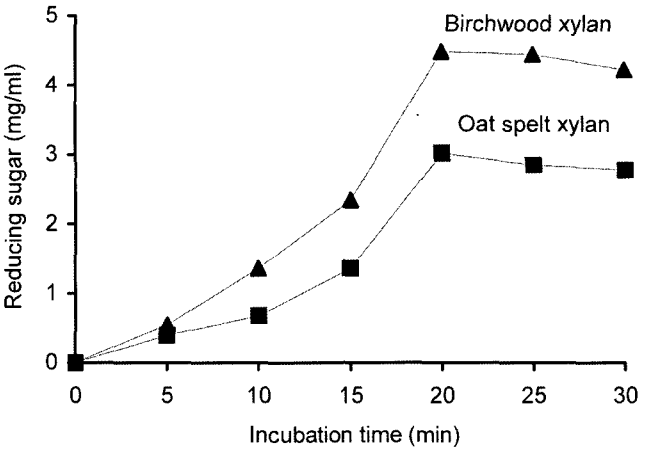

Fig. 5. Time course of hydrolysis of soluble xylans of xylanolytic enzymes in *Bacillus* sp. K-1.
Time course of hydrolysis of soluble xylans of xylanolytic enzymes shows initial hydrolysis of soluble xylans of the culture supernatant in oat spelt and birchwood xylans. The amount of hydrolysis in the culture supernatant in soluble oat spelt and birchwood shows a certain amount of reducing sugar (mg/ml) with indicated time. Filled circle and triangle indicate the hydrolysis rates of the xylanolytic enzymes in oat spelt and birch wood, respectively. This graph represents data from similar results of three independent experiments.

Hydrolysis of Lignocellulosic Materials and Kraft Pulps by Crude Enzyme

The effect of crude enzyme on the hydrolysis of lignocellulosic materials and unbleached kraft pulps is shown in Table 3. The enzyme was found to hydrolyze xylan in these substances. The amount of reducing sugars released from corn hull was greater than that of the other materials tested. It may be due to the xylan content and structural complexity. The enzymes also appeared to hydrolyze the xylan in sugarcane bagasse-, eucalyptus-, and pine-pulps.

DISCUSSION

Xylanolytic enzymes produced by *Bacillus* sp. K-1 were detected in 3 fractions: intracellular, pellet-bound, and extracellular. The extracellular xylanolytic enzymes consisted of two types of xylanases, β-xylosidase, and debranching

Table 2. Hydrolysis of insoluble and soluble xylans by extracellular enzymes and pellet-bound enzyme from *Bacillus* sp. K-1.

Oat spelt xylan	Enzyme	Reducing sugars (mg/ml)	Relative reducing sugars (%)
Insoluble xylan	Crude extracellular enzyme	0.82	100.00
	Unbound extracellular enzyme	0.38	46.34
	Bound extracellular xylanase	0.68	82.93
	Pellet-bound xylanase	0.35	42.68
Soluble xylan	Crude extracellular enzyme	0.80	100.00
	Unbound extracellular enzyme	0.44	55.00
	Bound extracellular xylanase	0.31	38.75
	Pellet-bound xylanase	0.16	20.00

Table 3. Hydrolysis of lignocellulosic materials and unbleached kraft pulps by xylanolytic enzymes from *Bacillus* sp. K-1.

	Reducing sugars (μg/U enzyme)
Lignocellulosic materials	
Corn hull	104.8
Sugarcane bagasse	60.6
Rice straw	49.3
Eucalyptus wood	41.3
Unbleached kraft pulps	
Sugarcane bagasse pulp	149.0
Eucalyptus pulp	70.5
Pine pulp	59.2

enzymes, such as arabinofuranosidase and acetyl esterase. One of these xylanases (23 kDa) was xylan-binding endoxylanase [22], and it seemed to be released from the residual xylan of the culture by the reducing sugars. It was found that the final hydrolysis product, xylose, could effect adsorption of the polysaccharide-binding region of xylanase A from *Clostridium stercorarium* to insoluble xylan; desorption occurs when the xylose concentration increases [28]. However, the pellet-bound xylanase (35 and/or 40 kDa) was strongly bound to the residual xylan, since it was eluted by 2% TEA instead of the usual 1% TEA. Some plant cell wall hydrolases, such as cellulose [7] and xylanase [20], comprise both catalytic and noncatalytic polysaccharides-binding domains. The noncatalytic polysaccharides-binding domains play an important role for the efficient hydrolysis of cellulosic substances [8, 11]. Our results also showed that xylan-binding endoxylanase and pellet-bound xylanase more effectively hydrolyzed insoluble xylan than soluble xylan. The enzyme was also able to hydrolyze xylans in lignocellulosic materials and kraft pulps in alkaline condition. Until recently, xylanases, comprising noncatalytic specific xylan-binding regions, have been described from a few microorganisms, such as *Thermomonospora fusca* [9], *Cellulomonas fimi* [4], *Streptomyces thermoviolaceus* [29], and *Bacillus* sp. K-1 [22]. Most xylanolytic microorganisms produce isoforms of xylanases because of the structural complexity of xylan [29]. *Bacillus* sp. K-1 produced four isoforms of xylanases having differerent molecular masses of 23, 35, and/or 40 and 45 kDa. The functions of these four isoenzymes might be synergistic hydrolysis of xylan by different modes of action due to substrate specificities.

Intracellular β-xylosidase seemed to hydrolyze short-chain xylooligosaccharides to xylose for the growth of cells. Biely *et al.* [3] found that xylobiose and xylotriose penetrate into the cell of *Cryptococus albidus* by the active transport system with β-xyloside permease. β-Xylosidase hydrolyzes xylobiose and xylotriose to xylose in the cell [2]. However, the process of xylose uptake in our bacterium is still unknown. In *Bacillus subtilis*, xylose has to be converted to xylulose and then to xylulose-5-phosphate through the pentose phosphate pathway [12]. Intracellular arabinofuranosidase of *Aspergillus niger* 5-16 hydrolyzes the branched chain of xylooligosaccharide to increase the hydrolysis of short-chain xylooligosaccharides by β-xylosidase [10, 16]. The enzymes located in different fractions seem to exhibit synergistic action on the hydrolysis of xylan to xylose for the energy of the cell.

The crude extracellular enzymes from *Bacillus* sp. K-1 may be used in hydrolysis of xylans in lignocellulosic materials and kraft pulps.

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