

# Purification and Characterization of a Thermostable Xylanase from *Paenibacillus* sp. NF1 and its Application in Xylooligosaccharides Production

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High levels of extracellular xylanase activity (211.79 IU/mg) produced by *Paenibacillus* sp. NF1 were detected when it was submerged-cultured. After three consecutive purification steps using Octyl-Sepharose, Sephadex G75, and Q-Sepharose columns, a thermostable xylanase (XynNF) was purified to homogeneity and showed a molecular mass of 37 kDa according to SDS-PAGE. The specific activity of the purified XynNF was up to 3,081.05 IU/mg with a 14.55-fold purification. The activity of XynNF was stimulated by Ca<sup>2+</sup>, Ba<sup>2+</sup>, DTT, and β-mercaptoethanol, but was inhibited by Fe<sup>3+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup>, Cu<sup>2+</sup>, SDS, and EDTA. The purified XynNF displayed a greater affinity for oat spelt xylan with the maximal enzymatic activity at 60°C and pH 6.0. XynNF, which was shown to be cellulose-free, with high stability at high temperature (70°C–80°C) and low pH range (pH 4.0–7.0), is potentially valuable for various industrial applications. The enzyme hydrolyzed oat spelt xylan to yield mainly xylooligosaccharides (95.8%) of 2-4 degree of polymerization (DP2-4). Moreover, the majority of the xylooligosaccharides (DP2-4) products was xylobiose (61.5%). The thermostable xylanase (XynNF) thus seems potentially useful in the production of xylooligosaccharides.

**Keywords:** Endoxylanase, *Paenibacillus* sp., purification, characterization, oat spelt xylan, xylooligosaccharides

## Introduction

Endo-1,4-β-D-xylanase (E.C. 3.2.1.8) is an important enzyme for the degradation of the major hemicellulosic polysaccharide of lignocellulosic materials, since they hydrolyze xylopyranosyl linkages of β-1,4-xylan [27]. As the key xylan-degrading enzyme, xylanases have attracted considerable attention because of their widespread application in various industrial processes such as pulp bleaching, animal feeding, baking and brewing, waste-treating, bioenergy converting, textiles, and food industry [1, 12]. Recently, *Paenibacillus* species is frequently isolated and identified from various sources for their ability to hydrolyze lignocellulosic materials. Many strains

of *Paenibacillus* species, like *P. campinasensis* G1-1 [27] producing a thermostable xylanase, *Paenibacillus terrae* HPL-003 producing a new bimodular xylanase [17], and alkalophilic *Paenibacillus* sp. 12-11 [26] producing an alkaline xylanase, showed various application potential.

Xylan, composed of a linear backbone of 1,4-β-linked D-xylose units, is the major constituent of hemicellulose, which is the second most abundant component of plant biomass [29]. It can be hydrolyzed to xylose by the synergistic effect of endoxylanase, exoxylanase, and β-xylosidase. When it was hydrolyzed by only endoxylanase, the major product was xylooligosaccharides (XOS). XOS are newly developed functional oligosaccharides that exhibit excellent prebiotic effect and health benefits, such as the stimulation

of human intestinal *Bifidobacteria* and *Lactobacillus* growth [5, 6, 13]. Currently, XOS are produced mainly by enzymatic hydrolysis owing to high specificity, negligible substrate loss, and side product generation [22, 29]. However, the xylanases to be used for XOS production should preferably be free of or have very little exoxylanase and  $\beta$ -xylosidase activities stable at high temperature to withstand xylan extraction conditions and be available at low cost [3, 13]. Therefore, thermophilic endoxylanases are best suited for XOS production.

In our previous report, a novel strain, *Paenibacillus* sp. NF1, producing high levels of extracellular thermostable xylanase was isolated from cow dung compost in China [20]. In order to characterize the xylanase from *Paenibacillus* sp. NF1, it was purified to homogeneity from culture supernatants by three kinds of chromatography. The attractive attributes and special hydrolysis products of this xylanase suggest that it is potentially valuable for various industrial applications, especially for xylooligosaccharides production.

## Materials and Methods

### Strain and Chemicals

*Paenibacillus* sp. NF1, which was isolated, identified, and cultivated for xylanase production as described earlier [20], was preserved in our laboratory. Oat spelt xylan, birchwood xylan, carboxymethyl cellulose (CMC), Avicel, xylose, xylobiose, xylotriose, xylotetraose, sodium dodecyl sulfate (SDS),  $\beta$ -mercaptoethanol, bovine serum albumin (BSA), and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Octyl-Sepharose, Sephadex G75, and Q-Sepharose were purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). All other chemicals were of analytical reagent grade purity and obtained from commercial sources.

### Production of Xylanase by *Paenibacillus* sp. NF1

For xylanase production, *Paenibacillus* sp. NF1 was grown in optimized medium [20] containing corncob 20 g/l, beef extract 28.2 g/l,  $K_2HPO_4$  5 g/l,  $MgSO_4$  0.5 g/l, and NaCl 7.1 g/l, at 28°C with shaking at 160 rpm. Culture samples were collected at 6 h intervals over the period of 96 h cultivation. The initial pH of the medium was 6.9 and the inoculum size was 2%.

### Purification of Extracellular Xylanase

The culture supernatants of *Paenibacillus* sp. NF1 were collected by centrifugation (8,000 rpm for 10 min at 4°C). The supernatants collected after centrifugation were salted out with ammonium sulfate at 70% saturation. After that, the precipitates were dissolved in sodium phosphate buffer (20 mM, pH 6.0), and solid ammonium sulfate was added to adjust to the final saturation of 40%. The

samples were loaded onto an Octyl-Sepharose FF column (2.6 × 20 cm) pre-equilibrated with sodium phosphate buffer (20 mM, pH 6.0) containing 40% saturation of ammonium sulfate. The target protein was eluted with a linear gradient (40%–0% saturation) of ammonium sulfate in sodium phosphate buffer (20 mM, pH 6.0). The active fractions were loaded onto a Sephadex G75 column (1.6 × 60 cm) equilibrated with sodium phosphate buffer (20 mM, pH 8.0). The xylanase active fractions were collected for further purification by a Q-Sepharose HP column (2.6 × 20 cm) using linear gradient elution from 0–1 M NaCl in sodium phosphate buffer (20 mM, pH 8.0). Finally, the purified xylanase was desalted by dialysis against deionized water and concentrated by freeze-drying. The protein concentration of the samples at each step of chromatography was determined spectrophotometrically at 280 nm. The protein concentration of the pooled enzyme solution from each purification step was determined according to the Bradford method [2] using BSA as the standard.

### SDS-PAGE and Zymogram Analysis

The purified and concentrated xylanase preparations were analyzed by SDS-PAGE using a 12% polyacrylamide gel containing 0.1% (w/v) oat spelt xylan. After electrophoresis, the gel was divided and the protein bands were visualized by staining with Coomassie Brilliant Blue G250 (Sigma) or detected for xylanase activity [9, 11]. For zymogram analysis, the gel was treated with 25% 2-propanol and incubated at 60°C for 30 min in 20 mM sodium phosphate buffer (pH 6.0). After incubation, the gel was subsequently incubated under agitation at room temperature in Congo red solution (1 mg/ml) for 10 min, washed with 1 M NaCl, and fixed with 5% (v/v) acetic acid [28]. Clear areas in the zymogram indicated enzyme activity. Molecular weight standards from Fermentas were used as molecular mass markers.

### Xylanase Assay

The activity of xylanase was measured by determining the amount of reducing sugars liberated from 1% oat spelt xylan using 3,5-dinitrosalicylic acid at 60°C and pH 6.0 [10]. The method was described previously by Zheng *et al.* [28]. The amount of enzyme that liberated 1  $\mu$ mol of reducing sugars per minute was defined as one unit (IU) of xylanase activity.

### Effects of Temperature and pH on Xylanase Activity and Stability

The optimum temperature of the xylanase was measured by incubating the reaction mixtures at different temperatures (40–80°C) and pH 6.0 for 10 min. The relative enzymatic activity was calculated as a percentage of the maximal activity. Its thermal stability was determined by incubating the purified xylanase in sodium phosphate buffer (20 mM, pH 6.0) at various temperatures (40–80°C) for 180 min. The residual enzyme activity was calculated as a percentage of the starting activity. The optimum pH of xylanase activity was measured at 60°C using acetic acid sodium buffer (pH 3.0–5.0), sodium phosphate (pH 6.0–7.0), Tris-HCl

(pH 8.0–9.0), and glycine-NaOH (pH 10.0), respectively, at the same concentration of 20 mM. The relative enzymatic activity was calculated as a percentage of the maximal activity. To assess its pH stability, the purified xylanase was preincubated in buffers at various pH values (pH 3.0–10.0) at 60°C for 180 min and the residual activities were determined at 30 min intervals.

#### Effects of Various Metal Ions and Other Additives on Xylanase Activity

The effects of various metal ions (KCl, CaCl<sub>2</sub>, BaCl<sub>2</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>, FeCl<sub>2</sub>, FeCl<sub>3</sub>, ZnCl<sub>2</sub>, and CuSO<sub>4</sub>), metal chelators (EDTA) and surfactants (DTT, β-mercaptoethanol, and SDS) on the purified xylanase activity were evaluated by including it in the reaction mixtures at the concentration of 1 mM and 10 mM. Enzyme solutions were incubated at 60°C for 60 min before the residual activity was measured. The activity of the enzyme assayed under the same condition without additives was defined as 100%.

#### Substrate Specificity and Kinetic Studies

The specificities and kinetic constants ( $K_m$  and  $V_{max}$ ) of xylanase were determined following the method of Zheng *et al.* [28]. For specificities determination, the activity of the purified xylanase against oat spelt xylan, birchwood xylan, rice hull xylan, bran xylan, CMC, and Avicel at concentrations of 1% (w/v) at 60°C and pH 6.0 were assayed. The enzyme preparations were incubated with oat spelt xylan and birchwood xylan at concentrations between 0.1% (w/v) and 1% (w/v) to estimate the kinetic parameters ( $K_m$  and  $V_{max}$ ) of the purified xylanase using a Lineweaver-Burk double reciprocal plot.

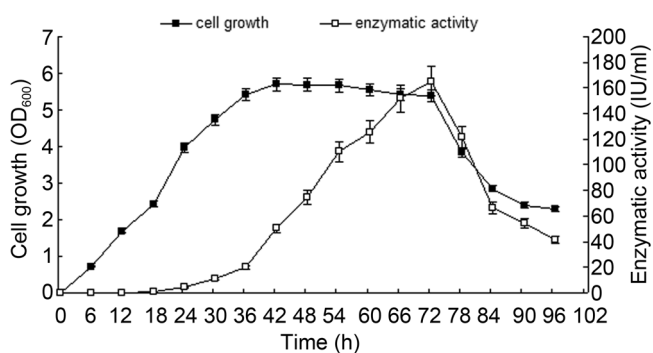
#### Analysis of Xylooligosaccharides in Xylanase Hydrolysates by High Performance Liquid Chromatography (HPLC)

One hundred fifty micrograms of oat spelt xylan in 1.5 ml sodium phosphate buffer (pH 6.0) was treated with 30 IU of the diluted purified xylanase at 60°C for 10 min, 1 h, and 2 h, respectively. The xylanase hydrolysates were analyzed by an HPLC (Agilent). HPLC analysis was done by using a chromatographic column (Shodex SUGAR KS-801, 8.0 × 300 mm) at 65°C with water as the mobile phase at 0.6 ml/min.

## Results and Discussion

#### Production of Extracellular Xylanase by *Paenibacillus* sp. NF1

When *Paenibacillus* sp. NF1 was cultured by the submerged method, the xylanase was secreted to the culture liquid. The production of extracellular xylanases by *Paenibacillus* sp. NF1 was investigated during 96 h of cultivation in the optimized production medium (detailed in Materials and Methods). As shown in Fig. 1, exponential growth of *Paenibacillus* sp. NF1 was observed until 42 h, and then the



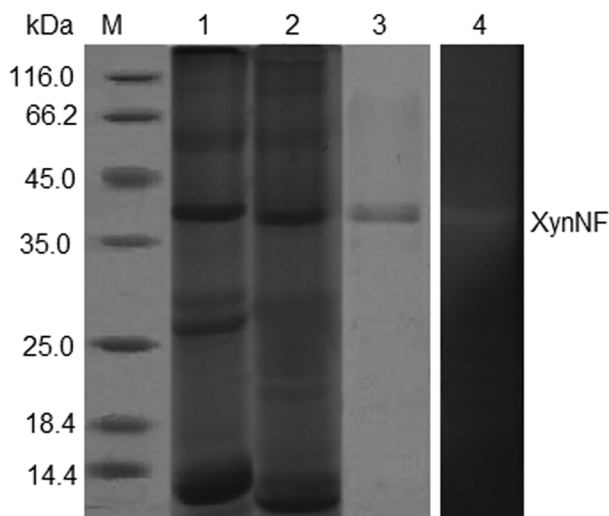
**Fig. 1.** Growth and extracellular xylanase production of *Paenibacillus* sp. NF1.

The culture growth was monitored by measuring the optical density at 600 nm with a 6 h interval, and the xylanase activity was also assayed using the DNS method. Data are presented as means ± SD ( $n = 3$ ).

stationary phase was ended after 30 h. Moreover, *Paenibacillus* sp. NF1 secreted extracellular xylanases at a low rate in the exponential phase and achieved the maximal xylanase production of up to 165.2 IU/ml at the end of the stationary phase (72 h, Fig. 1). After the stationary phase (72 h), the xylanase activity was gradually decreased because of hydrolysis by autologous protease in the decline phase of strain NF1. This course of xylanase degradation was also observed from *Fomitopsis pinicola* KMJ812 [15] and *Paenibacillus campinasensis* G1-1 [27] reported previously. *Paenibacillus* sp. NF1 producing relatively high levels of xylanases compared with those reported previously [21, 24, 25] could be an efficient and economical microorganism with potential applications in industrial production.

#### Purification of Xylanase XynNF

Under the optimized culture conditions, the specific activity of xylanase produced by *Paenibacillus* sp. NF1 reached to 211.79 IU/mg (Table 1). The xylanase was purified to electrophoretic homogeneity by the steps listed in Table 1. The specific activity of the purified xylanase was 3,081.05 IU/mg with a 14.55-fold purification, and the final yield for the enzyme was 10.63% (Table 1). As a single band on SDS-PAGE with an apparent molecular mass of about 37 kDa, the purified xylanase from culture supernatants was designated as XynNF (Fig. 2, lane 3), whereas zymogram analysis of the purified XynNF exhibiting a clear activity band on the corresponding zone further confirmed the presence of active xylanase (Fig. 2, lane 4). It also indicates that XynNF from *Paenibacillus* sp. NF1 was a monomeric



**Fig. 2.** SDS-PAGE and zymogram analysis of the purified xylanase from *Paenibacillus* sp. NF1.

The contents of each lane are as follows: Lane M: molecular weight of marker standard; Lane 1: crude extracellular xylanase from *Paenibacillus* sp. NF1; Lane 2: XynNF purified by hydrophobic-interaction chromatography; Lane 3: purified XynNF; Lane 4: zymogram analysis of the purified XynNF.

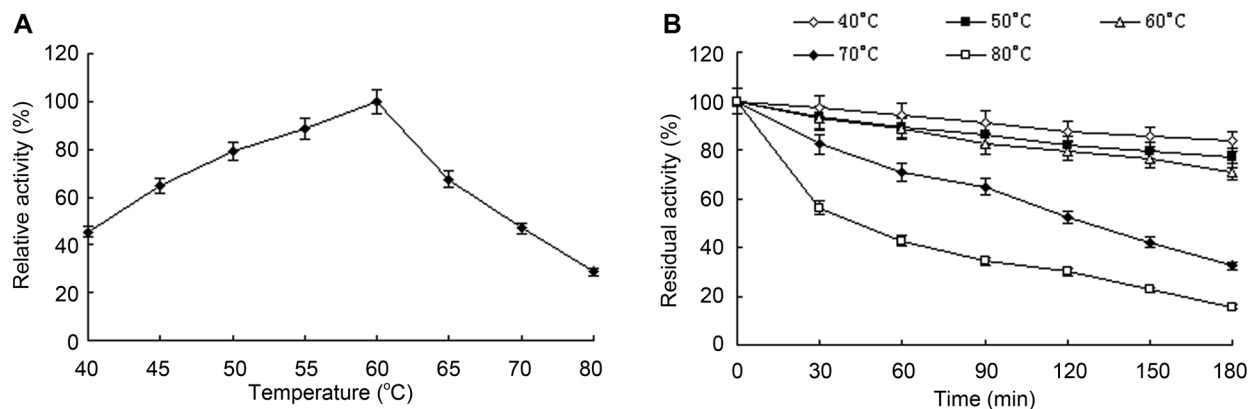
protein comparable to those previously reported for other xylanases [16, 21, 23].

### Effects of Temperature and pH on Enzyme Activity and Stability

The optimal reaction temperature of XynNF was 60°C, which is higher than that of *Paenibacillus* sp. 12-11 (55°C) [26], and the enzyme retained 46.88% of its maximum activity at 70°C (Fig. 3A). The thermal stability of XynNF was evaluated by incubation at temperatures from 40°C to 80°C for 180 min. The enzymatic activity retained at least 71.07% of the original activity after incubation at 40°C to 60°C for 180 min, and still retained about 70.65% and 42.66% of the original activity after incubation at 70°C and 80°C for 60 min, respectively (Fig. 3B). This thermal stability is better than earlier reports on most of the bacterial xylanases [1, 14, 19, 26]. The purified XynNF showed the maximum activity at pH 6.0, which is lower than that of XynG1-1 (7.5) from *P. campinasensis* G1-1 [28] and retained 59.78% of its maximum activity at pH 3.0 (Fig. 4A). It was stable between pH 4.0 and 7.0 for 180 min, retaining at least 59.11% of its activity at pH 6.0 (Fig. 4B),

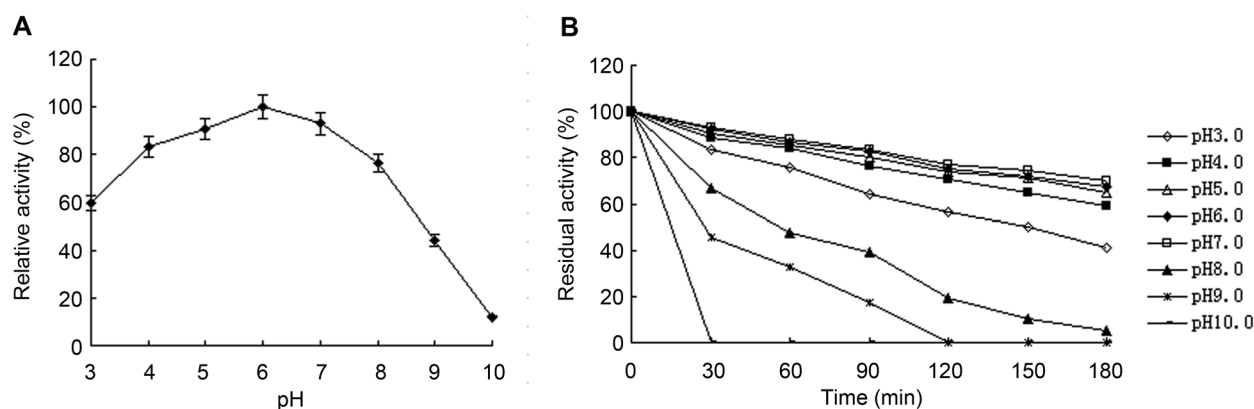
**Table 1.** Purification of xylanase (XynNF) from *Paenibacillus* sp. NF1.

Purification step	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg protein)	Yield (%)	Purification fold
Culture filtrate	82,600	390	211.79	100	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	62,025	117.5	527.87	75.09	2.49
Octyl-Sepharose	36,650	33.7	1,087.54	44.37	5.13
Sephadex G75	19,468	10.48	1,857.63	23.57	8.77
Q-Sepharose	8,781	2.85	3,081.05	10.63	14.55



**Fig. 3.** Effects of temperature on the activity (A) and stability (B) of the purified XynNF.

(A) Relative activities at different temperatures; the maximum activity observed at 60°C was taken as 100%. (B) Residual activities after incubation for various times at 40°C to 80°C, pH 6.0. The original activity without incubation was taken as 100%. Data are presented as means  $\pm$  SD ( $n = 3$ ).



**Fig. 4.** Effects of pH on the activity (A) and stability (B) of the purified XynNF. (A) Relative activities at different pH values; the maximum activity observed at pH 6.0 was taken as 100%. (B) Residual activities after incubation for various times at pH 3.0 to 10.0, 60°C. The original activity without incubation was taken as 100%. Data are presented as means  $\pm$  SD ( $n = 3$ ).

manifesting much greater pH stability at the condition of lower pH than those of most bacterial xylanases reviewed [1, 19, 26]. Its stability at high temperature (70°C–80°C) and activity at the condition of lower pH (pH 4.0–7.0) (Figs. 3 and 4) make XynNF valuable for various industrial applications, such as animal feeding, bioenergy converting, and food industry.

#### Effects of Various Metal Ions and Additives on Xylanase Activity

Addition of 1 mM or 10 mM of  $K^+$ ,  $Mg^{2+}$ , and  $Mn^{2+}$  had no significant effect on the purified XynNF (Table 2). However, the purified XynNF was activated by  $Ca^{2+}$  and  $Ba^{2+}$  and was inhibited by certain metal ions in the following order:  $Fe^{3+} > Zn^{2+} > Fe^{2+} > Cu^{2+}$  (Table 2). The reducing agents, DTT and  $\beta$ -mercaptoethanol, stimulated the activity of XynNF (Table 2). The strong inhibition by SDS and EDTA (Table 2) indicates that hydrophobic interactions may be important in maintaining the structure of XynNF and certain metal ions were required for its activity.

#### Substrate Specificity and Kinetic Studies

Specificity studies indicate that XynNF could hydrolyze all the tested xylans, but did not act on CMC and Avicel. The purified XynNF exhibited the highest xylanase activity when oat spelt xylan or birchwood xylan was used as the substrate (Table 3), with relatively lower activities being measured for rice hull xylan (82.23%) and bran xylan (79.86%). In addition, the purified XynNF showed  $K_m$  values of 5.64 and 6.32 mg/ml and  $V_{max}$  values of 3,364.57 and 3,216.44  $\mu$  mol/min/mg of protein for oat spelt xylan and birchwood xylan, respectively. The values of  $K_m$  for

these two substrates indicated that XynNF had greater affinity for oat spelt xylans. Strong specificity toward birchwood xylan and oat spelt xylan was also verified for xylanases from *P. campinasensis* BL11 [8], *T. clypeatus* [18], and *P. sclerotiorum* [7].

#### Analysis of XOS in Xylanase Hydrolysates by HPLC

When oat spelt xylan was incubated with the purified

**Table 2.** Effects of metal ions and other additives on the activity of the purified XynNF.

Agents	Relative activity (%)	
	1 mM	10 mM
Control <sup>a</sup>	100 $\pm$ 3.22	100 $\pm$ 4.23
KCl	97.22 $\pm$ 1.34	98.05 $\pm$ 2.11
CaCl <sub>2</sub>	122.14 $\pm$ 4.42	132.41 $\pm$ 5.26
BaCl <sub>2</sub>	111.03 $\pm$ 3.46	119.55 $\pm$ 4.53
MgCl <sub>2</sub>	96.34 $\pm$ 2.46	96.59 $\pm$ 2.55
MnCl <sub>2</sub>	98.16 $\pm$ 3.22	97.54 $\pm$ 4.06
FeCl <sub>2</sub>	55.56 $\pm$ 2.36	52.43 $\pm$ 2.66
FeCl <sub>3</sub>	23.17 $\pm$ 2.12	20.34 $\pm$ 2.67
ZnCl <sub>2</sub>	34.66 $\pm$ 2.17	31.92 $\pm$ 2.83
CuCl <sub>2</sub>	83.24 $\pm$ 4.12	80.64 $\pm$ 4.36
DTT	126.94 $\pm$ 3.46	128.42 $\pm$ 4.05
$\beta$ -Mercaptoethanol	145.29 $\pm$ 3.32	147.12 $\pm$ 3.65
EDTA	46.56 $\pm$ 2.11	41.21 $\pm$ 1.99
SDS	25.43 $\pm$ 2.24	20.64 $\pm$ 2.32

<sup>a</sup>The activity of the enzymes assayed under the optimum condition in the absence of additives was defined as 100%. Data are presented as means  $\pm$  SD ( $n = 3$ ).

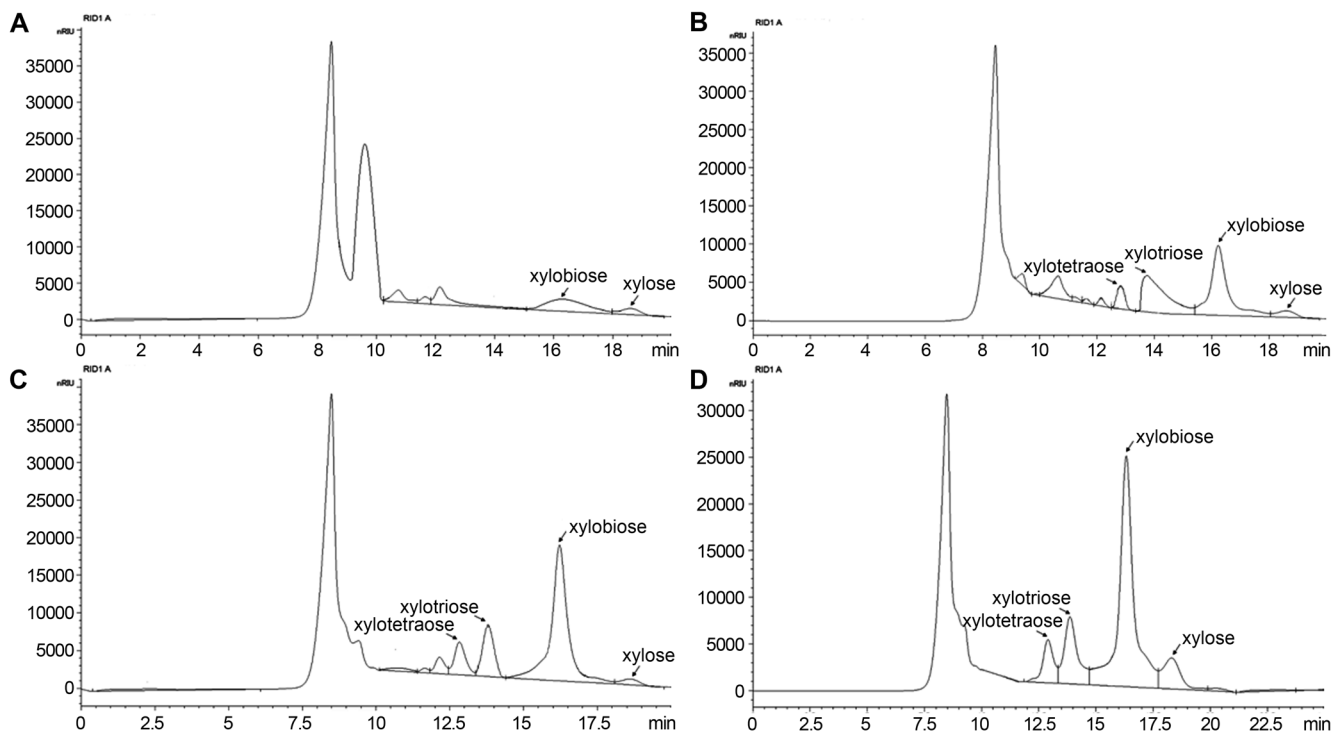


**Table 3.** Substrate specificity and kinetic parameters of the purified XynNF.

Substrate	Specific activity (IU/mg)	Relative activity <sup>a</sup> (%)	V <sub>max</sub> (μmol/min/mg)	K <sub>m</sub> (mg/ml)
Oat spelt xylan	3,081.05 ± 4.12	100 ± 2.11	3,364.57 ± 4.65	5.64 ± 0.97
Birchwood xylan	3,045.93 ± 4.34	98.86 ± 2.07	3,216.44 ± 4.43	6.32 ± 1.02
Rice hull xylan	2,533.55 ± 5.66	82.23 ± 2.65		
Bran xylan	2,460.53 ± 6.74	79.86 ± 2.86		
CMC	ND <sup>b</sup>	ND		
Avicel	ND	ND		

<sup>a</sup>Relative activity (%): the maximum specific activity was taken as 100%.

<sup>b</sup>ND: not detectable. Data are presented as means ± SD (*n* = 3).

**Fig. 5.** Chromatograms from HPLC analysis of the oat spelt xylan hydrolysates by the purified xylanase (XynNF).

Enzyme-substrate mixtures containing 1.5 ml of 1% (w/v) oat spelt xylan and 30 IU of the diluted purified xylanase were incubated at 60°C and pH 6.0 for 0 min (A), 10 min (B), 1 h (C), and 2 h (D), respectively. The oligomeric state (DP2-4) of each peak was determined by comparing it with the retention time of the xylooligomer standard marker.

XynNF for 2 h at 60°C and pH 6.0, the major hydrolysis products were xylobiose, identified by HPLC (Table 4 and Fig. 5). After a 10 min reaction time, about half of the larger polysaccharides ( $\geq$ xylopentaose) was hydrolyzed to xylobiose, xylotriase, and xyloetraase, but no xylose was liberated (Table 4). Xylobiose and xylotriase contents increased steadily during further incubation for 1 h; however, xyloetraase and xylose were not increased (Table 4). The amount of xyloetraase remained almost constant from 10 min to 2 h reaction and there were no higher xylooligosaccharides

**Table 4.** HPLC analysis of the products liberated by the action of the purified xylanase (XynNF) on oat spelt xylan.

Sugars	Percentage of sugars liberated after reaction time (%)			
	0 min	10 min	1 h	2 h
Xylose (X <sub>1</sub> )	0.4	0.4	0.4	4.2
Xylobiose (X <sub>2</sub> )	0.6	24.4	46.5	61.5
Xylotriase (X <sub>3</sub> )	0	23.2	29.4	24
Xyloetraase (X <sub>4</sub> )	0	10.5	10.1	10.3
$\geq$ Xylopentaose (X <sub>5</sub> )	99	41.5	13.6	0

( $\geq$ xylopentaose) in the hydrolysis products until the end of the hydrolysis at the reaction time of 2 h (Table 4, Fig. 5D). It indicates that XynNF was more efficient in conversion than *S. thermophile* xylanase [13], which took 8 h for complete hydrolysis of oat spelt xylan. By HPLC analysis of xylanase hydrolysates against oat spelt xylan, xylo-tetraose and higher xylooligosaccharides were found to be efficiently hydrolyzed mainly to xylobiose, a small part of xylo-triose appeared to be further hydrolyzed to xylobiose and xylose until 2 h, while xylo-tetraose was mainly hydrolyzed to xylobiose, but no xylobiose were hydrolyzed to xylose (Table 4 and Fig. 5). Therefore, XynNF was confirmed to be an endoxylanase rather than a  $\beta$ -xylosidase that hydrolyzes xylobiose to xylose. When the reaction time was increased from 10 min to 2 h, the xylooligosaccharides (DP2-4) content finally increased to 95.8% (Table 4) and xylobiose was the majority of the xylooligosaccharides (DP2-4) products (Fig. 5). With the time course of hydrolysis, the amount of xylobiose increased further reaching to 61.5% in 2 h (Table 4). The high catalytic efficiency and enrichment effect of xylobiose makes XynNF a highly potential enzyme in xylooligosaccharides production.

In conclusion, the endoxylanase (XynNF) of *Paenibacillus* sp. NF1 was purified to homogeneity by multistep chromatographies. Its stability at high temperature (70°C–80°C) and low pH range (pH 4.0–7.0) makes it a suitable biocatalyst for various industrial applications. In particular, its application in oat spelt xylan hydrolysis, showing 95.8% xylooligosaccharides (DP 2-4) products, makes it a promising candidate for xylooligosaccharides production.

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