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Partial characterization of extracellular xylanolytic activity derived from *Paenibacillus sp.* KIJ1

Inkyung Park[2] and Jaiesoon Cho[1,2]*

[1]Department of Animal Sciences and Environment, College of Animal Bioscience and Technology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Korea.
[2]Animal Resources Research Center, College of Animal Bioscience and Technology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Korea.

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An Antarctic bacterial isolate was found to exhibit the extracellular xylanolytic activity. Based on 16S rRNA gene sequence analysis, the strain was named *Paenibacillus* sp. KIJ1. The maximum xylanase production was achieved by growing *Paenibacillus s*p. KIJ1 in media with 0.5% carboxymethylcellulose and 0.5% yeast extract, which were found to be the best sources of carbon and nitrogen, respectively. Optimal enzyme activity occurred at 50°C and pH 6.0. The xylanase was very specific for xylan with little or no activity on other carbohydrates and synthetic aryl-glycosides substrates. Enzyme activity was enhanced by Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+} and strongly inhibited by SDS. The KIJ1 xylanase may offer potential for use as a microbial feed additive.

Key words: *Paenibacillus* sp., antarctic, xylanase, xylan, microbial.

INTRODUCTION

Hemicellulose primarily consists of xylan, which is the second most abundant natural polysaccharide and a polymer with a linear backbone of β-1,4-linked xylose residues (Lee et al., 2008) . Xylan carries attachment points for different chemical moieties, such as arabinofuranosyl and glucuronyl groups, that mediate linkages with the other components of lignocellulose (Lee et al., 2008). In the field of non-ruminant animal nutrition, cereals such as barley, rye and wheat are major ingredients in poultry diets (Sorensen et al., 2004) . However, arabinoxylan, one of the most representative non- starch polysaccharides (NSPs) found in cereal-based diets functions as an anti-nutritive factor on poultry (Ward and Marquardt, 1987; Choet and Annison, 1990). When such a component is present in soluble form, it increases the viscosity of ingested feed in the small intestines, interfering with the mobility and absorption of other nutrients (Polizeli et al., 2005). This can ultimately lead to physiological disorders and depressed production per formances of the bird (Silversides et al., 2006). β-1,4-xylanases (EC 3.2.1.8) are glycosidases that catalyze the endohydrolysis of 1,4- β-D-xylosidic linkages in xylan into short xylooligosaccharides (Ning et al., 2008). The xylanases have potential applications in the food, animal feed, paper, pulp and biofuel industries (Khandeparker and Numan, 2008). To date, a number of xylanases have been isolated from fungi and bacteria. Commercial xylanases are industrially produced worldwide. The principal microorganisms used to obtain these enzymes are fungi such as *Aspergillus niger* , *Trichoderma* sp. and *Humicola insolens* (Polizeli et al., 2005). Nevertheless, commercial xylanases can also be obtained from bacteria (Polizeli et al., 2005). However, only a few reports on xylanases from the distinct genus *Paenibacillus*, which is newly separated from the conventional *Bacillus* genus, have been published (Berge et al., 2002; Rattiya et al., 2009). Supplementing microbial xylanases in animal feed decreases the viscosity of arabinoxylans and alleviates the anti-nutritive effects imposed by the feedstuffs, resulting in enhanced availability of the overall nutrients or in the release of prebiotic low molecular weight xylan fragments

*Corresponding author. E-mail: chojs70@konkuk.ac.kr. Tel: +82-2-450-3375. Fax: +82-2-455-1044.

(Sorensen et al., 2004). Furthermore, the enzymes ameliorate excreta (Phosphorus, Nitrogen, Copper and Zinc) problems and contribute significantly to reducing waste problems and manure output, which is beneficial to the environment (Bedford, 1995). The present study describes the general properties of extracellular xylanoly-tic activity found in an Antarctic bacterial isolate, *Paenibacillus* sp. KIJ1.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The bacterial isolate derived from the Antarctic soil samples was a donation from KOPRI (Korea Polar Research Institute) operating the King Sejong Station (South Korea) in Antarctica. Screening for xylanase activity was performed on selective agar [0.5% xylan (birchwood; Sigma), 0.45% $(NH_4)_2SO_4$, 0.05% yeast extract (Difco), 0.07% KH_2PO_4, 0.01% $MgSO_47H_2O$, 0.01% NaCl, 0.01% $CaCl_2$ $2H_2O$, 0.001% $MnSO_44H_2O$, 0.001% $FeSO_47H_2O$ and 1.5% bacto agar (Difco); (pH 6.5)] on the basis of the Congo red plate assay previously described (Teather and Wood, 1982) at 30°C.

Growth and xylanase production were investigated in 100 ml of a xylanase production medium [0.5% carboxymethylcellulose(Sigma), 0.5% yeast extract (Difco), 0.07% KH_2PO_4, 0.01% NaCl, 0.01% $CaCl_22H_2O$, 0.01% $MgSO_47H_2O$, 0.001% $MnSO_44H_2O$ and 0.001% $FeSO_47H_2$ O; (pH 6.5)] in a 500 ml Erlenmeyer flask aerobically incubated with vigorous shaking (220 rpm) by monitoring the absorbance ($O.D._{600nm}$) and xylanase activity of the culture supernatant at 30°C at various time points.

Taxonomic identification of strain KIJ1

Genomic DNA was extracted from strain KIJ1 using the FastDNA kit (Qbiogene) according to the manufacturer's protocol. The 16S rRNA gene was amplified from genomic DNA by polymerase chain reaction (PCR) using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG- 3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (William et al., 1991). The amplified 1,449 bp sequences were determined by an automated ABI PRISM 3730 XL DNA analyzer (Applied Biosystems). The resulting se-quences were compared with the GenBank database (NCBI) using BLAST (Altschul et al., 1990). Sequences showing a relevant degree of similarity were imported into the CLUSTAL W program (Thompson et al., 1994) and aligned. The evolutionary distances with other strains of *Paenibacilli* were computed using the maximum composite likelihood method (Tamura et al., 2004) and the phylo-genetic relationships were determined using the software MEGA version 4.0 (Tamura et al., 2007).

Nucleotide sequence accession numbers

The nucleotide sequence of the 16S rRNA gene has been deposited in the GenBank database under Accession No. GQ906940.

Effect of carbon and nitrogen sources on enzyme activity

The effects of carbon and nitrogen compounds on extracellular xylanase production by strain KIJ1 were examined over 96 h cultivation. A basal medium (0.5% yeast extract, 0.07% KH_2PO_4, 0.01% NaCl, 0.01% $CaCl_22H_2O$, 0.01% $MgSO_47H_2O$, 0.001% $MnSO_44H_2O$ and 0.001% $FeSO_47H_2O$) containing 0.5% carboxy-methylcellulose was adjusted to pH 6.5 before sterilization, unless otherwise stated. The effect of different nitrogen sources on xylanase production was investigated by replacing the yeast extract in the basal medium with peptone, tryptone, beef extract, or casamino acid. Then, the effect of different carbon sources on xylanase production was investigated by replacing the carboxyme-thylcellulose in the medium with mannan, xylan, avicel, mannose, fructose, lactose, xylose, or glucose. Xylanase activity in culture supernatant sampled at 96 h culture time was measured under the standard assay conditions.

Partial purification of the enzyme

One (1) L of xylanase production medium [0.5% carboxy-methylcellulose(Sigma), 0.5% yeast extract (Difco), 0.07% KH_2PO_4, 0.01% NaCl, 0.01% $CaCl_2H_2O$, 0.01% $MgSO_47H_2$ O, 0.001% $MnSO_44H_2$ O and 0.001% $FeSO_47H_2O$; (pH 6.5)] in two Erlenmeyer flasks of 2 L capacity was aseptically inoculated with single colony of strain KIJ1 and aerobically cultivated with vigorous shaking (220 rpm) for 96 h at 30°C. The culture medium containing secreted xylanase was centrifuged (9,000 x g; 30 min; 4°C) to remove cell, and then protein in the supernatant was precipitated with ammo-nium sulfate (75% saturation). The pellet was dissolved in 50 mM Tris -HCl (pH 8.0) and dialyzed overnight against 50 mM Tris-HCl (pH 7.4) at 4°C. The dialyzed solution was used as the xylanase source throughout this work to examine its catalytic properties.

Zymography

The enzyme was subjected to non-denaturing 10% PAGE by using a Modular Mini-Protein II Electrophoresis System (Bio-Rad) according to the manufacturer's instructions. The gel was then incubated in a 0.5 % solution of xylan for 12 h at 50°C, followed by staining in 1% Congo red solution (Sigma) for 1 h and detained for 6 h in 1 M NaCl. The gel was further immersed in ethanol or rinsed in 5% acetic acid to distinguish between clear colorless zones of xylan hydrolysis and the dark blue color of unhydrolyzed xylan.

Measurement of enzyme activity and substrate specificity

Xylanase activity was measured at 50°C by assaying the release of reducing sugar from birchwood xylan in 1 ml of 50 mM Bis-Tris (pH 6.0) by the dinitrosalicylic acid (DNS) method (Miller, 1959), unless otherwise stated. Activity on beechwood xylan, carboxymethyl-cellulose, avicel, mannan and chitosan was determined by the DNS method under standard assay conditions with a final concentration of 1.8%. Activity on *para*-nitrophenyl (*p*Np) conjugated substrates such as *p*Np-D-xylopyranoside and *p*Np-D-cellobioside was deter-mined in 1 ml of 50 mM Bis-Tris (pH 6.0) at 50°C with a final concentration of 0.5%. The reaction was then stopped by the addition of 1 ml of 1 M Na_2CO_3 and color development was mea-sured at $O.D._{400\ nm}$. One unit (U) of enzyme activity was defined as the amount of enzyme to produce 1 μmol of reducing sugar equivalent, or *p*-nitrophenol per minute under the assay conditions described.

Effect of pH and temperature on enzyme activity

Xylanase activities were investigated in the pH range of 3 - 8.5 [50 mM glycine- HCl (pH 3); 50 mM sodium acetate (pH 4-5); 50 mM Bis-Tris-HCl (pH 6-7); 50 mM Tris -HCl (pH 7.4-8.5)] at 30°C and temperatures between 20 and 80°C at the optimum pH.

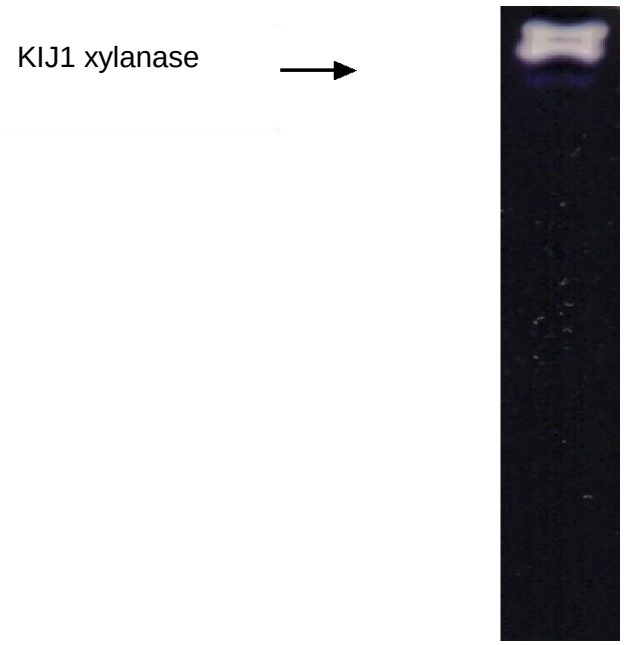


Figure 1. Zymogram analysis of xylanase activity in the enzyme preparation.

Effect of metal ions and chemicals on enzyme activity

The effect of different metal ions and chemicals on xylanase activity was determined under standard enzyme assay conditions in the pre-sence of 5 mM of Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, EDTA, SDS, or PMSF (phenylmethylsulfonyl fluoride).

RESULTS AND DISCUSSIONS

Identification of isolated strain KIJ1, xylanase production and partial purification of the enzyme

To identify the isolated strain KIJ1 showing xylanase activity (Figure 1), we cloned the 16S rRNA gene and compared the sequence with those available in the data-base. A phylogenic tree based on the 16S rRNA gene sequences from 9 members of bacterial *Paenibacillus* strains showed that the strain KIJ1 shared 98.5% sequence identity with the type strain, *Paenibacillus odorifer* LMG 19079T (Figure 2). Therefore, it was named *Paenibacillus* sp. KIJ1.

The time courses of cell growth and xylanase activity were shown in Figure 3. Xylanase activity steeply increased for the first 24 h of incubation and then reached a plateau, exhibiting maximal activity (1.52 U/ml) at 96 h of incubation, while cell growth reached a peak (1.39 of $O.D_{600nm}$) at 48 h of incubation and drastically decreased to 0.77 of $O.D_{600\ nm}$ by 120-h of incubation. In our observation, *Paenibacillus* sp. KIJ1 did not grow on minimal medium M9 (Sambrook et al., 1989) containing carboxymethylcellulose or xylan. This is similar to previous result from other Antarctic xylanase-producing bacteria, *Pseudoalteromonas haloplanktis* which was unable to utilize xylan as a carbon source on xylan-supplemented minimal media (Collins et al., 2002). Therefore, the KIJ1 xylanase may not be involved in the production of sugars for cellular metabolism. In the Antarctic environment, sources of xylan and of other polysaccharides are extremely limited (Collins et al., 2002), with the main source being the cell walls of green and red algae belonging to microphytobenthos (Turkiewiz et al., 2000). The xylanase may contribute to weakening the cell wall structure of algae, thereby allowing better access to the cellulose in the cell walls as well as to the storage polysaccharides (Collins et al., 2002).

As shown in Table 1, the extracellular xylanase from *Paenibacillus* sp. KIJ1 was partially purified from the culture supernatant by ammonium sulfate precipitation.

Effect of carbon and nitrogen sources on enzyme activity

Different carbon sources and nitrogen sources were tested to determine the ones best suited for xylanase production. Among the carbon sources tested, optimal enzyme activity was induced when the cells were cultivated in the basal medium containing CMC (carboxymethylcellulose) (Figure 4A). However, very low levels of xylanase activity were detected in media con-taining other Carbon sources such as lactose, glucose, fructose, mannose, xylose, or mannan, while yeast extract was found to be the best source of nitrogen (Figure 4B).

Similar observation is previously made that much lower activities of a fungal strain, *Thermomyces lanuginosus* producing high levels of an extracellular thermostable xylanase were induced after growth on glucose, sucrose, fructose, xylose, or mannan than on corncobs (Singh et al., 2000).

Effect of pH and temperature on enzyme activity

The enzyme showed optimal activity at pH 6.0 and exhi-bited near-optimal activities over a fairly broad range of pH 5.0 - 8.5 (Figure 5A), which is close to the phy-siological pH of chicken digestive tract (pH 4.5 - 7.5) with the exception of pH 3.5 in the proventriculus (Cho et al., 2006).

This suggests that the enzyme can be applied as a feed additive. This result is similar to that for the Antarctic bacterium, *Pseudoalteromonas haloplanktis*, from which the xylanase had a wide pH activity range with maximum activity occurring between pH 5.3 and 8 (Collins et al., 2002) and a recent bacterial xylanase-producer, *Paenibacillus curdlanolyticus* B-6 derived from an anaerobic fermentor, in which more than 80% activity was retained at pH 5.0 - 9.0 (Rattiya et al., 2009).

The optimal xylanase activity occurred at 50°C, while

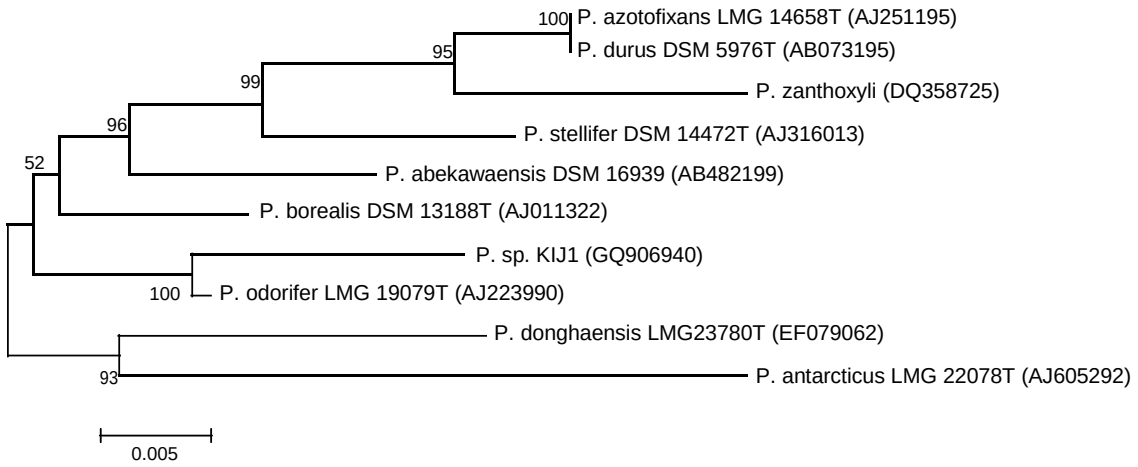


Figure 2. Phylogenic relationship of the 16S rRNA sequences of *Paenibacillus sp*. KIJ1 with other type strains of *Paenibacillus.* Bootstrap values (based on 1,000 trials and only values > 50%) are shown at the nodes. The GenBank accession numbers are indicated in parentheses. *Bar,* 5-base substitutions per 1,000 nucleotide position. P. represents the abbreviation of *Paenibacillus*.

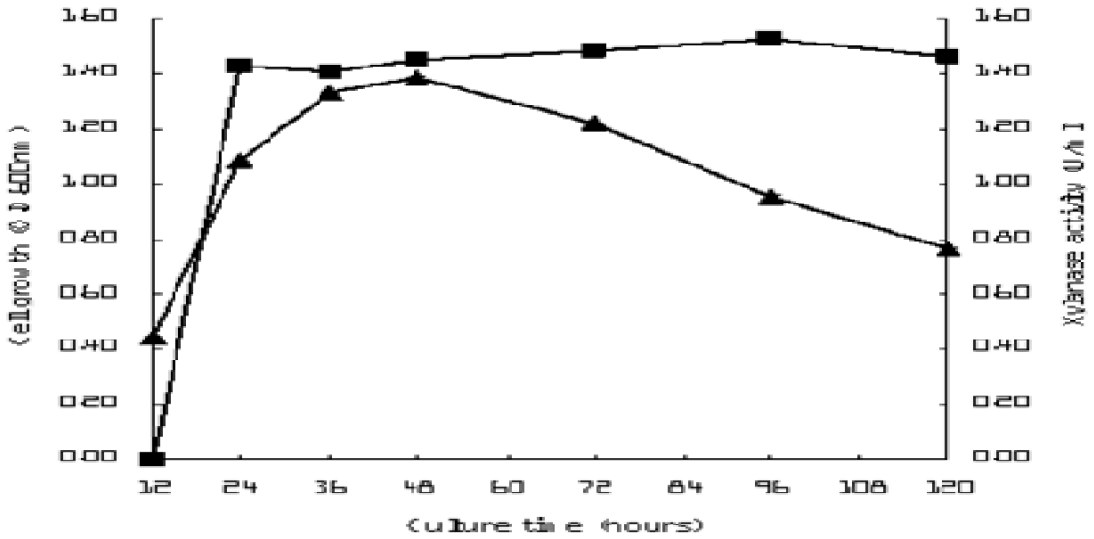


Figure 3. Growth and xylanase production by *Paenibacillus* sp. KIJ1. Symbols represent xylanase activity (filled square) and growth (filled triangle). For the production of xylanase, the strain was grown in 100 ml liquid medium in an Erlenmeyer flask (500 ml) containing 0.5% carboxymethylcellulose, 0.5% yeast extract, 0.07% KH_2PO_4, 0.01% NaCl, 0.01% $CaCl_2 2H_2O$, 0.01% $MgSO_4 7H_2O$, 0.001% $MnSO_4 4H_2O$, and 0.001% $FeSO_4 7H_2O$ (pH 6.5).

more than 80% of the activity was found at 40 – 60 °C and 60% lost at 70 °C (Figure 5B). The thermostability of the enzyme was tested after pre-incubation of the enzyme at 40 - 80 °C for 30 min. The xylanase was stable up to 40 °C, but 40% of the activity was lost at 50 °C and no activity remained at 60 °C and higher temperatures (data not shown).

Generally, enzymes isolated from microorganisms native to cold environments exhibit higher catalytic efficiency at low temperatures and greater thermo-sensitivity than their mesophilic counterparts (Gerday et al., 1997). For instance, CelG from the Antarctic bacterium, *P. haloplanktis* is a heat-labile cellulase and its half-life during incubation at 45 °C is approximately 40 min (Garsoux et al., 2004). Interestingly, a cold-active cellulase, CelG can be produced from the mesophilic ruminal anaerobe, *Fibrobacter succinogenes* S85, with an optimal temperature of 25 °C and complete inactivation even after

Table 1. Summary of partial purification profile for extracellular xylanase produced in *Paenibacillus* sp. KIJ1.

Purification step	Total activity (U)[a]	Total proteins(mg)	Specific activity(U/mg)	Purification (fold)	Recovery (%)
Culture supernatant	1520	28700	0.05	1	100
Ammonium sulfate precipitation	1290	22754	0.06	1.2	85

[a]One unit (U) of xylanase activity was defined as the amount of enzyme to produce 1 μmol of reducing sugar equivalent from birchwood xylan(Sigma) per minute at 50 °C and pH 6.0.

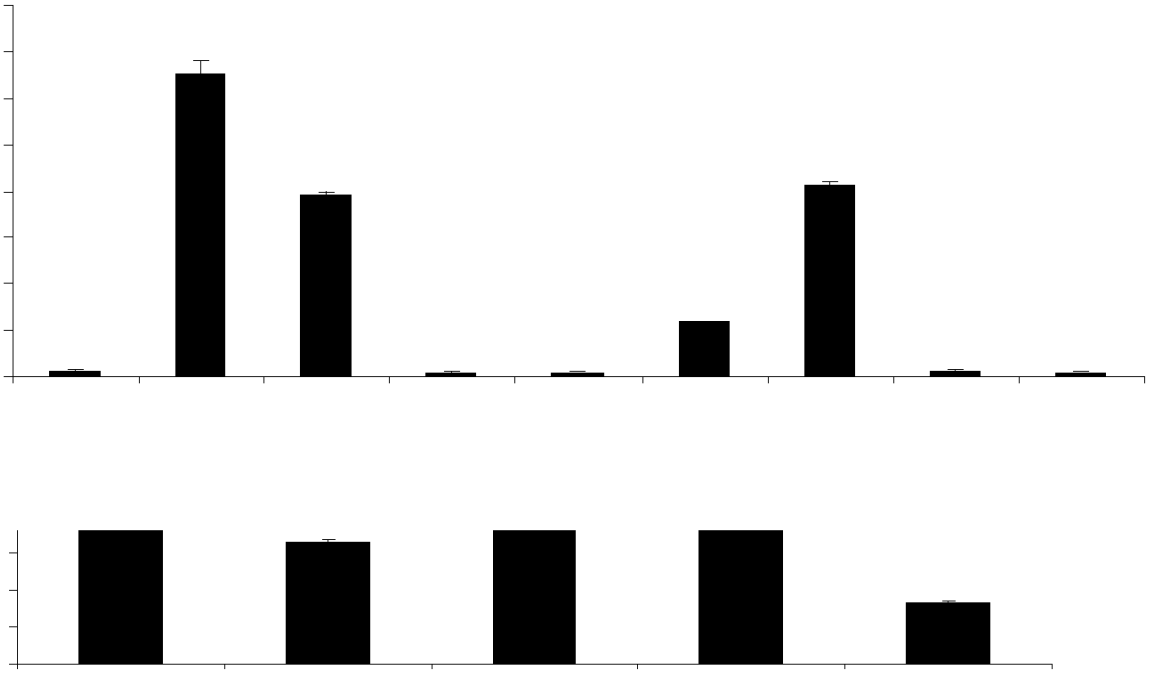


Figure 4. Effect of carbon (A) and nitrogen (B) sources on xylanase activity by *Paenibacillus* sp. KIJ1 during 96-h cultivation. Data represent the means ± SE from three experiments.

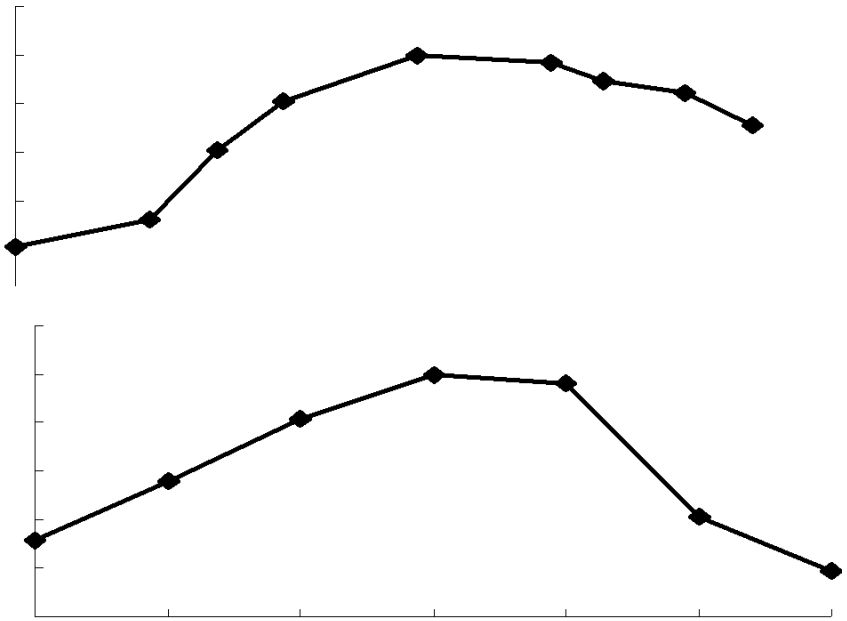
20 min of exposure at 50°C (Iyo and Forsberg, 1999). However, with regard to temperature optimum and thermostability, the KIJ1 xylanase seems to be a mesophilic enzyme, despite the enzyme being produced from an Antarctic isolate.

Substrate specificity

As shown in Table 2, the enzyme was very specific for xylan. It showed little activity against other polysaccharides such as carboxymethylcellulose, avicel, mannan and chitosan. In addition, little or no activity was detected on synthetic substrates such as *p*Nitrophenyl-D-xylopyranoside and *p*Nitrophenyl-D-cellobioside, indicating that the enzyme was not a β-xylosidase. In the Antarctic bacterial strain, *P. haloplanktis* that produce true xylanase, the enzyme also shows no activity against the synthetic (*p*Nitrophenyl - or 4-methylumbelliferyl – conjugated) substrates (Collins et al., 2002) . Never-theless, the specific activity of xylanase B from another *Paenibacillus* sp. BP-23 on *p*Nitrophenyl- D-xylopyranoside and *o*Nitrophenyl-D- xylopyranoside is similar to that found on the main substrate, xylan (Gallardo et al., 2003).

Effect of various reagents on enzyme activity

Xylanase activity in the presence of different metal ions or chemicals was shown in Table 3. Amongst metal ions, the



Temperature (°C)

Figure 5. Optimal pH (A) and temperature (B) activity profiles. (A) Relative activity at 30°C and various pHs where 100% equates to 19.8 U/ml. (B) Relative activity at pH 6 and various temperatures where 100% equates to 37 U/ml. The assays were performed at a final concentration of 1.8% birchwood xylan

Table 2. Effect of metal ions and chemicals on xylanase activity

Reagent[a]	Relative activity (%)[b]
No addition	100.0
Mg^{2+}	84.5
Ca^{2+}	128.2
Co^{2+}	113.1
Zn^{2+}	108.2
Ni^{2+}	88.0
Cu^{2+}	123.8
Mn^{2+}	123.4
EDTA	76.3
SDS	58.7
PMSF	90.6

[a] The final concentration of each reagent was 5 mM in the assay buffer.
[b]100% was assigned to the activity in the absence of all reagents and equates to 39.1 U/ml.

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activity of *Paenibacillus* sp. KIJ1 xylanase was enhanced by 5 mM Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, or Mn^{2+}. In a previous report, 5 mM of Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Mn^{2+} stimulate the *Bacillus licheniformis* xylanase, increasing activity by 16, 16, 25, 20 and 27%, respectively, (Lee et al., 2008).

In contrast, it has been reported that xylanase from the Antarctic bacterium, *P. haloplanktis* is sensitive to Cu^{2+} (16% inhibition) and Zn^{2+} (14% inhibition) even at the lower concentration of 1 mM (Collins et al., 2002). The KIJ1 xylanase was slightly inhibited by Mg^{2+} and Ni^{2+}. The

Table 4. Comparison of other bacterial strains producing xylanase presented in this article with *Paenibacillus* sp. KIJ1.

Organism	Optimum temperature (°C)	Optimum pH	Substrate specificity	Stimulatory metal ions	References
Paenibacillus sp. KIJ1	50	6.0	Specific	Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}.	This study
Pseudoalteromonas haloplanktis	35	5.3-8.0	Specific	ND	(Collins et al., 2002)
Bacillus licheniformis	40-50	6.0	Specific	Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}.	(Lee et al., 2008)
Paenibacillus curdlanolyticus B-6	60	7.0	Broad	ND	(Rattiya et al., 2009)
Paenibacillus sp.BP-23	40	5.5	Broad	ND	(Gallardo et al., 2003)

ND, not determined.

presence of the chelating agent, EDTA moderately inhibited xylanase activity, suggesting the enzyme may be a metalloenzyme (Li et al., 2008). Enzyme activity was slightly inhibited by PMSF which is a well-known inhibitor of serine proteases. Xylanase activity strongly decreased with the common anionic surfactant SDS, indicating that hydrogen bonds may be crucial in maintaining enzyme activity. Currently, the global market for industrial enzymes is divided into three application segments: technical enzymes, food enzymes and animal feed enzymes (Hasan et al., 2006). Particularly, the growth of the animal feed enzyme segment is somewhat higher, with an expected 4% average annual growth rate (Hasan et al., 2006). The use of microbial enzymes in feed production is an important sector of agribusiness, with annual world production exceeding 600 million tons and a turnover of more than 50 billion dollars (Polizeli et al., 2005). Generally, xylanases are used in animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytases, galactosidases, and lipases (Polizeli et al., 2005).

In terms of microbial feed biotechnology, the KIJ1 xylanase may be a promising candidate because of its strict substrate specificity and its favorable activity levels at physiologically relevant pH and temperature, compared with those from other bacterial sources (Table 4). Future scientific works such as gene cloning, protein engineering and downstream fermentation technology will be planned to maximize catalytic efficiency and productive yield of the enzyme.

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