Available online at www.sciencedirect.com
SCIENCE DIRECT®
CARBOHYDRATE RESEARCH
ELSEVIER
Carbohydrate Research 338 (2003) 1881–1890
www.elsevier.com/locate/carres

Note

Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of *Sporotrichum thermophile*

Petros Katapodis,[a] Maria Vršanská,[b] Dimitris Kekos,[a] Wim Nerinckx,[c] Peter Biely,[b] Marc Claeyssens,[c] Basil J. Macris,[a] Paul Christakopoulos[a,*]

[a] *Department of Chemical Engineering, National Technical University of Athens, 5 Iroon Polytechniou Ave, Zografou Campus, 15700 Athens, Greece*
[b] *Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava, Slovak Republic*
[c] *Department of Biochemistry, Fysiology, Microbiology, Faculty of Sciences, Ghent University, K.L., Ledeganckstraat 35, B-9000 Ghent, Belgium*

Received 1 November 2002; received in revised form 9 June 2003; accepted 21 June 2003

Abstract

An endo-β-1,4-xylanase (1,4-β-D-xylan xylanoxydrolase, EC 3.2.1.8) present in culture filtrates of *Sporotrichum thermophile* ATCC 34628 was purified to homogeneity by Q-Sepharose and Sephacryl S-200 column chromatographies. The enzyme has a molecular mass of 25,000 Da, an isoelectric point of 6.7, and is optimally active at pH 5 and at 70 °C. Thin-layer chromatography (TLC) analysis showed that endo-xylanase liberates mainly xylose (Xyl) and xylobiose (Xyl_2) from beechwood 4-*O*-methyl-D-glucuronoxylan, *O*-acetyl-4-*O*-methylglucuronoxylan and rhodymenan (a β-(1→4)-β(1→3)-xylan). Also, the enzyme releases an acidic xylo-oligosaccharide from 4-*O*-methyl-D-glucuronoxylan, and an isomeric xylotetraose and an isomeric xylopentaose from rhodymenan. Analysis of reaction mixtures by high performance liquid chromatography (HPLC) revealed that the enzyme cleaves preferentially the internal glycosidic bonds of xylooligosaccharides, [1-^{3}H]-xylooligosaccharides and xylan. The enzyme also hydrolyses the 4-methylumbelliferyl glycosides of β-xylobiose and β-xylotriose at the second glycosidic bond adjacent to the aglycon. The endoxylanase is not active on pNPX and pNPC. The enzyme mediates a decrease in the viscosity of xylan associated with a release of only small amounts of reducing sugar. The enzyme is irreversibly inhibited by series of ω-epoxyalkyl glycosides of D-xylopyranose. The results suggest that the endoxylanase from *S. thermophile* has catalytic properties similar to the enzymes belonging to family 11.

Keywords: Endoxylanase; Family 11; *Sporotrichum thermophile*

Xylan found in almost all parts of green plant cell walls is the second most abundant polysaccharide in nature. In recent years, microbial enzymes depolymerizing this polysaccharide found applications in food industry, animal feed, and paper and pulp industries.[1–3]

The enzymes acting on the xylan backbone are classified in two groups: endo-β-1,4-xylanases (E.C. 3.2.1.8, xylan xylanohydrolase, EX) and exo-β-1,4-xylanases (E.C. 3.2.1.37, D-xylan xylanohydrolase). Based on hydrophobic cluster analysis and amino acid sequence homologies EXs have been classified into two groups mainly, families 10 and 11.[4,5] Previous studies with endo-xylanases of *Streptomyces lividans* (families 10 and 11),[6] *Trichoderrma reesei* (two family 11 xylanases),[7] *Cryptococcus albidus* (family 10),[8] *Thermomyces lanuginosus* (family 11)[9] and two family 10 endoxylanases from *Thermoascus aurantiacus*[10,11] suggested that the enzymes belonging to family 10 exhibit greater catalytic versatility or lower substrate specificity than enzymes of family 11. In addition, the activity of EXs of family 10 is less hampered by the presence 4-*O*-methyl-D-glucuronic acid (MeGlcA), acetic acid and α-L-arabinofuranose on the xylan chains.[6,10,11] Furthermore, the presence of β-(1→3) linkages, such as in rhodymenan, leads to a more serious steric barrier for EXs of family 11 than for family 10 EXs.[6,9] In

* Corresponding author. Tel.: +30-3210-7723231; fax: +30-3210-7723163.
E-mail address: hristako@orfeas.chemeng.ntua.gr (P. Christakopoulos).

0008-6215/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0008-6215(03)00291-X

consonance with these considerations, EXs of family 10 liberate from 4-*O*-methyl-D-glucuronoxylan, rhodymenan and, with some exceptions, also from acetylxylan and arabinoxylan, smaller products than those formed with EXs of family 11.[6,10,11]

Almost in most cases, the glycosidase catalytic mechanism involves two essential carboxyl residues: a proton donor and a nucleophile. The importance of two completely conserved and catalytically important glutamate residues in family 11 glycoside hydrolases[5,12] has been demonstrated previously by site-directed mutagenesis. Similarly, two conserved glutamate residues are involved in family 10 glycosidic hydrolases.[13]

Epoxyalkyl glycosides used as affinity labeling compounds of glycoside hydrolases target mainly for the nucleophylic residue,[14] yielding a covalent ester bond between protein and the glycoside. ω-Epoxyalkyl D-xylopyranosides were shown to only inactivate enzymes from family 11.[15]

The thermophilic fungus, *Sporotrichum thermophile* produces high levels of xylanases, when grown on a cheap carbon source such as corn cob. The main purpose of the present study was to investigate the biochemical and catalytic properties of a purified endoxylanase from this organism and to establish its relationship with the recognized endo-xylanase families.

Production of xylanase—*S. thermophile* ATCC 34628, when grown on corn cob in a 7 L stirred tank fermentor at 50 °C and pH 5 for 4 days, produced 56 U of xylanase per mL of the culture filtrate. Also, among the five strains of *S. thermophile* tested in our laboratory, ATCC 34628 produced the highest xylanase activity.

Purification of endoxylanase—The crude enzyme was eluted from an anion exchanger (Q-Sepharose), at pH 8.5 using a linear gradient of 0–0.5 M NaCl. During chromatography, a major fraction X-I was separated. This fraction contained 35% of the total amount of endoxylanase applied to the column.

In the second purification step, the concentrated fraction X-I was applied on a Sephacryl S-200 column as described in Materials and Methods. At this stage, the enzyme had a specific activity of 875 U/mg protein towards birchwood xylan, was purified 13-fold, and contained 25% of the initial crude enzyme activity. The results of the purification are summarized in Table 1.

General properties—Isoelectric focusing in the pH range of 3.0–9.5 of the purified endo-xylanase revealed a pI of about 6.7 (Fig. 1(A)). The molecular mass of endo-xylanase was found to be 25 kDa as determined by SDS-PAGE electrophoresis (Fig. 1(B)).

The endo-xylanase was optimally active at pH 5 and 70 °C. Approximately, 24% of the maximum activity was retained at 80 °C. It was retained 80% of its activity after 24 h incubation at pH 5 and 50 °C. The enzyme kept 63 and 55% of its activity, at pH 4 and 6, respectively. Thermostability of xylanase was determined by measuring the residual activity, after incubation of the enzyme at 60 and 70 °C (pH 5) and the xylanase exhibited half lives of 150 and 15 min, respectively.

Mode of action on xylan, Xyl_n and MeUmb $(Xyl)_n$—The relationship between the change in specific fluidity and release of reducing sugar during hydrolysis of xylan is shown in Fig. 2. The enzyme mediated a rapid

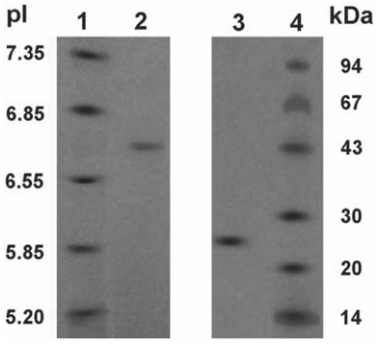

Fig. 1. Isoelectric focusing (A) and SDS-PAGE (B) of purified endo-β-1,4-xylanase from *S. thermophile* ATCC 34628. (A) Lane 1: standard protein markers in the order of increasing pI: β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin (6.85), horse myoglobin (7.35); lane 2: purified xylanase; (B) Lane 3: purified xylanase, lane 4: standard protein markers in the order of increasing molecular mass: trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), albumin (67 kDa) and phosphorylase b (94 kDa).

Table 1
Purification of a xylanase from *S. thermophile*

Step	Total protein [a] (mg)	Total activity [b] (U)	Specific activity [a] (U/mg)	Purification (fold)	Yield (%)
Culture filtrate	3400	224,000	66	1.0	100.0
Ultrafiltration	2750	201,000	73	1.1	89.7
Q-Sepharose	405	71,000	175	2.7	31.7
Sephacryl S-200	64	56,000	875	13.3	25.0

[a] Protein was measured by the bicinchoninic acid method according to Pierce instruction manual No 23235X.
[b] Xylanase activity was assayed using birchwood xylan with reducing sugar detection by the DNS method.

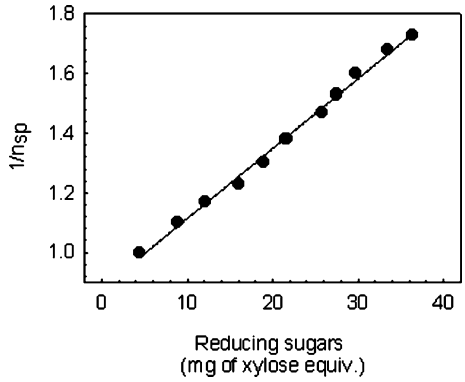

Fig. 2. Plot of increase in specific fluidity ($1/n_{sp}$) vs. the release of reducing sugars during the hydrolysis of birchwood xylan (4% w/v) by purified endo-β-1,4-xylanase of *S. thermophile* ATCC 34628 (2.5 U).

decrease in the viscosity of xylan solution, with the release of only small amounts of reducing sugar.

The hydrolysis pattern of xylotriose, xylotetraose, xylopentaose and xylohexaose at the concentration given and by the same amount of enzyme was studied by HPAEC–PAD analysis of the products released after 1 h, as described in Section 1. The enzyme effected almost complete degradation of xylohexaose but only 35 and 24% of xylopentaose and xylotetraose, respectively was hydrolyzed during the same reaction time. The enzyme was not active on xylotriose. Examination of the hydrolysis products of xylo-oligosaccharides indicates that the enzyme has an endo character (Table 2). Clearly, the xylanase attacks mainly the internal glycosidic bonds releasing xylotriose from xylohexaose, xylobiose and xylotriose from xylopentaose and xylobiose from xylotetraose.

Hydrolysis products generated by the action of xylanase on soluble beechwood xylan were analyzed by HPAEC–PAD (Fig. 3). Xylotriose, xylotetraose, xylopentaose and xylohexaose were the predominant end products, followed by higher xylooligosaccharides ($>X_6$).

Action on polysaccharides—The hydrolysis products released from polysaccharides by endo-xylanase were analyzed by TLC. From beechwood 4-*O*-methyl-D-glucuronoxylan the enzyme liberated mainly xylose, xylobiose, and acidic oligosaccharides which had the

Table 2
Hydrolysis products released from xylo-oligosaccharides by the purified xylanase

Substrate (0.5 mM)	X	X_2	X_3	X_4	X_5
Xylotriose	–	–			
Xylotetraose	20	62	18		
Xylopentaose	–	49	51	–	
Xyloexaose	–	23	56	21	–

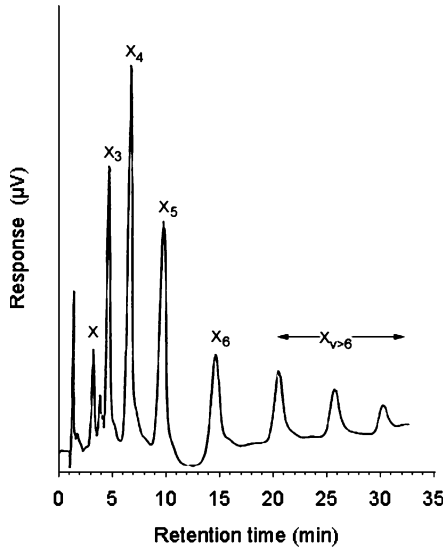

Fig. 3. Pattern of xylo-oligosaccharides released from soluble xylan by endo-β-1,4-xylanase from *S. thermophile* ATCC 34628.

R_{Xyl} values 0.36, a chromatographic mobility compatible with aldopentauronic acid (Fig. 4). Aldopentauronic acid, isolated from such polysaccharide hydrolysate, showed a ^{13}C NMR spectrum identical with that of the shortest acidic oligosaccharides liberated from glucuronoxylan by endo-xylanases of family 11, which is identical with Xylβ1-4(MeGlcAα-1,2)-Xylβ-1,4-Xylβ-1,4-Xyl.[6]

The main products of rhodymenan hydrolysis were xylose, xylobiose and an isomeric oligosaccharide with β-(1→3) linkage, which had a chromatographic mobility of an isomeric xylotetraose (R_{Xyl} 0.43) (Fig. 5). This isomeric xylotetraose does not occur among the products of rhodymenan hydrolysis by EXs of family 10.[8,10,11]

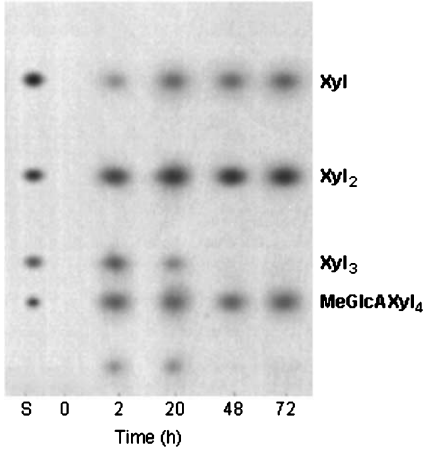

Fig. 4. TLC analysis of the hydrolysis products released from 4-*O*-methyl-D-glucuronoxylan by endo-β-xylanase from *S. thermophile* ATCC 34628. Position of stantdards (S) and time of incubation time (h) are indicated.

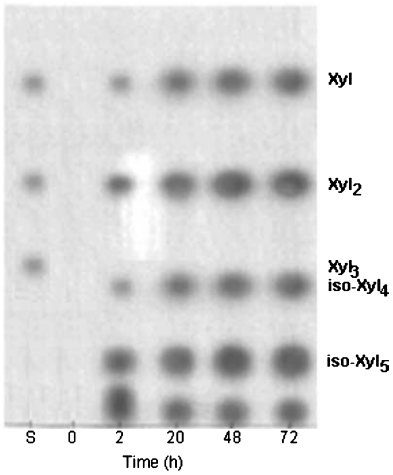

Fig. 5. TLC analysis of the hydrolysis products released from rhodymenan (β-(1→4)-β-(1→3)-xylan) by endo-β-1,4-xylanase from *S. thermophile* ATCC 34628. Position of stantdards (S) and time of incubation time (h) are indicated. $IsoXyl_4$ correspond to isomeric tetrasaccharide containing at least one β-(1→3) linkage.

When endo-xylanase was tested on beechwood *O*-acetyl-4-*O*-methylglucuronoxylan, the acetylated xylo-oligosaccharides which showed chromatographic mobility higher than Xyl and found in the hydrolysates by xylanase of family 10,[8,10,11] were not observed among the hydrolysis products (Fig. 6). The liberated acetylated and non-acetylated products could be resolved by two-dimensional TLC, with deacetylation of compounds between the two developments (Fig. 7). This pattern of products is similar to that obtain with Xln B and Xln C from *S. lividans*, EXs of family 11.[6] Non-acetylated products, mainly Xyl and Xyl_2, lay on diagonal (slope approx 1.0) and monoacetylated products (acetylated xylotriose; ACX_3) lying on the line with slope approx

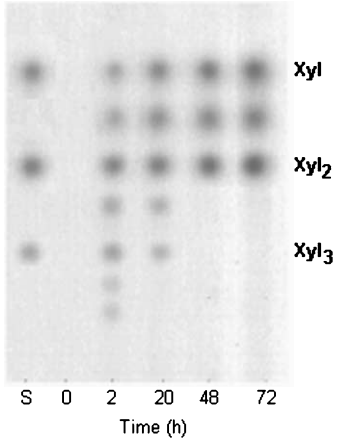

Fig. 6. TLC analysis of the hydrolysis products released from *O*-acetyl-4-*O*-methyl-D-glucuronoxylan by endo-β-1,4-xylanase from *S. thermophile* ATCC 34628. Position of standards (S) and time of incubation time (h) are indicated.

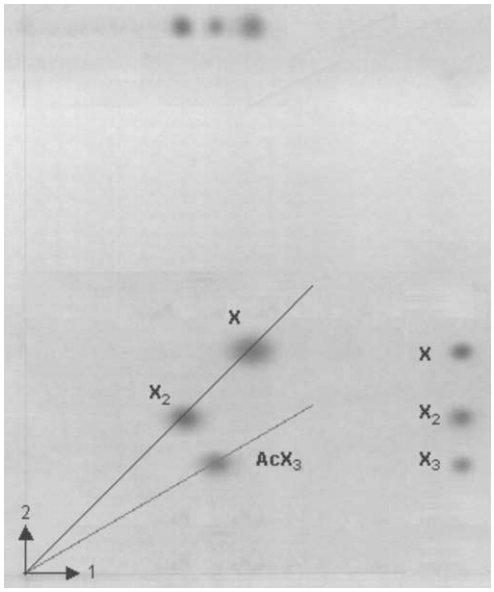

Fig. 7. Two-dimensional chromatography of products of hydrolysis of beechwood acetylxylan by endo-β-1,4-xylanase from *S. thermophile* ATCC 34628. The hydrolysate was chromatographed first in direction 1, then the dried chromatogram was exposed to ammonium hydroxide vapours to deacetylated all acetylated products of hydrolysis, and then the compounds were chromatographed in direction 2.

0.65. The acetylated products like acetylated Xyl_2 generated in significant quantities by EXs of family 10 were not observed.[8,11]

Action on oligosaccharides and the architecture of the substrate binding site—Bond-cleavage frequencies of oligosaccharides determined as initial product ratios of degradation of [1-^{3}H]-xylooligosaccharides at 0.25 mM concentration, are shown in Fig. 8. The enzyme attacks xylotriose exclusively at the first glycosidic bond from the reducing end. In higher oligosaccharides, like xylotetraose and xylopentaose, the enzyme shows a strong preference to attack the second glycosidic bond from the reducing end.

The k_o/K_m parameters (Fig. 8) indicate that the enzyme hydrolyzes xylotriose about 70 times slower

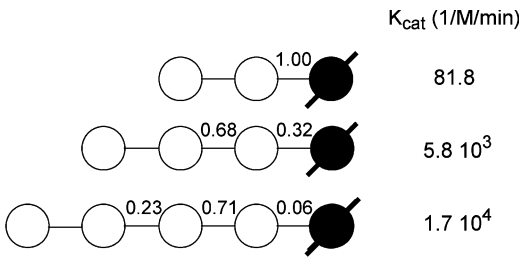

Fig. 8. Bond cleavage frequencies of [1-^{3}H]-xylooligosaccharides and their k_o/K_m parameters during initial attack by endo-β-1,4-xylanase from *S. thermophile* ATCC 34628. Symbols (○) and (●) correspond to xylopyranosyl and labeled reducing-end D-xylose residues, respectively.

than xylotetraose and xylotetraose about 2.5 times slower that xylopentaose. These data suggest that the enzyme substrate binding site consists of five distinct subsites. This assumption was confirmed by calculating the affinities for several putative subsites around the catalytic groups (Fig. 9).The enzyme has an asymmetric distribution of binding affinities right and left of the catalytic groups. Strong affinity to bind xylopyranosyl residues is exhibited by subsites −3, −2 and +2. No affinity was found at the hypothetical subsite +3. The affinities of subsites −1 and +1 were not determined due to lack of additional experimental data. The non-appreciable rate of hydrolysis of xylobiose by the enzyme suggests that the sum of affinities of the two subsites adjacent to the catalytic groups will be negative. Hence, endo-β-1,4-xylanase of *S. thermophile* appears to have a substrate binding site interacting with five xylopyranosyl residues (Fig. 10). The structure of the shortest acidic fragment liberated from glucuronoxylan suggests that only subsite −3 and +2 are able to accommodate xylopyranosyl residues carrying 4-*O*-methylglucuronosyl substituents (Fig. 10). Recently, the structure of a family 15 carbohydrate-binding module (CBM) in complex with xylopentaose has been solved by X-ray crystallography.[16] The data show that xylopentaose adopts a threefold helical structure in the binding cleft of CBM, consistent with the known

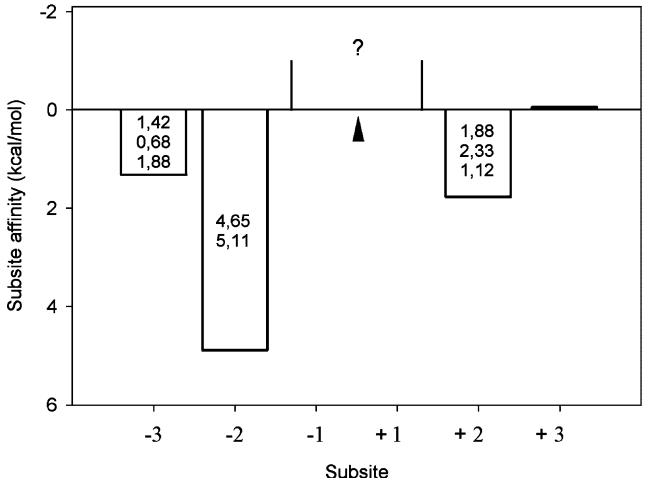

Fig. 9. Subsite affinities of *S. thermophile* ATCC 34628 endo-β-1,4-xylanase. The subsite affinities were calculated by the method of Suganuma and coworkers (1978) using the bond-cleavage frequencies of all determined endo-β-1,4-xylanase cleavage modes. The histogram of the subsite interaction energy with xylopyranosyl residues of linear 1,4-β-xylooligosaccharides shows the average values. The calculated values for individual subsites are given in the corresponding columns. 1 cal = 4.184 J. The negative numerals are used to mark the subsites left of catalytic groups and positive numeral those right of catalytic groups. The arrow indicates the position of catalytic groups.

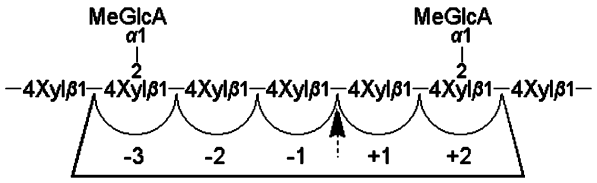

Fig. 10. Schematic representation of the productive enzyme glucuronoxylan complex of *S. thermophile* ATCC 34628 endo-β-1,4-xylanase. Only subsites −3 and +2 can accommodate a substituted xylopyranosyl residue.

structure of xylan (three xylopyranosyl units in one turn).

Action on artificial substrates—The useful criterion for differentiating the xylanases of families 10 and 11 is their action on 4-nitrophenyl-β-xylopyranoside and 4-nitrophenyl-β-cellobioside. Both these substrates were hydrolyzed at the aglyconic by xylanases of family 10. The action of endo-xylanase from *S. thermophile* was tested on both substrates at low and high substrate concentrations. No evidence has been obtained for the liberation of 4-nitrophenol from any of the two substrates either at 40 or 100 mM. The enzyme hydrolysed MeUmb-Xyl_2 at the aglyconic linkage while MeUmb-Xyl_3 is hydrolysed mainly in the middle. Kinetic parameters determined for their hydrolysis are summarised in Table 3. The affinity of XYLI towards MeUmb-Xyl_3 was 1000 times higher than that towards MeUmb-Xyl_2 (Table 3) in contrast with the family 10 xylanase from *T. aurantiacus* which exhibited almost the same affinity for the two substrates.[11] The affinity of the enzyme towards $CNPX_2$ was in the same range with that calculated for MeUmb-Xyl_2 (Table 3).

Inactivation experiments—As an example, the inactivation kinetics of the *S. thermophile* xylanase by (*S*)-4,5-epoxypentyl-β-D-xyloside are shown in Fig. 11(a). Semi-logarithmic plotting of the residual activity against time is shown in Fig. 11(b), giving the values of the apparent rate constants (k_i) for different inhibitor concentrations. The inactivation constants (k_i, K_i) were calculated graphically either by hyberbolic (Fig. 11(c)) or linear (Fig. 11(d)) fitting of the data obtained.[17] The results are summarized in Table 4. All the ω-epoxyalkyl glycosides used were found to inactivate the enzyme.

This is the first report on the purification and characterization of an endoxylanase from the culture

Table 3
Kinetic constants of endo-xylanase from *S. thermophile* for model substrates

Substrate	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m
$CNPX_2$	0.50	8	16
$MeUX_2$	0.45	4	9
$MeUX_3$	0.53	3995	7538

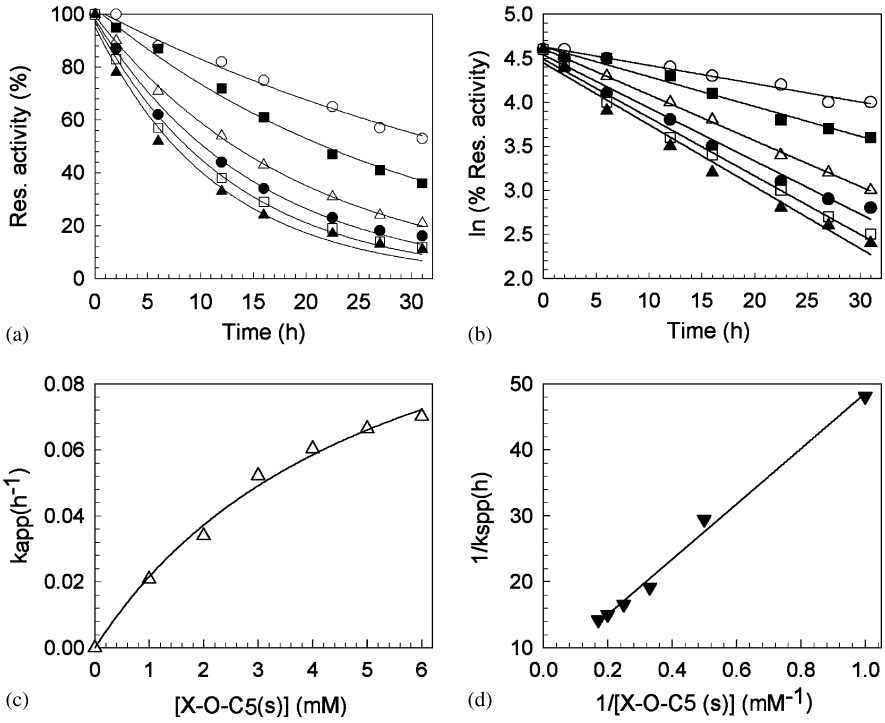

Fig. 11. Inactivation kinetics of the *S. thermophile* endo-β-1,4-xylanase with (*S*)-4,5-epoxypentyl-β-D-xyloside [$XOC_5(s)$]. (a) Residual activity as a function of time and inhibitor concentration. The enzyme (80 μM) was incubated with different concentration of (*S*)-4,5-epoxypentyl-β-D-xyloside [1 (open circles), 2 (black squares), 3 (open triangles), 4 (black circles), 5 (open squares), 6 mM (black triangles) in 50 mM phosphate buffer, pH 5.6, at 25 °C]. Samples were withdrawn at the indicated time intervals for measurement of residual (Res.) activity; (b) semi-logarithmic plot; (c) hyperbolic plot; (d) linearized plot.

medium of *S. thermophile* ATCC 34628 grown on corn cob as carbon source. The level of xylanase produced by *S. thermophile* on corn cob as carbon source was approx 60 higher than that produced on wheat straw.[18]

The enzyme was purified to homogeneity by a simple procedure which included mainly ion-exchange and gel filtration chromatographies. The molecular mass (25 kD) of purified endo-xylanase purified from *S. thermophile* was within the range detected for xylanases belonging to the family 11.[19] A low-molecular mass xylanase can be useful in pulp bleaching, since smaller enzymes can more readily access the fiber wall structure and alter more efficiently pulp properties.[20]

Like fungal xylanases from *Neocallimastix frontalis*,[21] *Trichoderma reesei*,[22] *Aspergillus oryzae*[23] and *Fusarium oxysporum*[24,25] the presently studied enzyme showed low activity on xylotriose. The xylanase hydrolyzes xylo-oligosaccharides containing at least four xylose residues and attacks mainly the internal glycosidic bonds.

The xylanase from *S. thermophile* hydrolyzes beechwood xylan to a mixture of xylo-oligosaccharides with xylotriose, xylotetraose, xylopentaose and xylohexaose being the major products. This makes the purified xylanase potentially suitable for use in the production of xylo-oligosaccharides.[26] Xylo-oligosaccharides prepared from various sources of xylans such as wheat bran,[27] birchwood, or corncob[28] can selectively be used by the beneficial intestinal microflora, *Bifidobacteria*, and are thus expected to be used as a valuable food additives. These sugars comprise mainly xylobiose, xylotriose, xylotetraose, and higher oligosaccharides.

The nature of the fragments liberated from 4-*O*-methylglucuronoxylan, rhodymenan, and acetyl-4-*O*-methylglucuronoxylan is fully compatible with the fact

Table 4
Inactivation of the xylanase from *S. thermophile*

	X–O–C_3(S)	X–O–C_3(R)	X–O–C_4(S)	X–O–C_4(R)	X–O–C_5(S)	X–O–C_5(R)	X_2–O–C_3
$1/K_i$	110	168	43	54	125	205	240
k_i	0.0047	0.0031	0.0220	0.0310	0.0030	0.0116	0.0067

Association constants ($1/K_i$, M^{-1}) and inactivation rates (k_i, h^{-1}) were determined for the alkyl epoxides derived from D-xylose and xylobiose.

that the purified xylanase from *S. thermophile* belongs to family 11. The enzyme does not liberate short fragments from the polysaccharides, as in the case of the xylanases from family 10. This is in agreement with its relatively low activity towards short xylo-oligosaccharides such as MeUmb-Xyl_2 a substrate around 1000 times less preferred than MeUmb-Xyl_3. The identification of the shortest acidic fragment released from 4-*O*-methylglucuronoxylan leads to the assumption that the enzyme, in contrast to EXs of family 10, cannot attack the xylosidic linkage towards the non-reducing end next to the branch. Similarly to other xylanases of family 11, the *S. thermophile* xylanase requires three unsubstituted consecutive xylopyranosyl residues (Fig. 10) for productive binding.

It was reported that low concentrations (2–5 mg/L) of acidic xylooligomers formed from hardwood xylan with fungal xylanases affect the growth of tissue cultures of aspen (*Populus tremuloides*)[29] and pine (*Pinus thunbergii*).[30] Furthermore, small quantities of acidic xylan hydrolysate stimulate rooting of conifer cuttings in hydroponic culture solution.[31] The addition of purified aldoterauronic acid obtained from birchwood xylan by treatment with a *T. aurantiacus* family 10 endoxylanase at a concentration of 1.6 mg/L demonstrated recognizable and high promotive action towards the induction of callus and somatic embryogenesis in culture explants of common mallow (*Malva silvestris* L) and cotton (*Gossypium hirsutum*).[31] The liberation of aldopentauronic acid from beechwood 4-*O*-methyl-D-glucuronoxylan by the endoxylanase from *S. thermophile* makes it a potential candidate for the production of acidic xylo-oligosaccharides.[32]

Since the isomeric xylotetraose, Xylβ1-4Xylβ1-3Xylβ1-4Xyl, occurs among the products of rhodymenan hydrolysis by the xylanase of *T. aurantiacus*, it can be proposed that the enzyme similarly as other EXs of family 11,[33] attacks in the polysaccharide β-(1→4) linkages which follow a β-(1→3) linkage towards the reducing end.

The action pattern on acetylxylan becomes an additional criterion for a more defined classification of EXs. No acetylated Xyl_2 was found in the hydrolysates by xylanase from *S. thermophile* a characteristic fragment of hydrolysis of acetylxylan by EXs of family 10.[8] Similar results were observed with XlnB and XlnC of *S. lividans*,[6] EXII of *T. reesei*[8] and EXI of *Schizophyllum commune*,[34] all members of family 11.

All the ω-epoxyaxyalkyl glycosides used were found to inactivate the enzyme. Similar results were obtained with two glycoside hydrolases of family 11 (*T. lanuginosus* Xyn and *T. reesei* Xyn II).[15] In striking contrast with the family 11 enzymes, the family 10 glycoside hydrolases from *T. aurantiacus* (Xyn) and *C. thermocellum* (Xyn Z) were inactivated by none of the ω-epoxyalkyl-derived xylo-oligosaccharides, even at high concentration and with prolonged incubation times.[15]

Since ω-epoxyalkyl xylosaccharide inactivations must occur via prior recognition of the carbohydrate moiety at the minus subsites,[15] hydrolases belonging to family 11 also should have a good ligand-recognition capability at the minus subsites (in comparison with those of family 10). This is in agreement with the fact that the enzymes belonging to family 10 exhibit lower glycon specificity than enzymes of family 11 since cellobiosides, lactosides and D-glucopyranosyl-β-(1,4)-D-xylopyranosides containing a cleavable aglycon are hydrolysed with considerable turn-over numbers.[35]

Finally, based on the catalytic properties and the partial subsite mapping, the endo-xylanase purified from *S. thermophile* can be classified in family 11 of glycosyl hydrolases.

1. Experimental

1.1. Materials

S. thermophile ATCC 34628 was purchased from ATCC and maintained on potato dextrose-agar (PDA) medium. Q-Sepharose and Sephacryl S-200 were from Pharmacia, Sweden. Beechwood 4-*O*-methyl-D-glucuronoxylan was isolated from sawdust as described.[7] *O*-Acetyl-4-*O*-methyl-D-glucuronoxylan was obtained by the extraction of beechwood holocellulose with dimethylsulphoxide.[36] Rhodymenan, a β-(1,4)-β-(1,3)-xylan from *Rhodymenia stenogona* was a gift from Dr A.I. Usov (Russian Academy of Sciences, Moscow, Russia). Xylo-oligosaccharides and arabinoxylan were purchased from Megazyme (Ireland). Methylumbelliferyl β-glycosides of xylobiose (MeUmb-Xyl_2) and xylotriose (MeUmb-Xyl_3) were synthesized as will be described elsewhere (Vršanská, M., Nerinckx, W., Biely, P. and Claeyssens, M., unpublished data). [1-^{3}H]-Reducing-end labelled xylo-oligosaccharides with specific radioactivity of approx 10 MBq/μmol, were obtained by catalytic titration of unlabelled compounds by the method of Evans and coworkers.[37] The (*R*,*S*)-ω-epoxyalkyl-β-xylopyranosaccharides used in this study as well as the chromogenic substrate 2-chloro-4-nitrophenyl-β-xylobioside ($CNPX_2$) were synthesized as described previously.[15] All other chemicals used were analytical grade and purchased either from Sigma, or other companies.

1.2. Production of xylanase

The mineral medium used for the production of xylanolytic enzymes contained per litre: KH_2PO_4, 3.0; K_2HPO_4, 2.0; $MgSO_4 \cdot 7\ H_2O$, 0.5; $CaCl_2 \cdot 2\ H_2O$, 0.1; $FeSO_4 \cdot 7\ H_2O$, 0.005; $MnSO_4 \cdot 4\ H_2O$, 0.0016; $ZnSO_4 \cdot 7\ H_2O$, 0.0014; $CoCl_2 \cdot 6\ H_2O$, 0.0002 g. A 5 mL mycelia

and spore suspension of *S. thermophile* from a 6-day old culture, grown on PDA slope at 45 °C, was inoculated to five 500 mL Erlenmeyer flasks each containing 200 mL of the above mineral medium supplemented with corn cob (27 g/L) as carbon source and $(NH_4)_2HPO_4$ (7.5 g/L) as nitrogen source. The flasks were incubated at 50 °C in an orbital shaker operating at 200 rpm for 2 days. The preculture (500 mL) was used to inoculate a 7 L stirred tank fermentor containing 5 L mineral medium having above composition. Maximum xylanase production has obtained when using corn cob (unpublished data). The fungus was cultured for 4 days at 50 °C and pH 5.0. Oxygen concentration was controlled to a minimum of 20% saturation by varying agitation speed. The fermentation broth was first centrifuged at 10,000*g* for 30 min at 4 °C and the clear supernatant was assayed for xylanase as described below and concentrated by ultra-filtration on a Minitan system (Millipore, Bedford, MA, USA) with Minitan membrane plates (10 kD cut off). The retentate, containing endo-xylanase activity, was used for further purification of the enzyme.

1.3. Assay of xylanase

Endo-β-1,4-xylanase activity was assayed using 1% (w/v) birchwood glucuronoxylan in 100 mM citrate–phosphate buffer, pH 5.0 and 15 min incubation at 50 °C. The release of reducing sugars was determined by the DNS method[38] and 1 unit (U) of activity was defined as the amount of the enzyme that liberates 1 μmol of reducing sugar (xylose equivalent), per minute. The relationship between reducing sugars and fluidity of a xylan solution in the presence of endoxylanase, was determined as follows: A 20 mL reaction mixture containing 4.0% (w/v) birchwood xylan and the enzyme (2.5 U) in 100 mM citrate–phosphate buffer pH 5 was incubated at 45 °C. The relative viscosity (Oswald viscometer) and the amount of reducing sugars released was estimated at different time intervals. The reciprocal of the specific viscosity ($1/n_{sp}$) was calculated by the formula $1/n_{sp} = t_o/(t - t_o)$, where t and t_o represent the time of outflow of the reaction mixture and buffer, respectively. This was plotted against the reducing sugars released.

1.4. Protein determination

The protein concentration was determined by the bicinchoninic acid method according to Pierce instruction manual No 23235X using bovine serum albumin (BSA) as a standard. A_{280} was used to monitor protein in column effluents.

1.5. Purification of endoxylanase

1.5.1. Anion exchange chromatography. Two thousand seven hundred and fifty milligram of crude enzyme in 50 mM histidine–HCl buffer, pH 8.5, was loaded on a Q-Sepharose column (20 × 15 cm). After washing with 20 mL of the equilibrating buffer, a linear gradient of 0–0.5 M NaCl in 100 mL of the same buffer was applied. Elution with this buffer (flow rate of 60 mL/h) yielded two endo-xylanase active fractions, the main fraction X-I (62% of the total eluted xylanase activity), and the minor fraction X-II. Fraction X-I was desalted on a PD-10 column (G-25 Sephadex) and concentrated by freeze-drying.

1.5.2. Gel filtration. Fraction X-I, dissolved in 2.5 mL 5 mM citrate–phosphate buffer pH 5 was applied on a Sephacryl S-200 (Pharmacia) column (2.5 × 100 cm) pre-equilibrated and eluted with the same buffer (flow rate of 60 mL/h). This purification step yielded one endoxylanase active fraction and its homogeneity was checked by SDS- (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and IEF-PAGE.

1.6. Biochemical characterization of purified endoxylanase

1.6.1. Determination of purity and molecular mass. These were determined by SDS-PAGE using the PhastSystem electrophoresis unit (Pharmacia, Sweden) and a 10–15% polyacrylamide gel.

1.6.2. Determination of isoelectric point (pI). The IEF-PAGE and the PhastSystem electrophoresis unit (Pharmacia, Sweden) were employed. The gel contained ampholytes covering a pH range of 3.0–9.5.

1.6.3. Effect of pH and temperature on the activity and stability of endoxylanase. The enzyme activity was studied in the pH 3.0–9.0 range: 100 mM citrate–Na_2HPO_4 buffer, pH 3.0–7.0; 100 mM phosphate buffer, pH 6.0–8.0; 100 mM Tris–HCl buffer, pH 7.0–9.0. The temperature effect was followed between 30 and 80 °C. The stability of the endoxylanase was studied at pH 3.0–9.0 range, using the above buffers at 0 and 50 °C.

1.6.4. Hydrolysis of polysaccharides. The 2.0% (w/v) solutions of polysaccharides in 50 mM citrate–Na_2HPO_4 buffer, pH 5.0 were incubated with appropriately diluted enzyme at 50 °C. The aliquots were analyzed at different time intervals, for hydrolysis products, by thin-layer chromatography (TLC) on microcrystalline cellulose (DC-Alufolien Cellulose, Merck) in the solvent system 3:2:1 EtOAc–AcOH–

water (by vol.). Reducing sugars were visualized by the aniline–hydrogen phthalate reagent.

1.6.5. Isolation and identification of products of hydrolysis of 4-*O*-methyl-D-glucuronoxylan. A solution of beechwood 4-*O*-methyl-D-glucuronoxylan (0.5 g in 25 mL of distilled water, pH 5.0) was incubated for 2 days at 50 °C with 5 U of *S. thermophile* xylanase. After this time the polysaccharide was found to be almost completely hydrolyzed into a mixture of D-xylose, xylobiose, small amount of xylotriose and an acidic oligosaccharide migrating on TLC identically as an aldopentauronic acid. To isolate the acid, the mixture was passed through a column of Dowex 1 (acetate form), which was washed first with distilled water until no more neutral sugars were found in the eluate. Aldopentauronic acid was liberated from the column by 3 M solution of AcOH. The AcOH was eliminated by evaporation in vacuum and the resulting syrup (75 mg) was used for ^{13}C NMR spectroscopy (Bruker AVANCE DPX 300, Germany).

1.6.6. Bond cleavage frequencies and kinetic parameters of [1-^{3}H]-xylo-oligosaccharides. The initial bond cleavage frequencies of reducing-end labeled oligosaccharides, identical with relative rates of the cleavage of individual glycosidic linkages, were determined using the method of Allen and Thoma[39] at 0.25 mM substrate concentration and appropriate enzyme dilutions. The reactions were performed in 0.02 M acetate buffer (pH 5.0) at 50 °C. Aliquots of the mixtures were removed at time intervals and subjected to TLC in 3:2:1 EtOAc–AcOH–water (by vol.). After detection of guide strips with standards with aniline–hydrogen phthalate reagent, the radioactivity in substrate and products was measured in a liquid scintillation counter. The ratio of radioactivity in the substrate and products was determined and used for graphic determination of the initial bond cleavage frequencies, i.e., frequencies corresponding to the zero reaction time. From the same experiments k_o/K_m parameters were determined using a linearized form of the Michaelis–Menten equation according to the procedure of Suganuma and coworkers.[33] The two parameters obtained for oligosaccharides up to [1-^{3}H]-xylopentaose were sufficient to calculate the number of substrate-binding subsites and subsite affinities as described by Suganuma and coworkers.[33]

1.6.7. Activity towards xylan, Xyl$_n$. Reaction mixtures (1.0 mL) consisting of 0.9 mL of 0.1% (w/v) xylan or 0.5 mM Xyl$_n$, $n = 2$–5, in 20 mM buffer citrate–Na_2HPO_4, pH 5.0 and 0.1 mL enzyme (10 μg protein) were incubated at 50 °C for 1 h. Quantitative data were obtained by analysis of samples (20 μL) using an HPLC system (Waters 600E) with CarboPac PA1 column and Dionex ED-40 electrochemical detector (Dionex, USA). A AcONa gradient (0–300 mM in 60 mM NaOH) was applied during 40 min at a flow rate of 1 mL/min. The products were quantified on the basis of peak height using standard (Xyl)$_n$ solutions.

1.6.8. Determination of kinetic parameters for the hydrolysis of aryl glycosides. Hydrolysis of aryl glycosides was carried out in 50 mM citrate–phosphate buffer, pH 5.6 at 40 °C using a SPECTRAmax 250 Microplate Spectrophotometer (Molecular Devices, USA). Reaction after temperature equilibration were initiated by the addition of 10 μL solution of pure xylanase (20 μg protein) in a total volume of 210 μL. The substrate concentrations ranges used were: 0.1–100 mM for 2-chloro-4-nitrophenyl-β-xylobioside ($CNPX_2$), 4-nitrophenyl-β-D-xylopyranoside (pNPX), 4-nitrophenyl-β-D-cellobioside (pNPC). The release of pNP or CNP was monitored spectrophotometrically at 405 nm. Kinetic constants (K_m and k_{cat}) were determined based on the initial rates of hydrolysis.

1.6.9. Determination of kinetic parameters for the hydrolysis of MeUmb(Xyl)$_n$. Reaction mixtures (1.0 mL) consisting of 0.9 mL of 0.1–20 mM MeUmb(Xyl)$_n$; $n = 2$–3, in 50 mM buffer citrate–phospate, pH 5 and 0.1 mL enzyme (appropriate enzyme dilutions) were incubated at 25 °C for 4 h. Samples (50 μL) were removed at different time intervals and analyzed by HPLC using a NH_2-μBondapak column (300 × 3.9 mm) (Waters) with 70:30 MeCN–water as mobile phase at a flow rate of 1.0 mL/min. MeUmb and MeUmb(Xyl)$_n$, $n = 1$–3, were identified using a Waters UV detector (Model 440) at 313 nm. The products were quantified on the basis of peak height using standard MeUmb and MeUmb(Xyl)$_n$ solutions. Analysis of the frequency of bond cleavage of MeUmb(Xyl)$_n$ was carried out as described previously.[13]

1.6.10. Inactivation experiments. Sets of reaction mixtures containing different concentrations of the epoxides (0–10 mM) and a constant concentration of enzyme (0.5 μM) in a total volume of 100 μL were incubated at 25 °C in 50 mM citrate–phosphate buffer at pH 5. At 30 min intervals, 10 μL aliquots were taken to measure the residual activity using 2-chloro-4-nitrophenyl β-xylobioside ($CNPX_2$; 200 μL; 500 mM) as substrate. The product formation (CNP, 405 nm) was followed continuously at 20 °C in a microtiterplate reader. The activity of an enzyme blank (without epoxide) was followed in the same way. Apparent inactivation constants were obtained and the relevant association ($1/K_i$) and inactivation (k_i) constants were calculated by conventional analysis of the data.[15]

Acknowledgements

The authors acknowledge the financial support of the commission of European Communities through the INCO Copernicus Programme Project number IC15-CT-96-1000.

References

1. Royer, J. C.; Nakas, J. P. *Enzyme Microb. Technol.* **1989**, *11*, 405–410.
2. Viikari, L.; Kantelinen, A.; Sundquist, J.; Linko, M. *FEMS Microbiol. Rev.* **1994**, *13*, 335–350.
3. Gilbert, H. J.; Hazlewood, G. P. *J. Gen. Microbiol.* **1993**, *139*, 187–194.
4. Gilkes, N. R.; Henrissat, B.; Kilburn, D. G.; Miller, R. C., Jr.; Warren, R. A. *J. Microbiol. Rev.* **1991**, *55*, 303–315.
5. Davies, G.; Henrissat, B. *Structure* **1995**, *3*, 853–859.
6. Biely, P.; Kluepfel, D.; Morosoli, R.; Shareck, F. *Biochim. Biophys. Acta* **1993**, *1162*, 246–254.
7. Biely, P.; Vršanská, M.; Kremnicky, M.; Tenkanen, M.; Poutanen, K.; Hayn, M. In *Trichoderma* Cellulases and Other Hydrolases; Pouminen, P.; Reinikainen, T., Eds.; Fagepaino Oy: Helsinki, 1972; pp 125–135.
8. Biely, P.; Vršanská, M.; Tenkanen, M.; Kluepfel, D. *J. Biotechnol.* **1997**, *57*, 151–166.
9. Bennett, N. A.; Ryan, J.; Biely, P.; Vrsanska, M.; Kremnicky, L.; Macris, B. J.; Kekos, D.; Christakopoulos, P.; Katapodis, P.; Claeyssens, M.; Nerinckx, W.; Ntauma, P.; Bhat, M. K. *Carbohydr. Res.* **1998**, *306*, 445–455.
10. Kalogeris, E.; Christakopoulos, P.; Kekos, D.; Macris, B. J. *J. Biosci. Bioeng.* **1999**, *876*, 819–821.
11. Kalogeris, E.; Christakopoulos, P.; Kekos, D.; Biely, P.; Vršanska, M.; Macris, B. J. *J. Molec. Catalysis (B: Enzymatic)* **2001**, *1146*, 491–501.
12. Torronen, A.; Kubicek, C. P.; Henrissat, B. *FEBS Lett.* **1993**, *321*, 135–139.
13. Gebler, J. C.; Gilkes, N. R.; Claeyssens, M.; Wilson, D. B.; Beguin, P.; Wakarchuk, W. W.; Kilburn, D. G.; Miller, R. C.; Warren, R. A. J.; Withers, S. G. *J. Biol. Chem.* **1992**, *267*, 12559–12561.
14. Svensson, B.; Sogaard, M. *J. Biotechnol.* **1993**, *29*, 1–37.
15. Ntarima, P.; Nerinckx, W.; Klarskov, K.; Devreese, B.; Bhat, M. K.; Van Beeumen, J.; Claeyssens, M. *Biochem. J.* **2000**, *347* (8), 65–73.
16. Szabó, L.; Jamal, S.; Xie, H.; Charnock, S. J.; Bolam, D. N.; Gilbert, H. J.; Davies, G. J. *J. Biol. Chem.* **2001**, *276* (52), 49061–49065.
17. Segel, I. H. *Biochemical Calculations*; Wiley & Sons: Chichester, 1975; pp 208–323.
18. Sugden, C.; Bhat, M. K. *World J. Microbiol. Biotechnol.* **1994**, *10*, 444–451.
19. Torronen, A.; Rouvinen, J. *J. Biotechnol.* **1997**, *57*, 137–149.
20. Filho, E. X. F. Hemicellulases and biotechnology. In: Pandalai, S. G. (Ed.), Recent Research Developments in Microbiology. Research Signopost. Trivandrum, India, pp. 165–176.
21. Garcia-Campayo, V.; McCrae, S. I.; Wood, T. M. *World J. Microbiol. Biotechnol.* **1994**, *10*, 64–68.
22. Tenkanen, M.; Puls, J.; Poutanen, K. *Biotechnol. Appl. Biochem.* **1991**, *13*, 380–389.
23. Bailey, M.; Puls, J.; Poutanen, K. *Biotechnol. Appl. Biochem.* **1991**, *13*, 380–389.
24. Christakopoulos, P.; Nerinckx, W.; Kekos, D.; Macris, B. J.; Claeyssens, M. *J. Biotechnol.* **1996**, *51*, 181–189.
25. Christakopoulos, P.; Nerinckx, W.; Kekos, D.; Macris, B. J.; Claeyssens, M. *Carbohydr. Res.* **1997**, 191–195.
26. Saha, B. C. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 762–766.
27. Chen, C.; Chen, J. L.; Lin, T. Y. *Enzyme Microb. Technol.* **1997**, *21*, 91–96.
28. Yamada, H.; Itoh, K.; Morishita, Y.; Taniguchi, H. *Cereal Foods World* **1993**, *38* (7), 490–492.
29. Okazaki, M.; Fujikawa, S.; Matsumoto, N. *J. Jpn. Soc. Nutr. Food Sci.* **1990**, *43*, 395–401.
30. Ishi, K.; Mohri, T.; Kinoshita, I.; Ishihara, M. *Trans. Jpn. For. Sci.* **1993**, *104*, 609–610.
31. Ishihara, M.; Nagao, Y.; Shimizu, K. In: Proc. 8th Int. Symp. Wood Pulping Chem., Helsinki, vol. 2, 1995, pp. 17–22.
32. Katapodis, P.; Kavarnou, A.; Kintzios, S.; Pistola, E.; Kekos, D.; Macris, B. J.; Christakopoulos, P. *Biotechnol. Lett.* **2002**, *24*, 1413–1416.
33. Suganuma, T.; Matsuno, R.; Ohnishi, M.; Hiromi, K. *J. Biochem.* **1978**, *84*, 293–316.
34. Biely, P.; MacKenzie, C. R.; Schneider, H.; Puls, J. In *Wood and Cellulosics: Industrial Utilization Biotechnology, Structure and Properties*; Kennedy, J. F.; Phillips, G. O.; Williams, P. A., Eds.; Wiley: New York, 1987; pp 283–289.
35. Claeyssens, M.; Henrissat, B. *Protein Sci.* **1992**, *1*, 1293–1297.
36. Bouveng, H. O.; Garegg, P. J.; Lindberg, B. *Acta Chem. Scand.* **1960**, *14*, 742–748.
37. Evans, E. A.; Sheppard, H. C.; Turner, J. C.; Warrell, D. C. *J. Labelled Compounds* **1974**, *10*, 569–587.
38. Miller, G. L. *Anal. Chem.* **1959**, *31*, 426–428.
39. Allen, J. D.; Thoma, J. A. *Biochemistry* **1978**, *17*, 2338–2344.