

Action of acetylxyylan esterase from *Trichoderma reesei* on acetylated methyl glycosides

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Abstract Substrate specificity of purified acetylxyylan esterase (AcXE) from *Trichoderma reesei* was investigated on partially and fully acetylated methyl glycopyranosides. Methyl 2,3,4-tri-*O*-acetyl- β -D-xylopyranoside was deacetylated at positions 2 and 3, yielding methyl 4-*O*-acetyl- β -D-xylopyranoside in almost 90% yield. Methyl 2,3-di-*O*-acetyl β -D-xylopyranoside was deacetylated at a rate similar to the fully acetylated derivative. The other two diacetates (2,4- and 3,4-), which have a free hydroxyl group at either position 3 or 2, were deacetylated one order of magnitude more rapidly. Thus the second acetyl group is rapidly released from position 3 or 2 after the first acetyl group is removed from position 2 or 3. The results strongly imply that in degradation of partially acetylated β -1,4-linked xylans, the enzyme deacetylates monoacetylated xylopyranosyl residues more readily than di-*O*-acetylated residues. The *T. reesei* AcXE attacked acetylated methyl β -D-glucopyranosides and β -D-mannopyranosides in a manner similar to the xylopyranosides.

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Key words: Acetylxyylan esterase; Substrate specificity; Acetylated methyl glycoside; Mode of action; *Trichoderma reesei*

1. Introduction

Acetylxyylan esterases (AcXEs) have been recognized as components of microbial cellulolytic and hemicellulolytic systems. The enzymes liberate acetic acid from partially acetylated 4-*O*-methyl-D-glucuronoxylan, the main component of hardwood hemicellulose [1–4]. Our recent investigations [5,6] regarding the action of two AcXEs on partially and fully acetylated methyl glycopyranosides provided the first insight into their function in acetylxyylan degradation.

AcXE from *Schizophyllum commune* preferentially deacetylated the 3 position in partially and fully acetylated methyl xylopyranosides affording 2,4-di-*O*-acetyl- and 2- and 4-*O*-monoacetyl derivatives from Me- β -Xylp [5]. Likewise, 2,4,6-tri-*O*-Ac-Me- β -Glc and 4,6-di-*O*-Ac-Me- β -Glc were the principal products from 2,3,4,6-tetra-*O*-Ac-Me- β -Glc. The enzyme also catalyzed a two-step, double 2,3-deacetylation of 2,3,4,6-tetra-*O*-Ac-Me- β -Manp in a highly selective way.

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Abbreviations: AcXE, acetylxyylan esterase; 2,3,4-tri-*O*-Ac-Me- β -Xylp, methyl 2,3,4-tri-*O*-acetyl- β -D-xylopyranoside; 2,3,4,6-tetra-*O*-Ac-Me- β -Glc, methyl 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranoside; 2,3,4,6-tetra-*O*-Ac-Me- β -Manp, methyl 2,3,4,6-tetra-*O*-acetyl- β -D-mannopyranoside

The ability of the AcXE from *S. commune* to deacetylate the mannopyranoside suggested that the enzyme may not be an AcXE, but rather a more general polysaccharide or carbohydrate deacetylase [5].

AcXE from *Streptomyces lividans* did not show an ability to deacetylate acetylated mannopyranosyl residues and appears to be a more acetylxyylan-specific enzyme [6]. Also in contrast to *S. commune* AcXE, the *St. lividans* catalyzed an essentially simultaneous double deacetylation of 2,3,4-tri-*O*-Ac-Me- β -Xylp and 2,3,4,6-tetra-*O*-Ac- β -Glc at positions 2 and 3. The observed double deacetylation kinetically resulted from the fact that the enzyme deacetylated xylopyranosyl derivatives in which position 2 or 3 was free (non-acetylated) up to two orders of magnitude faster than derivatives in which both the 2 and 3 positions were occupied (acetylated) [6].

The objective of the present study was to examine on similar artificial substrates the mode of action of AcXE from *Trichoderma reesei* [7,8], one of the most extensively investigated cellulolytic microorganisms. The results show that the *T. reesei* AcXE possesses catalytic abilities intermediate between those of the two previously characterized AcXEs from *S. commune* and *St. lividans*.

2. Materials and methods

2.1. Enzyme and its assay

The investigated AcXE produced by *T. reesei* RUT C-30 was purified from a cellulose-spent culture medium as described by Sundberg and Poutanen [7]. Enzyme activity was determined on 4-nitrophenyl acetate at 25°C as described by Johnson et al. [9]. One unit is defined as 1 μ mol of 4-nitrophenol released in 1 min.

2.2. Substrates

Fully acetylated methyl glycopyranosides were obtained by acetylation of methyl glycosides purchased from Sigma Chemical Co. (St. Louis, MO, USA) with acetic anhydride in pyridine [10]. Diacetates (2,3- and 2,4- and 3,4-) of methyl β -D-xylopyranoside were generous gifts from Dr. P. Kováč (National Institutes of Health, Bethesda, MD, USA), Dr. J. Hirsch (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia) and Dr. A. Fernandes-Mayoral (Instituto de Química Orgánica General, CSIC, Madrid Spain).

2.3. Enzyme-substrate mixtures

Reactions were performed in homogeneous solutions containing 0.1 M sodium phosphate buffer (pH 6.0) at 40°C. Saturated solutions of substrates were used and their concentrations determined by the phenol-sulfuric acid reagent [11]. Measured concentrations were as follows: methyl 2,3,4-tri-*O*-acetyl- β -D-xylopyranoside 14.5 mM, methyl 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranoside 10.4 mM and methyl 2,3,4,6-tetra-*O*-acetyl- β -D-mannopyranoside 6.4 mM. Methyl 2,3-, 2,4- and 3,4-di-*O*-acetyl- β -D-xylopyranosides were reacted at 15 mM. All reactions were initiated by the addition of prewarmed enzyme solution. The enzyme concentration varied with different substrates and will be given in the figure captions and table heading.

2.4. Analysis of reaction mixtures

Aliquots from the incubation mixtures were taken periodically and evaluated by TLC on Silica gel 60 (Merck) in ethyl acetate–benzene–2-propanol (2:1:0.1, v/v). The sugars were visualized with *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent [12]. Chromatographic mobilities of various methyl *O*-acetyl-glycosides have been previously reported [5]. A separate set of aliquots were immediately frozen on dry ice and freeze-dried. Dried samples were subjected to quantitative analysis by GLC after conversion of reaction products to their trimethylsilyl ethers [5]. Initial rates of deacetylation were calculated from the plots of the ratio of deacetylated products versus time of incubation.

2.5. Identification of deacetylation products

Deacetylation products from various methyl *O*-acetyl-glycopyranosides were identified by GLC-MS and NMR spectroscopy as described earlier [5]. When necessary, individual derivatives were isolated from mixtures by preparative TLC using conditions described above, followed by elution with chloroform (triacetates), ethylacetate (tri- and diacetates) or ethanol (monoacetates). Details of all procedures have been described elsewhere [5].

3. Results

3.1. Action of AcXE on methyl per-*O*-acetyl-*D*-glycosides

Deacetylation of 2,3,4-tri-*O*-Ac-Me- β -Xylp by *T. reesei* AcXE leads to 4-*O*-Ac-Me- β -Xylp in a high yield (~88%) (Fig. 1). The monoacetate appeared in the reaction mixture as the major product at an early stage of the reaction. Due to this fact it was impossible to decide which of the two positions 2 and 3 was deacetylated as the first one or more rapidly. Of the two theoretical intermediates of the double deacetylation, only 3,4-di-*O*-Ac- β -Xylp transitionally appeared. The absence of 2,4-di-*O*-Ac- β -Xylp does not necessarily mean the enzyme was unable to first remove the acetyl group from position 3. Conversely, this observation could have resulted from fast conversion of 2,4-di-*O*-Ac- β -Xylp to 4-*O*-Ac-Me- β -Xylp. As will be shown below, 2,4-di-*O*-Ac- β -Xylp is a slightly better substrate for the second deacetylation than 3,4-di-*O*-Ac-Me- β -Xylp. A second detected diacetate, 2,3-di-*O*-Ac-Me- β -Xylp, appeared at later stages in the reaction mixture, due to a slow deacetylation at position 4. The removal of the acetyl group from the 4 position apparently resulted in a less reactive substrate which persisted in the reaction mixture. Similarly, subsequent conversion of 4-*O*-Ac-Me- β -Xylp to a completely deacetylated product is very slow (Fig. 1) further indicating that AcXE cannot hydrolyze the acetyl group in position 4 as fast as those in positions 2 and 3, regardless of the presence or absence of a neighboring carbon free hydroxyl group.

The AcXE exhibited similar regioselectivity as above towards 2,3,4,6-tetra-*O*-Ac-Me- β -Glc (Fig. 2). The enzyme removed first two acetyl groups from positions 2 and 3, afford-

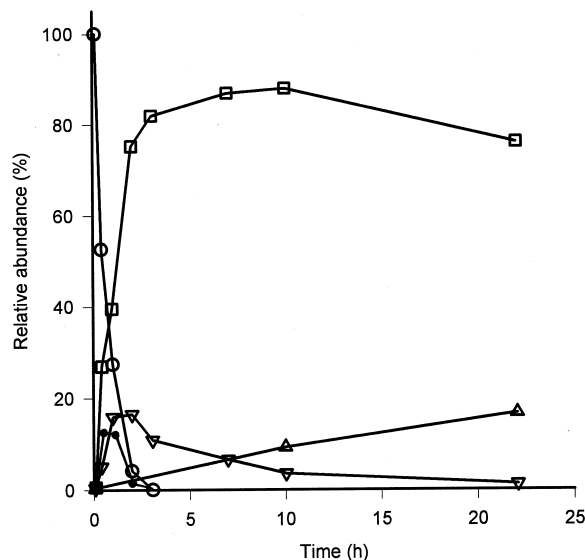


Fig. 1. Time course of 2,3,4-tri-*O*-Ac-Me- β -Xylp (14.5 mM) deacetylation by AcXE from *T. reesei* (0.05 U/ml) as evaluated by gas chromatography of trimethylsilyl ethers. Symbols: $\circ$, 2,3,4-tri-*O*-Ac-Me- β -Xylp; $\bullet$, 3,4-di-*O*-Ac-Me- β -Xylp; ∇ , 2,3-di-*O*-Ac-Me- β -Xylp; $\square$, 4-*O*-Ac-Me- β -Xylp; $\triangle$, fully deacetylated substrate.

ing 4,6-di-*O*-Ac-Me- β -Glc. The intermediates of this conversion, the 2,4,6- and 3,4,6-tri-*O*-acetates, were not detected in the reaction mixture, probably due to fast removal of the second acetyl group from position 3 or 2. The reaction mixture contained a low level of triacetates which were formed by removal of the acetyl group from positions 4 and 6, positions which are attacked by the enzyme at the slowest rate. Both 2,3,4-tri-*O*-Ac- and 2,3,6-tri-*O*-Ac-Me- β -Glc persisted in the reaction mixture for long durations, analogous to the behavior of the enzyme towards 2,3-di-*O*-Ac-Me- β -Xylp. In contrast to AcXEs of *St. lividans* and *S. commune*, 4,6-di-*O*-Ac-Me- β -Glc was further converted, mainly to 4-*O*-Ac-Me- β -Glc (60% yield) and partially to 6-*O*-Ac-Me- β -Glc.

T. reesei AcXE was also tested on several other per-*O*-acetylated methyl glycopyranosides. TLC demonstrated that the enzyme deacetylated 2,3,4,6-tetra-*O*-Ac-Me- α -Glc, 2,3,4,6-tetra-*O*-Ac-Me- β -Gal, 2,3,4,6-tetra-*O*-Ac-Me- β -Gal and 2,3,4,6-tetra-*O*-Ac-Me- α - and - β -Manp. However, rates were slower than those observed for 2,3,4,6-tetra-*O*-Ac-Me- β -Glc. In all cases, the deacetylation did not cease at the level of 4,6-di-*O*-Ac derivatives but continued further to monoacetates and even to completely deacetylated derivatives. The final deacetylation of monoacetates was more pronounced with β - as opposed to α -glycosides.

Table 1
Enzymic and spontaneous transformation of Me- β -Xyl tri- and di-*O*-acetates

Deacetylation by <i>T. reesei</i> AcXE				Spontaneous transformation	
Substrate	Initial rate		Major products	Rate	Products (4 h)
	mM/min	mM/min/U ml ⁻¹		mM/min	2,3-:2,4-:3,4-
2,3,4-tri-Ac	0.28	5.7	4-Ac, 3,4-di-Ac	not applicable	
2,3-di-Ac	0.15	3.0	4-Ac-, 2-Ac	0.0057	1:0.1:0.4
2,4-di-Ac	0.30	61.2	4-Ac	0.022	0.15:1:0.7
3,4-di-Ac	0.28	56.0	4-Ac	0.018	0.07:0.5:1

Substrates, 15 mM; concentration of AcXE of *T. reesei* 0.05 U/ml (2,3,4-tri-*O*-Ac and 2,3-di-*O*-Ac), 0.005 U/ml (2,4-di-*O*-Ac and 3,4-di-*O*-Ac).

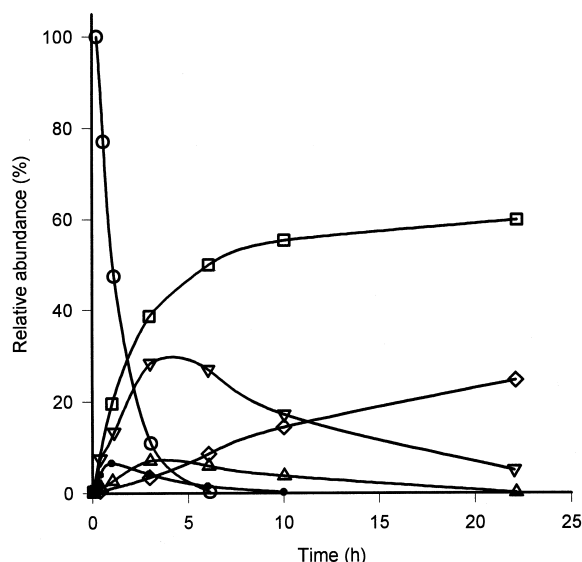


Fig. 2. Time course of 2,3,4,6-tetra-*O*-Ac-Me- β -Glcp (10.4 mM) deacetylation by AcXE of *T. reesei* (0.07 U/ml) as evaluated by gas chromatography of trimethylsilyl ethers. Symbols: $\circ$, 2,3,4,6-tetra-*O*-Ac-Me- β -Glcp; ∇ , 4,6-di-*O*-Ac-Me- β -Glcp; $\bullet$, 2,3,4-tri-*O*-Ac-Me- β -Glcp; Δ , 2,3,6-tri-*O*-Ac-Me- β -Glc; $\square$, 4-*O*-Ac-Me- β -Glcp; $\diamond$, 6-*O*-Ac-Me- β -Glcp.

3.2. Action on diacetates of Me- β -Xylp

The observation that 2,3,4-tri-*O*-Ac-Me- β -Xylp was deacetylated to 4-*O*-Ac-Me- β -Xylp without significant accumulation of di-*O*-acetylated intermediates suggested that the enzyme slowly hydrolyzed the first acetyl group and then more rapidly removed the second acetyl group. This consideration was substantiated by experiments in which the initial rate of deacetylation of 2,3,4-tri-*O*-Ac-Me- β -Xylp was compared with that of all three possible Me- β -Xylp diacetates (Table 1). 2,3,4-Tri-*O*-Ac-Me- β -Xylp was converted mainly into 4-*O*-Ac-Me- β -Xylp, but relatively slowly. The 4-acetate was also the sole product of a more rapid AcXE action on 2,4-di-*O*-Ac-Me- β -Xylp and 3,4-di-*O*-Ac-Me- β -Xylp. Thus, the enzyme rapidly deacetylated positions 2 and 3 when one of the two positions was not esterified. When both positions 2 and 3 were acetylated and position 4 was underivatized, as it is in 2,3-di-*O*-Ac-Me- β -Xylp, the initial deacetylation products were 4-*O*-Ac-Me- β -Xylp and 2-*O*-Ac-Me- β -Xylp. 4-*O*-Ac-Me- β -Xylp likely was

a result of spontaneous or enzyme-mediated acetyl migration from position 3 to position 4.

Table 1 presents the initial rates of deacetylation of the four substrates, and also shows the major initial products. Two substrates, 2,3,4-tri-*O*-Ac-Me- β -Xylp and 2,3-di-*O*-Ac-Me- β -Xylp, were hydrolyzed 10 and 20 times more slowly than 2,4- and 3,4-di-*O*-Ac-Me- β -Xylp. The rates of deacetylation were greater than the rates of spontaneous transformation of the diacetates (Table 1), measured without enzyme under identical experimental conditions [5]. Thus, product formation was not significantly influenced by spontaneous acetyl group migration and resulted from enzyme action.

4. Discussion

Like AcXEs from *S. commune* [5] and *St. lividans* [6], the *T. reesei* AcXE displays a different regioselectivity for deacetylation of methyl glycosides when compared to lipases used for carbohydrate deacetylation [13–18]. The deacetylation regioselectivity for positions 2 and 3 corresponds to the function of the enzyme in acetylxylen degradation.

It is interesting to compare in more detail the yet known catalytic abilities of AcXE from *T. reesei* with those of AcXE from *S. commune* [5] and *St. lividans* [6]. Some of the catalytic properties of the AcXE from *T. reesei* are similar to the properties of the *S. commune* enzyme, while others resemble those of the *St. lividans* AcXE. The action of *T. reesei* AcXE on 2,3,4-tri-*O*-Ac-Me- β -Xylp is similar to that of *St. lividans* AcXE, both yielding 4-*O*-Ac-Me- β -Xylp. The *S. commune* enzyme yielded a mixture of 2-*O*-Ac-Me- β -Xylp and 4-*O*-Ac-Me- β -Xylp. However, while the *St. lividans* AcXE removes both acetyl groups almost simultaneously, the double deacetylation of the *T. reesei* enzyme is more of a two-step process. Experiments with Me- β -Xylp diacetates explain the slight difference in the action of *St. lividans* and *T. reesei* AcXEs on 2,3,4-tri-*O*-Ac-Me- β -Xylp. The AcXE from *S. lividans* has an unusually high deacetylation preference for the 2 or 3 position when the 3 or 2 position, respectively, is not acetylated [6]. Derivatives in which both these adjacent positions are acetylated, 2,3,4-tri-*O*-Ac-Me- β -Xylp and 2,3-di-*O*-Ac-Me- β -Xyl, are very poor substrates of the *St. lividans* enzyme in comparison with the other two diacetates. Initial rates of deacetylation between these two pairs of substrates differ by two orders of magnitude in the case of *St. lividans* enzyme [6]. The differ-

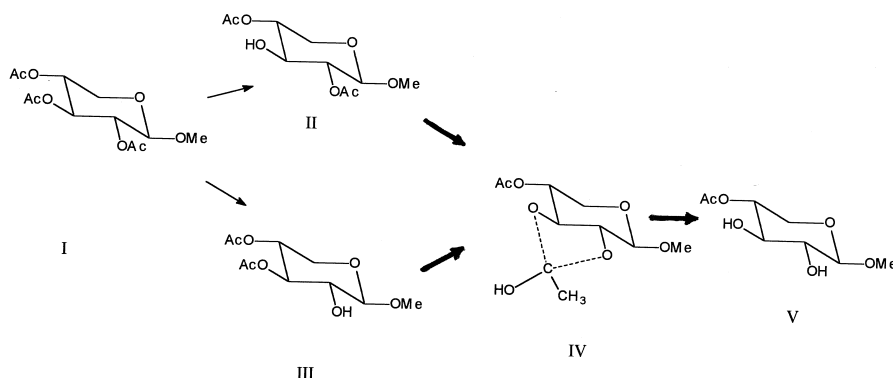


Fig. 3. Steps proposed for the conversion of 2,3,4-tri-*O*-Ac-Me- β -Xylp (I) to 4-*O*-Ac-Me- β -Xylp (V) by AcXE from *T. reesei*. The diacetate (II) was not detected in the reaction mixture probably due to its fast deacetylation to the monoacetate. The five-membered intermediate (IV) is proposed to be common for deacetylation of both positions 2 and 3. Thin arrows mark slow steps; thick arrows fast steps.

ence was only one order of magnitude for the *T. reesei* enzyme. Despite this slight difference it may be suggested the *T. reesei* AcXE, similar to the *St. lividans* AcXE, deacetylates monoacetylated xylopyranosyl residues of acetylxylan much faster than doubly acetylated residues. The deacetylation mechanism of positions 2 and 3, when the neighboring positions 3 and 2 are non-acetylated, might involve an enzyme-catalyzed formation of a five-membered transition state (Fig. 3), from which the acetyl group is finally released. Such intermediates are believed to be involved in the spontaneous migration of acetyl groups along the glycopyranoid ring [19]. The ability of another AcXE to deacetylate equally well positions 2 and 3 via the five-membered intermediate makes the question on regiospecificity of AcXEs for positions 2 and 3 irrelevant.

Of the three discussed AcXEs, only the *T. reesei* and *S. commune* enzymes hydrolyze aryl acetates, such as 4-nitrophenyl- or 4-methylumbelliferyl-acetate. Also, similarly to the *S. commune* AcXE, but in contrast to the *S. lividans* enzyme, the *T. reesei* AcXE deacetylates 2,3,4,6-tetra-*O*-Ac-Me- β -Manp. However, deacetylation does not stop at the stage of 4,6-di-*O*-Ac-Me- β -Manp, as it does with the *S. commune* AcXE, but continues to monoacetyl and fully deacetylated derivative. The ability of *T. reesei* enzyme to deacetylate derivatives of mannose suggested that the enzyme is not specific for acetylxylan and could be a more general acetylpolysaccharide esterase. Recent data of Tenkanen [20] do not support this assumption because the enzyme showed no activity on acetylgalactoglucomannan. This observation, however, does not exclude the capability of the enzyme to operate on acetylated manno oligosaccharides.

Correlation of the catalytic properties with the secondary structure of the three investigated AcXEs is still not possible, because there is no amino acid sequence homology between the AcXEs of *St. lividans* [21] and *T. reesei* [22]. Additionally, a sequence of 50 NH₂-terminal amino acids of the AcXE from *S. commune* (P. Biely, M. Hrmová, G.L. Côté and G.B. Fincher, unpublished results) reveals no homology with the other two enzymes. Apparently, all three enzymes probably evolved from different ancestors, as proposed earlier for other AcXEs by Margoles-Clark et al. [22]. However, the NH₂-terminal sequence of *S. commune* AcXE displays 65–68% homology with NH₂-terminal sequences of acylesterase and acetylxylan esterase from *Aspergillus* species [23–25]. A 50% sequence identity was reported between the xylan-specific acylesterase domain of the bifunctional xylanase from *Cellulomonas fimi* and AcXE from *St. lividans* [26]. No doubt with more data it will be possible to group these enzymes into families.

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References

- [1] Biely, P., Puls, J. and Schneider, H. (1985) FEBS Lett. 186, 80–84.
- [2] Biely, P., MacKenzie, C.P., Puls, J. and Schneider, H. (1986) Bio/Technology 4, 731–733.
- [3] Poutanen, K., Ratto, M., Puls, J. and Viikari, L. (1987) J. Biotechnol. 6, 49–60.
- [4] Christov, L.P. and Prior, B.A. (1993) Enzyme Microbiol. Technol. 15, 460–475.
- [5] Biely, P., Côté, G.L., Kremnický, L., Weisleder, D. and Greene, R.V. (1996) Biochim. Biophys. Acta 1298, 209–222.
- [6] Biely, P., Côté, G.L., Kremnický, L., Greene, R.V., Dupont, C. and Kluepfel, D. (1996) FEBS Lett. 396, 257–260.
- [7] Sundberg, M. and Poutanen, K. (1991) Biotechnol. Appl. Biochem. 13, 1–11.
- [8] Poutanen, K., Sundberg, M., Korte, H. and Puls, J. (1990) Appl. Microbiol. Biotechnol. 33, 506–510.
- [9] Johnson, K.G., Fontana, J.D. and MacKenzie, C.R. (1988) Methods Enzymol. 160, 551–560.
- [10] Horton, D. (1963) Methods Carbohydr. Chem. 2, 368–373.
- [11] Dubois, M., Gillis, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350–356.
- [12] Bounias, M. (1980) Anal. Biochem. 106, 291–293.
- [13] Sweets, H.M. and Wang, C.H. (1986) J. Am. Chem. Soc. 108, 6421–6422.
- [14] Shaw, J.-F. and Klivanov, A.M. (1977) Biotechnol. Bioeng. 29, 648–651.
- [15] Henne, W.J., Sweets, H.M., Wang, Y.-F. and Wong, C.-H. (1988) J. Am. Chem. Soc. 53, 4939–4945.
- [16] Chen, H.-P., Hsiao, K.-F., Wu, S.-H. and Wang, K.-T. (1995) Biotechnol. Lett. 17, 305–308.
- [17] Lopez, R., Perez, C., Fernandez-Mayoralas, A. and Conde, S. (1993) J. Carbohydr. Chem. 12, 165–171.
- [18] Druckhammer, D.H., Hennen, W.J., Pederson, R.L., Barbas III, C.F., Gautheron, C.M., Krach, T. and Wang, C.-H. (1991) Synthesis 1991, 499–525.
- [19] Hsiao, K.-F., Lin, H.-J., Leu, D.-L., Wu, S.-H. and Wang, K.-T. (1994) Bioorg. Med. Chem. Lett. 4, 1629–1632.
- [20] Tenkanen, M. (1998) Biotechnol. Appl. Biochem. (in press).
- [21] Shareck, F., Biely, P., Morosoli, R. and Kluepfel, D. (1995) Gene 153, 105–109.
- [22] Margoles-Clark, E., Tenkanen, M., Söderlund, H. and Penttilä, M. (1996) Eur. J. Biochem. 237, 553–560.
- [23] Linden, J., Samara, M., Decker, S., Johnson, E., Boyer, M., Pecs, M., Anney, W. and Himmel, M. (1994) Appl. Biochem. Biotechnol. 45/46, 383–393.
- [24] de Graaff, L.H., Visser, J., van den Broeck, H.C., Strozyk, F., Kormelink, F.J.M. and Boonman, J.C. (1992) EP 0507 369 A3.
- [25] Koseki, T., Furuse, S.S., Kimio, I.I., Sakai, H.H. and Matuzawa, H.H. (1996) DDBJ/EMBL/GenBank.
- [26] Laurie, J.L., Clarke, J.H., Ciruela, A., Faulds, C.B., Williamson, G., Gilbert, H.J., Rixon, J.E., Millward-Sadler, J. and Hazlewood, G.P. (1997) FEMS Microbiol. Lett. 148, 261–264.