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ORIGINAL PAPER

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Thermostable xylanase10B from *Clostridium acetobutylicum* ATCC824

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Abstract The *Clostridium acetobutylicum* xylanase gene *xyn10B* (CAP0116) was cloned from the type strain ATCC 824, whose genome was recently sequenced. The nucleotide sequence of *C. acetobutylicum xyn10B* encodes a 318-amino acid protein. Xyn10B consists of a single catalytic domain that belongs to family 10 of glycosyl hydrolases. The enzyme was purified from recombinant *Escherichia coli*. The Xyn10B enzyme was highly active toward birchwood xylan, oat-spelt xylan, and moderately active toward avicel, carboxymethyl cellulose, polygalacturonic acid, lichenan, laminarin, barley-β-glucan and various *p*-nitrophenyl monosaccharides. Xyn10B hydrolyzed xylan and xylooligosaccharides to produce xylobiose and xylotriose. The pH optimum of Xyn10B was 5.0, and the optimal temperature was 70°C. The enzyme was stable at 60°C at pH 5.0–6.5 for 1 h without substrate. This is one of a number of xylan-related activities encoded on the large plasmid in *C. acetobutylicum* ATCC 824.

Keywords Biomass · Hemicellulose · Xylan · Enzyme · Xylose

Introduction

Biomass can serve as a natural source for chemical production. The short production cycle is a positive feature for its potential in meeting future demands for fuel and chemicals. Naturally occurring plant biomass consists of 20–30% hemicellulosic materials [7, 27]. In recent years, bioconversion of hemicellulose has received much attention because of its practical applications in various agro-industrial processes, such as efficient conversion of hemicellulosic biomass to fuels and chemicals [7]. Digestion of hemicellulose is also important in delignification of paper, pulp, digestibility enhancement of animal feed, clarification of juices, and improvement in the consistency of beer [14, 29, 30, 34]. Xylans are the major constituent of hemicellulose and are heterogeneous polysaccharides consisting of a main chain of 1,4-linked β-D xylopyranosyl residues that often carry acetyl, arabinosyl and glucuronosyl substituents [27].

Microbial endoxylanases have generated considerable research interest mainly due to their prospective application in food, animal feed, paper and pulp industries [3, 5, 30]. Thermostable xylanases are especially attractive for industrial applications [28, 30, 31, 34].

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) is to convert the polymer xylan to xylosaccharides.

Many xylanase and xylosidase genes from fungi and bacteria have been analyzed and their encoded proteins have been characterized [2, 9, 10, 13, 23]. On the basis of amino acid sequence similarity, xylanases can be substantially divided into two groups of glycosyl hydrolases: family 10 (formerly family F) and family 11 (formerly family G) [8]. The catalytic domains comprising these families of xylanases are quite different from each other in their structures and enzymatic properties [5, 8, 11].

The clostridia are a diverse group of Gram-positive, rod shaped anaerobes that include several toxin-producing pathogens (*Clostridium difficile*, *C. botulinum*, *C. tetani*, *C. perfringens*) and a large number of terrestrial species that produce acetone, butanol, ethanol, isopropanol, and organic acids through fermentation of a variety of carbon sources [12, 24, 25, 33]. *C. acetobutylicum* ATCC 824 has been utilized by industry for acid and solvent production, and acetone and butanol, which are produced in large amounts, have been used as fuel sources, precursors for explosives production, and various

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other uses [33]. *C. acetobutylicum* has a four megabase pair chromosome and harbors a large plasmid, 192 kb pairs in size, which encodes many solvent-related genes and also carries a number of genes encoding polysaccharide degradative enzymes [22]. Strain ATCC 824 can grow on xylan, and the isolation and characterization of two xyalanases has been reported [17, 18]. Based on the genomic sequence of this strain, it appears to encode a number of xylanases [22].

Here we describe the cloning and expression of *xyn10B*, the second xylanase gene encoding a single domain thermostable enzyme classified in family 10 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 strain used was DH10B [F^- *mcr*A Δ (*mrr*$^-$ *hsd*RMS$^-$ *mcr* BC) ϕ 80d *lac*ZΔ M15Δ, *lac*X74 *end*A1 *rec*Al *deo*R Δ (*ara*, *leu*) 7697 araD139 *gal*U *gal* K *nup*G *rps*Lλ^-]. *Escherichia coli* M15 (Qiagen, Valencia, Calif.) was used as the host for a derivative of plasmid pQE-30T, which generates a protein containing a six-His tag at its *N*-terminus. Plasmid pQE-30T was used as the cloning vector to construct truncated derivatives of Xyn10B. All bacterial culture media reagents were supplied by either Difco (Detroit, Mich.) or Sigma (St. Louis, Mo.). Restriction enzymes and reagents for PCR were purchased from New England Biolabs (Beverly, Mass.).

Genomic DNA (80–100 ng) was used as a template for PCR with two synthetic oligonucleotide primers, primer 1 containing a *Bam*HI recognition sequence (underlined), 5′-CGC GGA TCC GCG ACT GCT AAA GCT GCA ATG-3′, and primer 2 containing a *Kpn*I recognition sequence (underlined) and a stop codon, 5′-CGG GGT ACC CCG TTA ATG ACT AGC TAA ATA GAA T-3′. The amplified DNA fragment was digested with *Bam*HI and *Kpn*I, and inserted between the *Bam*HI and *Kpn*I sites of pQE-30T, to generate pQE30T -*xyn10B*, designated pMA2. The recombinant molecules were introduced into *E. coli* DH10B and xylanase-producing colonies were selected by the Congo red method [26]. Plasmid (pMA2) DNA was isolated from xylanase-positive colonies and was characterized to show it had the expected sequence, and it was then introduced into *E. coli* M15 for protein preparation. The plasmid pMA2 contains a thrombin-recognition sequence (LVPRGS) between the Xyn10B polypeptide and the six-His tag.

Purification of Xyn10B

To produce the recombinant protein, a 1 l culture of *E. coli*/pMA2 was grown to mid-log phase (absorbance at 600 nm, 0.7) and then 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. The incubation was continued for 4 h to allow cells to produce the recombinant protein. Cells were harvested, suspended in 10 mM sodium phosphate buffer (pH 7.4), and disrupted by sonication for 15 min at 4°C. Cell debris was removed by centrifugation at 14,000 *g*. The cell-free extract was used for purification of Xyn10B. The majority of the *E. coli* proteins was removed by treatment of the crude extract at 60°C for 15 min followed by sedimentation of the precipitate by centrifugation. Chromatography on a HiTrap ChelatingHP column (5 ml; Amersham-Pharmacia, Piscataway, N.J.) was performed according to the supplier's protocol. Active fractions were identified by the assay of Miller [21], combined and desalted by dialysis against 20 mM Tris-HCl (pH 7.5). The His_6 tag was removed from the recombinant protein by thrombin protease digestion (10 U/mg Xyn10B) and the digested protein was chromatographed on the HiTrap column as before. Xyn10B was further purified on a MonoQ HR5/5 column (0.5 ×5 cm; Amersham-Pharmacia) 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 0–0.5 M linear gradient of NaCl in the same buffer. The purified Xyn10B without His_6tag, was used for analysis of 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). The pH was adjusted with 1 *N* NaOH in the presence of 1% oat-spelt xylan (Sigma) and the reaction was performed for 10 min at 60°C. Activities against avicel (1%), carboxymethyl cellulose (CMC; 0.5%, w/v), laminarin (1%, w/v), lichenan (1%, w/v), polygalacturonic acid (1%), arabinogalacturonic acid (1%), 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 [21], and endoglucanase activities were measured by the Somogyi-Nelson method [32]. One unit of xylanase and endoglucanase activities was defined as the amount of enzyme releasing 1 μmol xylose or glucose equivalent per minute from the substrate. β-Xylosidase, β-cellobiosidase, β-glucosidase, β-mannoside 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-galactopyranoside (PNPGL), *p*-nitrophenyl-β-D-glucopyranoside (PNPG), *p*-nitrophenyl-β-D-mannoside (PNPM), and *p*-nitrophenyl-α-L-arabinofuranoside (PNPA) at 410 nm, respectively. *p*-Nitrophenyl substrates were obtained from Sigma. 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 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 *p*-nitrophenol per minute.

We determined the concentration of protein by the Bradford method [4] with a protein assay kit from Bio-Rad (Hercules, Calif.), using bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) used the method of Laemmli [15]. Zymogram analysis was performed as described by Ali et al. [1] using a 10% SDS-PAGE gel containing 0.1% oat-spelt xylan.

Analysis of hydrolysis products

Xylan and xylooligosaccharides (xylobiose to xylohexaose, each 10 μg) were incubated with 0.1 U purified enzyme at 60°C overnight. Thin-layer chromatography of the hydrolysis products was performed on a DC-Fertigplatten SIL G-25 (Macherey-Nagel, Düren, Germany) developed with a solvent of nitroethane:ethanol:H_2O (1:3:1). Xylooligosaccharides were stained by spraying the plate with a solution of 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 databases as part of the pSOL1 sequence AE001438. The protein sequence is available at NCBI (NP_149279) and at EMBL (Q97TI5).

Results

Cloning of the *xyn10B* gene

Approximately 550 colonies were screened for xylanase production by the Congo red method. As a result, one was selected as a xylanase-producing recombinant. The plasmid isolated from this colony was confirmed by restriction enzyme site pattern and its DNA sequence. The plasmid pMA2 contained an insert encoding the xylanase gene, *xyn10B*, which conferred high xylanase activity on the *E. coli* host.

Purification and characterization of the recombinant Xyn10B

The recombinant Xyn10B was purified 8-fold from the cell-free extract of *E. coli* M15/pMA2 by Ni-Trap affinity and MonoQ column chromatography. The final preparation gave a single band on SDS-PAGE. The molecular weight of the enzyme was estimated to be 34 kDa (Fig. 1, lane 2), which is in good agreement with the value calculated from the deduced amino acid sequence of recombinant Xyn10B. The purified enzyme had high specific activity toward birchwood xylan (β1–4) (1,431 U/mg), oat-spelt xylan (β1–4) (1,128 U/mg) and low activity toward several other substrates i.e., lichenan (β1–4; β1–3) (35 U/mg), laminarin (β1–4) (25 U/mg). Analysis of its ability to hydrolyze other substrates showed activity towards the following compounds: polygalacturonic acid, CMC (β1–4), and PNPX, PNPC, PNPGL, PNPG, PNPM, PNPA *p*-nitrophenyl-β-D-fucopyranoside (PNPF) (Table 1).

The enzyme was maximally active around pH 5.0 when the enzyme activity was assayed at 70°C in Britton and Robinson's universal buffer solutions at various pH values (Fig. 2a). The enzyme was stable in the pH range of 3.0–10.0 when incubated at 25°C for 24 h in the same buffer solutions without the substrate. The effect of temperature on the activity and stability of the enzyme

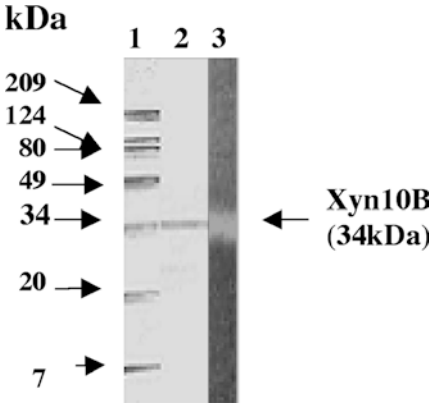

Fig. 1 SDS-PAGE and Zymogram of purified Xyn10B from *Escherichia coli*. Lanes: *1* Protein molecular weight size markers, *2* purified Xyn10B, *3* gel was stained for xylanase activity as described [1]

Table 1 Substrate specificity of Xyn10B. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol xylose, glucose, or *p*-nitrophenol (*p*-NP) equivalent per minute from the polysaccharide or *p*-NP substrate. Assays were conducted in 50 mM phosphate buffer at pH 6.3 at 60°C

Substrate	Specific activity (U/mg)
Birchwood xylan (β1-4)	1,431
Oat-spelt xylan (β1-4)	1,128
Carboxymethyl cellulose (β1-4)	101
Avicel (β1-4)	170
Lichenan (β1-4; β1-3)	35
Laminarin (β1-4)	25
Polygalacturonic acid (β1-4)	179
Arabinogalacturonic acid (β1-3; β 1-6)	N[a]
Barley-β-glucan (β1-4; β1-3)	24
p-Nitrophenyl-β-D-xylopyranoside	102
p-Nitrophenyl-β-D-cellobioside	133
p-Nitrophenyl-β-D-glucopyranoside	49
p-Nitrophenyl-β-D-galactopyranoside	22
p-Nitrophenyl-β-D-mannoside	40
p-Nitrophenyl-α-D-arabinofuranoside	92
p-Nitrophenyl-β-D-fucopyranoside	38

[a]Not active

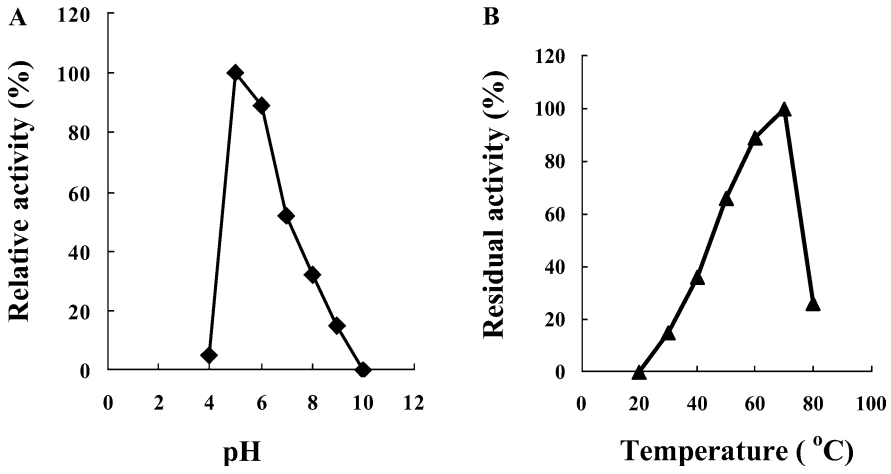

Fig. 2a, b Enzymatic properties of Xylanase 10B. For determination of the optimum pH, Xyn10B enzyme was incubated in Britton and Robinson's universal buffer at various pH values at 60°C (**a**). The temperature optimum, was determined by incubating the enzyme and substrate for 10 min in the range of 10–80°C using phosphate buffer, pH 6.3 (**b**). Oat-spelt xylan was used as substrate for these experiments

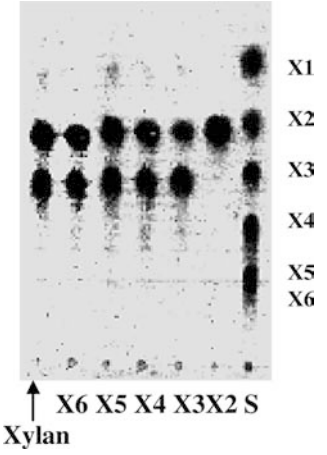

Fig. 3 Thin layer chromatography of hydrolysis products from xylan and xylooligosaccharides. Oat-spelt xylan, or each oligosaccharide (10 μg, X2–X6), was incubated with purified Xyn10B (0.1 U) overnight in phosphate buffer, pH 6.3 at 60°C, and the hydrolyzates were analyzed by thin layer chromatography in the vertical dimension. After digestion, a portion of the hydrolysate was spotted near the bottom of the plate at the location indicated. Lanes: *X2* xylobiose hydrolysate, *X3* xylotriose hydrolysate, *X4* xylotetrose hydrolysate, *X5* xylopentose hydrolysate, *X6* xylohexose hydrolysate, *S* a mixture of authentic oligosaccharides containing: xylose (*X1*); xylobiose (*X2*); xylotriose (*X3*); xylotetrose (*X4*); xylopentose (*X5*); xylohexose (*X6*)

was examined. The optimum temperature for activity was found to be 70°C (Fig. 2b). The enzyme was completely stable at 60°C for 1 h at pH 5.0–6.5 when buffers in this pH range were examined. The enzyme activity was strongly inhibited by $HgCl_2$ and was partly inhibited by $FeCl_3$, $CuSO_4$, and $CdCl_2$ at a concentration of 1 mM.

The action of the enzyme on xylan (oat-spelt and birchwood xylan) and xylooligosaccharides was analyzed. As shown in Fig. 3, the products of Xyn10B were mainly xylobiose and xylotriose. Xyn10B was not active on xylobiose and less active with xylotriose than on longer xylooligosaccharides. The recombinant Xyn10B hydrolyzed xylan to yield xylobiose and xylotriose. The hydrolysis products from birchwood xylan were similar to the hydrolysis products from oat-spelt xylan.

Discussion

The crystal structures of several xylanases have been determined [6, 11, 19]. The catalytic domain of thermophilic *Thermoascus aurantiacus* xylanase I has been analyzed and shown to have an 8-fold α/β-barrel fold (TIM-barrel) and two catalytic residues were identified [19]. Residue Glu-237 was identified as the nucleophile, and Glu-131 was identified as the acid/base amino acid involved in catalysis. In *C. acetobutylicum* the corresponding Glu residues are conserved in xylanases 10A and 10B. The positioning of active site residues has been discussed in a *Bacillus circulans* xylanase [16]. Two histidine residues, His83 and His209, are involved in a hydrogen-bond network in the vicinity of the active site glutamate residues of *T. aurantiacus* xylanase I. In *C. acetobutylicum* the corresponding residues, His97 and His220, are conserved.

Xyn10B, in addition to degrading xylan, also hydrolyzed avicel, polygalacturonic acid, barley β-glucan, CMC, PNPX, PNPC, PNPGL, PNPG, PNPM, PNPA, and PNPF. The wide substrate specificity is common to enzymes in family 10, e.g., *Rhodothermus marinus*, Xyn1 [30], *T. aurantiacus* xylanase I [19, 20], *Clostridium stercorarium* XynC (, renamed Xyn10B) [2], *Clostridium thermocellum* XynX [13]. Xyn10B also can degrade lichenan and laminarin, a property that is limited to family 10. To our knowledge Xyn10B shows the widest substrate specificity reported to date.

In the hydrolysis experiments with xylooligomers, xylobiose and xylotriose were formed as the final products. The hydrolysis pattern generated by xylanase 10B (deposited with NCBI as CAP0116, NP_149279) is different from that found for Xyn10A (NCBI: CAP0053, NP_149217) from this *C. acetobutylicum*. *C. acetobutylicum* Xyn10A was active on xylotriose, which was not completely cleaved by *C. acetobutylicum* Xyn10B. These two xylanases of *C. acetobutylicum* should act on xylan in a concerted manner to hydrolyze it more effectively. Lee et al. [18] reported that *C. acetobutylicum* endoxylanase XynA (molecular weight 65 kDa) had an opti-

mum pH of 5.0 and an optimum temperature of 50°C, and could hydrolyze larchwood xylan randomly, yielding xylohexaose, xylopentaose, xylotetraose, xylotriose, and xylobiose as the end products. The other enzyme reported from *C. acetobutylicum*, XynB, (molecular weight 29 kDa) had an optimum pH of 5.5–6.0 and an optimum temperature of 60°C. This lower molecular weight enzyme also hydrolyzed xylan randomly, yielding xylotriose and xylobiose as the end products. The N-terminal amino acid sequence was not determined for these enzymes. There appear to be differences in substrate specificity between both the enzymes reported and xylanse 10B [18].

A thermostable xylanase, Xyn10B, from *C. acetobutylicum* belonging to glycosyl hydrolase family10 has been isolated and shown to have broad substrate specificity. This xylanase 10B of *C. acetobutylicum* may be important for hydrolysis of xylan in plant cell wall in cooperation with Xyn10A.

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References

1. Ali BRS, Romaniec MM, Hazlewood GP, Freedman RB (1995) Characterization of the subunits in an apparently homogeneous subpopulation of *Clostridium thermocellum* cellulosomes. Enzyme Microb Technol 17:705–711
2. Ali MK, Fukumura M, Sakano K, Karita S, Kimura T, Sakka K, Ohmiya K (1999) Cloning, sequencing, and expression of the gene encoding the *Clostridium stercorarium* xylanase C in *Escherichia coli*. Biosci Biotechnol Biochem 63:1596–1604
3. Biely P (1985) Microbial xylanolytic systems. Trends Biotechnol 3:286–290
4. Bradford MM (1997) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of dye binding. Anal Biochem 86:142–146
5. Coutinho PM, Henrissat B (1999) Carbohydrate-active enzymes: an integrated database approach. In: Recent advances in carbohydrate bioengineering. Royal Society of Chemistry, Cambridge, pp 3–12
6. Derewenda U, Swenson L, Green R, Wei Y, Morosoli R, Shareck F, Kluepfel D, Derewenda ZS (1994) Crystal structure at 2.6-Å resolution, of the *Streptomyces lividans* xylanase A, a member of the F family of β-1,4-D-glycanases. J Biol Chem 269:20811–20814
7. Grohmann K, Wymann CE, Himmel ME (1992) Potential for fuels from biomass and waste. In: Rowell RM, Schults TP, Narayan R (eds) Emerging technologies for materials and chemicals from biomass. American Chemical Society, Washington, D.C., pp 354–392
8. Henrissat B, Bairoch A (1960) Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316:695–696
9. Karlsson NE, Roxa EB, Holst O (1997) Cloning and sequence of a thermostable multidomain xylanase from the bacterium *Rhodothermus marinus*. Biochim Biophys Acta 1353:118–124
10. Karlsson NE, Dahlberg L, Torto L, Gorton L, Holst O (1998) Enzymatic specificity and hydrolysis pattern of the catalytic domain of the xylanase Xyn1 from *Rhodothermus marinus*. J Biotechnol 60:23–35
11. Katsube Y, Hata Y, Yamaguchi H (1990) Estimation of xylanase active site from crystalline structure. In: Ikehara M (ed) Protein engineering, Japan Scientific Societies Press, Tokyo, pp 91–96
12. Keis S, Bennett CF, Ward VK, Jones DT (1995) Taxonomy and phylogeny of industrial solvent-producing clostridia. Int J Syst Bacteriol 45:693–705
13. Kim H, Jung KH, Pack MY (2000) Molecular characterization of XynX, a gene encoding a multidomain xylanase with a thermostabilizing domain from *Clostridium thermocellum*. Appl Microbiol Biotechnol 54:521–527
14. Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411–456
15. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
16. Lawson SL, Wakarchuck MW, Withers SG (1997) Positioning the acid/base catalyst in a glycosidase: studies with *Bacillus circulans* xylanase. Biochemistry 36:2257–2265
17. Lee SF, Forsberg CW, Gibbins LN (1985) Xylanolytic activity of *Clostridium acetobutylicum*. Appl Environ Microb 50:1068–1076
18. Lee SF, Forsberg CW, Rattray M (1987) Purification and characterization of two endoxylanases from *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 53:644–650
19. Lo Leggio L, Kalogiannis S, Bhat MK, Pickersgill RW (1999) High resolution structure and sequence of *T. aurantiacus* xylanase I: implications for the evolution of thermostability in family 10 xylanases and enzymes with (beta) alpha-barrel architecture. Proteins 36:295–306
20. Lo Leggio L, Kalogiannis S, Eckert K, Teixeira SCM, Bhat MK, Andrei C, Pickersgill RW, Larsen S (2001) Substrate specificity and subunit mobility in *T. aurantiacus* Xylanase 10A. FEBS Lett 509:303–308
21. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
22. Nolling JG, Breton G, Omelchenko MV, Makarova KS, Zeng Q, Gibson R, Lee HM, Dubois J, Qiu D, Hitti J, Wolf YI, Tatusov RL, Sabathe F, Doucette-Stamm L, Soucaille P, Daly MJ, Bennett GN, Koonin EV, Smith DR (2001) Genome sequence and comparative analysis of the solvent producing bacterium *Clostridium acetobutylicum*. J Bacteriol 183:4823–4838
23. Ohmiya K, Sakka K, Karita S, Kimura T (1997) Structure of cellulases and their applications. Biotechnol Genet Eng Rev 14:365–414
24. Stackebrandt E, Rainey FA (1997) Phylogenetic relationships. In: Rood J, McClane BA, Songer JG, Titball RW (eds) The Clostridia: molecular biology and pathogenesis. Academic, San Diego pp 3–19
25. Stackebrandt E, Kramer IJ, Swiderski J, Hippe H (1999) Phylogenetic basis for a taxonomic dissection of the genus Clostridium. FEMS Immunol Med Microbiol 24:253–258
26. Teather RM, Wood PJ (1982) Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl Environ Microbiol 43:777–780
27. Timell TE (1967) Recent progress in the chemistry of wood hemicelluloses. Wood Sci Technol 1:45–70
28. Tomme P, Warren AJ, Miller RC Jr, Kilburn DG, Gilkes NR (1995) Cellulose-binding domains-classification and properties. In: Sadder JM, Penner M (eds) The enzyme degradation of insoluble polysaccharides. American Chemical Society, Washington, D.C. pp 142–162
29. Viikari L, Tenkanen M, Buchert J, Ratto M, Baily M, Siikaho M, Linko M (1993) Hemicelluloses for industrial applications. In: Saddler JN (ed) Bioconversion of forest and agricultural plant residues. CAB, Oxford, pp 131–182
30. Wong KKY, Saddler JN (1992) Trichoderme xylanases: their properties and application. In: Visser J, Beldman G, Spmeren MAK, Voragen AGJ (eds) Xylans and xylanases. Elsevier, Amsterdam, pp 171–186
31. Wong KKY, Tan LUL, Saddler JN (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and application. Microbiol Rev 52:305–317

32. Wood WA, Bhat KM (1988) Methods for measuring cellulase activities. Methods Enzymol 160:87–112
33. Woods DR (ed) (1993) The clostridia and bio/technology. Butterworth-Heinemann, Boston
34. Zeikus JG, Lee C, Lee YE, Shaha BC (1991) Thermostable saccharidases: new sources, uses and biodesign. In: Leatham GF, Himmel ME (eds) Enzymes in biomass conversion. American Chemical Society, Washington, D.C., pp 36–51