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Purification and characterization of two cellulase free xylanases from an alkaliphilic *Bacillus firmus*

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Abstract

Two xylanases from *Bacillus firmus* were purified to homogeneity by gel filtration and ion-exchange chromatography. These enzymes have molecular weights of 45 kDa and 23 kDa, respectively, and both show enzymatic activity over the pH range of 5.0–11.0 at 37°C. These enzymes hydrolyzed xylan from birchwood to release mainly the products of xylose, xylotriose and xylohexose, thus indicating that the xylanases act preferentially toward the internal glycosidic bonds of xylo-oligosaccharides. However, the two xylanases show different modes of action, and a combination of both is likely to lead to concerted degradation of xylan down to the mono- and disaccharides.

Keywords: Xylanases; *Bacillus firmus;* Enzyme purification; Xylanase action

1. Introduction

Xylan is the most abundant of the hemicelluloses which are heteropolysaccharides having a chain of β-1,4-linked xylopyranose residues. The complete hydrolysis of xylan requires the combined action of various enzymes such as endoxylanase (EC 3.2.1.8), exoxylanase (β-D-xylan xylohydrolase), and β-D-xylosidase (EC 3.2.1.37) etc. [1]. Xylanases randomly hydrolyze the β-1,4-glycosidic bonds of xylan to produce several xylo-oligomers. In recent years, xylanases have received attractable research interest due to their potential industrial applications [2]. However, such applications require xylanase(s) with particular properties, the bio-bleaching of paper pulp requires a xylanase that remains active even above pH 9.0 and lacks all cellulase activity.

The bacterium used in this study, *Bacillus firmus*, was previously isolated from a wastewater treatment plant of pulp and paper industry at Bang-Pre-In at Prankornsriayuttaya province, Thailand [3,4]. *Bacillus firmus* is capable of growth at pH values, ranging from 10–12. Normally growth is at temperature of 37°C; the cultures were thermolabile at temperatures above 55°C. Moreover, this strain produces two major extracellular xylanases, with molecular weights of 45 kDa and 23 kDa, respectively. No other hemicellulose-degrading enzyme activities were detected in the culture medium with xylan, CMC or avicel as the sole carbon source, suggesting that this strain produces xylanase mainly. The mode of action of xylanase and cellulase has been reported. It was considered that the cellulose/xylan binding domain (CBD/XBD) was an important factor in the degradation process of insoluble cellulosic materials [5,6]. For xylanases the concept of substrate recognition and induction, i.e. the control of transcription by the cooperative actions of an activator and a repressor, have generally been accepted. Xylanase synthesis is induced by natural xylan and other β-1,4-xylo-oligosaccharides isomers [7,8].

In this study, we describe the purification and characterization of two major xylanases from *Bacillus firmus* bacteria. These enzymes are active over a wide range of pH. In addition other properties presented here suggest that these xylanases could be of commercial interest.

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2. Materials and methods

2.1. Bacterial strain and culture conditions

The bacterial strain, used in this study, was isolated from a wastewater treatment plant of a pulp and paper manufacturer [3,4] and identified by the Micro-IS System as *Bacillus firmus*. It was grown at 37°C in Berg's mineral salts medium [9] supplemented with 0.2% $NaNO_3$, 0.1% yeast extract, 0.05% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.002% $MnSO_4.H_2O$, 0.002% $FeSO_4.7H_2O$, 0.002% $CaCl_2.2H_2O$ and 0.5% birchwood xylan (>90% xylose residues, Sigma Chemical Co., St. Louis, Mo.). The pH was initially adjusted to 10.5 with 1% Na_2CO_3. After 4 days of incubation, the culture supernatant was recovered by centrifugation at 8,000 rpm for 15 min.

2.2. Purification of xylanases

All steps were performed at 4°C unless otherwise noted. Solid ammonium sulfate was added to the culture supernatant to 65% saturation. The precipitate was recovered by centrifugation and dissolved in an appropriate volume of 10 mM Tris-HCl (pH 8.0). The final solution was dialyzed three times against 5 liters of the same buffer for overnight standing. The dialyzed solution was applied to a DEAE-Toyopearl 650F column (1.6 by 25 cm) previously equilibrated with 10 mM Tris-HCl buffer. The column was first washed with 140 ml of 10 mM Tris-HCl buffer and then eluted with a linear gradient of 0 to 0.5 M NaCl in 500 ml of the same buffer at a flow rate of 1 ml/min. The eluted fractions with xylanase activity were pooled, dialyzed to remove any salt contaminants and lyophilized into powder. This partially purified protein was dissolved in 2 ml of 10 mM Tris-HCl buffer and applied onto a TSK-Fractogel 55F column (2.6 by 140 cm). Elution was carried out with the same buffer containing 0.3 M of NaCl

2.3. Xylanase assay

The assay mixture consisted of 40 μl of enzyme solution and 160 μl of a 0.5% birchwood xylan suspension in 100 mM Na_2CO_3-$NaHCO_3$ buffer (pH 9.0). The reaction was incubated at 37°C for 10 min and 0.4 ml of DNS reagent (1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, 1% sodium hydroxide) [10] was added to stop the reaction, then boiled for 5 min. The absorbance at 500 nm was measured after adding 2.4 ml of water. One unit was defined as the amount of enzyme required to produce reducing sugars equivalent to 1 μmol of xylose per min under the conditions described above; 1 U corresponds to 16.7 nkat in Systeme International d' Unites units [11].

2.4. Effect of pH on activity of xylanases

The pH values of various reaction solution were adjusted with 100 mM of following buffer systems: acetate buffer (pH 4.0 to 5.5), phosphate buffer (pH 6.0 to 6.5), Tris-HCl buffer (pH 7.0 to 9.0), glycine-NaOH buffer (pH 10.0 to 11.4), and Na_2HPO_4-NaOH buffer (pH 11.0 to 12.0). The substrate, 0.5% birchwood xylan in various pH buffer, was incubated with 0.5 U of 45 kDa xylanase or 0.15 U of 23 kDa xylanase for 30 min at 37°C and 0.4 ml of DNS reagent was added to stop the reaction, and then boiled for 5 min. The absorbance at 500 nm was measured after adding 2.4 ml of water.

2.5. Enzymatic hydrolysis of xylan

Enzymatic hydrolysis of birchwood xylan was performed according to the method of Matte and Forsberg [12]. Separation of the products of hydrolysis was performed by isocratic gradient of acetonitrile:water (78:22) in a HPLC system equipped with Microsorb Amine column and a RI detector 8110. Further characterization of the collected products were carried out by mass spectrometry analysis and monitored by xylo-oligomer standards.

2.6. Zymogram analysis for xylanase activity

This analysis was performed by the method of Morag et al. with slightly modification [6]. Samples were subjected to electrophoresis on a SDS-PAGE containing 0.1% xylan. After electrophoresis, the gel was washed three times for 30 min at 4°C in 100 mM Na_2CO_3-$NaHCO_3$ buffer (pH 9.0) containing 25% isopropanol for the first two washes to remove SDS, then incubated in the same buffer for 10 min at 37°C. The zymogram was prepared by soaking the gel in 0.1% Congo red solution for 15 min at room temperature, then washed with 1 M NaCl and introduced 0.5% acetic acid to expose two xylanase active bands that contrasted the dark background

2.7. General protein techniques

Protein concentration was estimated by the method of Bradford [13] using bovine serum albumin as a standard. SDS-

Table 1
Summary of purification of xylanases from *Bacillus firmus*

Source	Specific activity (U/mg protein)	Purification
Crude filtrate	1.75	1
45 kDa xylanase	34.08	19.5
23 kDa xylanase	3.67	2.1

Unit definition: 1 U was defined as the amount of enzyme which produced reducing sugars equivalent to 1 μmol of xylose per minute under the conditions described.

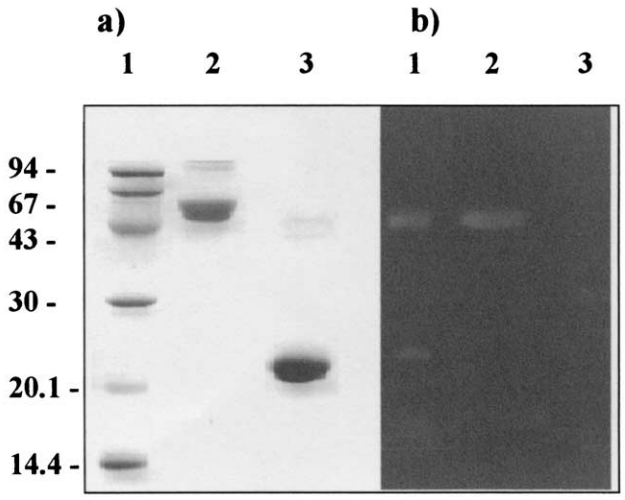

Fig. 1. (A) SDS-PAGE analysis of xylanases from *Bacillus firmus*. Lane 1, molecular mass markers: phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa. Lane 2, 45 kDa xylanase (10 μg); lane 3, 23 kDa xylanase (10 μg). (B) Zymogram of the corresponding clear band showing xylanase activity. Lane 1, crude filtrate (50 μg); lane 2, 45 kDa xylanase (10 μg); lane 3, 23 kDa xylanase (10 μg).

PAGE was performed by the method of Laemmli [14]. Isoelectric focusing was done on PhastGEL IEF (pH 3 to 9) by automatically controlled of PhastSystem (Pharmacia). Purified xylanases were subjected to N-terminal microsequencing using automated Edman degradation in an Applied Biosystem model 467A sequencer under standard conditions.

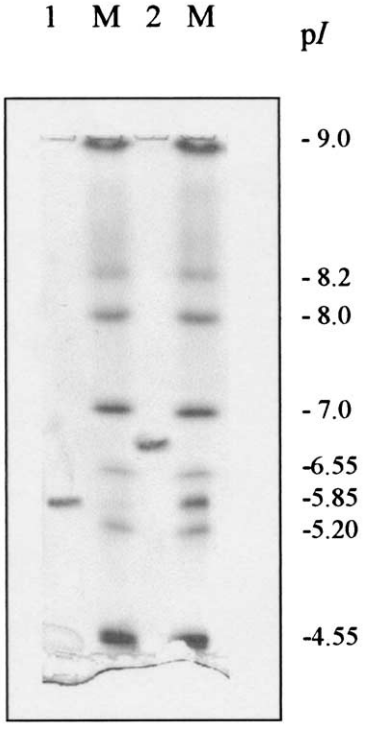

Fig. 2. IEF-PAGE of purified xylnase from *Bacillus firmus*. Lane 1, 45 kDa xylanase; lane 2, 23 kDa xylanase; lane M, p*I* markers.

3. Results and discussion

3.1. Purification of xylanases

After 4 days of cultivation of *Bacillus firmus* in an alkaline medium with the presence of birchwood xylan as the sole carbon source, the extracellular xylanase was detected at 1.75 U/mg protein in the culture supernatant (Table 1). The crude extract from the culture medium was taken through the two-step purification of ion-exchange chromatography and gel filtration. Two purified xylanase proteins appeared as single bands on SDS-PAGE and had the molecular masses of 45 kDa and 23 kDa, respectively (Fig. 1). Both proteins also showed relatively clear bands on the zymogram gel, detected by Congo red staining, indicating they were active xylanases (Fig. 1). However, the xylanase activity band for purified 23 kDa protein was very faint. A summary of the purification is presented in Table 1. Final purification of the xylanases increased their specific activi-

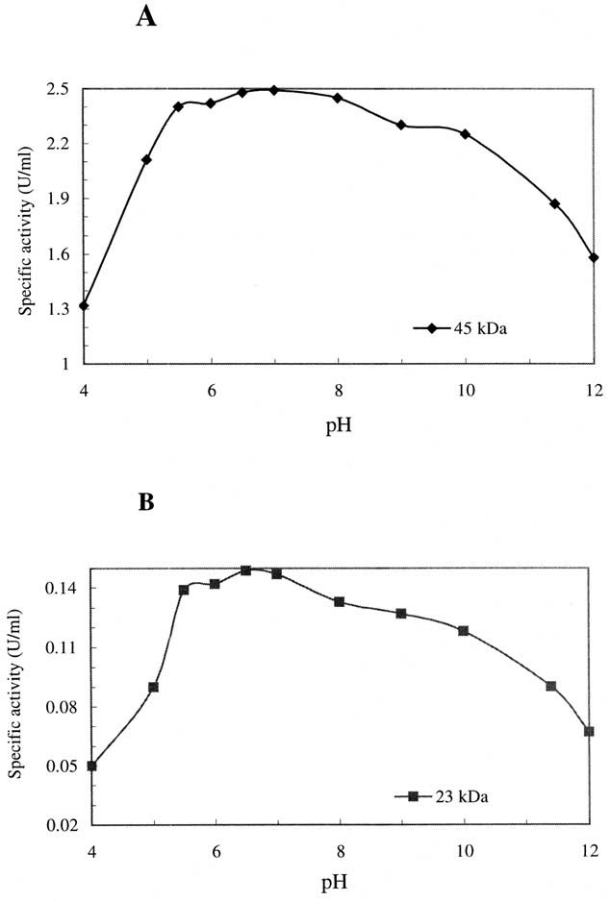

Fig. 3. The enzymatic activity of the purified 45 kDa (A) and 23 kDa (B) xylanases measured under different pH conditions. The reaction pHs were adjusted to 4.0 to 12.0 with various buffers at 100 mM. The substrate was incubated with purified enzymes for 30 min at 37°C. For the assay of 45 kDa xylanase each reaction contained 0.5 U of enzyme which corresponding to 14 μg of protein. The amount of 23 kDa xylanase used in each reaction was 0.15 U which corresponding to 40 μg of protein.

(A)

46	NDQPFAWQVAS L SER	60	Ba. C-125 (45 kDa)
	ND PFAW VA L R		(10/15 identical)
1	**NDSPFAWSVAKLXXR**	15	**45 kDa xylanase** (This study)
	N PFAW VA L R		(9/15 identical)
51	NVQPFAWQVASLADR	65	Ba. NG-27 (45 kDa)

(B)

28	ATDYWQYWTVGGGMV	42	Ba. st. (23 kDa)
	+T Y QY T G G V		(8/14 identical)
1	**GT -YG QY XTDGQGXV**	14	**23 kDa xylanase** (This study)
	G Y + TDG QG V		(8/14 identical)
43	GY- Y Y S FWTDGQG NV	56	Ch. gr. (23 kDa)

Fig. 4. The N-terminal sequences of the purified xylanases compared with those of other xylanases. Ba. C-125, xylanase A from *Bacillus* sp. C-125 [19]; Ba. NG-27, an alkaline thermostable xylanase from *Bacillus* sp. NG-27 [20]; Ba. st., xylanase A from *Bacillus stearothermophilus* [21]; Ch. gr., a xylanase from *Chaetomium gracile* [22]. The partial sequences of the xylanases were aligned relatively to (A) 45 kDa and (B) 23 kDa xylanase to indicate identical amino acid residues. The numerical number showed the position of amino acid residue in its entire sequence. The X and "+" symbols represent undecided and conserved amino acid residues, respectively.

ties considerably, to 34.08 and 3.67 U/mg protein for the 45 kDa and 23 kDa xylanases, respectively. The former showed an activity that appeared to be 10-fold higher than the latter. This may also reflect the barely detected band on the zymogram gel for the 23 kDa xylanase (Fig. 1)

3.2. General properties of xylanases

Purified xylanases were homogeneous when further examined by isoelectrofocusing (IEF)(Fig. 2). The 45 kDa xylanase had a p*I* value of 5.8 whereas a p*I* value of 6.8 for the 23 kDa xylanase.

The activities of both xylanases at various pH values were measured by using birchwood xylan as the substrate. The reaction pHs were adjusted to 4.0 to 12.0 with various buffers at 100 mM. Both xylanases showed enzyme activity over a broad pH range of 4.0 to 12.0 at 37°C (Fig. 3). The pH optimum is at 6–8 for 45 kDa xylanase and at 6–7 for 23 kDa xylanase, respectively. The 45 kDa xylanase was slightly more alkaline resistant than that of 23 kDa enzyme with higher relative activity remained at pH 11. The pH profile of the purified enzymes indicated that the xylanase activities of both proteins remained considerable in the alkaline pH range. The properties of these two enzymes may be advantageous in the application of prebleaching of kraft pulps.

3.3. Analysis n-terminal sequences

The N-terminal amino acid sequences of the purified xylanases were compared with protein sequence data available at National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and European Bioinformatics Institute (EBI, http://srs.ebi. ac.uk/) databases us-

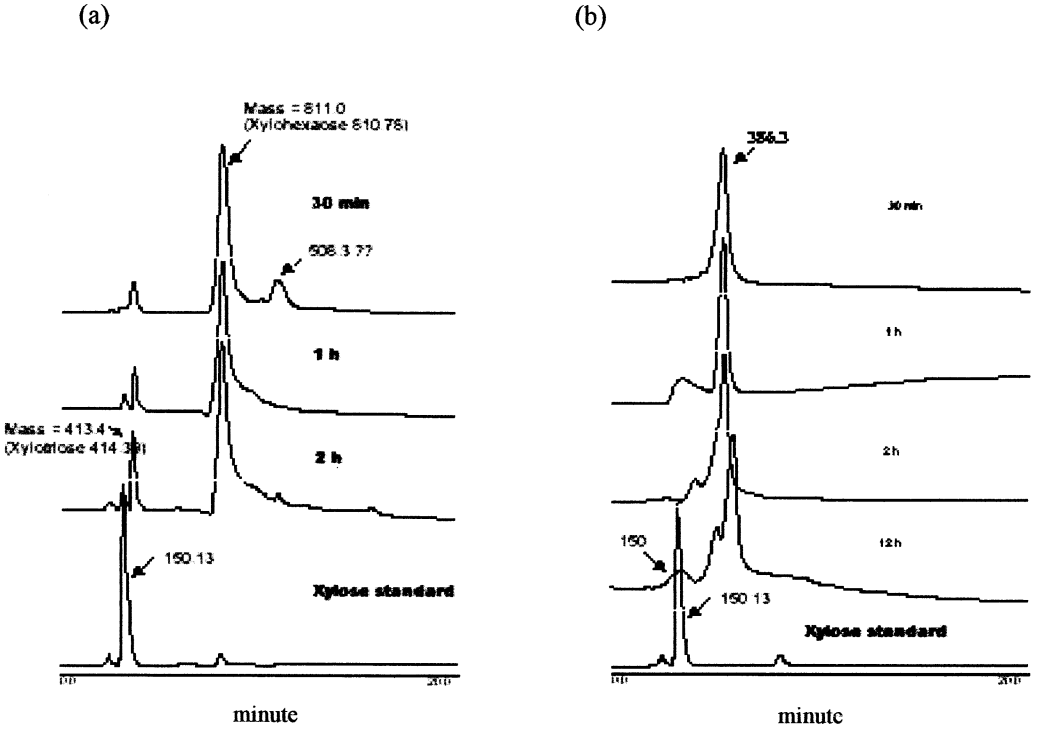

Fig. 5. Hydrolysis of xylan with purified 45 kDa (a) and 23 kDa (b) xylanase. The reaction mixture of 10 ml containing 1 mg of enzyme and 0.2 g birchwood xylan in 100 mM Tris-HCl buffer (pH 8.5), and incubated at 37 C for indicated time. The products were analyzed using a Microsorb Amine column with acetonitrile: water (78:22) as a mobile phase and were measured in the eluent by using a Waters refractive index (RI) detector. The molecular mass and identity of each product were determined by a mass spectrometer.

ing the on-line BLAST network service. The sequencing data of 45 kDa xylanase showed >50% identical with the N-terminal amino acid sequences of other microbial xylanases as well as that for 23 kDa xylanase, as shown in Fig. 4. The sequencing data of 23 kDa xylanase also shown good homology with the N-terminal amino acid sequences following signal peptides of other 23 kDa xylanases from *Bacillus* sp. YA-14 (8/14 identical) [15], *Bacillus subtilis* (8/14 identical) [16] and *Bacillus circulans* (8/14 identical) [17](Data not shown). Note that the xylanases which showed high homology with the 23 kDa xylanase of *B. firmus* all belong to family 11 glycosyl hydrolases as classified by Gilkes et al. [18]. This result indicates that the two purified xylanases possess at least a common signal peptide of 28–35 amino acid residues long, as shown by the sequence comparison and the similarity of molecular weights of the xylanases from the compared bacteria. We, therefore, conclude that the purified enzymes were indeed xylanases.

3.4. Hydrolysis of native xylan

In order to better understand the mode of action of the purified xylanases, the hydrolytic products of the insoluble birchwood xylan (Sigma) incubated with xylanases were identified by xylose standards and mass spectrometry. As expected, both xylanases effectively hydrolyzed xylan but showed different modes of action. The hydrolysis by 45 kDa xylanase produced mainly the xylotriose (mass = 413.4) and xylohexaose (mass = 811). Under prolonged incubations, a trace amount of xylose (mass = 150) was detected (Fig. 5a). Whereas the 23 kDa xylanase even after long period of incubation produced only an oligosaccharide derivative of molecular mass of 386.3 (Fig. 5b) which is presumed to be that of a xylooligosaccharide with side chains. However, there is a trace amount of xylose appeared after 12 h of incubation with 23 kDa xylanase. These data indicating that these purified xylanases were both endoxylanases that randomly cleave xylan as a substrate. It was interesting that co-incubation of the two xylanases with the substrate resulted in the releasing of xylose, xylobiose and an unknown product with a molecular mass of 375.3 (Fig. 6). The production of xylose also shown to be increasing with time. This result is quite different from those of individual action of the xylanases, suggesting a cooperative relationship of the two xylanases in the degradation of the polysaccharide into simple oligosaccharides.

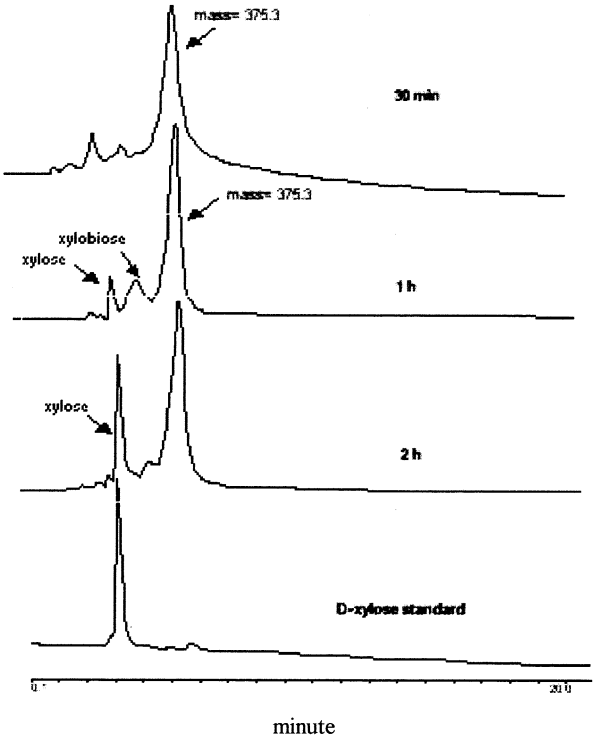

Fig. 6. Hydrolysis of birchwood xylan by purified 45 kDa and 23 kDa xylanases in parallel. The reaction mixture of 10 ml containing 45 kDa:23 kDa:xylan = 1 mg:1 mg:100 mg in 100 mM Tris-HCl buffer (pH 8.5) was incubated at 37°C for the indicated times. Products were analyzed using a Microsorb Amine column with acetonitrile:water (78:22) as a mobile phase and measured as in Fig. 5.

4. Conclusion

Two xylanases with molecular masses of 45 kDa and 23 kDa, respectively, were purified from the culturing medium of an alkalophilic *Bacillus firmus* which was isolated from a wastewater treatment plant of pulp and paper industry. Both enzymes showed a broad pH activity profile and relatively high activity under alkaline conditions toward birchwood xylan. Both xylanases had endo character on hydrolyzing substrate but showed different modes of action. They cooperatively released simple sugars of xylose, xylobiose and an unknown product with a molecular mass of 375.3 that was quiet different from the hydrolyzing pattern of individual xylanase. Thus, the crude xylanases extract from this bacterium may potentially applicable in enzymatic hydrolysis of xylan especially in kraft pulp prebleaching process.

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