Genetic organization, sequence and biochemical characterization of recombinant β -xylosidase from *Thermoanaerobacterium saccharolyticum* strain B6A-RI

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Endoxylanase (*xynA*) and β -xylosidase (*xynB*) genes from *Thermoanaerobacterium saccharolyticum* were subcloned from a cosmid clone (pXDM1) to generate pXPH3. The nucleotide sequence of a *Pst*I–*Hind*III fragment in pXPH3 that contained *xynB* revealed an open reading frame (ORF) of 1500 bp encoding a 55 kDa protein. Another open reading frame (ORF1) of unknown function was found 21 bp downstream from the first stop codon of *xynB*. *xynB*, ORF1 and *xynA* had the same direction of transcription. *xynB* from *T. saccharolyticum* strain B6A-RI exhibited 45% amino acid similarity, with 18% amino acid identity to *xynA* of *T. saccharolyticum* strain B6A-RI, and 61% similarity and 37% identity with the β -xylosidase gene from *Caldocellum saccharolyticum*. Recombinant β -xylosidase was purified from *E. coli* (pXPH3) cells. The enzyme was a monomer with a molecular mass of 55 kDa. The specific activity and pH and temperature optima for hydrolysis of *p*-nitrophenyl- β -D-xylopyranoside (pNPX) were 5.53 U mg⁻¹, 5.5 and 70 °C, respectively. The β -xylosidase was stable at 65 °C, but lost activity at 85 °C. The purified enzyme had hydrolytic activity towards xylopentose, xylotriose, xylobiose and pNPX, but had no activity toward xylan.

Introduction

Xylan is the second most abundant renewable polysaccharide in nature and is comprised of a β -1,4-linked D-xylose polymer with arabinofuranose, glucuronic acid, methylglucuronic acid and acetyl side-groups (Whistler & Richards, 1970). Complete breakdown of xylan requires the action of several hydrolytic enzymes of which endoxylanase (EC 3.2.1.8) and β -xylosidase (EC 3.2.1.37) are the most important (Biely, 1985). Endoxylanase randomly cleaves the β -1,4 bonds in the xylan backbone to yield oligosaccharides, xylobiose and xylose. The resulting xylo-oligosaccharides are attacked by β -xylosidase at the non-reducing end (Wong *et al.*, 1988).

Microbial β -xylosidases occur in both bacteria and fungi, with the latter group being the most studied. Relatively little work has been done on β -xylosidases from thermophilic bacteria (Bachmann & McCarthy, 1989; Hudson *et al.*, 1991; Nanmori *et al.*, 1990; Shao & Wiegel, 1992). To date, only β -xylosidase genes from *Bacillus pumilus* (Panbangred *et al.*, 1984; Xu *et al.*, 1991), *Bacillus polymixa* (Sandhu & Kennedy, 1984), *Bacillus subtilis* (Bernier *et al.*, 1983), *Butyrivibrio fibrisolvens* (Sewell *et al.*, 1989), and *Caldocellum saccharolyticum* (Luthi *et al.*, 1990) have been cloned into *Escherichia coli*.

Thermoanaerobacterium saccharolyticum strain B6A-RI, a thermophilic anaerobic bacterium isolated from Yellowstone National Park, grows actively on xylan as a sole carbon source and does not have cellulase activity (Lee *et al.*, 1993a). In this organism the degradation of xylan to xylo-oligosaccharides is catalysed by secreted or cell-bound endoxylanases followed by hydrolysis to xylose by intracellular β -xylosidase. The enzymes are coregulated; their production is induced by xylan or xylose but not glucose (Lee *et al.*, 1993b).

To isolate genes encoding for xylanolytic enzymes a library of *T. saccharolyticum* strain B6A-RI chromo-

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M97883.

1 CTGCAGTATTGAGAATGGCAACACATCAAGGATAATACCTGCTGTAGAAGCATTGGTATATCCATATGTTGTAGGATATACTGACTTTGTAAGTGAAGA
 101 TGGTGTGTTTGGTGAGCTTATAAAGCCTTAAAGAAGCATGTAAATGACGATCATGAAGCCTGGTATATGCATAGATGAAGTATCTGGCGGTTGGAACTT
 201 TCGTCAACAGCAAGAATACATGGAATAGCAAAATTTCTTGTGCAATATGTGTATAAAGATGTGCTAAATATAGATTTTGGAGAAAAAGAGCGCAAT
 301 GGGACAAAGTACATGCCACATGGCAACAGGTGTCTTGCAGTGAAGATTGTGCTACAGATCAGGTAAACAGCGATACAGGTACGCCAAGAGGAGCGCGTT
 401 GTATCCTAGGCTTGTAAACAGCATACTGTGGATGAAATAGACTTTGGGGGGTTTAAACATGATTAAAGTAAGAGTGCCAGATTTTCCGATAAGAAGTTT
 S D R W R Y C V G T G R L G L A L Q K E Y I E T L K Y V K E N I D F
 501 CTGATAGATGGAGATATTGTGTGCGAACAGCGAGACTTGGCCTTGGCGCTCAAAGGAATACATCGAGACATTAAATATGTGAAGAAAAATATAGACTT
 K Y I R G H G L L C D D V G I Y R E D V V G D E V K P F Y N F T Y
 601 AAGTATATAAGAGGACATGGCCTTTGTGTGACGATGTAGGCATATACAGAGAAGATGTGTAGGAGATGAGTAAAGCCTTTTACAATTTTACCTAT
 I D R I F D S F L E I G I R P F V E I G F M P K K L A S G T Q T V
 701 ATTGACAGGATTTTGTACTATTTTAGAAATCGGAATAAGGCCATTGTGGAATCGGATTTATGCCTAAAAATTAGCATCTGGTACACAGACAGTAT
 F Y W E G N V T P P K D Y E K W S D L V K A V L H H F I S R Y G I E
 801 TTTATTGGGAGGGGAATGCTCACTCCTCCCAAGGATTATGAAAGTGGAGCGATCTTGTCAAAGCGGTTTGCATCACTTATTCTCGATATGGGATTGA
 E V L K W P F E I W N E P N L K E F W K D A D E K E Y F K L Y K V
 901 AGAAGCTTGAAGTGCCATTGTAGATATGGAATGAACCAAACTTAAAGAGTTTGGAAAGATGCTGATGAGAAAGAAATATTTAAGCTGTACAAGGTT
 T A K A I K E V N E N L K V G G P A I C G G A D Y W I E D F L N F
 1001 ACTGCAAGGCTATAAAGGAAGTAAATGAAATTTGAAGTAGGAGGGCCTGCTATATGCGGTGGTGTGCTGACTACTGGATAGAAGATTTTGAATTTCT
 C Y E E N V P V D F V S R H A T T S K Q G E Y T P H L I Y Q E I M P
 1101 GCTATGAAGAAATGTTCTGTAGATTTTGTATCGCGACCGCTACCACATCTAAGCAAGGTGAATATACGCCACATCTCATATACCAGGAGATTATGCC
 S E Y M L N E F K T V R E I I K N S H F P N L P F H I T E Y N T S
 1201 ATCTGAATACATGCTAAACGAATTTAAACAGTGAGAGAGATCATAAAACTCACATTTTCCGAACCTTCCGTTTCATATACTGAGTACAATACCTTCA
 Y S P Q N P V H D T P F N A A Y I A R I L S E G G D Y V D S F S Y
 1301 TATAGTCTCAAAATCCTGTACACGATACGCCATTTAATGCTGCTATATGCCAGGATTTTAAAGCAAGGGAGAGATTATGTTGATTCATTTCTTACT
 W T F S D V F E E R D V P R S Q F H G G F G L V A L N M I P K P T F
 1401 GGAGCTTTAGCGCGTTTTTGAAGAAAGAGATGTGCCGCGATCCCAATTCATGAGGAGATTGGACTTGTGCGATTGAATATGATACCAAGCGTACCTT
 Y T F K F F N A M G E M L Y R D E H M L V T R R D D G S V A L I
 1501 TTACACATTTAAATCTTTAATGCTATGGGAGAGGAAATGCTTTATAGAGATGAGCACATGCTTGTGACGAGGAGAGATGATGGCTCTGTTGCACTCATA
 A W N E V M D K T E N P D E D Y E V E I P V R F R D V F I K R Q L
 1601 GCTTGAATGAAGTTATGGATAAGACTGAAATCCAGATGAAGATTATGAAGTCGAGATACCAGTTAGATTAGAGATGTGTTATTAAAGACAATTGA
 I D E E H G N P W G T W I H M G R P R Y P S K E Q V N T L R E V A K
 1701 TTGATGAAGACATGGCAATCCATGGGAACGTGGATACACATGGGAAGCGGAGGTATCTAGCAAGAACAGGTAAATACATTGAGAGAGTTGCAAA
 P E I M T S Q P V A N D G Y L N L K F K L G K N A V V L Y E L T E
 1801 GCCAGAGATTATGACAAGTCAGCTGTGCGAATGACCGATACCTTAAATCTAAAGTTTAAATAGTAAAAATGCAGTTGATTTGATGAATTGACTGAG
 R I D E S S T Y I G L D D S K I N G Y *
 1901 AGAATTGATGAATCAAGCATATATAGGAATTTGATGATAGCAAGATAAATGGATATTTGATGCACATTAGGAGGATTGATATGGGACTTTTGGACATGC
 P L Q K L R E Y T G T N P C P E D F D E Y W D R A L D E M R S V D P
 2001 CACTGCAAAAGCTTAGAGAATACACTGGTACAAATCCATGCCCTGAAGATTTCGATGAGTATTGGGATAGGCTTTAGATGAGATGAGGTCAAGTATGATCC
 K I K M K K S S F Q V P F A E C Y D L Y F T G V R G A R I H A K Y
 2101 CAAAATTAATTAAGAAAAAGTAGCTTTCAAGTGCCCTTTTGCAGAGTGCTACGATTTGACTTTACAGGTGTTCTGTTGGTCGAGAAATTCATGCAAGATAT
 I R P K T E G K H P A L I R F H G Y S S N S G D W N D K L N Y V A
 2201 ATAAGACCTAAGACAGAAGGAAACATCCAGCGTTGATAAGATTTCATGGATATTCGTCAAATTCAGCGACTGGAACGACAAATTAATTAAGTACGAGCG
 A G F T V V A M D A R G Q G G Q S Q D V G G V N G N T L N G H I I R
 2301 CAGGCTTTACCGTTTGTGGCTATGGATGCAAGAGGTCAAGGAGGCGAGTCTCAAGATGTTGGCGGTGAAATGGGAACACTTTAAATGGGCATATTATAAG
 G L D D D A D N M L F R H I F L D T A Q L A G I V M N M P E I D E
 2401 AGGGTTAGACGATGATGCTGACAACATGCTTTTAGGCATATCTTCTAGATACTGCCAGTTGGCTGGAATAGTTATGAATATGCCAGAAATCGATGAA
 D R V A V M G P S Q G G G L S L A C A A L E P K I R K V V S E Y P
 2501 GATAGATTGCAAGTATGGGACCTTCTCAAGGTGGAGGTTTGTCAATTAGCGTGTGCAGCATTGGAACCCAGATACGCAAGTAGTATCAGATACCCAT
 F L S D Y K R V W D L D L A K N A Y Q E I T D Y F R L F D P R H E R
 2601 TCTGTGCTGATATAAAGAGTTTGGGATTAGACCTTGCAGAAATGCTTACCAAGAGATTACGGACTATTTTAGGCTTTTATGATCAAGGCATGAAAG
 E N E V F T K L G Y I D V K N L A K R I K G D V L M C V G L M D Q
 2701 AGAAAAAGGTTTACTAAGCTTGGCTATATAGATGTAAAGAATCTGCGCAAGAGGATAAAGCGCATGTGTTAATGTGCGCTTGGCGTTATGGATCA
 V C P P S T V F A A Y N N I Q S K K D I K V Y P D Y G H E P M R G
 2801 GTATGTCCGCCATCAACTGTCTTTGACGCTACAACAACATACAGTCAAGAGAGATATAAAGTGTATCCTGATTATGGACATGAGCCTATGAGAGGAT
 F G D L A M Q F M L E L Y S *
 2901 TTGGAGATTAGCGATGCAAGTTTATGTTGGAATATATCATAAGGAGATGCAAGTGCATCTCTTAAGTGTATTCTTTATTAATTTTTATATTCGCTT
 3001 ACGTTACGTTTAAAGCCTGTGTATTTTTAAACACGATGAAATAATTTGGGTCTGTATGCCTACAGACTTGGCGATTTCAAATGACTTCATGTTTTT
 3101 TTCTTTAATAAGGCTTATGGCTTATGGCTTTTCCATTGCGCTTATTGTGAGGATTTCTATGAAGTTTATGCGCTGTCTCTTTTGAATGTGG 3194

supernatant obtained by centrifugation (12000 *g*, 20 min) was used as the crude enzyme preparation.

(ii) *Heat treatment.* The extract of *E. coli* was heat-treated at 75 °C for 15 min in a water bath with shaking, and then chilled on ice. The denatured proteins were removed by centrifugation at 12000 *g* for 20 min.

(iii) *Anion exchange column chromatography.* The supernatant from the heat treatment step was loaded onto a column (2.5 cm × 18 cm) of Q-Sepharose (Pharmacia) equilibrated with 50 mM-sodium acetate buffer (pH 5.5). The enzyme was eluted with a linear gradient (0–1.0 M) of sodium chloride in a total volume of 500 ml. The active fractions were pooled and concentrated by ultrafiltration with a YM 30 membrane (Amicon).

(iv) *Gel filtration chromatography.* The concentrated sample was further purified using a Waters FPLC system with a Superose-12 column (Pharmacia) equilibrated with 50 mM-sodium acetate buffer (pH 5.5) containing 50 mM-sodium chloride.

N-terminal amino acid sequence determination of β -xylosidase. The protein was prepared by washing the purified β -xylosidase five times with double-distilled water using a Centricon-30 (Amicon) filtration device to remove contaminating salts and buffer from the solution. The samples were hydrolysed *in vacuo* at 110 °C for 24 h using 5.7 M-HCl. Amino acid composition analysis was done with a Pico-Tag amino acid analyser (Waters Associates). The N-terminal amino acid sequence was identified by a protein sequencer model 477A (Applied Biosystems) with an on-line phenylthiohydantoin analyser (Applied Biosystems) in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University.

Protein measurement and PAGE. Protein concentration was estimated by the method of Bradford (1976) using Protein assay reagent from Bio-Rad with BSA (Sigma) as a standard. SDS-PAGE was done as described by Laemmli (1970) using a 12% (w/v) polyacrylamide gel. Protein bands were visualized by Coomassie Brilliant Blue R250 staining. The molecular mass of the denatured protein was estimated by SDS-PAGE with low-range molecular mass standards (Bio-Rad) including phosphorylase (97 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Analysis of products of xylan hydrolysis. The xylan and xylo-oligosaccharides were dissolved in 50 mM-sodium acetate buffer (pH 5.5) and incubated at 60 °C with approximately 1 U purified enzyme. Samples were withdrawn periodically and the hydrolysis products were analysed by Whatman HP-K high performance silica gel plates (10 × 10 cm) using butanol/acetic acid/distilled water (2:1:1, by vol.), and were visualized using an orcinol spray reagent (100 mg orcinol in 100 ml 20% sulphuric acid in methanol).

Results

Subcloning and DNA sequencing

The cosmid clone, pZXA1, with a 36 kb DNA insert encoding for both endoxylanase and β -xylosidase activities, was unstable and after several transfers a stable deletion mutant (pXDM1) which retained activity was selected. This cosmid clone contained a 28 kb insert of *T. saccharolyticum* strain B6A-RI chromosomal DNA which carried the endoxylanase (*xynA*) and β -xylosidase (*xynB*) genes (Fig. 1a). The complete nucleotide sequence of *xynA* and its flanking regions had been determined

previously and have been deposited with GenBank and assigned the accession number M97882. A 2 kb *Pst*I–*Hind*III fragment containing *xynB* was subcloned from pXDM1 into pUC18 to produce pXPH3. Fig. 1(b) shows the physical map of *xynB*.

The nucleotide sequence of the β -xylosidase gene was determined and is shown in Fig. 2. The sequence, which is consistent with the observed restriction map, revealed an ORF of 1500 bp, which corresponded to *xynB*. The molecular mass of the enzyme deduced from the DNA sequence was 55 kDa.

A putative ribosome-binding sequence, 5'-GGGGGG-3', was observed 7 bp upstream from the initiation codon, ATG. Another ORF (ORF1) of unknown function was found 21 bp downstream from the first stop codon of *xynB*. Interestingly, *xynB*, ORF1 and *xynA* all have the same direction of transcription (Fig. 1).

Comparison of *xynB* with other genes

Comparison of the deduced amino acid sequence of the β -xylosidase gene from *T. saccharolyticum* strain B6A-RI to the endoxylanase gene from the same organism, and β -xylosidases from *Bacillus pumilus* IPO (Xu *et al.*, 1991), *Butyrivibrio fibrisolvens* (Sewell *et al.*, 1989) and *Caldocellum saccharolyticum* (Luthi *et al.*, 1990) revealed little similarity between the enzymes from the mesophiles (less than 16% identity) compared to the thermophilic enzymes. The endoxylanase and β -xylosidase from *T. saccharolyticum* strain B6A-RI exhibited 45% amino acid similarity and had 18% amino acid identity. Comparison of the β -xylosidase gene from *T. saccharolyticum* strain B6A-RI with GenBank and EMBL sequence libraries identified the β -xylosidase gene from *Caldocellum saccharolyticum* as being most similar, with 61% similarity and 37% identity. Using the PILEUP program from the GCG package (University of Wisconsin), significant regions of homology were found between β -xylosidase A from *C. saccharolyticum* and endoxylanase from *T. saccharolyticum* strain B6A-RI, and between β -xylosidase B from *C. saccharolyticum* and β -xylosidase from *T. saccharolyticum* strain B6A-RI (Fig. 3). The translated sequence of *xynB* did not share a strong identity with other endoxylanases. No proteins were identified which showed a high amino acid similarity to the putative protein encoded by ORF1 of *T. saccharolyticum* strain B6A-RI.

Purification and characterization of recombinant β -xylosidase

Recombinant β -xylosidase was purified from *E. coli* (pXPH3) cells to determine its physicochemical properties. A summary of the purification procedure is shown in Table 1. Heat treatment was a particularly effective

Fig. 3. Deduced amino acid sequence alignment of (1) β -xylosidase A from *Caldocellum saccharolyticum*, (2) endoxylanase from *T. saccharolyticum* strain B6A-RI, (3) β -xylosidase B from *C. saccharolyticum*, (4) β -xylosidase from *T. saccharolyticum* strain B6A-RI. The asterisks denote conserved amino acids between the two sequences of greatest homology and amino acids in bold are common to all four sequences.

step, resulting in a five-fold purification and 81 % yield. SDS-PAGE analysis (Fig. 4) revealed that the enzyme had an approximate molecular mass of 55 kDa. This is identical to the value (55 kDa) calculated from the deduced amino acid sequence. The molecular mass of the recombinant enzyme was estimated at 60 kDa by gel filtration, indicating that this enzyme is monomeric. The N-terminal amino acid sequence of the recombinant β -xylosidase was Met-Ile-Lys-Val-Arg-Val-Pro-Asp-Phe-Ser-Asp. This was identical to the N-terminal amino acid sequence deduced from the DNA sequence (Fig. 2).

The pH optimum for hydrolysis of pNPX was about 5.5, with the enzyme showing high activity from pH 5.0 to 6.6 (Fig. 5a). The optimum temperature for hydrolase activity was 70 °C, with activity decreasing rapidly at higher temperatures (Fig. 5b). To examine the thermostability of the enzyme, β -xylosidase was incubated at various temperatures for up to 60 min, and the residual activities were assayed and compared with the untreated sample. β -Xylosidase was stable at 65 °C, but lost activity at 85 °C. The half-life of the enzyme at 75 °C was 55 min (data not shown).

Table 1. Summary of purification of β -xylosidase from *E. coli* (pXPH3)

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Purification (-fold)	Yield (%)
Cell-free extract	165	391	0.422	1.0	100
Heat treatment	134	67	2.6	4.7	81.2
Q-Serpharose	101	21	4.81	11.4	61.2
Gel filtration	83	15	5.53	13.1	50.3

Fig. 4. SDS-PAGE analysis of the purification of recombinant β -xylosidase from *E. coli* (pXPH3) cells. Lane M, molecular mass markers; lane 1, crude *E. coli* (pXPH3) extract (200 μ g protein); lane 2, following heat treatment (10 μ g protein); lane 3, after anion exchange chromatography (5 μ g protein) and lane 4, after gel filtration chromatography (3 μ g protein).Table 2. Expression and localization of β -xylosidase

Cells were harvested from the late exponential phase at an OD₆₆₀ of 1.30 and 2.20 for *T. saccharolyticum* and *E. coli*, respectively.

Organism	Specific activity (U mg ⁻¹)	
	Supernatant	Intracellular
<i>E. coli</i> (pXPH3)	0	0.16
<i>T. saccharolyticum</i> strain B6A-RI	0	1.26

Localization studies revealed that β -xylosidase was intracellular in both the recombinant organism and *T. saccharolyticum* strain B6A-RI (Table 2). Lower levels of β -xylosidase were expressed in *E. coli* compared to *T. saccharolyticum*, and in both organisms levels of β -xylosidase were dependent on growth phase and increased in the stationary phase.

The activity of the purified β -xylosidase with various *para*- and *ortho*-nitrophenyl-glycosidic substrates was tested (Table 3). The β -xylosidase was highly specific, as

Fig. 5. The pH (a) and temperature (b) profile of recombinant β -xylosidase. Citrate/phosphate buffer (0.1 M) was used for determination of the pH optimum, and 50 mM-sodium acetate buffer (pH 5.5) for determination of the temperature optimum. The 100 % value represents 44 U and 17 U of β -xylosidase for pH and temperature profiles, respectively.Table 3. Hydrolysis of various nitrophenyl-glycosides by the purified β -xylosidase

Values are means of three independent experiments.

Substrate	Specific activity (U mg ⁻¹)
<i>p</i> -Nitrophenyl- β -D-xylopyranoside	5.529
<i>o</i> -Nitrophenyl- β -D-xylopyranoside	4.696
<i>p</i> -Nitrophenyl- β -D-fucopyranoside	0.045
<i>p</i> -Nitrophenyl- α -D-glucopyranoside	0.000
<i>p</i> -Nitrophenyl- β -D-glucopyranoside	0.141
<i>p</i> -Nitrophenyl- α -L-arabinofuranoside	0.073
<i>o</i> -Nitrophenyl- β -D-galactopyranoside	0.000
<i>p</i> -Nitrophenyl- β -D-lactopyranoside	0.000

it was active only on *p*-nitrophenyl- β -D-xylopyranoside and *o*-nitrophenyl- β -D-xylopyranoside. The low level of activity observed with *p*-nitrophenyl- β -D-fucopyranoside, *p*-nitrophenyl- α -L-arabinofuranoside and *p*-nitrophenyl- β -D-glucopyranoside may not be significant, and no activity was detected with the other substrates tested.

Fig. 6. Time course of hydrolysis of the soluble fraction of oat spelt xylan and xylo-oligosaccharides by the recombinant β -xylosidase. Oligosaccharides of xylose (xylobiose, xylo-oligosaccharides and xylopentose) and oat spelt xylan were incubated with purified β -xylosidase and analysed using TLC. Lane M, size markers of (vertical scale) xylose (X_1), xylobiose (X_2), xylo-oligosaccharides (X_3) and xylopentose (X_5). For the other lanes, xylan (X_n), xylobiose (X_2), xylo-oligosaccharides (X_3) and xylopentose (X_5) were hydrolysed, and samples were taken at time 0 (0), 15 min (1), 30 min (2), 1 h (3) and 5 h (4).

Oligosaccharides of xylose (xylobiose, xylo-oligosaccharides and xylopentose) and oat spelt xylan were incubated with purified β -xylosidase and analysed using TLC (Fig. 6). All of the xylo-oligosaccharides (polymers of two to five xylose residues) were degraded to xylose. Transferase activity was also detected by the appearance of xylo-oligosaccharides during the early stages of hydrolysis of xylobiose. No activity could be detected using oat spelt xylan as the substrate. No β -xylosidase activity was detected in control extracts from *E. coli*.

Discussion

The complete nucleotide sequence of the β -xylosidase gene of *T. saccharolyticum* strain B6A-RI was established, together with its flanking regions. In our other studies, the endoxylanase and β -xylosidase activity of *T. saccharolyticum* strain B6A-RI have been shown to be under co-ordinate control, induced by xylose or xylan (Lee *et al.*, 1993b), and the endoxylanase gene (*xynA*) has been cloned and sequenced (Y.-E. Lee, S. E. Lowe & J. G. Zeikus, unpublished data). In the cloned chromosomal DNA from *T. saccharolyticum* strain B6A-RI, *xynA* and *xynB* genes were clustered within a 20 kb region, and had the same transcriptional orientation. The proximity of *xynB*, ORF1 and *xynA* in the cloned DNA and the uniform direction of transcription of these genes suggests that they may be organized into an operon. This is further substantiated by the finding that no terminator or prominent stem-loop structure was identified in the sequence analysed. However, it remains

to be shown that *xynA* and *xynB* are part of the same operon in *T. saccharolyticum* strain B6A-RI. Studies using RNA, such as Northern blot analysis and primer extension experiments would address this question. Recent studies with *Bacteroides ovatus* (Whitehead & Hespell, 1990), *Bacillus pumilus* (Panbangred *et al.*, 1984), *Pseudomonas fluorescens* (Kellet *et al.*, 1990) and *Caldocellum saccharolyticum* (Luthi *et al.*, 1990) have reported the clustering of genes involved in hemicellulose degradation in these organisms.

Confirmation that the cloned *xynB* is from the *T. saccharolyticum* strain B6A-RI chromosomal DNA was obtained by Southern blot analysis. A single *Pst*I and *Xba*I fragment hybridized to the β -xylosidase probe, suggesting that there is only one chromosomal copy of the cloned gene. However, the possibility that *T. saccharolyticum* strain B6A-RI has more than one β -xylosidase cannot be ruled out, since multiple β -xylosidases have been reported in *Bacillus pumilus* (Panbangred *et al.*, 1984) and *C. saccharolyticum* (Luthi *et al.*, 1990).

Localization studies of the cloned β -xylosidase revealed that the enzyme was located within the *E. coli* cell. This is consistent with the observation that most of the β -xylosidase activity from *T. saccharolyticum* strain B6A-RI was intracellular, as has been found for the closely related thermophilic anaerobes *Thermoanaerobacter* strain B6A (Hespell, 1992) (now *Thermoanaerobacterium saccharolyticum* strain B6A) and *Thermoanaerobacter ethanolicus* JW200 (Shao & Wiegel, 1992). The deduced amino acid sequence of thermostable *T.*

saccharolyticum strain B6A-RI β -xylosidase exhibited a higher degree of homology to thermostable β -xylosidase from *C. saccharolyticum* (Luthi *et al.*, 1990) than to those of less thermostable β -xylosidases from *Bacillus* (Xu *et al.*, 1991) and *Butyrivibrio* (Sewell *et al.*, 1989) species.

The cloned β -xylosidase had a monomeric molecular mass of 55 kDa, which was similar to that of β -xylosidase from *Butyrivibrio fibrisolvens* (Sewell *et al.*, 1989) and *Bacillus pumilus* (Panbanged *et al.*, 1984) each single subunits of 60 and 62.6 kDa, respectively. The β -xylosidase from *Clostridium acetobutyricum* (Lee & Forsberg, 1987) is composed of two different subunits with molecular masses of 85 and 63 kDa, and the enzyme from *Thermoanaerobacter ethanolicus* JW200 is also a dimer, with an overall molecular mass of 165 kDa (Shao & Wiegel, 1992). Fungal β -xylosidases have relatively larger subunits of 116 to 118 kDa (Kitprechavabichi *et al.*, 1986; Matsuo & Yasui, 1984).

β -Xylosidase from *T. saccharolyticum* strain B6A-RI and *T. ethanolicus* JW200 (Shao & Wiegel, 1992) have the same pH optimum, although the enzyme from the latter organism was more thermostable. There was no significant similarity between the N-terminal sequences of the β -xylosidases from these two organisms.

The substrate specificity of β -xylosidase from *T. saccharolyticum* strain B6A-RI for xylo-oligosaccharides was similar to that of other bacterial β -xylosidases (Deleyn *et al.*, 1982; Lee & Forsberg, 1987; Panbanged *et al.*, 1984; Sewell *et al.*, 1989), with the exception of β -xylosidase from *Thermoanaerobacter ethanolicus*, which also possessed high arylarabinosidase activity, but had a lower affinity for *p*-nitrophenyl- α -L-arabinoside and *p*-nitrophenyl- β -D-xylopyranoside (Shao & Wiegel, 1992). Most β -xylosidases have little or no activity against xylan, and this was also found with β -xylosidase from *T. saccharolyticum* strain B6A-RI. These enzymes degrade xylo-oligosaccharides to xylose and also have transferase activity, which are considered to be the properties of a β -xylosidase (Reilly, 1981).

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