The xylan-degrading enzyme system of *Talaromyces emersonii*: novel enzymes with activity against aryl β -D-xylosides and unsubstituted xylans

Maria G. TUOHY,* Jürgen PULS,† Marc CLAEYSSSENS,‡ Mária VRŠANSKÁ§ and Michael P. COUGHLAN*||

*Department of Biochemistry, University College, Galway, Ireland, †Institut für Holzchemie, D-2050 Hamburg 80, Germany, ‡Laboratorium voor Biochimie, Rijksuniversiteit Gent, B-9000 Gent, Belgium, and §Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava, Czechoslovakia

Talaromyces emersonii, a thermophilic aerobic fungus, produces a complete xylan-degrading enzyme system when grown on appropriate substrates. In this paper we present the physico-chemical and catalytic properties of three enzymes, xylosidase (Xyl) I (M_r 181 000; pI 8.9), II (M_r 131 000; pI 5.3) and III (M_r 54 200; pI 4.2). Xyl I and II appear to be dimeric and Xyl III is a single-subunit protein. All three enzymes catalyse the hydrolysis of aryl β -D-xylosides and xylo-oligosaccharides.

Xyl I is a classic β -xylosidase (1,4- β -D-xylan xylohydrolase; EC 3.2.1.37), and Xyl II and III are novel xylanases (endo-1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) which we believe have not hitherto been reported. In addition to the above substrates, they also catalyse the extensive hydrolysis of unsubstituted xylans, and may have considerable biotechnological potential. The hydrolysis product profiles and bond-cleavage frequencies with various substrates are presented.

INTRODUCTION

Xylans, the most plentiful of the hemicelluloses, are present in the cell walls of all land plants and are particularly abundant in tissues that have undergone secondary thickening [1]. Extensive breakdown of xylans requires the synergistic action of a variety of hydrolytic enzymes, the xylan-degrading enzyme system [2–4]. Depending on the origin of the cell wall material, such enzymes include the main chain-cleaving enzymes, the endo-1,4- β -D-xylanases (EC 3.2.1.8) and the β -xylosidases (EC 3.2.1.37), and those that liberate side chain substituents, namely α -glucuronidases, acetylxyloesterases and α -L-arabinofuranosidases (EC 3.2.1.55).

β -Xylosidases cleave xylobiose, remove xylose residues from the non-reducing ends of xylo-oligosaccharides, and many can also liberate xylose from artificial substrates such as aryl β -xylosides [2–6]. Generally, β -xylosidases do not act against 'native' xylans although exceptions have been reported. An example of the latter is the enzyme from *Neocallimastix frontalis* [7]. Endoxylanase, as the name implies, cleaves β -1,4 linkages in the backbone of xylans, arabinoxylans, glucuronoxylans and related polymers [2–6]. However, depending on the enzyme, such action may require, or be hindered by, side chain substitution of the substrate (for a review see ref. [8]). Xylanases can also hydrolyse higher xylo-oligosaccharides, the affinity for such substrates increasing with increasing degree of polymerization (DP) [2,3]. By contrast with the β -xylosidases, they do not cleave xylobiose and most such enzymes investigated, including representatives from *Aspergillus niger* [9], *Aspergillus ochraeus* [10], *Bacillus subtilis* [11], *Humicola grisea* var *thermoidea* [12] and *Streptomyces lividans* [13,14], do not hydrolyse aryl β -D-xylosides. Again, however, some exceptions have been noted. Among these are Xyn A from *Caldocellum saccharolyticum* [15], a xylanase from *Cryptococcus albidus* [16] and one from *Thermoascus aurantiacus* [17], whereas Xyl I from *Streptomyces lividans* can hydrolyse *p*-nitrophenyl (PNP) β -D-glucoside but has no action against PNP β -D-xyloside [18]. In the case of the *C. albidus*

enzyme, it has been established that conversion of PNP β -D-xyloside occurs, not by direct hydrolysis, but by a complex transfer reaction sequence [19].

Talaromyces emersonii, a thermophilic fungus, produces a complete thermostable xylan-degrading enzyme system when grown on appropriate substrates ([20–23] and M. G. Tuohy, J. Puls and M. P. Coughlan, unpublished work). In this paper we report on the properties of three enzymes, Xyl I–III, from this system. Each of the three is active against aryl β -D-xylosides and xylo-oligosaccharides. Xyl I appears to be a typical β -xylosidase. However, Xyl II and III are unusual xylanases in that they also catalyse extensive endohydrolysis of xylans from which arabinose substituents have been removed. They may have considerable potential in specific biotechnological applications including pulp and paper manufacturing.

MATERIALS AND METHODS

Substrates for growth, enzyme induction and assay

Beet pulp (average size 0.2 cm $\times$ 0.8 cm) and wheat bran were obtained locally. Avicel was obtained from Merck, Darmstadt, Germany; arabinogalactan (larchwood), CM-cellulose (low-viscosity material), β -glucan (from barley), arabinan (beet pulp), pectin (citrus), polygalacturonic acid (sodium pectate; uronic acid content 85–90%), PNP α -L-arabinofuranoside, PNP α -L-arabinopyranoside, PNP α - or β -D (or L)-fucoside, PNP α - or β -D-galactoside, PNP α - or β -D-glucoside, PNP β -D-lactoside, PNP α - or β -D-mannoside or PNP α -L-rhamnopyranoside, *o*-nitrophenyl (ONP) and PNP β -D-xyloside, laminarin, oat spelt xylan and methylumbelliferyl β -D-xyloside were from Sigma Chemical Co.; 2-chloro-4-nitrophenyl (CNP) β -D-xylopyranoside was obtained from Lambda Probes and Diagnostics (Graz, Austria). PNP β -D-xylobioside [24] and xylo-oligomers (xylobiose to xyloheptaose) [25] were prepared as described previously. Reducing end-1- 3 H-labelled xylo-oligomers were prepared by catalytic tritiation of the unlabelled compounds [26]. Beechwood xylan from the viscose process, a virtually unsubstituted material

Abbreviations used: Xyl I, II and III, xylosidases I, II and III; DP, degree of polymerization; PNP, *p*-nitrophenyl or *p*-nitrophenol; ONP, *o*-nitrophenyl; CNP, chloronitrophenyl.

|| To whom all correspondence should be addressed.

(DP 30), was obtained from Lenzing AG. Arabinoxylan was prepared by extraction of delignified wheat straw using NaOH, in the course of which all ester-linked substituents were removed [25]. The xylose/arabinose/4-*O*-methylglucuronic acid ratio was 260:21:10 (by wt.). Agarose-gel support medium (GelBond) was from FMC Bio-Products (Rockland, ME, U.S.A.). Hydroxyapatite (Bio-Gel HTP) was from Bio-Rad Laboratories, Watford, Middx., U.K.

Organisms and culture conditions

The thermophilic aerobic fungus, *T. emersonii* strain CBS814.70, used in these studies was obtained from Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. It is routinely sub-cultured on Sabouraud dextrose-agar medium. Solid-state cultivation (final volume approx. 200 ml) was carried out under static conditions in 2-litre Erlenmeyer flasks at 45 °C. Media, adjusted to pH 4.5 with 1 M NaOH, contained mineral salts, corn steep liquor (0.5%, w/v), (NH₄)₂SO₄ (1.5%, w/v), KH₂PO₄ (0.5%, w/v), yeast extract (0.1%, w/v) plus inducing substrate (33% wet w/w) as described earlier [20,27].

Enzyme extraction and assay

After suitable periods of growth the solid-state cultures were mixed with 13 vol. of 0.1 M sodium acetate buffer, pH 5, containing 0.01% (v/v) Tween 80. The mixture was blended gently for 20 s in a homogenizer, then incubated with shaking at 140 rev./min room temperature for 2 h, and finally centrifuged for 1 h at 1300 *g*. The supernatant was filtered through glass-wool and used for assay of enzyme activity and protein concentration.

For routine purposes, β -xylosidase activity was measured by monitoring the increase in A_{410} resulting from the liberation of *p*-nitrophenol (PNP) after 15 min incubation at 50 °C of PNP β -D-xyloside (final concn. 0.5 mM) with a portion of enzyme in 100 mM sodium acetate buffer, pH 5 (final volume 0.6 ml). β -Glucosidase and β -galactosidase activities were measured as above using PNP β -D-glucoside (final concn. 0.9 mM) or PNP β -D-galactoside (final concn. 0.5 mM) as appropriate. Some xylosidase assays were carried out using microtitre plates with the filter appropriate to 405 or 410 nm and a 490 nm reference filter. Reaction mixtures contained 100 μ l of substrate in 100 mM sodium acetate buffer, pH 5, and 5–10 μ l of appropriately diluted enzyme. Reactions, in either type of assay, were stopped before reading by the addition of 1 vol. of 1 M Na₂CO₃. Enzyme activity is given as μ mol/min per ml of enzyme, i.e. as international units (IU). For comparative purposes, the yields of activity in solid-state cultures are expressed as IU/g of inducing substrate.

Because of the presence of phenolic and other interfering substances in crude extracts and fractions obtained during the early stages of purification, protein concentration could be determined with accuracy only after precipitation with 5% (w/v) trichloroacetic acid and redissolution in 0.1 M NaOH containing 2.0% (w/v) Na₂CO₃. Standard BSA was treated in the same way and protein concentration was determined by the method of Lowry et al. [28].

Electrophoresis and isoelectric focusing

Proteins in the extracts of solid-state cultures used for enzyme isolation were separated under denaturing conditions by SDS/PAGE [29]. For electrophoresis under non-denaturing conditions

the samples were not heated and SDS and 2-mercaptoethanol were excluded. Gels were stained for protein using Coomassie Brilliant Blue R250 and for glycoprotein using the periodic acid/Schiff reagent [30]. The M_r values of the purified enzymes were calculated by comparison of their mobilities with those of standard proteins (M_r range 14400–669000, as appropriate). Replicate gels were stained for β -xylosidase activity by incubating the developed gels with 2 mM methylumbelliferyl β -D-xyloside (room temperature for 4 min) or 20 mM PNP β -D-xyloside (50 °C for 20 min), in 100 mM sodium acetate buffer, pH 5.0. In the latter case, reactions were stopped by the addition of 1 M Na₂CO₃. The pI values of the purified enzymes were determined by isoelectric focusing on LKB ampholine PAG plates covering the pH range from 3.5 to 9.5, using the ampholytes (pH range 3 to 10) supplied by Pharmacia and the procedures recommended in the Pharmacia information booklet 1804. Standard pH marker proteins (Pharmacia), covering the range 3.5–9.3, were run in conjunction with the unknowns. Gels were stained for protein and activity as above.

Determination of products of substrate hydrolysis

H.p.l.c. gel filtration

The products of hydrolysis of the artificial aryl substrates, after incubation at 25 °C of 200 μ l of 50 mM sodium acetate buffer, pH 5.6, plus 20 μ l of substrate and a sample of enzyme, were fractionated by h.p.l.c. gel filtration on a column (25.0 cm $\times$ 0.43 cm) of Polyol RSil (13 μ m, Bio-Rad). Portions (10 μ l) of reaction mixture were mixed with 100 μ l of acetonitrile/water (7:3, v/v). Then 20 μ l of this mixture was applied to the column, fractions were eluted at 1.5 ml/min using acetonitrile/water (7:3, v/v) and detected spectrophotometrically at 313 nm.

High-performance anion-exchange chromatography

The products of hydrolysis of xylans (10 mg/ml) and unlabelled xylo-oligosaccharides (2.5–5.0 mg/ml), after incubation at 50 °C with shaking at 140 rev./min, with a sample of enzyme in 100 mM sodium acetate buffer, pH 5.0, were fractionated by high-performance anion-exchange chromatography using the Dionex system as described previously [31].

T.l.c.

The frequencies of bond cleavage with 1-³H-labelled xylo-oligosaccharides as substrates were determined as described previously [32]. To this end, the products of hydrolysis of the labelled xylo-oligosaccharides were fractionated by t.l.c. and the areas corresponding to the various products were excised. The amount of radioactivity in each area was then measured using the scintillation cocktail supplied by Beckman Instruments Inc. (Fullerton, CA, U.S.A.) and a Beckman LS 1801 scintillation counter.

Enzyme purification

A summary of the procedures used to isolate, to apparent homogeneity, the three enzymes with activity against PNP β -D-xyloside is given in Scheme 1. Because of their action against this substrate, they are termed xylosidases I, II and III (Xyl I, II and III). However, as is explained below, only Xyl I is a true β -xylosidase (EC 3.2.1.37), the other two being unusual xylanases (EC 3.2.1.8).

Scheme 1 Summary of the procedures used in the purification of Xyl I, II and III from *T. emersonii*

β -Xylosidase I (Xyl I)

Crude extract was concentrated 13-fold by hollow fibre (Amicon) filtration and freeze-dried. (It will be noted in Table 1 that ultrafiltration effected an increase in activity presumably by removal of low M_r inhibitors, possibly phenolic substances released from the growth substrates.) This material could be stored indefinitely at 4 °C without loss of activity. For routine purification procedures, one-fifth (about 1.62 g) of freeze-dried material was resuspended in 20 ml of 100 mM sodium acetate buffer, pH 5.0, and precipitated by the addition of 2 vol. of propan-2-ol pre-equilibrated at -70 °C. The precipitate was collected by centrifugation at 27500 g (13000 rev./min) for 40 min at 0 °C. Residual propan-2-ol was removed under a stream of air, and the precipitate was resuspended in sufficient 100 mM sodium acetate buffer, pH 5.0, to give an overall protein concentration 60-fold greater than that of the crude extract. The resuspended material was fractionated by gel filtration on a column (2.6 cm $\times$ 80.5 cm) of Sephacryl S-300 superfine. Xyl I activity was co-eluted with α -arabinofuranosidase, α -galactosidase I (two forms of this enzyme were detected, see below) and β -glucosidase and overlapped with a peak containing β -galactosidase, β -fucosidase and α -rhamnopyranosidase activities. Fractions 43–69 were pooled and concentrated approx. 6-fold by ultrafiltration using an Amicon with a PM-13 membrane and N_2 at 48.3–89.7 kPa. The concentrated material was dialysed for 8–13 h against two changes of 25 mM Mops buffer, pH 7.0.

The concentrated material was then subjected to anion-exchange chromatography on a column (1.9 cm $\times$ 6.0 cm) of DEAE-cellulose DE-52 pre-equilibrated with 25 mM Mops buffer, pH 7.0. Protein was eluted using a linear gradient (0–0.35 M) of NaCl in the above buffer. α -Rhamnopyranosidase was eluted

first followed by overlapping peaks of Xyl I, β -galactosidase, β -fucosidase and β -glucosidase, with α -galactosidase I and α -arabinofuranosidase in later fractions. Fractions 69–79, containing Xyl I of highest specific activity, were pooled and concentrated as before and dialysed against two changes of 50 mM ammonium acetate, pH 4.5.

The above material was fractionated by anion-exchange chromatography on a column (1.6 cm $\times$ 12.0 cm) of DE-52 pre-equilibrated with 50 mM ammonium acetate, pH 4.5. In this step, the Xyl I, β -galactosidase, β -fucosidase and β -glucosidase activities did not bind and were readily separated from residual α -galactosidase I and α -arabinofuranosidase activities as well as from unidentified protein. The fractions (5–15) containing Xyl I activity were pooled and brought to 25% (w/v) saturation with $(NH_4)_2SO_4$ and fractionated on a column (0.8 cm $\times$ 9.7 cm) of phenyl-Sepharose pre-equilibrated with 50 mM ammonium acetate, pH 4.5, containing 25% (w/v) $(NH_4)_2SO_4$. Protein was eluted using, simultaneously, a decreasing gradient (25–0%, w/v) of $(NH_4)_2SO_4$ and an increasing gradient (0–40%, v/v) of ethylene glycol. Under these conditions, Xyl I was separated cleanly from contaminating activities. Fractions 35–45, which contained Xyl I in an apparently homogeneous form (see below), were pooled.

β -Xylosidase/xylanase II (Xyl II)

Samples of resuspended propan-2-ol precipitates of concentrated crude extracts, prepared as described above, were subjected to gel filtration on a column (2.6 cm $\times$ 100.7 cm) of Sephacryl S-200. Fractions 91–109, which exhibited Xyl II activity in addition to α -galactosidase II, β -glucanase and xylanase activities, were pooled and concentrated using an Amicon with a PM-10 mem-

brane and dialysed against 50 mM ammonium acetate buffer, pH 5.0.

The enzyme preparation was then fractionated by anion-exchange chromatography on a column (1.6 cm $\times$ 20.0 cm) of DE-52 pre-equilibrated with 50 mM ammonium acetate buffer, pH 5.0. Fractions 33–43, which contained β -xylosidase, α -galactosidase II, traces of xylanase but no β -glucanase, were pooled and concentrated as before. The concentrated material was dialysed against 50 mM Mops buffer, pH 7.0.

The resulting preparation was again subjected to anion-exchange chromatography on a column (1.9 cm $\times$ 7.0 cm) of DE-52 pre-equilibrated with 50 mM Mops buffer, pH 7.0. In this step xylanase and other (unidentified) proteins were removed but β -xylosidase and α -galactosidase activities still overlapped to some extent. Fractions 36–42 were pooled, concentrated as before and dialysed against 1 mM sodium phosphate buffer, pH 7.2.

The preparation from above was then fractionated by adsorption chromatography on a column (1.3 cm $\times$ 13.0 cm) of hydroxyapatite. Protein was eluted by successive linear gradients: (a) 1–5 mM MgCl_2 (total volume 30 ml); (b) 1–13 mM sodium phosphate buffer, pH 7.2 (total volume 40 ml); (c) 13–300 mM of the same buffer (total volume 100 ml). Residual activity against arabinoxylan was eluted at fractions 10–16 in the first wash, while in the second, α -galactosidase II (fractions 73–78) separated cleanly from Xyl II (fractions 80–85). The latter were pooled and found to be apparently homogeneous (see below).

β -Xylosidase/xylanase III (Xyl III)

Fractions 127–147 from S-200, which exhibited Xyl III, β -glucosidase and CM-cellulase activities, were pooled, concentrated as before and $(\text{NH}_4)_2\text{SO}_4$ was added to 25% (w/v) saturation. This material was then fractionated by hydrophobic chromatography on a column (0.8 cm $\times$ 9.7 cm) of *n*-octyl-Sepharose. Protein was eluted by using a simultaneous decreasing gradient (25–0% saturation) of $(\text{NH}_4)_2\text{SO}_4$ and an increasing gradient (0–20%, v/v) of ethylene glycol. Fractions 25–28, which exhibited Xyl III plus a small amount of β -glucosidase and other unidentified protein, were pooled, concentrated as before and dialysed against 50 mM ammonium acetate buffer, pH 5.0. This material was fractionated by anion-exchange chromatography on a column (1.9 cm $\times$ 7.0 cm) of DE-52 pre-equilibrated with 50 mM ammonium acetate buffer, pH 5.0. Protein was eluted using a linear gradient (0–0.3 M) of NaCl. This removed residual contaminating β -glucosidase and xylanase activities. Apparently homogeneous Xyl III was then eluted as a single sharp peak by washing with 50 mM ammonium acetate buffer, pH 5.0, containing 0.4 M NaCl.

RESULTS AND DISCUSSION

Enzyme production and purification

Extracts of various solid-state cultures of *T. emersonii*, strain CBS814.70, exhibited β -xylosidase activity [20–22]. Because of the method of preparation used, such extracts could include cell-bound as well as extracellular enzymes. However, since filtrates of liquid cultures also contain high yields of xylanase and β -xylosidase activities [20–22], we conclude that most of the activity observed in solid-state extracts is of extracellular origin. Although wheat bran is routinely the best inducing substrate for β -xylosidase production, an equal mixture of wheat bran and beet pulp is the best medium for production (by *T. emersonii*) of

carbohydrases generally [20–22]. Thus extracts from such cultures harvested after 11 days of cultivation were used for the isolation of the enzymes exhibiting aryl β -D-xylosidase activity as detailed in the Materials and methods section. The yields and fold purification of each enzyme given in Table 1 reflect the fact that the bulk of the activity in crude extracts is due to the action of Xyl I.

Enzyme homogeneity, M_r and pI values

Xyl I, II and III each yielded single bands when stained for protein and activity after SDS/PAGE or isoelectric focusing (not shown). The relevant bands staining for protein were coincident with those staining for activity. Thus, by these criteria, each enzyme preparation would appear to be homogeneous. Staining for carbohydrate and tight binding to concanavalin A-Sepharose also indicated each enzyme to be a glycoprotein. The calculated M_r and pI values of each enzyme are listed in Table 2. Comparison of the M_r values obtained by gel filtration on Sephacryl S-300 with those obtained using SDS/PAGE suggested that Xyl I and II are dimeric proteins whereas Xyl III is a single-subunit enzyme.

The reported M_r values of β -xylosidases from bacterial and fungal sources cover quite a wide range. Examples include single-subunit proteins from *Aspergillus niger* (78000) [34], *Bacillus pumilus* (62600) [35], *Butyrivibrio fibrisolvens* (60000) [36], *Chaetomium trilaterale* (118000) [37], *Emericella nidulans* (116000) [38] and *Paecilomyces varioti* (67000) [39]. By contrast, the xylosidases from *Bacillus coagulans* (190000 by S-200; 212000 by ultracentrifugation; 26000 by SDS/PAGE) [40], *Clostridium acetobutylicum* (two subunits of 63000 and 85000, respectively) [41], *Neocallimastix frontalis* (180000 by gel filtration; 90000 by SDS/PAGE) [7] and *Trichoderma reesei* (100000) [42] are dimers or higher aggregates. Moreover, active dimeric forms of the xylosidases of *B. pumilus* (130000) [35] and *C. acetobutylicum* (240000) [41] have been reported. Endoxylanases, on the other hand, are generally found to be single-subunit proteins with M_r values ranging from 16000 to about 60000 and pI values ranging from 3.6 to 10.0 [3,5,43].

pH and temperature optima

The pH and temperature optima under assay conditions and thermal stabilities of Xyl I–III are listed in Table 2. As expected from earlier studies on the xylan-degrading enzyme system of *T. emersonii* [22], the three enzymes under discussion have acidic pH optima and have much higher temperature optima and greater thermal stabilities than the majority of xylan-degrading enzymes (especially those from fungal sources) reported in the literature [3,5,43]. However, thermostable xylanases and/or xylosidases from *Bacillus* sp. 11-IS [44], *Caldocellum saccharolyticum* [45], *Ceratocystis paradoxa* [46,47] and *Talaromyces byssoschlamyoides* [48] have been characterized. Like these enzymes, the thermostabilities of those reported here may be advantageous in biotechnological applications.

Substrate specificity

As judged by the release of reducing sugars, none of the purified enzyme preparations effected the hydrolysis of filter paper, Avicel, CM-cellulose, β -glucan (from barley), arabinan (beet pulp), arabinogalactan (larchwood), laminarin, pectin (citrus) or polygalacturonic acid. Neither did they catalyse the hydrolysis of PNP α -L-arabinofuranoside, PNP α -L-arabinopyranoside, PNP α - or β -D (or L)-fucoside, PNP α - or β -D-galactoside, PNP α - or

Table 1 Summary of the purification of Xyl I, II and III from *T. emersonii*

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)*	Purification (fold)	Yield (%)
Xyl I						
Crude extract	500	249.2	49.0	0.2	1.0	100.0
Amicon ultrafiltration†	25.0	126.6	113.7	0.9	4.5	232.0
Propan-2-ol pptn.	16.0	128.8	87.8	0.7	0.8	77.2
Sephacryl S-300	123.0	2.2	82.6	37.7	41.9	72.7
DE-52, pH 7.0	28.0	0.7	72.6	103.7	115.2	63.9
DE-52, pH 4.5	26.0	0.5	72.6	145.2	161.3	63.9
Phenyl-Sepharose	29.5	0.49	71.3	149.4	166.0	62.7
Xyl II						
Crude extract	1000	498.4	98.0	0.2	1.0	100.0
Amicon ultrafiltration†	32.0	480.0	227.5	0.47	2.4	232.2
Propan-2-ol pptn.	36.0	481.7	179.7	0.37	0.8	79.0
Sephacryl S-200	71.5	38.8	11.4	0.3	0.6	5.0
DE-52, pH 7.0	26.0	0.96	8.7	9.0	19.1	3.8
Hydroxyapatite	12.0	0.11	10.5	95.5	203.2	4.6
Xyl III‡						
Sephacryl S-200	86.0	8.6	51.2	6.0	12.7	22.5
n-Octyl-Sepharose	40.0	2.1	35.5	16.9	40.0	15.6
DE-52, pH 4.5	10.0	0.04	29.9	747.6	1590.4	13.1

* With PNP β -D-xyloside as substrate.† Ultrafiltration gave an increase in activity presumably by removal of inhibitory low- M_r material. Subsequent yield and fold purification values are based on activity after this step.

‡ The first three steps were as for Xyl II.

Table 2 Physicochemical properties of Xyl I, II and III from *T. emersonii*

Property	Xyl I	Xyl II	Xyl III
M_r (S-300)	181 000	131 000	49 800
M_r (SDS/PAGE)	97 500	74 850	54 200
pI	8.9	5.3	4.2
Optimum pH*	2.5	4.2	3.5
Optimum temperature (°C)†	60.0	78.0	67.0

* In constant-ionic-strength citrate phosphate buffer prepared as described by Elving et al. [33].

† In 100 mM sodium acetate buffer, pH 5.0.

β -D-glucoside, PNP β -D-lactopyranoside, PNP α - or β -D-mannoside or PNP α -L-rhamnopyranoside. However, each acted, with different degrees of efficiency, against ONP and PNP β -D-xyloside, CNP β -D-xyloside, PNP β -D-xylobioside, methylumbelliferyl β -D-xyloside, xylo-oligosaccharides and unsubstituted xylans (see below).

Hydrolysis of aryl β -D-xylosides

The ability of each enzyme to catalyse the hydrolysis of PNP β -D-xyloside and PNP β -D-xylobioside was measured by fractionation of the reaction products by h.p.l.c. gel filtration. For the convenience of the latter procedure, the enzyme incubations were carried out at 25 °C, i.e. considerably below the temperature optima of these enzymes (see above). Under these conditions, only Xyl I effected the release of PNP from PNP β -D-xyloside, and only Xyl II released PNP directly from PNP β -D-xylobioside. At higher temperatures, all three enzymes acted on both substrates and also catalysed the hydrolysis of ONP β -D-xyloside and CNP β -D-xyloside.

Table 3 Kinetic properties of Xyl I, II and III from *T. emersonii*

Reactions were carried out at 50 °C, in 100 mM sodium acetate buffer, pH 5. Abbreviations: PNPX, PNP β -D-xylopyranoside; PNPX₂, PNP β -D-xylobiopyranoside; CNPX, CNP β -D-xylopyranoside; ONPX, ONP β -D-xylopyranoside.

Substrate	Parameter	Xyl I	Xyl II	Xyl III
PNPX	Sp. activity (μ mol/min per mg)	3.0	2.9	0.2
	K_m (mM)	0.13	32.9	1.4
	V_{max} (μ mol/min per ml)	1.7	6.3	0.26
	k_{cat} (s^{-1})	426	898	61
	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	3275	27	43
PNPX ₂	Sp. activity (μ mol/min per mg)	3.6	287.5	0.4
	K_m (mM)	—	0.4	—
	V_{max} (μ mol/min per ml)	—	20.3	—
	k_{cat} (s^{-1})	—	2876	—
	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	—	7190	—
CNPX	Sp. activity (μ mol/min per mg)	1.5	232	0.5
	K_m (mM)	0.15	2.9	0.4
	V_{max} (μ mol/min per ml)	1.5	33.3	0.9
	k_{cat} (s^{-1})	359	4722	216
	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	2395	1628	539
ONPX	Sp. activity (μ mol/min per mg)	44.8	163.1	92.6
	K_m (mM)	0.1	1.8	3.3
	V_{max} (μ mol/min per ml)	0.4	3.7	1.5
	k_{cat} (s^{-1})	96	523	345
	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	1066	292	104

The kinetic parameters listed in Table 3 show that Xyl I has much lower K_m values for PNP, CNP and ONP β -D-xylosides than do Xyl II or III. Consistent with the possibility that such values reflect the affinity of each enzyme for such substrates is the fact that the k_{cat}/K_m values, measures of catalytic efficiency [49], for Xyl I are generally greater than those for Xyl II and III. That CNP and ONP β -D-xylosides are better substrates for Xyl II and III than is PNP β -D-xyloside may reflect the fact that they (CNP

Table 4 Products accumulating during reaction of Xyl I, II and III with xylo-oligosaccharides (DP 2–7)

Reaction mixtures, incubated at 50 °C with shaking at 140 rev./min, contained a portion of enzyme plus 5.0 mg of substrate (except in the case of xyloheptaose which was 2.5 mg) in 100 mM sodium acetate buffer, pH 5.0 (final volume 1.0 ml). Reactions were stopped after 3 h by boiling and the products were separated by high-performance anion-exchange chromatography using the Dionex system as described in the text. The concentrations of the individual products formed in each incubation are given as the molar percentage of the total xylo-oligosaccharides (DP 2–7) present in reaction mixtures at 3 h.

Enzyme	Substrate (DP)	[Product] (molar % of total xylo-oligosaccharides DP 1–7)						
		1	2	3	4	5	6	7
Xyl I	2	7.1	83.7	9.1	0	0	0	0
	3	1.7	5.0	88.1	2.7	0	0	2.5
	4	1.4	0.5	13.0	78.7	4.6	3.3	0
	5	3.6	0.9	4.5	22.4	64.9	3.7	0
	6	3.0	0.4	1.0	4.2	24.7	61.5	5.1
	7	3.3	0	0	0	15.9	23.0	57.8
	7	3.3	0	0	0	15.9	23.0	57.8
Xyl II	2	0	97.0	1.5	1.5	0	0	0
	3	0	2.5	97.5	0	0	0	0
	4	1.5	71.6	22.0	4.8	0	0	0
	5	3.5	41.5	46.9	0	0	0	8.1
	6	0.9	50.3	48.8	0	0	0	0
	7	21.5	35.1	43.5	0	0	0	0
	7	21.5	35.1	43.5	0	0	0	0
Xyl III	2	0.8	96.1	3.1	0	0	0	0
	3	0	2.0	92.5	1.5	1.5	0	2.5
	4	0	3	6.7	84.0	0	6.3	0
	5	0	5.2	10.8	9.4	73.2	1.4	0
	6	0	2.1	4.1	5.2	8.2	80.5	0
	7	0.6	2.6	7.0	8.2	7.4	8.8	65.4
	7	0.6	2.6	7.0	8.2	7.4	8.8	65.4

and ONP β -D-xyloside) provide better leaving groups to assist catalysis.

As stated above, only Xyl II effected the direct removal of PNP from PNP β -D-xylobioside (PNPX₂). Indeed, the kinetic

parameters listed in Table 3 show it to be the best of the aryl substrates tested with this enzyme. It was not possible to determine K_m and V_{max} values for Xyl I and III with PNPX₂ as substrate because plots of velocity versus substrate concentration were not linear. This non-linearity may, as has been suggested before for other enzymes [24], be due to the fact that the PNP is not liberated directly from PNPX₂ by Xyl I or III but from the intermediately formed PNPX, as depicted in eqns. (1) and (2). Alternatively, PNP may be liberated via a transferase reaction (see later) followed by hydrolysis, as shown in eqns. (3) and (4). It has been established that the xylanase from *C. albidus* effects conversion of aryl β -xylosides in such a manner [19].

Hydrolysis of xylo-oligosaccharides

The products accumulating in reaction mixtures during reaction of Xyl I, II and III with xylo-oligosaccharides (DP 2–7) were fractionated and quantified by high-performance anion-exchange chromatography using the Dionex system (Table 4). The experimental conditions used were chosen so as to minimize the extent of hydrolysis and so allow the identification of intermediates, if any. It is clear that with any one substrate the product profiles of each enzyme differ. Moreover, while Xyl I effected hydrolysis of xylobiose, Xyl III did not do so or did so much less efficiently, whereas no hydrolysis of xylobiose by Xyl II was observed. The appearance of products with lower and higher DP than the starting substrate, when acting on substrates up to xylohexaose, is a marked feature of the reactions catalysed by Xyl I and III. This indicates the occurrence, under the

Figure 1 Time course of hydrolysis of xylotetraose by Xyl I (a) and of xylohexaose by Xyl II (b, c)

(a) Reaction mixtures, incubated at 50 °C at 140 rev./min, contained 79.3 ng of Xyl I plus 5.1 mg of xylotetraose in 100 mM sodium acetate buffer, pH 5.0 (final volume 1.2 ml). Portions (100 μ l) were taken at intervals, boiled to stop further reaction, and separated by high-performance anion-exchange chromatography using the Dionex system as described in the text. For convenience of comparison, the products at each time interval are shown as molar % of total at that time. (b, c) Reaction mixtures contained 5.1 mg of xylohexaose and 52.8 ng of Xyl II. Otherwise, conditions were as in (a). (b) shows conversion of xylohexaose into xylose, xylobiose and xylotriose and (c) shows conversion of xylohexaose into xylotetraose, xylopentose and xyloheptaose. ●, xylose; ○, xylobiose; ■, xylotriose; □, xylotetraose; ▲, xylopentose; △, xylohexaose; ◆, xyloheptaose.

Table 5 Xylo-oligosaccharide products released from Lenzing and wheat straw xylans by Xyl I, II and III of *T. emersonii*

Reaction mixtures, incubated at 50 °C, 140 rev./min for 12 h, contained 10 mg of substrate plus a portion of enzyme in 0.1 mM sodium acetate buffer, pH 5.0 (final volume 1 ml). The amounts of enzyme used were: Xyl I, 66 ng; Xyl II, 44 ng; Xyl III, 344 ng. The values shown in parentheses were obtained after pretreatment (at 50 °C, 140 rev./min for 12 h) of wheat straw xylan with 209 ng of arabinofuranosidase I from *T. emersonii* before reaction with Xyl II or Xyl III.

	[Product] (% of the weight of starting substrate)					
	Lenzing xylan			Wheat straw xylan		
	Xyl I	Xyl II	Xyl III	Xyl I	Xyl II	Xyl III
Xylose	5.0	0.6	0.01	0.9	0 (6.2)	0.01
Xylobiose	0	37.5	19.4	0	0 (14.5)	0.22
Xylotriase	0	54.9	29.4	0	0 (20.4)	0
Xyloetraase	0	3.9	2.0	0	0	0
Xylopentaase	0	4.7	0.9	0	0	0
Xylohexaase	0	1.8	0	0	0	0
Xyloheptaase	0	1.5	0	0	0	0
Total	5.2	104.9	51.7	0.9	0 (41.1)*	0.23 (27.2)*

*Percentage hydrolysis as measured by reducing sugars released.

experimental conditions used, of both transfer and hydrolytic reactions. By contrast, transfer products were much less evident in the Xyl II reaction mixtures. One might also conclude from the results in Table 4 that both Xyl I and III are more exo-acting (less endo-acting) than is Xyl II and that the affinity for substrate (as judged by the extent of hydrolysis), especially in the case of Xyl II, increased with increasing DP. This is a feature that distinguishes xylanases from xylosidases.

As examples of the time course of substrate conversion, the action of Xyl I on xyloetraase and of Xyl II on xylohexaase are shown in Figure 1(a) and Figures 1(b) and 1(c) respectively. Hydrolysis of xyloetraase by Xyl I proceeded almost exclusively with conversion into xylose and xylotriase, little xylobiose accumulating (Figure 1a). This would indicate that the bond cleaved is that at the reducing or non-reducing ends rather than that in the middle (see below). Reaction of xylohexaase with Xyl II was accompanied by the transient appearance of xyloetraase and to a lesser extent of xylopentaase and xyloheptaase (Figure 1c). In the meantime, dimers and trimers accumulated throughout the incubation period, but there was little formation of monomers (Figure 1b). Although the transient appearance of xyloheptaase underlined the fact that transfer reactions occurred during the incubation, it would seem that cleavage at internal linkages was preferred (see below).

Hydrolysis of xylans

The ability of each enzyme to hydrolyse Lenzing xylan (a xylan with DP about 30 and low degree of substitution [50]) and wheat straw arabinoxylan was also investigated by measurement of the reducing sugars released and by fractionation of such products using high-performance anion-exchange chromatography. Xyl I effected little hydrolysis of either substrate and the only product observed after long-term incubation was xylose (Table 5). Moreover, pretreatment of arabinoxylan with arabinofuranosidase (from *T. emersonii*) did not effect an increase in the extent of hydrolysis of this substrate by Xyl I. This paucity of action against polymeric xylans is typical of β -xylosidase action, affinity for substrate decreasing with increasing DP of the substrate [8].

Xyl II and III each hydrolysed Lenzing xylan extensively and, to judge by the range of oligosaccharides produced, did so in endo-acting fashion (Table 5). By contrast, Xyl II had absolutely

no action against arabinoxylan from wheat straw, whereas Xyl III had but little effect, releasing only small amounts of xylose and xylobiose. However, each enzyme did catalyse extensive degradation of arabinoxylan that had been pretreated with arabinofuranosidase (Table 5). Comparison of the expected (i.e. the arithmetic sum of the reducing sugars released by the individual enzymes) and observed (i.e. actual amounts of reducing sugars released by their combined action) extents of hydrolysis showed a very marked degree of synergy between the arabinosidase and Xyl II or III. In the case of arabinosidase plus Xyl II, the arithmetically expected extent of hydrolysis (on the basis of reducing sugars produced) was 1.3 % but the observed value was 41.2 %. Moreover, Xyl II fragmented the pretreated straw xylan in a typical endo-wise fashion, releasing xylose, xylobiose and xylotriase, as major degradation products, in addition to arabinosylxylobiose as the main substituted xylan fragment (Table 5). In the case of arabinosidase plus Xyl III, the expected and observed extents of hydrolysis of arabinoxylan were 1.4 % and 27.2 % respectively.

Although endoxylanases may be classified in a number of ways, in one respect they fall into two classes depending on whether cleavage of the xylan backbone is (a) hindered by side chain substituents or (b) occurs only in the vicinity of such substituents [8]. Clearly, the arabinose substituents, which on average occur approximately every 12 xylose residues in the arabinoxylan used, completely prevented the action of Xyl II or III. Since arabinosidase pretreatment greatly enhanced hydrolysis of the xylan backbone, one may conclude that both Xyl II and III require arabinose-free stretches of at least 24 xylose residues for action. We are not aware of any endoxylanase in which catalysis is so completely dependent on the absence of substitution.

Bond-cleavage frequencies with [1-³H]xylo-oligosaccharides as substrates

The products of action of Xyl I, II or III with reducing end-labelled xylo-oligosaccharides as substrates were fractionated by t.l.c., followed by excision of the various fractions and counting the radioactivity contained therein, as described in the Materials and methods section. By sampling at various time intervals during incubation, bond-cleavage frequencies, as numbers cor-

Figure 2 Frequencies of glycosidic bond cleavage by Xyl I, II and III as determined with [1-³H]xylotriose, [1-³H]xylotetraose and [1-³H]xylopentaose as substrates

Xylotriose, xylotetraose and xylopentaose are represented by three, four and five joined circles respectively. The closed circle at the reducing end of each substrate represents the 1-³H-labelled residue. The arrows indicate the sites of hydrolysis of substrate by each enzyme and the numbers below each arrow represent the percentage frequency of hydrolysis of the indicated bond.

responding to the relative rates of cleavage of individual glycosidic bonds, for each enzyme with each substrate used were determined as initial product ratios, i.e. ratios at zero reaction time [16]. The values obtained are shown in Figure 2. With all three substrates used, namely xylotriose to xylopentaose, it is clear that Xyl I preferentially liberated xylose by cleaving the non-reducing end linkage. By contrast, Xyl II preferentially, if not exclusively, liberated xylobiose units from the non-reducing end of its substrates. With the exception of its action on xylotriose, the same may be said of Xyl III. The bond-cleavage frequencies shown in Figure 2 are consistent with the results given above (Tables 4 and 5) and confirm the fact that Xyl I behaves as a typical β -xylosidase, whereas Xyl II and Xyl III may be classified as endoxylanases, albeit with different specificities.

Determination of bond-cleavage frequencies of xylotriose to xylopentaose is not sufficient to ascertain the number of subsites within the active site of an enzyme. For this, one also needs to utilize longer oligosaccharides and to determine the relevant kinetic parameters with each substrate so as to calculate the number of subsites and the relevant contribution each makes to substrate binding [51]. We hope to carry out these additional investigations when appropriately labelled substrates become available in the required quantities. In the meantime, it is clear that the active sites of all three enzymes must contain a number of subsites with at least two possible ways of binding substrate relative to the location of the catalytic site.

Conclusion

From the properties of Xyl I reported here, one may conclude that it is typical of those β -xylosidases (EC 3.2.1.37) that cleave

aryl substrates and xylobiose and remove xylose units preferentially from xyloligosaccharides, with activity against the latter decreasing with increasing DP [3,8]. By contrast, Xyl II and Xyl III may be classified as endo- β -1,4-xylanases (EC 3.2.1.8) in that they catalyse the endo-wise cleavage of xylans and xyloligosaccharides, affinity for the latter substrates increasing with increasing DP. However, they are novel in that they also catalyse the hydrolysis of aryl substrates and in that they require such long stretches of unsubstituted xylan backbone for activity. Moreover, Xyl II is unusual in being a dimer, whereas endoxylanases are generally single-subunit proteins. Several other xylanases isolated from *T. emersonii* do not cleave aryl substrates but do effect considerable hydrolysis of arabinoxylan without the assistance of arabinofuranosidase (M. G. Tuohy, J. Puls and M. P. Coughlan, unpublished work). The synergistic interaction between Xyl II (or Xyl III) and arabinofuranosidase from *T. emersonii* (Table 5) underscores the complexity of these substrates and the fact that co-operation between different enzyme components is needed for their conversion.

From the viewpoint of its existence *in vivo*, one may conclude that possession of Xyl II and III and arabinofuranosidases would allow *T. emersonii* to compete effectively with other organisms. Thus selective removal of arabinose substituents from the xylans of monocotyledonous plants would yield a carbon source that had become less soluble in water and so less accessible to other xylanases but which would readily be cleaved by Xyl II and III. For the same reasons, Xyl II and III should be promising biocatalysts in the enzymic bleaching of pulps. For this purpose, xylanases with high affinity for, and high activity against, less soluble and non-substituted xylans are in great demand [52].

This work was funded by EC Contract MA1D-0017-IRL to M.P.C. M.G.T. thanks University College Galway for a Junior Teaching Fellowship and COST 84bis and COST 84ter for providing the Short Term Fellowship to carry out some of this work at Hamburg and Gent. M.V. gratefully acknowledges the funding provided by University College, Galway to carry out some of this work at Galway.

REFERENCES

- Horton, D. and Wolfrom, M. L. (1963) in *Comprehensive Biochemistry* (Florkin, M. and Stoltz, E. H., eds.), pp. 189–232. Elsevier, Amsterdam.
- Biely, P. (1985) *Trends Biotechnol.* **3**, 286–290.
- Wong, K. K. Y., Tan, L. U. L. and Saddler, J. N. (1988) *Microbiol. Rev.* **52**, 305–317.
- Puls, J. and Poutanen, K. (1989) in *Enzyme Systems for Lignocellulose Degradation* (Coughlan, M. P., ed.), pp. 151–165. Elsevier Applied Science, London.
- Dekker, R. F. H. and Richards, G. N. (1987) *Adv. Carbohydr. Chem. Biochem.* **32**, 277–352.
- Matheson, N. K. and McCleary, B. V. (1985) in *The Polysaccharides* (Aspinall, G. O., ed.), pp. 1–105. Academic Press, New York.
- Hebraud, M. and Fevre, M. (1990) *FEMS Microbiol. Lett.* **72**, 11–16.
- Coughlan, M. P. (1992) in *Xylans and Xylanases* (Visser, J., Beldman, G., Kusters-van Someren, M. A. and Voragen, A. G. J., eds.), pp. 111–139. Elsevier, Amsterdam.
- Vršanská, M., Gorbacheva, I. V., Krátky, Z. and Biely, P. (1982) *Biochim. Biophys. Acta* **704**, 114–122.
- Biswas, S. R., Jana, S. C., Mishra, A. K. and Nanda, G. (1990) *Biotechnol. Bioeng.* **35**, 244–251.
- Bernier, R. Jr., Desrochers, M., Jurasek, L. and Paice, M. G. (1983) *Appl. Environ. Microbiol.* **46**, 511–514.
- Monti, R., Terenzi, H. F. and Jorge, J. A. (1991) *Can. J. Microbiol.* **37**, 675–681.
- Morosoli, R., Bertrand, J.-L., Mondou, F., Shareck, F. and Kluepfel, D. (1986) *Biochem. J.* **239**, 587–592.
- Kluepfel, D., Vats-Mehta, S., Aumont, F., Shareck, F. and Morosoli, R. (1990) *Biochem. J.* **267**, 45–50.
- Lüthi, E., Love, D. R., McNulty, J., Wallace, C., Caughey, P. A., Saul, D. and Bergquist, P. L. (1990) *Appl. Environ. Microbiol.* **56**, 1017–1024.
- Biely, P., Vršanská, M. and Krátky, Z. (1980) *Eur. J. Biochem.* **108**, 313–321.
- Tan, L. U. L., Mayers, P. and Saddler, J. N. (1987) *Can. J. Microbiol.* **33**, 689–692.
- Mishra, C., Keskar, S. and Rao, M. (1984) *Appl. Environ. Microbiol.* **48**, 224–228.

- 19 Biely, P., Vršanská, M. and Krátky, Z. (1980) *Eur. J. Biochem.* **112**, 375–381
- 20 Tuohy, M. G., Buckley, R. J., Griffin, T. O., Connelly, I. C., Shanley, N. A., Filho, E. X. F., Hughes, M. M., Grogan, P. and Coughlan, M. P. (1989) in *Enzyme Systems for Lignocellulose Degradation* (Coughlan, M. P., ed.), pp. 293–312, Elsevier Applied Science, London
- 21 Tuohy, M. G., Coughlan, T. L. and Coughlan, M. P. (1990) in *Advances in Biological Treatment of Lignocellulosic Materials* (Coughlan, M. P. and Amaral-Collaco, M. T., eds.), pp. 153–175, Elsevier Applied Science, London
- 22 Tuohy, M. G. and Coughlan, M. P. (1992) *Bioresource Technol.* **39**, 131–137
- 23 Filho, E. X. F., Tuohy, M. G., Puls, J. and Coughlan, M. P. (1991) *Biochem. Soc. Trans.* **19**, 25S
- 24 Claeysens, M., van Leemputten, E., Loontjens, F. G. and De Bruyne, C. K. (1966) *Carbohydr. Res.* **3**, 32–57
- 25 Puls, J., Borchmann, A., Gottschalk, D. and Wiegel, J. (1988) *Methods Enzymol.* **160**, 528–536
- 26 Evans, E. A., Sheppard, H. C., Turner, J. C. and Wawell, D. C. (1974) *J. Labelled Compd.* **10**, 569–587
- 27 Moloney, A. P., Hackett, T. J., Considine, P. J. and Coughlan, M. P. (1983) *Enzyme Microbiol. Technol.* **5**, 260–264
- 28 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 29 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 30 Zacharius, R. M., Zell, T. E., Morrison, J. H. and Woodlock, J. J. (1969) *Anal. Biochem.* **30**, 148–152
- 31 Puls, J., Tenkanen, M., Korte, H. and Poutanen, K. (1991) *Enzyme Microbiol. Technol.* **13**, 483–486
- 32 Allen, J. D. and Thoma, J. A. (1978) *Biochemistry* **17**, 2338–2344
- 33 Elving, P. L., Markowitz, J. N. and Rosenthal, I. (1956) *Anal. Chem.* **28**, 1179–1180
- 34 John, M., Schmidt, B. and Schmidt, J. (1979) *Can. J. Biochem.* **57**, 125–134
- 35 Panbangred, W., Kondo, T., Negoro, S., Shinmyo, A. and Okada, H. (1983) *Mol. Gen. Genet.* **192**, 335–341
- 36 Sewell, G. W., Utt, E. A., Hespell, R. B., Mackenzie, K. F. and Ingram, L. O. (1989) *Appl. Environ. Microbiol.* **55**, 306–311
- 37 Uziie, M., Matsuo, M. and Yasui, T. (1985) *Agric. Biol. Chem.* **49**, 1159–1166
- 38 Matsuo, M. and Yasui, T. (1984) *Agric. Biol. Chem.* **48**, 1853–1860
- 39 Kelly, C. T., O'Mahoney, M. R. and Fogarty, W. M. (1989) *Biotechnol. Lett.* **11**, 885–890
- 40 Esteban, R., Chordi, A. and Villa, T. G. (1983) *FEMS Microbiol. Lett.* **17**, 163–166
- 41 Lee, S. F. and Forsberg, C. W. (1987) *Appl. Environ. Microbiol.* **53**, 651–654
- 42 Poutanen, K. and Puls, J. (1989) *Am. Chem. Soc. Symp. Ser.* **399**, 630–639
- 43 Woodward, J. (1984) *Top. Enzyme Fermentat. Biotechnol.* **8**, 7–30
- 44 Uchino, F. and Nakane, T. (1981) *Agric. Biol. Chem.* **45**, 1121–1127
- 45 Hudson, R. C., Scholfield, L. R., Coolbear, T., Daniel, R. M. and Morgan, H. W. (1991) *Biochem. J.* **273**, 645–650
- 46 Dekker, R. F. H. and Richards, G. N. (1975) *Carbohydr. Res.* **39**, 97–114
- 47 Dekker, R. F. H. and Richards, G. N. (1975) *Carbohydr. Res.* **42**, 107–123
- 48 Yoshioka, H., Nagato, N., Chavanich, S., Nilubol, N. and Hayashida, S. (1981) *Agric. Biol. Chem.* **45**, 2425–2432
- 49 Voet, D. and Voet, J. G. (1990) *Biochemistry*, Wiley, New York
- 50 Lenz, J., Schurz, J. and Bauer, J. (1984) *Das Papier* **38**, 45–54
- 51 Meagher, M. M., Tao, B. Y., Chow, J. M. and Reilly, P. J. (1988) *Carbohydr. Res.* **173**, 273–283
- 52 Puls, J., Poutanen, K. and Lin, J. J. (1990) in *Biotechnology in Pulp and Paper Manufacture: Applications and Fundamental Investigations* (Kirk, T. K. and Chang, H.-M., eds.), pp. 183–190, Butterworth-Heinemann, Boston