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Molecular cloning and characterization of a novel thermostable xylanase from *Paenibacillus campinasensis* BL11

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ABSTRACT

An open reading frame (XylX) with 1131 nucleotides from *Paenibacillus campinasensis* BL11 was cloned and expressed in *E. coli*. It encodes a family 11 endoxylanase, designated as XylX, of 41 kDa. The homology of the amino acid sequence deduced from XylX is only 73% identical to the next closest sequence. XylX contains a family 11 catalytic domain of the glycoside hydrolase and a family 6 cellulose-binding module. The recombinant xylanase was fused to a His-tag for affinity purification. The XylX activity was 2392 IU/mg, with a K_m of 6.78 mg/ml and a V_{max} of 4953 mol/min/mg under optimal conditions (pH 7, 60 °C). At pH 11, 60 °C, the activity was still as high as 517 IU/mg. Xylanase activities at 60 °C under pH 5 to pH 9 remained at more than 69.4% of the initial activity level for 8 h. The addition of Hg^{2+} at 5 mM almost completely inhibited xylanase activity, whereas the addition of tris-(2-carboxyethyl)-phosphine (TCEP) and 2-mercaptoethanol stimulated xylanase activity. No relative activities for Avicel, CMC and D-(+)-cellobiose were found. Xylotriose constitutes the majority of the hydrolyzed products from oat spelt and birchwood xylan. Broad pH and temperature stability shows its application potentials for biomass conversion, food and pulp/paper industries.

1. Introduction

Xylan is the second most common hemicellulose found in plant cell walls after cellulose. The xylans are complex heteropolysaccharides consisting of a backbone chain of 1,4-β-D-xylopyranose units with a variety of side linkages, including acetyl groups, arabinofuranose, ferulic acid, methyl glucuronic acid, and others [1–3]. Several enzymes are involved in the breakdown of xylan. Endo-1,4-β-xylanases (E.C.3.2.1.8) depolymerize xylan by random hydrolysis of the xylan backbone, whereas 1,4-β-D-xylosidases (E.C.3.2.1.37) remove successive D-xylose residues from the non-reducing end group. There are also several specific hydrolases that are able to release the aforementioned side-groups presented in xylan [4].

The potential uses of microbial xylolytic enzymes have garnered significant attention. Recently, there has been much industrial interest in using xylan and its hydrolytic enzymatic complex [4,5] as a supplement in animal feed; for the manufacture of bread, food and drinks; in textiles; and in ethanol and xylitol production, especially for pulp and paper processing [4–6].

Diverse microbes have been explored as invaluable xylanase resources, e.g., *Alicyclobacillus* sp. [7], *Arthrobacter* sp. MTCC 5214 [8], *Bacillus coagulans* [9], *Bacillus pumilus* [10], and *Neocallimastix patriciarum* [11]. Xylanases from thermophilic organisms have received the most attention due to their greater application potential rendered by their enhanced stability in wide temperature and pH ranges [6,12,13].

Paenibacillus species are capable of hydrolyzing plant materials and are currently isolated and identified from soil- and plant-related sources [14–17]. Several members of the genus *Paenibacillus* secrete diverse assortments of extracellular polysaccharide-hydrolyzing enzymes, and their xylanolytic systems are gradually being identified [18–22].

The *Paenibacillus campinasensis* BL-11 strain was identified and isolated from a high temperature and alkaline environment [23]. It is able to produce xylanase, cellulase, pectinase and cyclodextrin glucanotransferase [23]. In this work, gene cloning and expression of a xylanase (denoted as XylX) from *P. campinasensis* BL11 was conducted. However, the characterization of a purified xylanase

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from *P. campinasensis* BL11 has not yet been reported. A 6× histidine tag was fused to the recombinant XylX to facilitate its purification by affinity chromatography.

2. Materials and methods

2.1. Materials, bacterial strains and plasmids

All chemicals used were either from Sigma (St. Louis, USA) or of analytical grade from E. Merck (Darmstadt, Germany), unless specified otherwise. Bacteria were routinely cultured in Luria–Bertani (LB) medium. LB medium contained 10 g/L Bacto-tryptone, 5 g/L yeast extract, and 5 g/L NaCl. *P. campinasensis* BL11 was isolated from a high temperature, alkaline environment and then phylogenically identified [23]. The vector pBCKS(+) was from Stratagene (La Jolla, CA). Vector pET25b and *E. coli* HMS174 (DE3) were from Novagen (Madison, WI). The PCR primers were synthesized by Bio Basic, Inc. (Markham, Ontario, Canada).

2.2. DNA isolation, genomic library construction and screening

Genomic DNA of *P. compinasensis* BL11 was isolated [24]. Sau3AI-digested 3- to 5-kb fragment pools were recovered and cloned into the BamHI-digested vector pBCKS(+). Ligated DNA was used to transform *E. coli* NM 522 cells. Transformants able to degrade oat spelt xylan were identified by the Congo red assay [25].

2.3. DNA sequencing and protein analysis

The nucleotide sequences of both strands were determined by FS DNA polymerase fluorescent dye terminator reactions. Sequencing products were detected using an Applied Biosystems 377 stretch automated sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide and deduced amino acid sequences were analyzed with the sequence analysis tools of EMBL Computational Services (http://www.ebi.ac.uk/Tools/sequence.html). Related sequences were obtained from database searches (SwissPort, PIR, PRF, and GenBank) using the programs BLASTP 2.0 and FASTA. The XylX sequence determined in the present study has been deposited in the GenBank database under accession No. DQ241676.

Potential proteins encoded by the BL11 xylanase gene were analyzed using various software programs. The predicted signal peptides and their cleavage sites were analyzed using the NN (neural networks) and HMM (hidden Markov models) methods. Conserved domains were searched using InterProScan (EMBL-EBI) and PSI-CD (NCBI). Finally, multiple alignments of the deduced amino acid sequence of XylX with its related xylanases were performed using ClustalW (EMBL-EBI).

2.4. Construction of a xylanase expression system

For gene expression in *E. coli*, a pET25b expression system (Novagen, Madison, WI) was used. The DNA fragment containing the xylanase-encoding sequence was amplified from one of the correct xylanase-positive pBCKS(+) clones with primers xylX-F (5′-CTAGCCAGCATATGA AAATCTATGGGA-3′) and xylX-R (5′-AGAATTCACCGGATCTCGAGATAGTCA-3′). The underlined sequences are the NdeI (xylX-F), EcoRI and XhoI (xylX-R) sites, respectively. The 1.1-kb PCR-amplified product was subjected to digestion with NdeI and XhoI. The fragments were ligated between the NdeI and XhoI sites of pET25b, resulting in the plasmid pETBX. Plasmid pETBX was then used to transform *E. coli* strain HMS174 (DE3).

2.5. Expression and purification of cloned xylanase

One colony of the expression strain was inoculated into 2 mL of Luria–Bertani medium containing 100 μg of ampicillin/mL and allowed to grow overnight at 37 °C in a rotary shaker. The overnight culture was then transferred to 30 mL of the same medium and grown to an A_{600} of 0.4–0.5. Protein production was induced by the addition of IPTG (isopropyl-β-D-thio-galactopyranoside) to a final concentration of 1 mM and grown for an additional 3, 6 and 12 h at 28 °C, after which the cells were harvested by centrifugation, washed and disrupted by sonication in 50 mM PBS (sodium phosphate). A clear lysate from the extracts was loaded on a Ni-NTA agarose (Novagen, Madison, WI) column. The resulting protein was then eluted by addition of 200 μL elution buffer (300 mM sodium chloride, 50 mM sodium phosphate, 50 mM imidazole, pH 7.0). The protein concentration was analyzed by the Bradford assay using a spectrophotometer.

2.6. SDS-PAGE and zymogram

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 10% polyacrylamide gel [26]. Proteins were fixed in the gels by soaking in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid for approximately 1 h and subsequently visualized by Coomassie blue staining. Zymographic detection of xylanase activity was carried out by modifying the protocol of Blanco et al. [27].

2.7. Effects of pH and temperature on xylanase activity and stability

His-tagged XylX was used in the remainder of this study for characterization and application. The effect of pH and temperature on xylanase activity was studied in the presence of different buffers for 20 min under various conditions. The buffers used were 100 mM acetate buffer (pH 5–6), 100 mM phosphate buffer (pH 6–8) and 25 mM borate buffer (pH 8–11).

All of the xylanase assays were carried out by the protocols described by König et al. [28]. A concentration of 1.5% (w/v) oat spelt xylan (Sigma) was used as the substrate, and it was reacted with the xylanase solution at various pH and temperature values for 20 min. The amount of released sugar was then determined by the dinitrosalicylic acid (DNSA) method [28].

The effects of temperature and pH on xylanase stability were assessed by incubating the reaction mixtures from 5 min up to 8 h at different temperatures ranging from 40 to 80 °C at pH 7 and at pH values ranging from 4 to 11 at 60 °C. The residual activity of each sample was then quantified by the DNSA method at pH 7, 60 °C.

2.8. Effect of additives on xylanase activity

The effect of various additives on XylX xylanase activity was determined by the presence of metal ions and other reagents. The additives used in this study were $CaCl_2$, $CoCl_2$, $HgCl_2$, $MnCl_2$, KCl, $MgCl_2$, $FeCl_2$, $FeCl_3$, $SrCl_2$, $ZnCl_2$, $CuSO_4$, $NiCl_2$, $PbCl_2$, EDTA, tris-(2-carboxyethyl)-phosphine (TCEP), N-bromosuccinimide, 2-mercaptoethanol, Tween 20, Tritone X-100 and SDS at various concentrations. The reaction mixtures containing the various additives were incubated for 60 min at 60 °C, and the xylanase activity was assayed by the DNSA method. The presented values are the averages of triplicate assays.

2.9. Substrate specificity

To identify the substrate specificity of XylX under optimal conditions, substrates including cellobiose, laminarin, barley β-glucan, oat spelt xylan, birchwood xylan, laminarin, *p*-nitrophenyl-xylopyranoside and Avicel (Fluka) were employed at 1% (w/v) in an enzyme assay. The enzyme assays were run for 120 min under optimal conditions, and the enzyme activities were determined by measuring the generated reduced sugar using the DNSA method.

2.10. Kinetic parameters

Reactions were conducted at the optimal condition, pH 7 and 60 °C, using 5–40 mg/mL oat spelt xylan solutions. Double reciprocal Lineweaver–Burk plots for xylanase activity versus substrate concentration were constructed to estimate kinetic parameters (K_m and V_{max}) by linear regression.

2.11. Xylan hydrolysis and product analysis

A concentration of 10 mg/mL of oat spelt xylan and birchwood xylan were reacted with 10 IU/mL XylX solution in 100 mM phosphate buffer at pH 7 and 50 °C. Hydrolyzed products were analyzed by a HPLC system equipped with a RI detector (Jasco RI-930, Tokyo, Japan). A 250 mm × 4.6 mm Asahipak NH2P-50 4E column (Showa Denko, Tokyo, Japan) was employed. The mobile phase consisted of acetonitrile and distilled water (70/30) with a flow rate of 1 mL/min at room temperature. Xylo-oligomer standards (X2–X5) from Megazyme (Wicklow, Ireland) and xylose were used for system calibration.

3. Results and discussion

3.1. Isolation of xylanolytic clones

Genomic DNA of *P. compinasensis* BL11 was partially digested with Sau3A, recovered from the agrose gel, and ligated to BamHI-digested vector pBCKS(+). Recombinant plasmids were transformed into *E. coli* NM522 cells. Positive clones, presenting clear zones around colonies and suggesting xylan hydrolysis, were obtained. Harvested cells of the clone were subjected to zymographic analysis, and the results are shown in Fig. 1. The exhibited extracellular xylanase activity bands of about 41 kDa coincide perfectly with our former description [23].

3.2. DNA sequence analysis of the xylanase gene, XylX

The DNA fragments harboring xylanolytic activity were verified by restriction mapping, subcloning in pUC19 and sequencing. The determined fragments matched the complete nucleotide

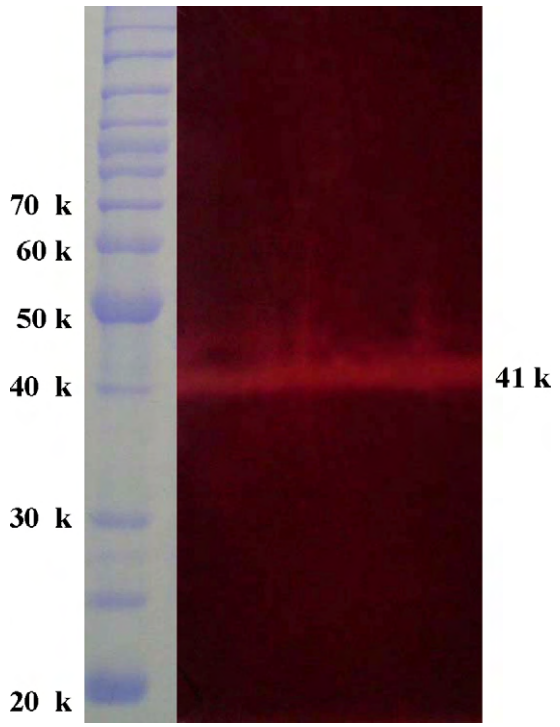

Fig. 1. The *E. coli* clones harboring plasmids containing xylanase genes exhibited xylanase activity. Lane M, molecular mass markers; lanes 1, 2 and 3, crude enzyme extract of 3 individual clones. Molecular weights of markers (left) and xylanase (right) are denoted next to the respective band.

sequences. One open reading frame (ORF) of 1131 bp, designated XylX (DQ241676), was identified to code for a polypeptide of 377 amino acids with a molecular mass of about 41 kDa. This ORF was translated to protein and analyzed *in silico* for its promoter, ribosome-binding site, signal peptide and functional domains. About 200 bp upstream of this ORF, there were two predicted promoters (sequence: tgaatttttcaaggtatgaccttaattttgataatggaataataaggcaa from the 45th to 90th base; sequence: tgataatggaataataaggcaaattttgataaattactaattgtaatcgc from the 73rd to 118th base) that might modulate the expression of XylX.

The deduced amino acid sequence of XylX is shown in the upper part of Fig. 2. The results suggest that the first 39 amino acids constitute a signal peptide (lower part in the schematics of Fig. 2). The signal peptidase cutting site was located between the 39th and the 40th residues and possessed a typical AXA motif for signal peptidase I. The full molecular mass of the 377-amino acid protein was predicted to be about 41 kDa, which corresponded to the results obtained by SDS-PAGE shown in Fig. 1.

Two functional domains were found in the enzyme, as shown in the schematic shown in Fig. 2: a catalytic domain (glycosyl hydrolases domain, family 11) located between 49 and 235 aa (amino acid) and a unique carbohydrate-binding domain (carbohydrate-binding module, family 6) located between 263 and 377 aa.

When comparing the amino acid sequence of XylX to those of other xylanases, the highest similarity scores were only 76% to *Bacillus* sp. YA-335 and 71% to *Paenibacillus curdlanolyticus*, as shown in Table 1. These results suggest that the cloned xylanase is a new xylanase. The difference in homology between xylanases of *P. compinasensis* BL11 and Xyn11A of *P. curdlanolyticus* B-6 was also verified in a recent study [22].

Table 1
Similarity of other xylanases in the databases based on the XylX amino acid sequence.

Strain	Similarity (%)	Amino acid differences/compared
Bacillus sp. YA-335	76	83/360
Paenibacillus curdlanolyticus	71	108/379
Bacillus pumilus	69	69/226
Bacillus sp. HBP8	67	74/225
Bacillus pumilus ATCC 7061	66	81/241
Clostridium papyrosolvens DSM 2782	65	80/233
Dictyoglomus turgidum DSM 6724	60	133/340
Dictyoglomus thermophilum H-6-12	56	148/340
Dictyoglomus thermophilum	55	151/340
Anaerocellum thermophilum DSM 6725	54	167/365
Caldicellulosiruptor sp. Rt69B.1	53	170/362
Clostridium thermocellum	52	155/325

3.3. Cloning, over-expression and purification of recombinant xylanase

The positive construct was confirmed and selected for PCR and sequencing analysis. A Ni-NTA histidine-binding resin was used for purification of the His-tagged recombinant XylX. The cell lysate and eluted fractions were analyzed by SDS-PAGE, as shown in Fig. 3. Under the induction of 1 mM IPTG at 28 °C, the yield of purified recombinant XylX demonstrated a minor portion of the total soluble protein, shown as the supernatant in the third lane of Fig. 3. Recombinant enzymes formed inclusion bodies, as shown in the fourth lane corresponding to the pellet. The soluble recombinant enzyme was effectively eluted from the column with 50 mM imidazole (lane E50) by loading the sample in the presence of 20 mM imidazole, shown in lane NB and lane W20. In a one-step purification, the majority of the induced recombinant enzyme was recovered, and a very high purity was demonstrated in lane E50 in Fig. 3. Purity was also verified by the observation that no further protein was eluted by 100 mM imidazole, as shown in the last lane (E100) in Fig. 3.

3.4. Effect of temperature and pH on xylanase activity

The activities of recombinant XylX at various pH and temperature values were measured using oat spelt xylan as the substrate. The reaction pH values were 4.0–11.0, and the temperature ranged from 40 to 75 °C. The purified XylX showed enzymatic activity over broad pH and temperature ranges, as shown in Fig. 4. The enzyme functioned reasonably well between 45 and 65 °C in the pH range of 5–9 (>60% activity). The activity optima were pH 7.0 and 60 °C, with the activity reaching 2392 IU/mg. The temperature and pH optima are rather consistent with those of xylanase isolated from all *Bacillus* spp. reviewed by Sá-Pereira et al. [29]. The optimal activity of XylX is higher than the values of xylanases from all *Bacillus* spp. [29] and of xylanases from the recently reported *Paenibacillus* sp. [20] and *Alicyclobacillus* sp. [7].

The activity of the enzyme decreased dramatically when the reaction was performed at pH 5. In contrast to acidic conditions, xylanase activity diminished gradually between pH 7 and pH 11. Compared to temperatures at or below 60 °C, the activity also decreased above 65 °C. However, minimal xylanase activity was observed at 78.7 IU/mg under very strict conditions of pH 11 and 75 °C.

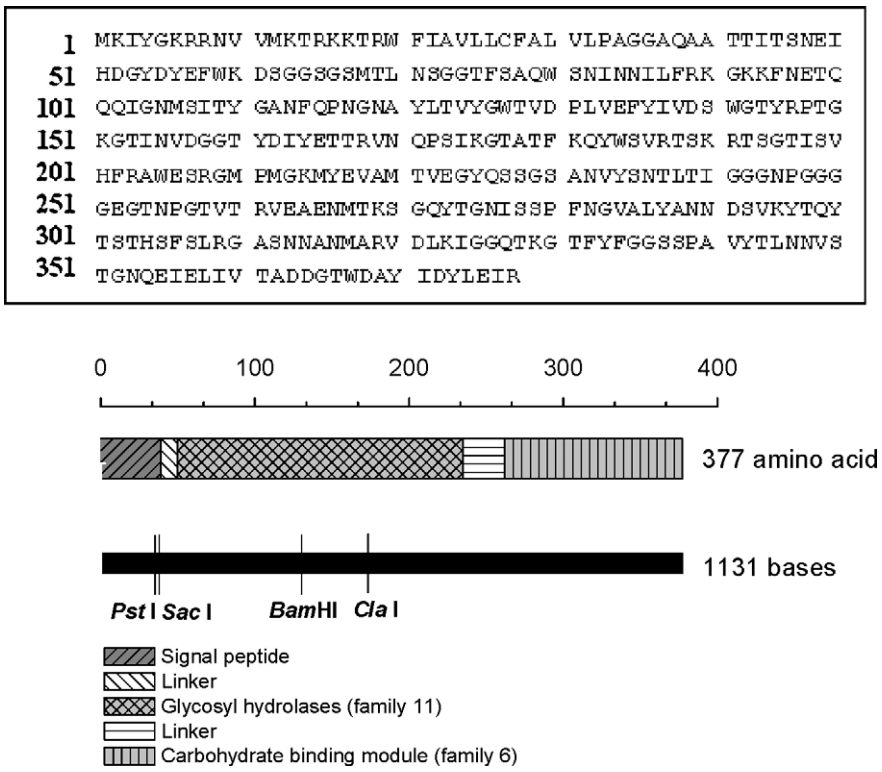

Fig. 2. Alignment of predicted amino acid sequences of the XylX xylanase (top). Schematic representation of the different domains and connecting sequences of XylX are indicated in the center. Cutting sites for restriction enzymes of the ORF are shown at the bottom.

3.5. Effect of temperature and pH on xylanase stability

The effect of temperature on recombinant XylX stability was investigated from 40 to 80 °C at pH 7 and is shown in the upper part of Fig. 5. Xylanase activity at pH 7 from 40 to 60 °C remained at more than 88.6% of its initial level for 8 h. At 70 and 80 °C, the retained activity of xylanase was around 11 and 8% of its initial level after 8 h, respectively. The projected half-lives of XylX xylanase activity from 40 to 60 °C are greater than those of most bacterial xylanases reviewed [30] and of the recently published *Alicyclobacillus* sp. A4 [7] and *Paenibacillus* sp. DG-22 xylanases [20]. Even at 70 and 80 °C, the interpolated half-lives were 120 and 50 min, respectively. XylX xylanase was more sensitive to metals than was the *Alicyclobacillus* sp. A4 xylanase.

The effect of pH on recombinant XylX stability was investigated from pH 4 to 11 at 60 °C and is shown in the lower part of Fig. 5. At the 8th hour, retained xylanase activity at 60 °C between pH 5 and pH 9 was more than 69.4% of the initial level. The enzyme stability at pH 9 was comparable to that of a 43-kDa xylanase from *Bacillus halodurans* S7 [31]. At pH 10 and 60 °C, the interpolated half-life of XylX was approximately 45 min, which is less than that value (3.5 h) of the aforementioned xylanase. Very little activity remained at both pH 4 and 11 at 60 °C. Again, the projected half-lives from pH 5 to 10 at 60 °C were much greater than those of most bacterial xylanases reviewed [30].

Wide pH adaptability and high thermostability render XylX an attractive candidate for biomass conversion applications in conjunction with acid or alkaline pretreatment and for many potential industrial applications. Stability at alkaline pH and 60–70 °C makes XylX particularly suitable for kraft pulp bleaching pretreatment,

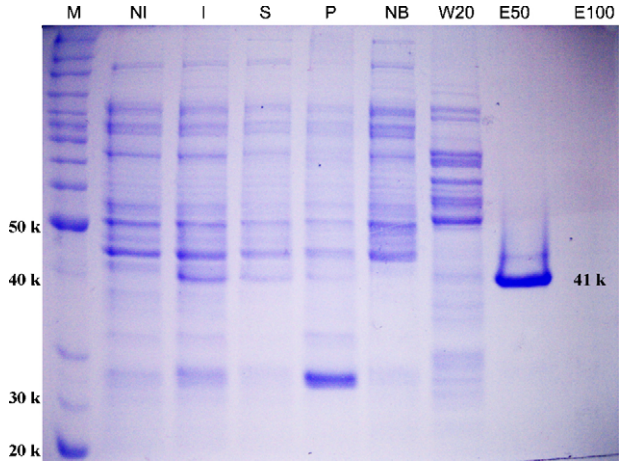

Fig. 3. XylX xylanase purification. M: marker; NI: non-induced; I: induced; S: supernatant; P: pellet; NB: non-binding protein; W20: washed by 20 mM imidazole; E50: eluted by 50 mM imidazole; E100: subsequently eluted by 100 mM imidazole. Molecular weights of markers (left) and xylanase (right) are denoted next to the respective band.

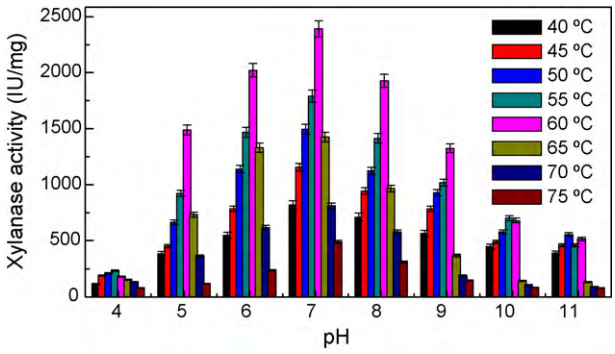

Fig. 4. Xylanase activities of recombinant XylX assayed under the respective pH values and temperatures.

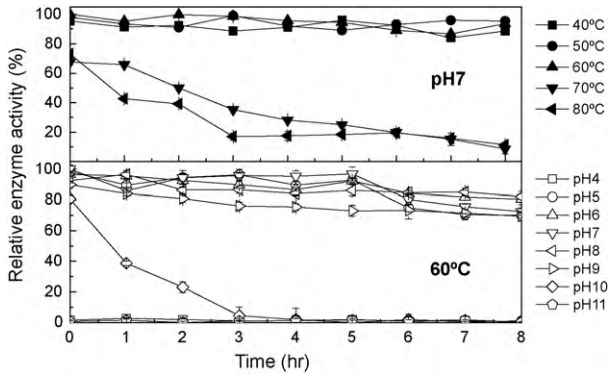

Fig. 5. Eight-hour stability at pH 7 (A) and at 60 °C (B) while assaying under optimal conditions for the His-tagged XylX xylanase.

because the conditions above are within the range of the practical prebleaching environment [12].

3.6. Effect of various additives on xylanase activity

The effects of various additives on XylX xylanase activity due to the presence of metal ions and other organic reagents are shown in Table 2. All metal ions used in the present study inhibited XylX xylanase activity to different extents. Mg^{2+}, K^{+} and Ca^{2+} slightly inhibited XylX xylanase activities. Cu^{2+}, Fe^{3+}, Zn^{2+}, Fe^{2+}, Pb^{2+} and Mn^{2+} also led to strong inhibition of XylX xylanase, with 20.29, 23.21, 29.01, 45.27, 48.75 and 65.44% activity remaining, respectively. XylX xylanase is more sensitive to metals than is *Alicyclobacillus* sp. A4 xylanase [7]. Hg^{2+} (5 mM) almost completely inhibited xylanase activity, and this effect might be due to the presence of the catalytically important cysteine [31]. Increasing stimulations with increasing TCEP and 2-mercaptoethanol are consistent with the observations of Sá-Pereira et al. for a *Bacillus subtilis* xylanase [32]. TCEP [33] and 2-mercaptoethanol counteract the oxidative effects of S–S linkages from cysteine residues, thereby stabilizing or even stimulating the xylanase [34]. Complete inhibition by N-bromosuccinimide, a tryptophan modifier, suggests the involvement of tryptophan residues in the active site of XylX, as reported for other xylanases [35,36].

3.7. Substrate specificity

By conducting a reducing sugar assay with different substrates, the substrate specificity of the purified XylX was investigated. The purified xylanase could degrade oat spelt xylan and birchwood xylan, with 100.00 ± 0.02% and 100.52 ± 0.04% relative activities. No relative activities for barley β-glucan, laminarin, Avicel, CMC and D-(+)-cellobiose were found. Trace relative activity for *p*-nitrophenyl-β-D-xylopyranoside was found to be 2.24 ± 0.01%. The absence of cellulase activity renders XylX an excellent candidate for pulp bleaching pretreatment [30].

Table 2
Effects of various additives on XylX xylanase activity by the presence of metals and other reagents.

Agent	Concentration	Residual activity (%)
Control	–	100 ± 1.77
Ca^{2+}	5 mM	88.18 ± 4.14
Co^{2+}	5 mM	85.30 ± 9.13
Hg^{2+}	5 mM	2.21 ± 0.77
Mn^{2+}	5 mM	65.44 ± 3.47
K^{+}	5 mM	96.39 ± 2.50
Mg^{2+}	5 mM	98.14 ± 1.21
Fe^{2+}	5 mM	45.27 ± 2.11
Fe^{3+}	5 mM	23.21 ± 2.31
Sr^{2+}	5 mM	85.43 ± 1.70
Zn^{2+}	5 mM	29.01 ± 1.16
Cu^{2+}	5 mM	20.29 ± 3.34
Ni^{2+}	5 mM	70.51 ± 3.19
Pb^{2+}	5 mM	48.75 ± 2.66
EDTA	5 mM	50.10 ± 3.23
Dithiothreitol	5 mM	67.93 ± 2.29
N-bromosuccinimide	5 mM	0.44 ± 0.03
TCEP[a]	5 mM	115.09 ± 23.51
TCEP[a]	0.5 mM	107.15 ± 20.63
2-Mercaptoethanol	0.5%	158.37 ± 10.23
2-Mercaptoethanol	0.05%	131.65 ± 16.20
Tritone X-100	0.25%	91.02 ± 7.78
SDS	0.25%	36.46 ± 0.90

[a] Tris-(2-carboxyethyl)-phosphine.

3.8. Kinetic parameters

The kinetic parameters of xylanase were determined from a Lineweaver–Burk double reciprocal plot of xylanase activity at 60 °C, pH 7. The K_m of the purified xylanase was 6.78 ± 0.59 mg/mL, and the V_{max} was 4953 ± 73 mol/min/mg. The kinetic parameters of XylX were higher than the values obtained for all *Bacillus* spp. xylanases reviewed by Beg et al. [4], except those from *Thermomyces* sp. and from *Thermotoga* sp.

3.9. Xylan hydrolysis product analysis

Hydrolysis products of oat spelt xylan and birchwood xylan by XylX were analyzed by HPLC, and the results are shown in Table 3. Fig. 6 shows the chromatogram of the hydrolysis products of oat spelt xylan by XylX at the 72nd hour. For oat spelt xylan, the concentrations of xylobiose (X2), xylotriose (X3), xylotetrose (X4), xylopentose (X5), and xylohexose (X6) increased with

Table 3
Xylan hydrolysis products (mg/mL) by XylX.

Reaction time (h)	X6	X5	X4	X3	X2	X1
Oat spelt xylan						
3	0.405	0.532	0.455	0.437	0.184	0.00
6	0.494	0.621	0.562	0.560	0.267	0.00
12	0.561	0.700	0.643	0.720	0.416	0.00
24	0.622	0.777	0.707	0.858	0.542	0.018
48	0.622	0.887	0.714	1.059	0.766	0.023
72	0.714	0.973	0.783	1.224	1.042	0.076
Birchwood xylan						
3	1.498	0.915	1.259	1.389	0.674	0.00
6	1.788	0.892	1.325	1.664	0.825	0.00
12	1.353	1.472	1.376	2.006	1.105	0.00
24	1.149	1.742	1.267	2.103	1.244	0.003
48	1.279	1.724	0.970	2.223	1.633	0.020
72	1.423	1.739	0.925	2.337	1.688	0.024

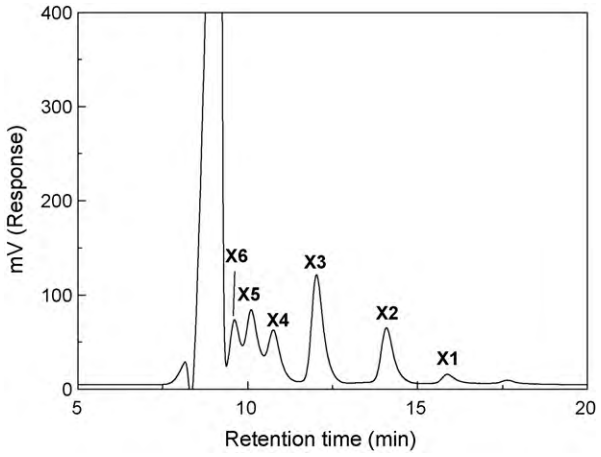

Fig. 6. Hydrolysis products of 10 mg/mL oat spelt xylan by XylX at the 72nd hour. X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetrose; X5: xylopentose; X6: xylohexose.

increasing hydrolysis time. Xylotriose was the major hydrolytic product of oat spelt xylan, with 1.224 mg/mL produced after 72 h of hydrolysis. Of the total hydrolysis products, 25.42% were xylotriose. The late appearance of xylose after the 12th hour correlated with the observation of trace relative activities for *p*-nitrophenyl-β-D-xylopyranoside in Section 3.7. More xylo-oligomers were produced by XylX hydrolysis from birchwood xylan than from oat spelt xylan, as shown in Table 3. Xylotriose was also the major hydrolytic product of oat spelt xylan, with 2.337 mg/mL produced after 72 h of hydrolysis. A total of 28.72% of the hydrolysis products were xylotriose. The above result demonstrates that XylX is an endo-type xylanase. It also validates that XylX can be employed to produce xylo-oligomers, which are increasingly important prebiotic food additives.

4. Conclusions

A high temperature, alkaline environment is best for isolating microorganisms and associated enzymes for specific industrial applications. This concept is also proposed in a recent study [37]. This is the first report of a 41-kDa xylanase identified from *Paenibacillus* sp. with only 73% homology to the next closest sequence in NCBI database. Wide pH adaptability, high thermostability, specific xylanase activity and a complete lack of cellulase activity render XylX an excellent candidate for many potential applications, especially kraft pulp bleaching pretreatment. The crude xylanase from *Paenibacillus* sp. BL-11 was already proved successful for application in hardwood kraft pulp bleaching pretreatment [38].

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