

β -Xylosidase activity, encoded by *xlnD*, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum

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(Received 18 November 1996) – EJB 96 1715/2

Two proteins exhibiting β -D-xylosidase activity were identified upon fractionation and purification of a culture filtrate of an arabinoxylan-grown *Aspergillus niger*. A single band of 110 kDa by SDS/PAGE was obtained in both cases and these were active on xylo-oligosaccharides and on xylan. Partial *xlnD* cDNA clones were immunochemically identified and isolated from a λ cDNA expression library. Sequence analysis showed that all cDNA clones correspond to a single gene. A genomic clone was isolated and overexpressed in *A. niger* and *A. nidulans*. The *xlnD* gene has an ORF of 2412 nucleotides, encodes a protein of 804 amino acids and contains a potential signal peptide of 26 amino acids. This results in a mature protein of 778 amino acids with a predicted molecular mass of 85 kDa and an isoelectric point of 4.5. The protein is N-glycosylated and contains 15 potential N-glycosylation sites. Sequence similarity is found with β -D-glucosidases both of bacterial and fungal origin. Both β -xylosidase proteins purified have high activity on the artificial substrate *p*-nitrophenyl β -D-xylopyranoside (XylNp) and a side activity on *p*-nitrophenyl α -L-arabinofuranoside and *p*-nitrophenyl β -D-glucopyranoside. *A. niger* strains in which the *xlnD* gene was disrupted accumulate mainly xylobiose and xylotriose when grown on xylan and have no significant β -xylosidase activity in the culture medium, indicating that this gene encodes the major extra-cellular β -xylosidase.

Keywords: *Aspergillus niger*; filamentous fungi; β -xylosidase gene cloning; induction; xylan hydrolysis.

Xylan, the major hemicellulose component in plant cell walls, is the most abundant polysaccharide after cellulose. Hence, hydrolysis of xylan plays an important role in the breakdown process of plant material in nature (Biely, 1985).

The structure of xylan is heterogeneous due to modification of the main chain which consists of β -1,4 linked D-xylose residues. At the O-2 and O-3 position these D-xylose moieties can be substituted with L-arabinofuranose and 4-O-methyl glucuronic acid residues or they can be esterified with acetic acid. Also, the L-arabinofuranosyl residues in the side chains can be esterified with ferulic and *p*-coumaric acid (Coughlan and Hazlewood, 1993). Complete degradation of this structure requires the synergistic action of a spectrum of different enzymes. The

major xylanolytic activities are those catalysed by endo-xylanases, which hydrolyse the β -1,4 bonds in the main chain, generating a mixture of xylo-oligosaccharides. Other activities are α -L-arabinofuranosidase, α -glucuronidase, acetyl (xylan) esterase, ferulic acid and *p*-coumaric acid esterase and β -xylosidase, each having a specific function in the cooperative degradation process. β -Xylosidase, which has been reported to be rate-limiting in xylan hydrolysis (Dekker, 1983; Poutanen and Puls, 1988), is the enzyme that cleaves off the terminal xylose units from the non-reducing end of the xylose oligomers arising from endo-xylanase activity. It is therefore important for complete hydrolysis of xylan.

In *Aspergillus* sp. it has been shown that expression of xylanolytic enzymes is subject to induction and carbon catabolite repression. *A. tubingensis* has three endo-xylanases encoded by *xlnA*, *xlnB* and *xlnC*. For the endo-xylanase encoding *xlnA* gene of *A. tubingensis* a regulatory region involved in the xylan specific transcription has been identified (de Graaff et al., 1994). Carbon catabolite repression also works via this fragment. In *A. niger* two endo-xylanases with isoelectric points of 4.5 and 8.2 are found (de Graaff et al., 1994), encoded by *xlnB* and *xlnC*, respectively. The major endo-xylanase in *A. niger* is encoded by *xlnB*. For *A. niger* the carbon sources known to induce the xylanolytic system are xylan, xylobiose and D-xylose, the latter two formed as a result of endo-xylanase and β -xylosidase activity. Although β -xylosidases of various *Aspergillus* sp. have been purified and biochemically characterised, as for example in the case of *A. niger* (Rodionova et al., 1983), *A. fumigatus* (Kiprechev et al., 1986) *A. awamori* (Kormelink et al., 1993)

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Abbreviations. CHEF, contour-clamped homogeneous electric field analysis; Xyl(MeUmb), 4-methylumbelliferyl β -D-xyloside; AraNp, *p*-nitrophenyl α -L-arabinofuranoside; GalNp, *p*-nitrophenyl β -D-galactopyranoside; GluNp, *p*-nitrophenyl β -D-glucopyranoside; XylNp, *p*-nitrophenyl β -D-xylopyranoside.

Enzymes. α -L-Arabinofuranosidase (EC 3.2.1.55); β -xylosidase (EC 3.2.1.37); endo-1,4- β -xylanase (EC 3.2.1.8); β -glucosidase (EC 3.2.1.21).

Note. The novel nucleotide sequence data published here have been submitted to the Genbank/EMBL sequence data bank and are available under accession number Z 84377.

and *A. nidulans* (Kumar and Ramon, 1996), up to now the cloning of a fungal β -xylosidase gene has not been reported.

Little is known about the molecular mechanism by which low-molecular-mass compounds trigger induction of the xylanolytic system. At the cellular level the inducing capacity of various (di)saccharides has been tested (Hrmova et al., 1991; Gosh and Nanda, 1994; de Graaff et al., 1994). Especially the β -xylosidase of *A. niger*, for which a strong transglycosylation activity has been described (Tavobilov et al., 1984; Shinoyama et al., 1988; Kizawa et al., 1991), could play an important role in the formation of signal molecules leading to transcription and expression of the xylanolytic genes. Cloning of the β -xylosidase gene of *A. niger* provides the possibility to determine the role of β -xylosidase in xylan hydrolysis and to assess its role in the induction of the xylanolytic system.

MATERIALS AND METHODS

Bacterial and fungal strains. All *Aspergillus niger* strains were derived from the wild-type N400 (CBS 120.49) as *A. niger* NW147, a mutant constitutive in xylanase expression, derived from *A. niger* NW205 (*argB13*, *cspA1*, *nicA1*, *pyrA6*). The *Aspergillus nidulans* wild-type strain used was *A. nidulans* WG096 (*pabaA1*, *yA2*) of FGSC 187. The *A. niger* strain N902 (*argB15*, *cspA1*, *fwnA1*, *metB10*, *pyrA5*), and *A. niger* NW155 (*argB13*, *cspA1*, *nicA1*, *pyrA6*, *prtF28*), derived from *A. niger* NW228, (van den Hombergh et al., 1995) and *A. nidulans* G191 (*pabaA1*, *pyrG89*, *fwnA1*, *uaY9*) (Ballance and Turner, 1985) were used as recipient strains in transformation experiments.

Escherichia coli strains used in propagation of λ phages or plasmids were XL1-Blue MRF', BB4 (Stratagene), LE392 and DH5 α .

Media and growth conditions. All media were based on *Aspergillus* minimal medium (Pontecorvo et al., 1953), with different carbon sources, as indicated in the text, and a starting pH of 6.0. Spores were inoculated at 10^6 ml $^{-1}$; *A. niger* strains were grown at 30°C and *A. nidulans* strains at 37°C. In transfer experiments, the first culture with 1% (mass/vol.) fructose was supplemented with 0.05% (mass/vol.) yeast extract and 0.1% (mass/vol.) casamino acids. Mycelia were, after overnight growth, recovered by filtration and washed with sterile saline prior to their addition to media containing various carbon sources. Xylans used were oat speltz xylan (Sigma), birchwood xylan (Roth), rye arabinoxylan (Megazyme) and a crude wheat arabinoxylan preparation (Latenstein, The Netherlands). Mycelia were harvested, washed with saline, and frozen using liquid nitrogen. Where necessary, media were supplemented with 10 mM L-arginine, 10 μ M nicotinamide, 10 μ M *p*-aminobenzoic acid, 2 mM L-methionine and 10 mM uridine.

General cloning and sequencing techniques. The following plasmid vectors were used in DNA subcloning: pGEM (-T) (Promega), pEMBL19 (Dente and Cortese, 1987), pBluescript (Stratagene), and pUC vectors (Yanisch-Perron et al., 1985). Standard methods were used for DNA manipulations, propagation and purification of (phage) DNA, plaque lift, (sub)-cloning etc. (Sambrook et al., 1989). Sequencing was performed by the dideoxy termination method (Sanger et al., 1977) using the T7 sequencing kit (Pharmacia). An ALFexpressTM DNA sequencer (Pharmacia) was used in fluorescent labelled sequencing. Also the Cy5TM AutoreadTM sequencing kit and the Cy5TM-dATP labelling mix (Pharmacia) with gene-specific oligonucleotides or the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham) with 5'-Cy5TM-labelled general sequencing primers were used, all according to the supplier's protocol. All nucleotide sequences were determined for both

strands. The coding region was also determined by sequencing cDNA. Sequence analysis was performed using the PC/GENE programme (Intelligenetics).

For the construction of plasmid pIM204, used to inactivate *xlnD*, an internal *xlnD* fragment, amplified by PCR with the gene-specific oligonucleotides xylos001 (1610–1628, Fig. 2) and xylos004 (3599–3616), was ligated in the pGEM-T vector, resulting in pIM202. Plasmid pIM204 was constructed by ligation of the *NsiI*–*XbaI* fragment of pIM130, containing the *pyrA* gene (EMBL, X96734) under the control of the UAS of the *xlnA* promoter of *A. tubingensis* (de Graaff et al., 1994), in the *SpeI*/*NsiI*-digested pIM202 vector.

Purification, amino acid sequencing, enzyme assays and enzymatic deglycosylation of *A. niger* β -xylosidase. For β -xylosidase purification, the columns of DEAE-Sephadex A-50, DEAE-Sephacrose Fast Flow and Mono Q HR 5/5 used were all obtained from Pharmacia LKB. Amino acid sequences were analysed using a gas-phase sequencer (HP G1005A), equipped with a phenylthiohydantoin analyser as described by Amons (1987).

The substrates 4-methylumbelliferyl β -D-xyloside [Xyl-(MeUmb)], *p*-nitrophenyl β -D-xylopyranoside (XylNp), *p*-nitrophenyl α -L-arabinofuranoside (AraNp), *p*-nitrophenyl β -D-galactopyranoside (GalNp) and *p*-nitrophenyl β -D-glucopyranoside (GluNp) were obtained from Sigma. Xylo-oligosaccharides were obtained from Megazyme. *p*-Nitrophenyl release was assayed as described by Rodionova et al. (1983), the endo-xylanase assay as described by Bailey et al. (1992).

N-glycanase from Genzyme was used according the manufacturer's protocol. ABF A was used as a positive control (Flipphi et al., 1994). Protein was analyzed by SDS/PAGE using 12% SDS/polyacrylamide gels (Laemmli, 1970) and stained with Coomassie brilliant blue R250.

DNA and RNA isolation and analysis. Fungal genomic DNA was isolated as described by de Graaff et al. (1988); total RNA was isolated from powdered mycelia using TRIzolTM Reagent (Life Technologies), according to the supplier's instructions. Poly(A)-rich RNA was isolated from total RNA by oligo(dT)-cellulose chromatography (Stratagene).

For northern analysis 20 μ g total RNA was separated on a formaldehyde agarose gel and, after capillary blotting to Hybond-N (Amersham), hybridised in 50% (by vol.) formamide at 42°C.

Screening of *A. niger* genomic and cDNA libraries and contour-clamped homogeneous electric field (CHEF) analysis. The *A. niger* N400 (Harmsen et al., 1990) genomic library was screened by plaque filter hybridisation, according to the standard protocol (Sambrook et al., 1989).

The *A. niger* NW147 cDNA library, of poly(A)-rich RNA of mycelia cultivated for 69 h and 81 h on 2% (mass/vol.) crude wheat arabinoxylan as carbon source, constructed with the ZAPTM-cDNA synthesis kit (Stratagene) and packaged using Packagene extracts (Promega), was immunochemically screened for β -xylosidase-expressing clones essentially as described by Flipphi et al. (1993). Antibodies against β -xylosidase I and II were raised in two Balb/c mice according to the protocol described by van der Veen et al. (1991), with booster intervals of four weeks. The polyclonal antibodies raised showed cross-reactivity with both β -xylosidase I and II. The serum raised against β -xylosidase II was used in the immunochemical screening. Positive cDNA clones were converted to Bluescript phagemids after superinfection with the helper phagemid ExAssistTM, according to the instructions of the manufacturer (Stratagene).

Assignment of *xlnD* to one of the eight chromosomes in *A. niger* was based on the CHEF/Southern analysis together with a

set of five tester strains with linkage-group-specific electrophoretic mobility variation (Verdoes et al., 1994).

Aspergillus transformation. *Aspergillus* strains were transformed according to the protocol described by Kusters-van Someren et al. (1991), using the requirement of uridine of the various strains. The plasmid pGW635 contains the *pyrA* gene of *A. niger* (EMBL X96734) and was used in (co)transformation, selecting for uridine prototrophy.

RESULTS

Purification of *A. niger* β -xylosidase. For the purification of β -xylosidase, *A. niger* NW147 was grown for 96 h in medium containing 1.5% (mass/vol.) crude wheat arabinoxylan. The pH of the culture filtrate was adjusted to pH 6.0 with sodium hydroxide, after which the culture filtrate was diluted threefold. After equilibration using 50 mM sodium acetate pH 5.0, DEAE-Sephadex A50 was added to the culture filtrate to adsorb and concentrate the proteins. Proteins were pulse-eluted with 50 mM sodium acetate pH 5.0 containing 0.5 M sodium chloride. Fractions containing activity against the chromogenic substrate Xyl-(MeUmb) were pooled, desalted and equilibrated against 20 mM piperazine/HCl pH 5.0. After loading on a DEAE-Sephadex Fast Flow column and washing with 3 vol. 20 mM piperazine/HCl pH 5.0, elution was performed with a linear gradient of 0–0.5 M sodium chloride in the same buffer. Fractions containing β -xylosidase activity, as measured with the artificial substrate XylNp, were pooled, dialysed against 20 mM piperazine/HCl pH 6.0 and applied on a Mono Q HR 5/5 column. Protein was eluted using a linear gradient of 0–1 M sodium chloride in the same buffer. Two peaks containing β -xylosidase activity were found, β -xylosidase I was eluting at 0.19 M sodium chloride and β -xylosidase II eluted at 0.29 M sodium chloride. SDS/PAGE of the fractions corresponding to the both peaks showed that both contained a single protein with an apparent molecular mass of 110 kDa. Both enzymes were equally active on xylo-oligosaccharides as determined by high-performance anion-exchange chromatography, resulting in the release of smaller oligosaccharides and xylose. Incubation of (low-substituted) birchwood xylan, contrary to rye arabinoxylan, with β -xylosidase did show release of xylose and xylo-oligosaccharides (data not shown).

After N-glycanase treatment, the molecular mass of both proteins decreased to about 85 kDa upon SDS/PAGE analysis (Fig. 1). The specific activities of both β -xylosidases against *p*-nitrophenyl substrates are listed in Table 1. Both enzymes are also competitively inhibited by D-xylose.

Immunochemical screening and isolation of *xlnD* cDNA clones. About 50 000 plaque-forming units of the cDNA library were immunochemically screened for expression of β -xylosidase with *E. coli* BB4 as plating bacteria. This resulted in the isolation of four positive clones. Restriction analysis of the cDNA clones revealed insert sizes of 1.4, 1.5, 2.4 and 2.5 kb. Sequence analysis revealed that all four cDNA clones correspond to the same gene.

Cloning and sequence of the *xlnD* gene. The largest cDNA insert (2.5 kb) was used to screen the *A. niger* genomic library. Southern hybridization with three of the isolated clones showed that they all shared fragments from the same genomic region. Both a 3.6-kb single hybridising *Pst*I fragment and a 4-kb *Kpn*I fragment were subcloned, resulting in pIM200 and pIM201, respectively. Since plasmid pIM201 contains only part of the structural gene, this plasmid was only used to subclone part of the 5' non-coding region for sequence analysis. The sequence

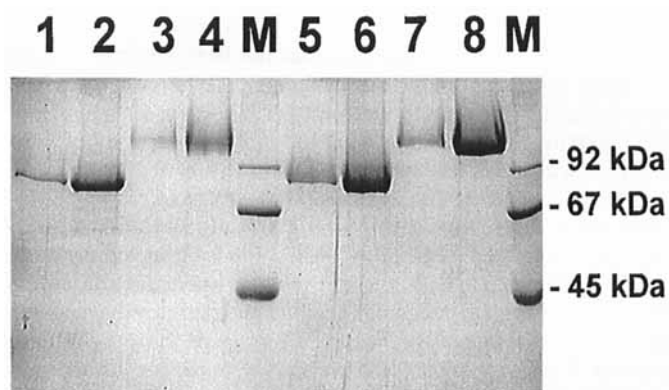


Fig. 1. SDS/PAGE with Coomassie staining of N-glycanase treatment of purified β -xylosidase I and β -xylosidase II. Lanes 1 and 2, 0.4 μ g and 3 μ g, respectively, N-glycanase-treated β -xylosidase I; lanes 3 and 4, 0.4 μ g and 3 μ g, respectively, β -xylosidase I untreated; lanes 5 and 6, 0.5 μ g and 4 μ g, respectively, N-glycanase-treated β -xylosidase II; lanes 7 and 8, 0.5 μ g and 4 μ g, respectively, β -xylosidase II untreated. The molecular masses of the markers (M) are shown on the right.

Table 1. Activity and inhibition of β -xylosidase I and β -xylosidase II.

Substrate	Activity	
	β -xylosidase I	β -xylosidase II
	U/mg	
XylNp	60.2	60.9
GluNp	0.2	0.3
GalNp	0.0	0.0
AraNp	2.8	3.4
Inhibitor	K_i for	
	β -xylosidase I	β -xylosidase II
	mM	
D-Xylose	9.8	13.2

determined for pIM200, also used in *Aspergillus* transformation experiments, was 3583 bp long, with 329 bp in the 5' non-coding region and 841 bp in the 3' non-coding region. A putative TATAA box is found 68 bp before the putative translation start codon (Fig. 2). No introns were found by sequence comparison of cDNA and genomic DNA. The longest cDNA clone is not full length but starts 37 bp after the putative translation initiation site found in the genomic DNA sequence. This translation start site is followed by an open reading frame of 2412 bp. Hence, the predicted peptide is 804 amino acids in length, with a molecular mass of 87 211 Da. N-terminal amino acid sequencing of purified β -xylosidase II failed, indicating that the N-terminus might be blocked. Analysis of the N-terminal amino-acid sequence, according to von Heijne (1986), predicted a signal peptidase cleavage site most likely located between Ala at position -1 and Gln at position 1. Other less likely cleavage sites were predicted before Asn3, and before Ala2. Therefore the predicted mature protein is most likely 778 amino acids long, with a molecular mass of 84 682 Da and an isoelectric point of 4.5. Four different polyadenylation sites were found on the basis of the four cDNA clones, 117, 132, 135 and 149 bp downstream of the stop codon.

Fig. 2. Nucleotide sequence of the *xlnD* gene and flanking regions and the deduced amino acid sequence of the β -xylosidase protein. The nucleotides are numbered on the left (with the coding nucleotides shown in capitals) along with the amino acids of β -xylosidase, which are numbered on the right. The most likely signal peptide is shown in italic and numbered negatively. The potential signal peptidase cleavage sites are shown by arrows (∇). Potential N-glycosylation sites are marked by (s). Flanking regions are shown in lower case, in which a TATAA element is marked by double underlining, four polyadenylation sites by arrows ($\downarrow$), putative CREA sites (context-dependent consensus 5'-SYGGRG-3' as presented by Cubero and Scazzocchio, 1994; Espeso and Peñalva, 1994) in capitals, and *Pst*I sites by single underlining.

A.										B.									
S.f1	alYlwpFads	Vr.agvgsVM	csYNrvNnty	acensyMMnh	l.LkeELGF.	.qGfVvSDWg	Aq			alkvAeEsiV	LLKNenntLP	ispek							
S.f2	ElYlwpFads	Ir.agvgsVM	csYNrvNnty	scensyMinh	l.LkeELGF.	.qGfVvSDWa	Aq			alkvAeEsiV	LLKNekntLP	ispnk							
H.an	EiYlwpFaeA	Vr.ggvssIM	csYNklNgsh	acqnsyLLny	l.LkeELGF.	.qGfVmtDWg	Al			alrsAvEgV	LLKNehetLP	lgrek							
A.tu	EiYlwpFaeA	VkkagvkaVM	ssYNklNgty	tseppwLLtk	v.LreEWGF.	.dGvVmsDWf	gs			irqlgaEgaV	LLKNdg.vLP	Lakss							
K.ma	EiYlwpFria	VkhanpvcIM	taYNkvNgdh	csqskkLLid	i.LrdEWkV.	.dGmlmsDWf	gt			lreiAaDsiV	LLKNknnlyLt	skerr							
C.tm	EiYfasFenA	VkkarpwvVM	caYNklNgey	csenryLLte	v.LkneEwmh.	.dGfVvSDWg	Av			arqaAaEsmV	LLKNeddvLP	Lkksq							
A.ac	EmYlwpFadA	Vr.agvgaIM	csYNqinNsY	gcqnsytLnk	l.LkaELGF.	.qGfVmsDWg	Ah			irklgaDstV	LLKN.nnaLP	Ltgke							
B.fi										aldaAkDsfV	LLKNd.gLP	Lnkkd							
T.re	ElYtwpFadA	V.qanvasVM	csYNkvNttw	acedqytLqt	v.LkdQLGF.	.pGyVmtDwn	Aq			...iArDgiV	LLKNdaniLP	Lkkpa							
S.ly	ElYlwpFanA	Vr.sgvassVM	csYNrlNqty	acensklMng	i.LkgELGF.	.qGfVvSDWY	At			vrkvAaagtV	LLKNvnnvLP	LkepK							
E.co	ndYmpPY.kA	gidagsgaVM	valNslNgtp	atsdswLLkd	v.LrdQWGF.	.kGitvSDhg	Ai			arevArEslV	LLKNrletLP	Lkksa							
T.br	EvFlypFaeA	VkeaklssIM	pgYheldgvp	chkskkLLnd	i.LrkDWGF.	.eGiVvSDYf	Ai			aykiAqEsiV	LLKNennlLP	Lkkdl							
M.bi	EyYeaPFraA	ItagaatgVM	taYNlvNgrp	atvnpdLndt	vrtwtDrld.	...LnvtDag	Ap			aretAakamV	LLKNergtLP	Ldpgk							
A.we						aZLGF.	.zGfVmsDWa	Ah											
A.ni	EyYtpqFhva	ardakvgsVM	caYNNavNgvp	acadsyFLqt	l.LrdtFPGV	dhGyVsSDcd	Aa			syqaAtqgiV	LLKNSnnvLP	Lteka							
Con	E-Y---F--A	-----VM	--YN--N---	-----L--	--L---GF-	--G-V-SD--	A-			----A-E--V	LLKN----LP	L----							

C.										D.									
S.f1	nVtAivwagp	lGdrsGtaia	nILFGkanPs	GhLpfTiakt						lvdYryFeek	nieprYaFGY	GLSYneYevs							
S.f2	nVtAivwagp	lGdrsGtaia	nILFGnanPs	GhLpfTvaks						lvdYryFeek	nieprYaFGY	GLSYneYkvs							
H.an	nVtAVIyssY	lGqdfGtvla	kVLFgdenPs	GkLpfTiakd						yvdYryFdky	nkprYefGY	GLSYsnFsls							
A.tu	kVrAVlqmwY	pGqelGnaia	dVLFgdePa	GrLpqtFtpka						fvgYrhhdtR	eielpFpFGf	GLgYtrFtwg							
K.ma	eanAlvqawY	gGnelGnaia	dVLYGdvvpN	GkLslswpfk						fvgYryYekl	qrkvaFpFGY	GLSYtttFeld							
C.tm	kVksVleayL	gGqalGgrw.	rmcYsvksiv	GkLaeTfpvk						fvgYryYdtk	gieplFpFGH	GLSYtkFeys							
A.ac	nVtAilwagL	pGqesGnsia	dVLYGgrvnPg	akspftWgkt						fidYrgFdkr	netpiYeFGH	GLSYtttFnys							
B.fi	nVnAImqcwY	pGargGraia	eVLFgkasPg	GkMplTfyas						nrtYryFk..	.gtplYpFGY	GLgYskidyl							
T.re	qVKAivwagL	psqesGnalv	dVLWGdvpsPs	GkLvyTiaks						fidYkhFdda	nitprYeFGY	GLSYtkFnys							
S.ly	nVtAilaahY	pGqenGnsim	dILYGdvnPs	GrLpyTipkl						lidYrhFdar	nitplYeFGY	GLSYtttFeie							
E.co	qadAiletwF	aGtegGnaia	dVLFgdyvPs	GkLpmsfprs						.ytsryFdea	n.galYpFGY	GLSYtttFtvs							
T.br	kIpAileawL	pGeegGrava	dViFGdynPg	GkLpisipqs						shwkgdYvel	stkplYpFGY	GLSYtttFtvs							
M.bi	hVpAilwtth	aGaetGnaia	dVLYGdvnPa	GrLqtTwylg						drtYlyFk..	.gtplYpFGH	GLSYtttFryq							
A.ni	nVsAllwggY	pGqsgGfair	dIitGkknPa	GrLvtTgyPa						..tYkwYt..	.geavYeFGH	GLfYttFaes							
Con	-V-A-----Y	-G---G-a--	----G---P-	G-L--T----						---Y-----	-----Y-FG-	GL-Y--F---							

Fig. 3. Regions of similarity between β -xylosidase and family 3 β -glucosidases. Amino acid sequence alignment of β -glucosidase BGL1 and BGL2 of *Saccharomycopsis fibuligera* (accession numbers P22506, P22507), BGLS of *Hansenula anomala* (*Candida pelliculosa*) (P06835), BGLS of *Agrobacterium tumefaciens* (P27034), BGLS of *Kluyveromyces marxianus* (*fragilis*) (P07337), BGLB of *Clostridium thermocellum* (P14002), BGL1 of *Aspergillus aculeatus* (P48825), ORF1 of *Butyrivibrio fibrisolvens* (B49776), BGLS of *Trichoderma reesei* (U09580), B2Tom of *Septoria lycopersici* (U24701), BGLS of *Escherichia coli* (P33363), BGLT of *Thermoanaerobacter brockii* (Z56279), BGLA of *Microbispora bispora* (L06134), BGLA₃ of *Aspergillus wentii* (P29090) and β -xylosidase of *A. niger*. Capital letters indicate at least 11 identical or similar residues and **con** (consensus) includes β -xylosidase within the comparison. Region A comprises Glu233–Ala293 (amino acid position within β -xylosidase) and was identified as the active-site region with the Asp (*) residue in BGLA₃ of *Aspergillus wentii* (Bause and Legler, 1980). Region B, C and D comprise Ser389–Ala413, Asn550–Ala589 and Thr612–Ser636 respectively. Searches were performed at the EPFL using the BLAST network service and sequences have been aligned by introducing gaps indicated by dots (.) to maximise similarity.

A homology search with the deduced β -xylosidase amino acid sequence revealed sequence identity with β -glucosidases of bacterial and fungal origin (Fig. 3). CHEF/Southern analysis assigned *xlnD* to linkage group II (data not shown). Hybridisation of *A. niger* genomic DNA under heterologous hybridisation conditions (56°C, washed to 0.3 M sodium chloride, 30 mM sodium citrate, 0.1% SDS) gave no additional signal. Southern hybridisation with DNA of other black *Aspergillus* sp. and *Trichoderma reesei* revealed one or more hybridising bands indicating the presence of a β -xylosidase counterpart(s).

Induction and overexpression of the *A. niger xlnD* gene. In *A. niger* N902, which is a wild-type strain for *xlnD*, transcription of the *xlnD* gene is subject to induction and carbon catabolite repression. Upon cultivation on xylan, induction of *xlnD* resulted in a maximal transcript level before the endo-xylanase gene *xlnB*, but the transcript also disappeared more rapidly (Fig. 4).

The uridine auxotrophy of *A. niger* NW155, having a strongly reduced extracellular protease activity and therefore useful for overexpressing *xlnD* for protein isolation, was used in a cotransformation experiment using pIM200 as cotransforming plasmid and the *pyrA*-containing plasmid pGW635 as selectable marker. The resulting *PYR*⁺ transformants were screened on plates containing 1% (mass/vol.) oat spelt xylan and 0.5 mM

Xyl(MeUmb). Of 26 transformants tested, five transformants which had an elevated level of Xyl(MeUmb) activity were selected. These transformants, with a *PYR*⁺ transformant as reference, were grown on minimal medium containing 1% (mass/vol.) oat spelt xylan for 27 h. Results of β -xylosidase activity in the medium towards XylNp are given in Fig. 5. Among the transformants, up to 20-fold elevated activity could be found compared to the reference strain. Western analysis showed that the 110-kDa protein was overexpressed in these transformants (Fig. 5). Southern hybridisation of genomic DNA of these transformants revealed that for all transformants analysed, one or multiple copies of the pIM200 plasmid were integrated into the genome (data not shown).

In a similar experiment, cotransformants were selected for *A. niger* N902 and *A. nidulans* G191 (Table 2), which had an increased Xyl(MeUmb) activity as described above.

Inactivation of *xlnD* expression by insertion of an internal *xlnD* fragment. To analyse the role of *xlnD* in the induction of the xylanolytic system of *A. niger*, plasmid pIM204 was constructed to inactivate *xlnD* by homologous recombination. This plasmid contains an internal fragment of *A. niger xlnD* of about 1600 bp and the *pyrA* gene as the selection marker in transformation. *A. niger* N902 was transformed with pIM204 select-

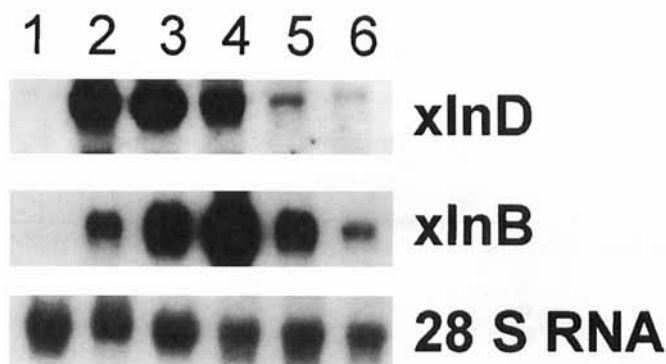


Fig. 4. Expression of β -xylosidase in *A. niger*. Northern analysis of the time course of induction on xylan of *xlnD* and *xlnB* (encoding endo-xylanase B) in *A. niger* wild-type N902; the 28S RNA probe was used to check the RNA amount and the quality of the 15 μ g total RNA loaded per lane. Lane 1, 3% (mass/vol.) glucose/2% (mass/vol.) birchwood xylan at 22 h; lanes 2–6, 2% (mass/vol.) birchwood xylan at 22 h, 30 h, 46 h, 70 h and 96 h, respectively.

A

<i>A. niger</i> strain	Activity (mU/ml)
1-NW155::pGW635	16
2-NW155::pIM200-A1	86
3-NW155::pIM200-A4	112
4-NW155::pIM200-A8	239
5-NW155::pIM200-A9	110
6-NW155::pIM200-A12	295

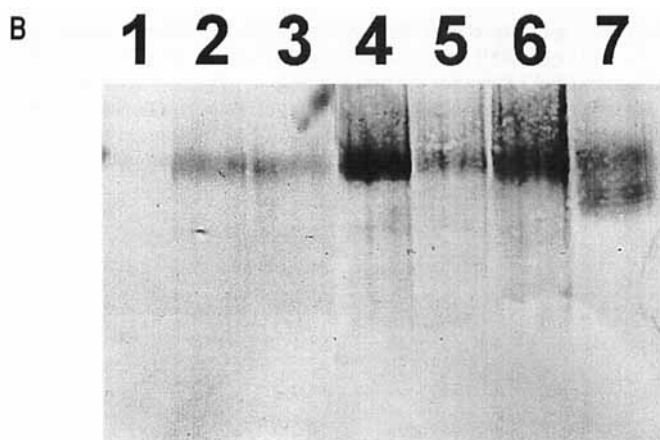


Fig. 5. Analysis of β -xylosidase expression produced by *A. niger* transformants. (A) β -Xylosidase activity of culture filtrate after growth on oat spelt xylan for 27 h determined on XylNp. (B) Immunological (β -xylosidase II antiserum) detection of β -xylosidase upon SDS/PAGE and western blotting; in lanes 1–6, 22 μ l culture filtrate was loaded from strains 1–6 listed in A; lane 7, 175 ng β -xylosidase II.

ing for uridine prototrophy. The resulting transformants were screened for Xyl(MeUmb) activity on a 1% (mass/vol.) oat spelt xylan plate. One out of 20 transformants tested was found to have a severely decreased Xyl(MeUmb) activity after 24 h of growth. Southern analysis of this particular pIM204 transformant demonstrated a single homologous integration event at the *xlnD* locus (data not shown).

Effect of overexpression and inactivation of *xlnD* on the xylanolytic system of *A. niger*. To determine the effect of *xlnD* expression on the overall expression of the xylanolytic spectrum, *A. niger* strain N902 (wild type for xylanolytic expression and recipient strain in transformation), three transformants overex-

Table 2. β -Xylosidase activities produced by *A. nidulans* strains transformed with pIM200. Activity in culture filtrate determined towards XylNp after growth for 26 h with 3% (mass/vol.) D-xylose or 2% (mass/vol.) xylan.

<i>A. nidulans</i> strain	Activity	
	3% D-xylose	2% xylan
	mU/ml	
WG096	0	16
G191::200-5	48	725
G191::200-7	11	96
G191::200-9	40	249
G191::200-13	33	520
G191::200-15	210	1525

pressing *xlnD*, the *xlnD*-disruptant strain and a *pyrA*⁺ transformant as control strain were all grown in liquid culture. After growth on fructose, all strains were transferred to xylan and D-xylose, as the latter carbon source can compensate for variation in xylan degradation and, for example, biomass accumulation, due to alteration of β -xylosidase activity.

For the *A. niger* N902 transformants, the integration of pIM200 copies is paralleled by an increase in *xlnD* transcript level on D-xylose and xylan compared to wild type (Fig. 6). β -Xylosidase activity however does not follow transcript levels of *xlnD*, since activity after 9 h on D-xylose is low compared to the activity on xylan. β -Xylosidase overexpression had a slight negative effect on endo-xylanase activity and *xlnB* transcription with both carbon sources. HPLC analysis of xylan culture media showed that initially relatively more D-xylose was formed and fewer oligosaccharides, compared to wild type, as would be expected by an increase in β -xylosidase activity (data not shown).

The β -xylosidase deficient transformant had, after transfer to xylose and xylan, no XylNp activity and had a truncated *xlnD* messenger. The absence of β -xylosidase did not alter the induction of *xlnB* on D-xylose but greatly increased the induction level of *xlnB* in xylan cultures. The induction was slow, compared to wild type, but the lag time in various experiments was dependent on the amount of biomass transferred. The culture filtrate of the truncation transformant was analyzed by HPLC and showed an accumulation of xylobiose and xylotriose upon degradation of xylan (Fig. 7), thereby confirming the inducing nature of these saccharides.

DISCUSSION

Two proteins β -xylosidase I and β -xylosidase II, purified from culture filtrate of *A. niger*, grown on crude arabinoxylan, have the same specificity pattern towards *p*-nitrophenyl substrates, xylo-oligosaccharides and also the same apparent molecular mass of 110 kDa upon SDS/PAGE. Both β -xylosidase I and β -xylosidase II are N-glycosylated, as can be concluded from the reduction in apparent molecular mass to 85 kDa after N-glycanase treatment and the change from a diffuse (glycosylated) band to a sharp (deglycosylated) band upon SDS/PAGE.

The activity of β -xylosidase I and β -xylosidase II against xylo-oligosaccharides, but also against xylan, indicates that the cloned β -xylosidase could be an exo-acting xylanase. However, the presence of a contaminating endo-xylanase activity in the β -xylosidase preparation is likely due to the production of xylo-oligosaccharides. The molecular mass of 110 kDa is in the same order of magnitude as reported before for β -xylosidases of *A.*

A

<i>A. niger</i> strain	Activity on					
	1% D-xylose 9 h		2% xylan 9 h		2% xylan 28 h	
	XylNp	endo-Xyl	XylNp	endo-Xyl	XylNp	endo-Xyl
	mU/ml	U/ml	mU/ml	U/ml	mU/ml	U/ml
N902	0	8	55	18	320	106
N902::204-11	0	5	0	10	0	78
N902::635	0	8	58	18	340	92
N902::200-5	0	7	200	10	1270	74
N902::200-20	10	7	382	8	3100	83
N902::200-18	0	7	330	8	3350	41

B

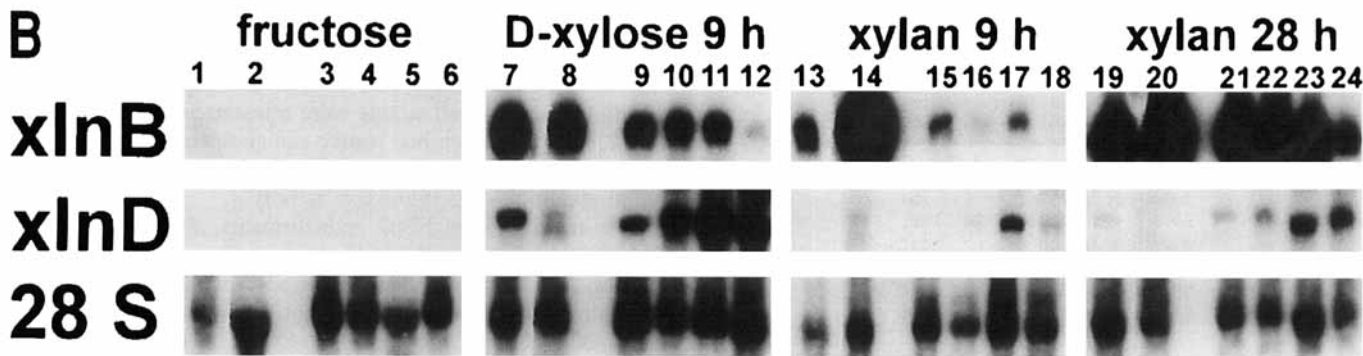


Fig. 6. Expression of the xylanolytic system of different *A. niger* transformants. 3 g of mycelia of the N902 wild-type, a *xlnD*-disruptant strain (pIM204), a strain transformed with *pyrA* (pGW635) and strains overexpressing *xlnD* (pIM200) were cultured for 18 h in 2% (mass/vol.) fructose and then transferred to 50 ml medium containing 1% (mass/vol.) D-xylose or 2% (mass/vol.) xylan for the times indicated. (A) Endo-xylanase (endo-xyl) and β -xylosidase (XylNp) activities of the culture filtrates. (B) Northern analysis of *xlnB* (encoding endo-xylanase B) and *xlnD* transcription of mycelia cultured and transferred as indicated. Lanes 1–6, 7–12, 13–18 and 19–24, 20 μ g of total RNA of N902, N902::204–11, N902::635, N902::200-5, N902::200-20 and N902::200-18, respectively. The 28S RNA probe of *Agaricus bisporus* was used to check the RNA amount and quality.

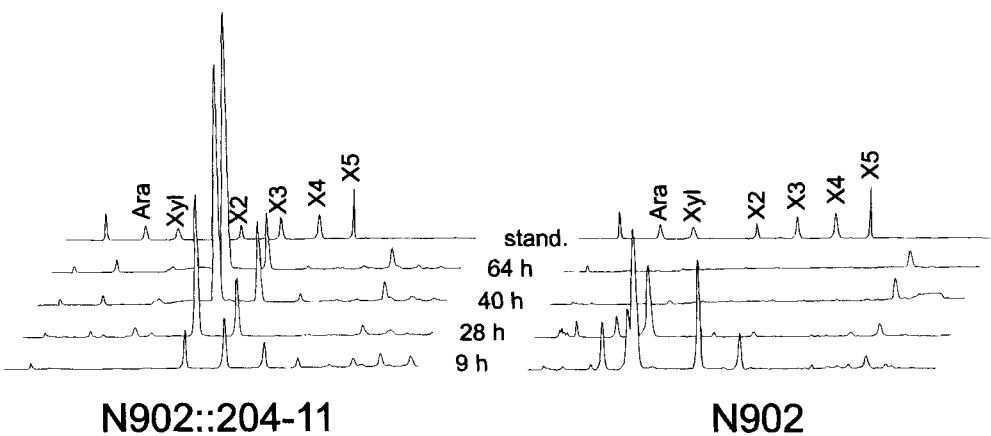


Fig. 7. Saccharification of xylan by *A. niger* N902 and β -xylosidase-deficient strain N902::204–11. 50 μ l of fivefold-diluted heat-inactivated (5 min, 100°C) culture filtrate was analysed by high-performance anion-exchange chromatography on a Dionex system with a Carbpac PA-100 column and pulsed amperometrical detection, using a gradient of 0.05–0.90 M NaOH suitable for xylose oligosaccharide separation. Cultures were transfers from 18 h growth on fructose into xylan media for the time indicated. Standards used are: Ara, L-arabinose; Xyl, D-xylose; X2–X5, xylobiose–xylopentose (Megazyme).

niger (122 kDa; Rodionova et al., 1983), *A. awamori* (110 kDa; Kormelink et al., 1993), *A. fumigatus* (90 kDa; Kitpreechavanich et al., 1986) and *A. nidulans* (85 kDa; Kumar and Ramón, 1996). Besides XylNp activity, β -xylosidase I and β -xylosidase

II of *A. niger* also exhibit activity towards AraNp ($\pm 5\%$), towards GluNp ($< 1\%$) but not against GalNp. Substrate ambiguity of β -xylosidases, exhibiting arabinofuranosidase activity, β -galactosidase activity, and/or β -glucosidase activity, has also

been reported before for β -xylosidases of *A. niger* (Rodionova et al., 1983), *T. reesei* (Poutanen and Puls, 1988) and bacteria (Utt et al., 1991; Sakka et al., 1993). The specific activities and the substrate specificities however differ from those previously reported (Rodionova et al., 1983; Poutanen and Puls, 1988).

The two *A. niger* β -xylosidases appear to be encoded by a single gene, *xlnD*. The complete nucleotide sequence of this gene has been determined and analysis of the isolated cDNAs revealed no introns and at least four functional polyadenylation sites. The translation start codon is postulated from the nucleotide sequence as being the first AUG after a presumed TATAA box and the beginning of a long open reading frame. A translational start at this point conforms to the consensus sequence (a purine, A, at position -3 and G at position $+4$) in eukaryotic genes (Kozak, 1991). Initiation of translation at the second in-frame AUG, however, cannot be completely ruled out (Kozak, 1995), as described for the *areA* gene of *Aspergillus nidulans* (Arst and Sheerins, 1996). The deduced amino acid sequence of β -xylosidase has a N-terminal extension of 26 amino acids. This would conform to the $(-3, -1)$ rule with a small, non-aromatic/charged amino acid (Ala) at the -3 position and a small amino acid (Ala) at the -1 position (von Heijne, 1986). Edman degradation of the N-terminus of purified β -xylosidase II gave no evidence for the correct open reading frame, since the protein appeared to be blocked, most likely due to the modification of the predicted N-terminal glutamine into a 5-oxoprolyl residue which is resistant to Edman degradation (Ooi et al., 1990). Another explanation, although less likely, could be an alternative processing site of the signal peptide between residues, $+2$ and $+3$. In this case protection against Edman degradation could be the result of N-glycosylation of the N-terminal Asn (Fig. 2). β -Xylosidase I and β -xylosidase II, which behave differently on Mono Q, could be products of different (N-terminal) processing, or N-glycosylation events, but other modifications, like limited proteolysis, cannot be excluded.

The *A. niger xlnD* encodes a novel β -xylosidase, not homologous to known β -xylosidase genes from family 43 of glycosyl hydrolases (Henrissat and Bairoch, 1993). The highest sequence similarity is found with β -glucosidases of the BGB group (Béguin, 1990) or family 3 (Henrissat, 1991). The active-site residue Asp, as determined for the *A. wentii* β -glucosidase A3 (Bause and Legler, 1980), is conserved within all glycosyl hydrolases of this family. β -Xylosidase of *A. niger* therefore is a new member of family 3 glycosyl hydrolases, possibly sharing the catalytic mechanism. Whether the two-amino-acid insertion in β -xylosidase in the active-site region causes the different specificity is unclear. The similarity found to β -glucosidases might also be the basis for the low β -glucosidase activity of β -xylosidase. Southern hybridisation under heterologous conditions suggests the presence of *xlnD* counterparts in other *Aspergillus* sp. and *T. reesei*. The availability of more sequences will therefore be only a matter of time.

The expression of the *xlnD* gene is regulated at the level of transcription (Fig. 4). In the presence of glucose the transcription is repressed. In the *xlnD* 5' non-coding region, as indicated in the sequence (Fig. 2) putative CRE binding sites can be found. This indicates that upstream repressing sequences may control *xlnD* transcription directly. Whether this operates by a double lock mechanism as proposed for the *A. tubingensis xlnA* endo-xylanase gene expression (de Graaff et al., 1994), is not yet known.

The reason for the discrepancy between the β -xylosidase activity and the transcription level, upon cultivation on D-xylose remains unclear. In part it might be due to association of β -xylosidase with the mycelial cell wall and enzyme leakage (Stålbrand et al., 1992), which is carbon-source-dependent, as was

shown for β -glucosidase of *Aspergillus phoenicis* (Réczey et al., 1989). β -Xylosidase is rate-limiting in enzymatic xylan hydrolysis (Dekker, 1983; Poutanen and Puls, 1988), probably due to the enzyme being located in the cell wall and therefore low recovery from the culture medium. Overexpression provides a higher amount of the β -xylosidase in the culture medium. Cultivation on xylan shows that, for β -xylosidase overproducers, the balance at the initial stages of degradation is indeed more towards lower-molecular-mass saccharides as D-xylose. Later on in the cultivation no saccharides are detected, indicating that β -xylosidase in this stage is not rate-limiting.

A saccharification system, which has some parallels, concerns the degradation of cellulose by *T. reesei* by the concerted action of cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) releasing cello-oligosaccharides and β -glucosidase. Here, overexpression of the *bgl1* gene, encoding an extracellular and mainly cell-wall-bound β -glucosidase (Messner and Kubicek, 1990), led to an increased cellulase activity suggesting a role for BGL1 in the induction cascade (Barnett et al., 1991; Kubicek et al., 1993). Overexpression of β -xylosidase gives no positive effect on *xlnB* transcription either on D-xylose or on xylan. The decrease of *xlnB* transcription on D-xylose, and also on xylan in some *xlnD* multi-copy transformants, could be due to limitation of the transcriptional activation machinery. Considering the end products of the glucohydrolases and xylohydrolases, glucose and D-xylose respectively, there is a major difference in that D-glucose represses cellulase formation (Kubicek et al., 1993), whereas D-xylose induces the xylanolytic system.

Disruption of *xlnD* in *A. niger* abolished β -xylosidase activity, thereby indicating that *xlnD* codes for the major extracellular β -xylosidase activity. This is confirmed by the accumulation in the culture media of xylobiose and xylotriose, products of endo-xylanase activity and substrates for β -xylosidase. In this β -xylosidase-deficient strain the total endo-xylanase activity was lower than in the wild-type strain, but *xlnB* transcription increased with the accumulation of xylobiose and xylotriose, which induce the xylanolytic system. Direct cultures of the β -xylosidase-deficient strain on xylan lead to big differences in biomass accumulation compared to the wild-type strain, thereby explaining the lower endo-xylanase activity measured. In the case of deletion of the *bgl1* gene in *T. reesei*, a lag in cellulase induction is found but this does not affect growth on cellobiose (Fowler and Brown, 1992).

The existence of an intracellular β -xylosidase in *A. niger* has not been shown yet, although mycelia-associated β -xylosidases are sometimes called intracellular (Uchida et al., 1992). Therefore, in contrast to *bgl1*, β -xylosidase is necessary for complete hydrolysis of xylan by *A. niger*, since it seems the only xylanolytic activity, capable of sufficiently rapid processing of xylobiose and xylotriose.

The exact nature of the inducer(s) remains unclear since degradation of xylose oligosaccharides by enzymes with a low activity, providing a slow feed of e.g. D-xylose, cannot be excluded. Also the presence of other undegradable (substituted) residues due to the absence of β -xylosidase can play a role in this higher induction. High transglycosylation activity has been described for *A. niger* β -xylosidase (Tavobilov et al., 1984; Shinoyama et al., 1988; Kizawa et al., 1991). However, this does not seem to play a role in induction since, using D-xylose as an inducer, the wild-type level of induction is found in the β -xylosidase-deficient strain. Both the β -xylosidase substrates xylobiose and xylotriose and the product D-xylose are inducing compounds for the xylanolytic system in *A. niger* and therefore β -xylosidase is not essential for xylanolytic induction.

This project was financed by a grant from the European Commission in the Biotech programme (BIO2-CT93-0174) to J. Visser. The authors

thank Hetty van den Broeck for performing the experiments on enzyme kinetics, Marco Gielkens for the cDNA library construction, J. Verdoes for providing the tester strains for CHEF analysis and Peter van de Vondervoort for performing the CHEF analysis and the construction of the strain NW155.

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