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Characterization of thermostable Xyn10A enzyme from mesophilic *Clostridium acetobutylicum* ATCC 824

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Abstract A thermostable xylanase gene, *xyn10A* (CAP0053), was cloned from *Clostridium acetobutylicum* ATCC 824. The nucleotide sequence of the *C. acetobutylicum xyn10A* gene encoded a 318-amino-acid, single-domain, family 10 xylanase, Xyn10A, with a molecular mass of 34 kDa. Xyn10A exhibited extremely high (92%) amino acid sequence identity with Xyn10B (CAP0116) of this strain and had 42% and 32% identity with the catalytic domains of *Rhodothermus marinus* xylanase I and *Thermoascus aurantiacus* xylanase I, respectively. Xyn10A enzyme was purified from recombinant *Escherichia coli* and was highly active toward oat-spelt and Birchwood xylan and slightly active toward carboxymethyl cellulose, arabinogalactouronic acid, and various *p*-nitrophenyl monosaccharides. Xyn10A hydrolyzed xylan and xylooligosaccharides larger than xylobiose to produce xylose. This enzyme was optimally active at 60°C and had an optimum pH of 5.0. This is one of a number of related activities encoded on the large plasmid in this strain.

Keywords Xylanase · *Clostridium acetobutylicum* · Biomass · Thermostable · Hemicellulose · Xylose

Introduction

Hemicelluloses are low-molecular-mass non-cellulosic polysaccharides that are found in plant cell walls, where they are closely associated with the main structural polysaccharide, cellulose, and with the aromatic polymer, lignin. Xylan is the major component of the plant cell wall and the most abundant renewable hemicellulose [34]. Xylans are heterogeneous polysaccharides consisting of a main chain of 1,4-linked β-D-xylopyranosyl residues that often carry acetyl, arabinosyl, and glucuronosyl substituents [3]. Xylan is now regarded as a usable biomass component which can be converted to biofuels and chemicals.

Microbial endoxylanases have attracted considerable research interest in recent years, mainly due to their potential application in the food, animal feed, paper, and pulp industries [3, 6, 31, 34]. However, such applications generally prefer a thermostable and cellulase-free xylanase with broad pH and temperature optima [35, 36, 38].

Two kinds of enzymes are generally involved in microbial hydrolysis of the main chain, i.e., endoxylanase (1,4-β-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidase (β-D-xyloside xylohydrolase; EC 3.2.1.37) [3]. The action of the main xylanolytic enzyme, β-endoxylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is to convert the polymer xylan to xylosaccharides [3]. Many xylanase and xylosidase genes from fungi and bacteria have been analyzed and their encoded enzymes have been isolated and characterized [2, 7, 12, 15, 17, 18, 22, 27, 28, 30]. On the basis of amino acid sequence similarity, xylanases can be substantially divided into two groups, family 10 (formerly family F) and family 11 (formerly family G) of glycosyl hydrolases [6, 9]. The domains comprising these families of xylanases are quite different from each other in their structures and enzymatic properties [7, 9, 14]. It was reported that endoxylanases of family 10 liberate aldotetrauronic acid as the shortest acidic oligosaccharide from glucuroxylans, while family 11 endoxylanases release aldopentauronic acid as the shortest acidic oligosaccharide [4]. Similarly, family 10 endoxylanases release an isomeric xylotriose (Xyl_3) as the shortest product containing one β-(1–3)-linkage from rhodymenan, a seaweed xylan with β-(1–3), β-(1–4)-linkage, while endoxylanase of family 11 release an isomeric tetrasaccharide [4]. Also, family 10 xylanases hydrolyze acetyl xylan to a higher degree and release short acetylated oligosaccharides, whereas the degree of hydrolysis of acetyl xylan by

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family 11 xylanases is rather low; and neither acetylated xylobiose (Xyl_2) nor xylotriose (Xyl_3) is found to any extent among the products of hydrolysis [4, 35].

The acetone-producing and butanol-producing capabilities of several strains of *Clostridium* are well known. Solventogenic clostridia were once used as the foremost industrial producers of acetone and butanol. Such chemicals can find application as fuels, fuel extenders, or commodity chemicals [8, 11, 29]. During the 1950s and 1960s, the rapid expansion in the petrochemical industry resulted in a substantial reduction in the use of clostridial fermentation for solvent production, as crude oil-derived acetone and butanol can be produced more economically [25].

Genome sequence information for one of the best known solventogenic bacteria, *Clostridium acetobutylicum* ATCC 824, shows that it harbors a mega plasmid, 192 kb in size, which encodes many solvent-related genes [26]. The large plasmid also carries a number of polysaccharide-degradative genes. This strain encodes at least 12 xylanase enzymes; and the characterization of two of these enzymes has been reported [20, 21].

Here, we describe the cloning and expression of *xyn10A*, a first report on a xylanase gene encoding a single domain thermostable enzyme from *C. acetobutylicum*.

Materials and methods

Bacterial strain and plasmids

C. acetobutylicum ATCC 824 was used as the source of genomic DNA. The *Escherichia coli* host was DH10B [F^- *mcr*A Δ(*mrr*$^-$ *hsd*RMS$^-$ *mcr* BC) φ 80d *lac*ZΔM15, Δ *lac*X74 *end*A1 *rec*Al *deo*R Δ (*ara*, *leu*) 7697 araD139 *gal*U *gal* K *nup*G *rps*Lλ^-]. *E. coli* M15 (Qiagen, Valencia, Calif., USA) was used as the host for a derivative of plasmid pQE-30T, which yields a protein containing a six-His tag at its N-terminus (Qiagen). Plasmid pQE-30T, a derivative of pQE-30 (Qiagen), was used as the cloning vector to construct truncated derivatives of Xyn10A.

Construction of pMA1 and screening of xylanase-producing recombinants

Genomic DNA (80–100 ng) was used as a template for PCR with two synthetic oligonucleotide primers: primer 1 (5′-CGC*GGATCC*GCGGGTGTATTCCTTCTAACT-3′), containing a *Bam*H1 recognition sequence (in italics) , and primer 2 (5′-GG*CTGCAG*CTAATGTGATGCTAAGTAAGATCTT-3′), containing a *Pst*I recognition sequence (in italics) and a stop codon. The amplified DNA fragment was digested with *Bam*HI and *Pst*I and was cloned between the *Bam*HI and *Pst*I sites of pQE-30T. The recombinant DNA was introduced into *E. coli* DH10B and xylanase-producing recombinants were identified by the Congo red method [32]. Plasmid (pMA1) DNA was isolated from a xylanase-positive colony and was introduced into *E. coli* M15 for protein preparation. Plasmid pMA1 was characterized by sequence analysis to confirm that it contained the expected fragment. The plasmid contained a thrombin-recognition sequence (LVPRGS) between the Xyn10A polypeptide and the six-His tag.

Purification of Xyn10A

Xyn10A protein was purified from *E. coli* M15 harboring pMA1. Cultures were grown at 37°C in 2 l of LB medium supplemented with ampicillin (50 mg/l) and kanamycin (25 mg/l). When the optical density at 600 nm was 0.7, isopropyl-β-D-thiogalactopyranoside was added to a concentration of 1 mM. The incubation was continued for an additional 4 h and the cells were then harvested, suspended in 10 mM sodium phosphate buffer (pH 7.4), and disrupted by sonication for 15 min at 4°C. Cell debris were removed by centrifugation at 14,000 *g* and the cell-free extract was used for purification of Xyn10A. The majority of the protein was removed by precipitation at 55°C for 30 min. Chromatography on a Hi-Trap chelating HP column (5 ml; Amersham–Pharmacia Biotech, Piscataway, N.J., USA) was performed according to the supplier's protocol. The fractions were analyzed for xylanase activity [24]. Active fractions were combined and desalted by dialysis against 20 mM Tris-HCl (pH 7.5). The sample was treated with thrombin protease (10 units/mg of Xyn10A) and chromatographed on the Hi-Trap column. The affinity tag was removed and the active xylanase was collected, desalted by dialysis as described above, and further purified on a MonoQ HR5/5 column (0.5×5.0 cm; Amersham–Pharmacia Biotech) equilibrated with 20 mM Tris-HCl buffer (pH 8. 0). The column was washed with the same buffer and eluted at 0.5 ml/min with a linear gradient of 25.5 ml of 0–0.5 M NaCl in the same buffer. The purified Xyn10A enzyme without the six-His tag was used for a comparison of the enzymatic properties.

Protein analysis

Xylanase activity was measured in 50 mM sodium phosphate buffer (pH 6.3) or Britton and Robinson's universal buffer (50 mM phosphoric acid, 50 mM boric acid, 50 mM acetic acid, with the pH adjusted using 1 N NaOH in the presence of 1% oat-spelt xylan; Sigma Chemical Co., St. Louis, Mo., USA) and the reaction was performed for 10 min at 60°C. Activities against avicel (1%), carboxymethylcellulose (0.5%), laminarin (1%), lichenan (1%), and barley β-glucan (1%) were also determined. Reducing sugars released from the substrate were measured with the 3,5-dinitrosalicylic acid reagent as described by Miller for xylanase [24] and endoglu-

canase activities were measured by the Somogyi–Nelson method [39]. One unit of xylanase or endoglucanase activity was defined as the amount of enzyme releasing 1 μmol/min of xylose or glucose equivalent from the substrate. Chromogenic substrates were purchased from Sigma Chemical Co. β-Xylosidase, β-cellobiosidase, β-glucosidase, β-mannosidase, and β-furanosidase activities were assayed at 60°C with *p*-nitrophenyl-β-D-xylopyranoside (PNPX), *p*-nitrophenyl-β-D-cellobioside (PNPC), *p*-nitrophenyl-β-D-glucopyranoside (PNPG), *p*-nitrophenyl-β-D-mannoside (PNPM), and *p*-nitrophenyl-α-L-arabinofuranoside (PNPA) at 410 nm, respectively. Assay mixtures containing each substrate at 1 mM in 50 mM phosphate buffer (pH 6.3) were incubated for 10 min at 60°C; and the reactions were stopped by the addition of 1 M Na_2CO_3. One unit of enzyme activity toward PNP-derivatives was defined as the amount of enzyme liberating 1 μmol/min of *p*-nitrophenol.

We determined the concentration of protein by the Bradford method [5] with a protein assay kit from Bio-Rad (Hercules, Calif., USA), using bovine serum albumin as a standard.

SDS-PAGE was carried out using the method of Laemmli [19]. Zymogram analysis was done as described by Ali et al. [1], using a 10% SDS-PAGE gel containing 0.1% oat-spelt xylan.

Analysis of hydrolysis product

Xylooligosaccharides (xylobiose to xylohexaose, each 10 μg) were incubated with 0.15 units of the purified enzyme at 60°C overnight in phosphate buffer, pH 6.3. The hydrolysis products were separated by thin-layer chromatography, using DC-Fertigplatten SIL G-25 plates (Macherey–Nagel, Düren, Germany) developed with a solvent of nitroethane/ethanol/H_2O (1:3:1); and xylooligosaccharides were stained by spraying the plate with 20% sulfuric acid in ethanol.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper appears in the Genome Therapeutics Corporation web site and in the NCBI (NP_149217), and EMBL (Q97TP5) databases.

Results

Cloning of the *xyn10A* gene

Approximately 1,000 colonies were screened for xylanase production by the Congo red method. As a result, one clone was selected as a xylanase-producing recombinant. The structure of the plasmid isolated from this clone was confirmed by restriction enzyme site pattern and its DNA sequence. The plasmid, pMA1, appeared to encode a novel xylanase gene, *xyn10A*.

Homology of Xyn10A

Comparison of the amino acid sequence of Xyn10A with entries in the SWISSPROT database indicated the mature Xyn10A is a single-domain enzyme composed of a family 10 catalytic domain typical of glycosyl hydrolases. Figure 1 shows schematically the molecular architecture of Xyn10A, along with several related enzymes. The glycosyl hydrolase domain of Xyn10A, covering amino acid positions 36–318, had extensive sequence similarity with the catalytic domains of the other xylanases of family 10 (Fig. 2), e.g., 92% sequence identity with *C. acetobutylicum* Xyn10B, NP_149279 (NCBI), Q97TI5 (EMBL) [26].

Purification and characterization of the recombinant Xyn10A

The recombinant Xyn10A protein was purified six-fold from the cell-free extract of *E. coli* M15 (*p*MA1) by Hi-Trap affinity and MonoQ column chromatography. The final preparation yielded a single band on SDS-PAGE; and the molecular mass of the enzyme was estimated to be 34 kDa (Fig. 3a, lane 2), which is in good agreement with the value calculated from the deduced amino acid sequence of Xyn10A. The purified enzyme had high specific activity toward oat-spelt xylan (213 units/mg) and low activity toward several substrates, i.e., lichenan (55 units/mg), laminarin (16 units/mg). Analysis of its

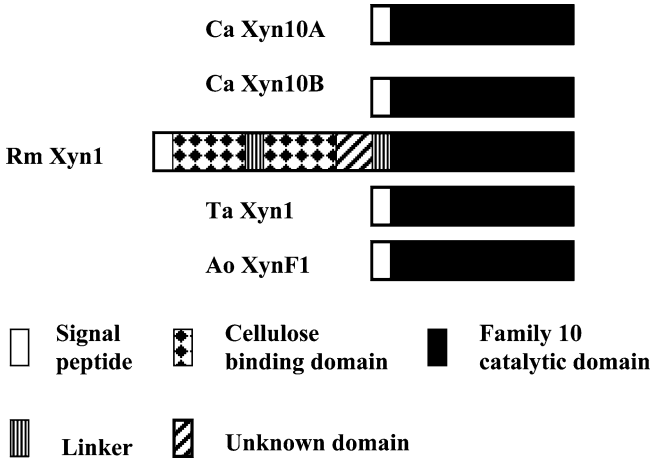

Fig. 1 Schematic diagram of protein domains and motifs in Xyn10A and some related enzymes: *C. acetobutylicum* (*Ca*) Xyn10A [26], *C. acetobutylicum* (*Ca*) Xyn10B [26], *Rhodothermus marinus* (*Rm*) Xyn1 [12], *Thermoascus aurantiacus* (*Ta*) Xyn1 [22], *Aspergillus oryzae* (*Ao*) XynF1 [16]. *Boxes* Domains with significant amino acid sequence similarity display the *same pattern*. The signal peptide indicates the common N-terminal secretion signal on these xylanases. The family 10 catalytic domain indicates the glycosyl hydrolase domain

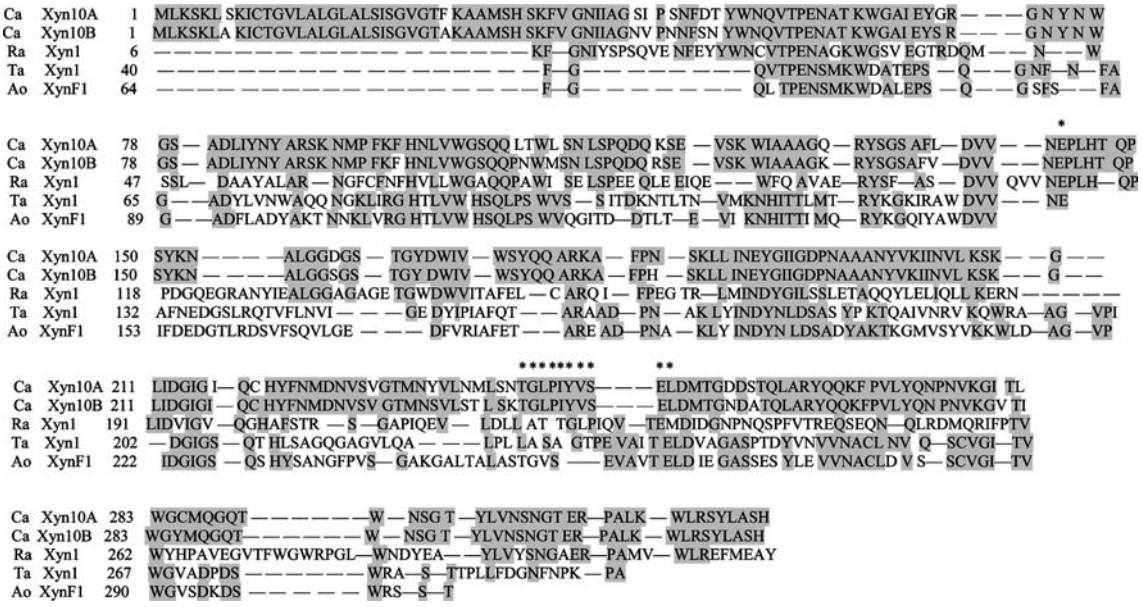

Fig. 2 Alignment of family 10 catalytic domains: *C. acetobutylicum* (*Ca*) Xyn10A, *C. acetobutylicum* (*Ca*) Xyn10B, *R. marinus* (*Rm*) Xyn1, *T. aurantiacus* (*Ta*) Xyn1, *A. oryzae* (*Ao*) XynF1. Amino acids which are conserved in at least two of five sequences are *highlighted*. – Gap left to improve alignment. *Numbers* refer to the position within the protein of the first amino acid of the respective line. Sequences are numbered from Met-1 of the translated protein. The consensus active site motif (GLPIYVSELD) is marked with *asterisks* and contains the conserved Glu that acts as a nucleophile in the active site [22]. The additional conserved Glu residue (Glu-142 of the *C. acetobutylicum* sequence) involved in the catalytic site is also indicated with an *asterisk*

ability to hydrolyze *p*-nitrophenol derivatives of various sugars showed activities on the following substrates: arabinogalacturonic acid (0.34 units/mg), carboxymethyl cellulose (0.10 units/mg), PNPX (0.16 units/mg), PNPG (0.11 units/mg), PNPM (0.1 units/mg), and PNPA (0.63 units/mg). The purified Xyn10A was unable to degrade polygalacturonic acid and had no galactopyranosidase and fucopyranosidase activities (Table 1).

The action of the enzyme on oat-spelt xylan, Birchwood xylan, and xylooligosaccharides was analyzed. As shown in Fig. 5, Xyn10A hydrolyzed xylan to yield the monosaccharide xylose. When xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose were treated with the enzyme, the only major product was xylose. The enzyme was less active on xylobiose. The hydrolysis product from Birchwood xylan was similar to the hydrolysis product from oat-spelt xylan. The enzyme activity was strongly inhibited by $HgCl_2$ and was partly inhibited by $FeCl_3$, KCl, $CuSO_4$, $MgCl_2$, $AlCl_3$, and $CdCl_2$ at concentrations of 1 mM. The enzyme was maximally active around pH 5.0 when the enzyme activity was assayed by 10-min incubation at 60 °C in

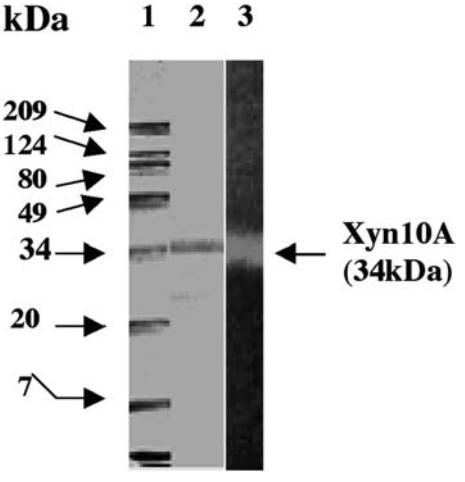

Fig. 3 SDS-PAGE and zymogram of the purified Xyn10A from *E. coli*. *Lane 1* Protein molecular mass size markers, *lane 2* purified Xyn10A, *lane 3* gel stained for xylanase activity [1]

Table 1 Substrate specificity of Xyn10A. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol/min of xylose, glucose, or *p*-nitrophenol equivalent from xylan, cellulose, or *p*-NP substrates, respectively. Assays were conducted in 50 mM phosphate buffer at pH 6.3 at 60°C. *N* Not active

Substrate	Specific activity (units/mg)
Birchwood xylan (β 1–4)	266
Oat-spelt xylan (β 1–4)	213
Avicel (β 1–4)	N
Carboxymethyl cellulose (β 1–4)	0.10
Lichenan (β 1–4; β1–3)	55
Laminarin (β 1–4)	16
Polygalactouronic acid (α1–4)	N
Arabinogalacturonic acid (β1–3; β1–6)	N
Barley-β-glucan (β 1–4; β1–3)	N
PNPX	0.16
PNPC	3.5
PNPG	0.11
p-nitrophenyl-β-D-galactopyranoside	N
PNPM	0.10
PNPA	0.63
p-nitrophenyl-β-D-fucopyranoside	N

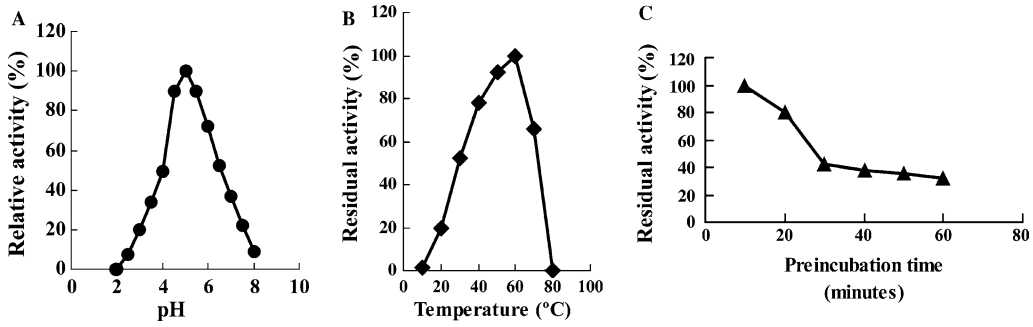

Fig. 4 Enzymatic properties of Xyn10A. For determination of optimum pH, the enzyme was incubated in Britton and Robinson's universal buffer at various pHs (**A**). The temperature optimum was determined by incubating the enzyme and substrate for 10 min in the range 10–80°C, using phosphate buffer at pH 6.3 (**B**). The temperature stability of the enzyme was determined by incubating the enzyme for 10–60 min at 60 °C in phosphate buffer (pH 6.3) without substrate, prior to assaying the residual activity of the enzyme (**C**). Oat-spelt xylan was used a substrate in all assays in this figure

Britton and Robinson's universal buffer solutions at various pHs (Fig. 4 a).

The enzyme was stable in the pH range 3.0–8.0 when incubated at 25°C for 12 h without substrate. The effects of temperature on the activity and stability of the enzyme were examined. The optimum temperature for activity was found to be 60°C (Fig. 4 b). The enzyme was completely stable at 60°C for 10 min at pH 5.0 (Fig. 4 c). Xyn10A was also stable at 25°C for 36 h in the absence of substrate.

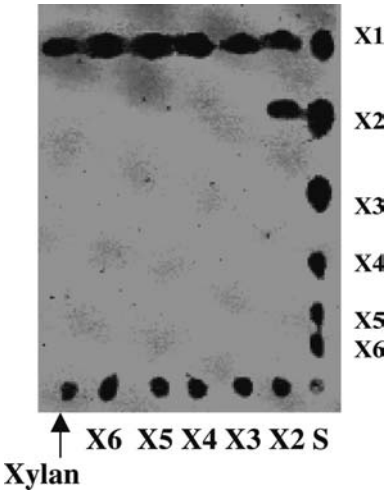

Fig. 5 Thin-layer chromatography of hydrolysis products from xylan and xylooligosaccharides. *Lane S* is composed of a standard mixture of xylose (*X1*), xylobiose (*X2*), xylotriose (*X3*), xylotetraose (*X4*), xylopentaose (*X5*), and xylohexaose (*X6*). Incubation of each standard under these conditions without enzyme yielded the initial compound. Each oligosaccharide (10 μg, *X2–X6*) was incubated with purified Xyn10A (0.15 units) overnight in phosphate buffer, pH 6.3, at 60°C and the hydrolyzates were analyzed by thin-layer chromatography as described in the Materials and methods. The first six lanes correspond to treatments of xylan, X6, X5, X4, X3, and X2, respectively, with the enzyme

Discussion

There are two endoxylanases, xylanase 10A and xylanase 10B, encoded on the megaplasmid, pSOL1, of *C. acetobutylicum* ATCC 824 [26]. Microbes that are strong utilizers of xylan are known to produce xylanases of different families, i.e., families 10 and 11, with different enzyme properties [6]. The gene *Xyn10A* encoded a xylanase consisting of a N-terminal signal peptide [37] followed by a single glycosyl hydrolase catalytic domain (Fig. 2). The function of the domain was confirmed by expression of active truncated Xyn10A in *E. coli*. The domain contains the consensus pattern (GLPIYVELD) of the active site (Fig. 2) of family 10 glycosyl hydrolases, according to the classification of Henrissat [9]. The length of the catalytic domain is 279 amino acids and it shows 42% identity with *Rhodothermus marinus* Xyn1 [12], 32% identity with the catalytic domain of *Thermoascus aurantiacus* xylanase I [22], and 32% identity with *Aspergillus oryzae* xylanase F1 [16].

The crystal structures of xylanases have been determined [7, 14, 22]. The catalytic domain of thermophilic *T. aurantiacus* xylanase I has an eight-fold α/β-barrel fold and two catalytic residues have been identified [22]: Glu-237 (identified as the nucleophile) and Glu-131 (identified as the acid/base amino acid involved in catalysis). In *C. acetobutylicum*, these residues correspond to Glu-250 and Glu-143, respectively (Fig. 2).

Recombinant Xyn10A, in addition to degrading xylan, also hydrolyzed carboxymethylcellulose, PNPX, PNPG, PNPC, and PNPM. This wide substrate specificity is common to all the enzymes in family 10, e.g., *R. marinus* Xyn1 [13] and *T. aurantiacus* xylanase I [23]. Xyn10A also can degrade lichenan and laminarin, which is less commonly reported for family 10 enzymes.

In the hydrolysis experiments with xylooligomers, xylose was formed as the final product (Fig. 5) This hydrolysis pattern is very similar to many previously analyzed xylanases [10, 15, 28, 33, 40], including two from the same glycosyl hydrolase family [10, 15]. This hydrolysis pattern differs from that found for *R. marinus* Xyn1 [13], where xylobiose and xylotriose were the main hydrolysis products reported. The previously reported *C. acetobutylicum* XynA (molecular mass 65,000 Da) hydrolyzed larchwood xylan randomly and yielded xylohexaose, xylopentaose, xylotetraose, xylotriose, and

xylobiose as end-products, while *C. acetobutylicum* XynB (molecular mass 29,000 Da) also hydrolyzed xylan randomly and produced xylotriose and xylobiose as end-products [21]. Observations outlined in those findings are quite different from those found for the *C. acetobutylicum* Xyn10A studied here.

In conclusion, a thermostable xylanase, Xyn10A, from *C. acetobutylicum* belonging to glycosyl hydrolase family 10 has been characterized and shown to have a wide substrate specificity. Xyn10A may serve an important role in the hydrolysis of xylan from plant cell walls.

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