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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