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INDUCTION OF D-XYLAN-DEGRADING ENZYMES IN *Trichoderma lignorum* BY NONMETABOLIZABLE INDUCERS. A SYNTHESIS OF 4-THIOXYLOBIOSE

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ABSTRACT

The D-xylan-degrading enzymes of *Trichoderma lignorum* can be induced by several oligo- and poly-saccharides. The induction is optimal when L-arabino-D-xylan is used as carbon source. The enzymes were also produced by cells grown on glycerol in the presence of 4-thioxylobiose (4-*S*-β-D-xylopyranosyl-4-thio-D-xylopyranose) and methyl β-D-xylopyranoside. These inducers were not taken up by the cells. The extent of induction was, however, lower than with xylobiose. Thioxylobiose was obtained in an overall yield of 55–60% from the reaction of 1,2,3-tri-*O*-benzoyl-4-*O*-trifluoromethylsulfonyl-β-L-arabinopyranose with the sodium salt of 1-thio-β-D-xylopyranose in *N'*,*N''*,*N'''*-hexamethylphosphoramide, followed by *O*-deacylation of the resulting 4-thiodisaccharide.

INTRODUCTION

D-Xylanases and β-D-xylosidases are widely distributed among mycelial fungi and bacteria[1], and are of central importance in view of the current interest in the hydrolytic conversion of lignocellulosic materials. Although there is still some confusion in the literature as to whether xylanases are produced constitutively or inductively[1], some recent reports demonstrate clearly that at least some bacterial[2], yeast[3], and fungal[4] enzymes are inducible. Soluble fragments of D-xylan, such as xylobiose and higher, homologous oligosaccharides, were effective as inducers of xylanase in the yeast *Cryptococcus albidus*, but the inductive effect may be partly hidden by catabolic repression[3]. 4-Thiocellobiose, as a nonmetabolizable substrate analog, was found to be an excellent inducer of cellulases, as well as of xylanases in the basidiomycete *Schizophyllum commune* without giving rise to catabolic repression[4]. It was of interest to investigate such properties with 4-thioxylobiose (**4**)

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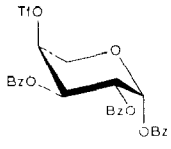

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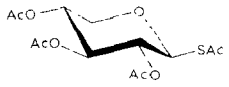

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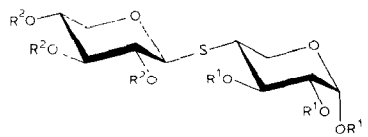

3 R^1 = Bz, R^2 = Ac
4 R^1 = R^2 = H

and a soil hyphal fungus, *Trichoderma lignorum*, known as a productive source of xylanases[5]. In addition, the induction spectrum of this species was ascertained.

EXPERIMENTAL

Microorganism and growth conditions. — *T. lignorum* was isolated from soil of the African equatorial forest and was kept on wort-agar slants at 8°. Unless otherwise stated, the strain was grown in a medium containing a 2% carbon source, 0.68% KH_2PO_4, 0.25% $(NH_4)_2SO_4$, 0.1% $NaNO_3$, 0.03% $CaCl_2 \cdot 2\ H_2O$, 0.03% $MgSO_4 \cdot 7\ H_2O$, 0.06% tryptone, 0.03% yeast extract, and 3 mL/L of a trace metal solution containing 0.15% $FeSO_4 \cdot 7\ H_2O$, 0.12% $MnCl_2 \cdot 4\ H_2O$, 0.1% $ZnSO_4 \cdot 7\ H_2O$, and 0.05% $CoCl_2 \cdot 6\ H_2O$. The initial pH of the medium was adjusted to 5.2. Large-scale growth (8 L) was performed at 29° with 2% L-arabino-D-xylan in a 12-liter Biostat V fermenter (Braun, Melsungen). The pH was kept constant with 1.5M KOH and 1.5M HCl. Other conditions were the same as those described[6].

Enzyme assays. — Xylanase activity was determined by addition of 0.02 mL of clear culture supernatant liquid to 2.0 mL of a buffered arabinoxylan suspension. This suspension was prepared by suspending arabinoxylan (10 mg/mL) in 0.1M acetate buffer (pH 4.3), followed by an ultrasonic treatment. After incubation for 3–15 min at 37°, the reaction was stopped by adding alkaline 2,4-dinitrosalicyclic acid reagent, and the insoluble substrate was removed by centrifugation. Reducing sugars were measured by the 2,4-dinitrosalicyclic acid method[7] using D-xylose as a standard. Units are defined as μmol of D-xylose/min. β-D-Xylosidase and α-L-arabinofuranosidase activities were measured by the amount of *p*-nitrophenol released (A_{405}) from the appropriate *p*-nitrophenol glycosides (2mM) at 30° in 0.1M acetate buffer (pH 4.5, final vol. 1.22 mL). Units are defined as μmol of *p*-nitrophenol/min. The reactions were terminated by the addition of 2M Na_2CO_3 (2 mL).

Induction experiments. — These assays were performed with *T. lignorum* grown on a rotary shaker at 29° in the medium described earlier, but containing 0.5% glycerol as carbon source. Inducer was added after 24 h, when the organism was in the exponential phase of growth. Samples were withdrawn at appropriate times and centrifuged, and the supernatant solutions were assayed for enzyme activities and concentration of residual inducer. Protein was determined by the method of Bradford[8] using the Bio-Rad protein assay with bovine gamma globulin

as a standard. Mono- and oligo-saccharides were analyzed by l.c. on a column of Aminex HPX-42 (Ca^{2+}) (ref. 9). The method was modified by use of the orcinol–sulfuric acid reagent in an automated analyzing system for monitoring the column effluent[10]. Cell dry-weight and the turbidity of the D-xylan suspension were determined as described[6].

Natural inducers and substrates. — L-Arabino-D-xylan from oat spelt (Roth, Karlsruhe, F.R.G.) was purified from contaminating α-D-glucans (16%) by successive treatment with *Bacillus subtilis* α-amylase (Boehringer) and *Aspergillus niger* amyloglucosidase (Boehringer). The glucan-free arabinoxylan was removed from the digest by precipitation with ethanol (3 vol.) and washed twice with ethanol. Xylobiose was prepared by incubation of arabinoxylan with xylanase II_A of *A. niger* and was isolated[6] from the hydrolyzate by gel chromatography on a column of Bio-Gel P-2. D-Xylan from larchwood, L-arabino-D-galactan, methyl β-D-xylopyranoside, *p*-nitrophenyl β-D-xylopyranoside, and *p*-nitrophenyl α-L-arabinofuranoside were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and cellulose MN 300 was from Macherey–Nagel & Co. (Duren, Germany).

1,2,3-Tri-O-benzoyl-4-O-trifluoromethylsulfonyl-β-L-arabinopyranose (**1**). — Trifluoromethanesulfonic anhydride (1.40 mL, 4.4 mmol) was added with stirring to a solution of 1,2,3-tri-*O*-benzoyl-β-L-arabinopyranose[11] in dichloromethane (8 mL) and pyridine (2 mL) at 0°. The mixture was stirred at room temperature for 0.5 h, dichloromethane (100 mL) added, and the solution successively washed with cold aqueous solutions of $KHSO_4$ (30%, w/v) and saturated $NaHCO_3$, and finally with water. Evaporation of the dried (Na_2SO_4) solution below 40° gave a solid foam which was used without purification in the following step.

1,2,3-Tri-O-benzoyl-4-S-(2,3,4-tri-O-acetyl-β-D-xylopyranosyl)-4-thio-α-D-xylopyranose (**3**). — To a solution of 2,3,4-tri-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-xylopyranose[12] (**2**; 1.22 g, 3.65 mmol) in methanol (15 mL) was added sodium (50 mg), and the solution kept for 12 h at room temperature under nitrogen. After evaporation of the solvent at reduced pressure, the residue was dissolved in *N,N',N''*-hexamethylphosphoramide (20 mL) and added to the crude triflate **1**. The mixture was stirred for 5 h at room temperature. Acetic anhydride (10 mL) and pyridine (10 mL) were added and the mixture was kept for 3 h at 60°. Addition of ice–water gave a precipitate that was filtered off, with the aid of Celite, and dissolved in dichloromethane. The solution was washed with water, dried (Na_2SO_4), and evaporated to give an oil which showed in t.l.c. (1:1, v/v, ethyl acetate–hexane) one major component (R_F 0.31). It was isolated by preparative column chromatography on silica gel (100 g, same eluent); syrup (1.615 g, 60%), $[\alpha]_D^{20}$ +65° (*c* 0.74, chloroform); ^{1}H-n.m.r. (chloroform-*d*, 250 MHz, $SiMe_4$): δ 8.13–7.24 (m, 15 H, PhCO), 6.74 (d, $J_{1,2}$ 3.5 Hz, H-1), 5.95 (t, $J_{3,4}$ 10.5 Hz, H-3), 5.57 (dd, $J_{2,3}$ 10.5 Hz, H-2), 5.17 (t, $J_{3',4'}$ 8 Hz, H-3'), 4.94 (t, $J_{2',3'}$ 8 Hz, H-2'), 4.86 (dd, $J_{4',5'e}$ 3.7 Hz, H-4'), 4.81 (d, $J_{1',2'}$ 8 Hz, H-1'), 4.14 (m, 2 H, H-5a,b), 4.06 (dd, $J_{4',5'e}$ 8 Hz, H-5'*e*), 3.54 (td, $J_{4,5a}$ 11, $J_{4,5e}$ 6 Hz, H-4), 3.31 (dd, $J_{5'a,5'e'}$ 12 Hz, H-5'*a*), and 2.04–1.76 (3 s, 9 H, OAc).

Anal. Calc. for $C_{37}H_{36}O_{14}S$: C, 60.27; H, 4.99; S, 4.35. Found: C, 60.51; H, 5.09; S, 4.38.

4-S-β-D-Xylopyranosyl-4-thio-D-xylopyranose (4-thioxylobiose) (**4**). — To a solution of the acylated 4-thiodisaccharide (**3**; 840 mg, 1.13 mmol) in anhydrous methanol (10 mL) was added sodium methoxide in methanol (M, 5 mL). The mixture was kept overnight at room temperature and then treated with Amberlite IR-120 (H^+). The residue obtained after filtration and concentration was purified by column chromatography on silica gel (60 g) using 3:3:2 (v/v) 2-propanol–ethyl acetate–water as the eluent, to yield **4** (300 mg, 90%) as a foam. Traces of impurities were removed by l.c. [30 cm × 7.8 mm (i.d.) stainless-steel column packed with Waters Micro-Bondapack NH_2, eluent acetonitrile–water, 21 MPA, differential-refractometer detector Waters R 401]; $[\alpha]_D^{20}$ −22.9° (*c* 2.6, methanol).

Anal. Calc. for $C_{10}H_{18}O_8S \cdot 1.5\ H_2O$: C, 38.83; H, 6.79; S, 10.35. Found: C, 38.31; H, 6.92; S, 10.07.

RESULTS AND DISCUSSION

Synthesis of 4-thioxylobiose (**4**). — Recent results obtained by one of our laboratories (Grenoble) introduced stereoselective methods of access to 1,2-*cis*-, as well as 1,2-*trans*-, 1-thioglycoses by employing the concept of nucleophilic activation of a thiolate anion in an aprotic polar solvent; this approach was extended to the synthesis of 4-thiocello-[4] and 4-thiomalto-oligosaccharides[13]. Based on these results, the 4-*O*-triflyl ester (**1**) of the readily available 1,2,3-tri-*O*-benzoyl-β-L-arabinopyranose[11] was treated with the sodium salt of 1-thio-β-D-xylopyranose,

TABLE I

EFFECT OF CARBON SOURCE ON THE PRODUCTION OF D-XYLAN-DEGRADING ENZYMES[a]

Carbon source	*Cell dry-weight (g/L)*	*Extracellular protein (mg/mL)*	*D-Xylanase activity (U/mL)*	*β-D-Xylosidase activity (mU/mL)*	*α-L-Arabino-furanosidase activity (mU/mL)*
L-Arabino-D-xylan	12,0	0,34	104	215	357
D-Xylan	10,7	0,10	65	80	48
Cellulose	13,7	0,11	14	50	68
Soluble starch	10,5	0,19	12	16	77
L-Arabino-D-galactan	2,4	0,11	5	12	173
Cellobiose	8,4	0,14	4	13	68
Sucrose	1,7	0,08	4	12	36
D-Xylose	6,0	0,19	4	23	107
Xylitol	8,9	0,12	3	13	186
D-Glucose	8,2	0,16	5	12	60
No addition	1,6	0,07	0	0	26

[a] *Trichoderma lignorum* was grown in a rotary shaker at 29° in the medium described in the Experimental section. Enzyme activities, cell dry-weight, and extracellular protein were measured after 72 h.

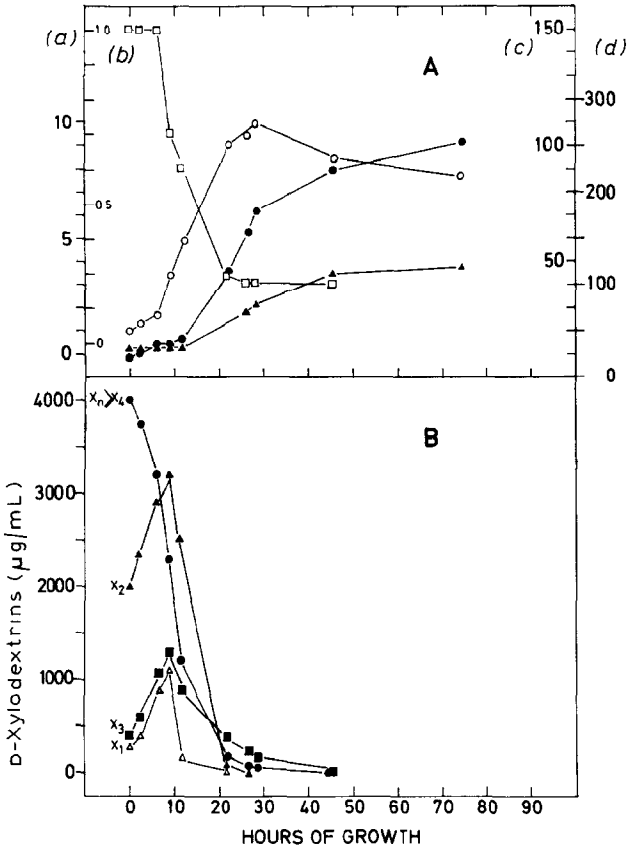

Fig. 1. (A) Relationship between extracellular D-xylanase activity [(*c*), U/mL, —●—] and β-D-xylosidase activity [(*d*), mU/mL, —▲—] production, growth of organism [cell dry-weight: (*a*), g/L of culture, —○—), and relative turbidity of the D-xylan suspension [(*b*), —□—]; and (B) formation and uptake of D-xylose and D-xylooligosaccharide in *T. lignorum* (12-L batch fermenter). The uptake of liberated D-xylose and D-xylooligosaccharides was followed by l.c.; X_1, X_2, X_3, etc. represent D-xylose, xylobiose, xylotriose, etc. The samples for l.c. were taken out at specified times indicated by the symbols.

which had been obtained by the action of sodium methoxide in methanol on 2,3,4-tri-*O*-acetyl-1-*S*-acetyl-1-thio-β-D-xylopyranose[12] (**2**). The condensation gave, after *O*-acetylation of the product for convenient isolation, a 60% yield of the acylated 4-thiodisaccharide **3**. Its ^{1}H-n.m.r. spectrum showed distinctly the signals for both D-xylopyranosyl constituents in the $^4C_1$ conformation. 4-Thioxylobiose (**4**) was obtained from **3** by conventional *O*-deacylation.

Induced production of enzymes. — D-Xylan-degrading enzymes of *T. lignorum* can be induced when the microorganism is grown on various carbohydrates as carbon sources (Table I). However, by far the most effective inducers were L-arabino-D-xylan and xylan. Enzyme excretion starts during the first half of the logarithmic phase of growth (Fig. 1A), when the polysaccharide degradation is almost complete (Fig. 1B).

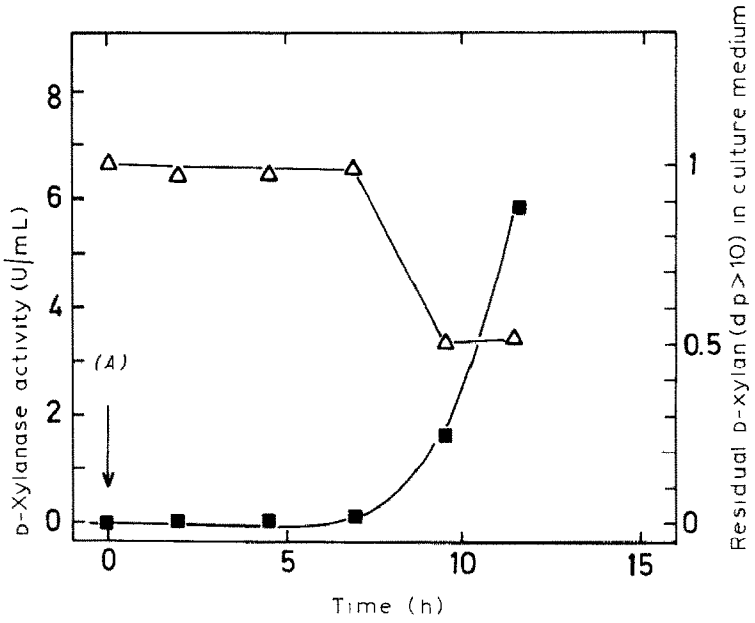

Fig. 2. Induction of D-xylanase by the degradation products of L-arabino-D-xylan: D-Xylanase activity (—■—), residual L-arabino-D-xylan in the culture medium (—△—). (A) Addition of 2 mg/mL of L-*arabino*-D-xylan.

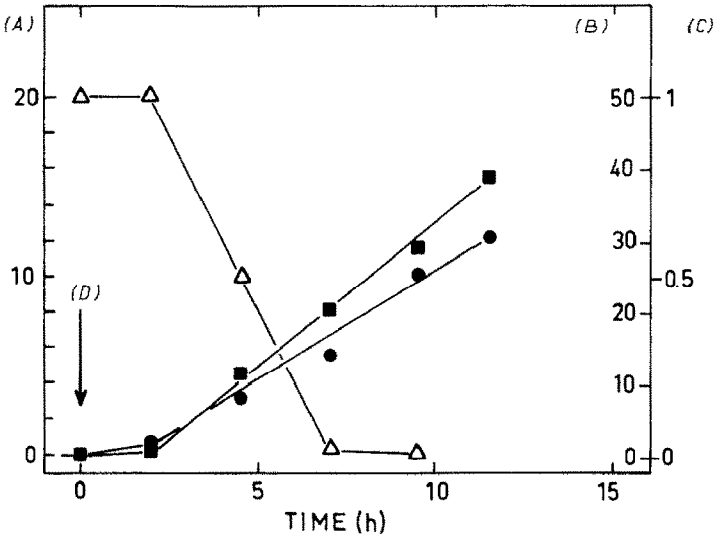

Fig. 3. Induction of D-xylanase and D-xylosidase by xylobiose: (A) D-Xylanase activity (U/mL, —■—), (B) β-D-xylosidase activity (mU/mL, —●—), (C) residual xylobiose in the culture medium (—△), and (D) addition of 4mM xylobiose.

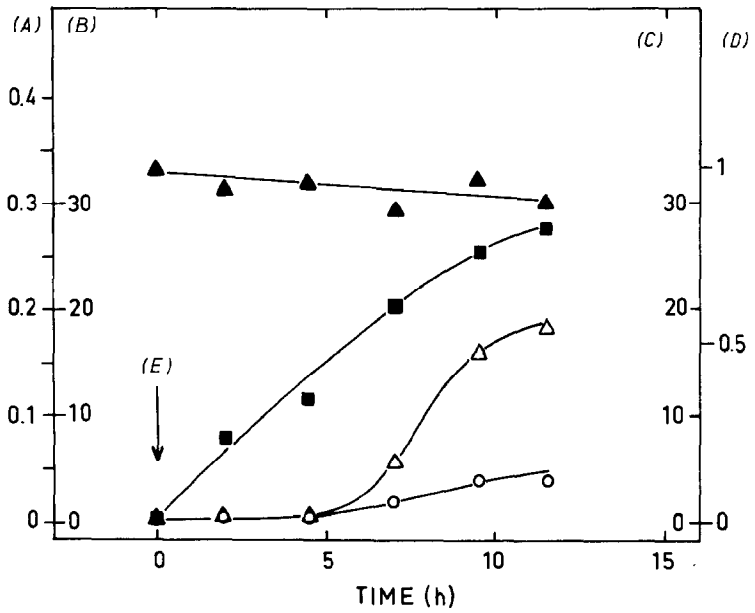

Fig. 4. Induction of D-xylanase, β-D-xylosidase, and α-L-arabinofuranosidase by 4-thioxylobiose (**4**): (A) D-Xylanase activity (U/mL, —■—), (B) β-D-xylosidase activity (mU/mL, —○—), (C) α-L-arabinofuranosidase activity (mU/mL, —△—), (D) residual 4-thioxylobiose (**4**) in the culture medium (—▲—), and (E) addition of 2mM **4**.

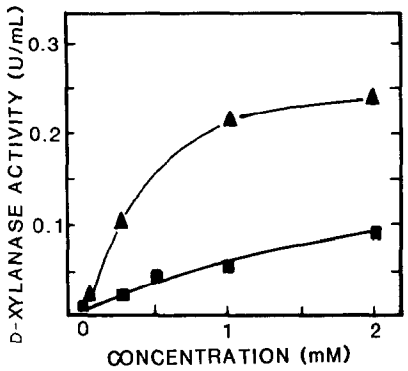

Fig. 5. Comparative D-xylanase production as an effect of inducer concentration with xylobiose (—▲—) and 4-thioxylobiose (**4**) (—■—). D-Xylanase activity was measured 6 h after the addition of inducers.

In order to characterize more accurately the induction spectrum of this strain and its kinetics, and ascertain possible regulatory mechanisms, *T. lignorum* was grown on a mineral medium with glycerol as the carbon source. When arabinoxylan was added to the culture medium in the log phase of growth of the organism, D-xylanase excretion started with a 7-h lag phase (Fig. 2); this lag phase was reduced to 2 h with xylobiose (Fig. 3). In both cases, xylanase excretion started with simultaneous decrease of the inducer concentration. These results indicate that the in-

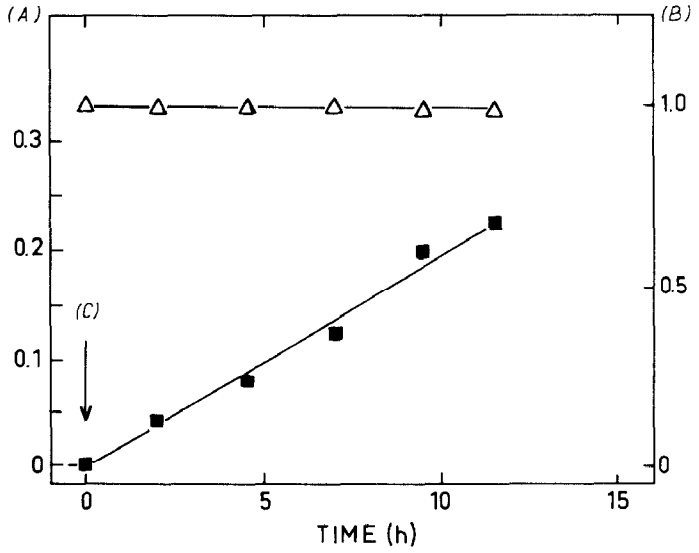

Fig. 6. Induction of D-xylanase by methyl β-D-xylopyranoside: (A) D-Xylanase activity (U/mL, —■—), (B) residual methyl β-D-xylopyranoside in the culture medium (—△—), and (C) addition of 4mM β-D-xylopyranoside.

ducing effect of arabinoxylan was caused by degradation products of that glycan and they may suggest, furthermore, that a β-D-xyloside-transport system is activated, as described for the yeast *C. albidus*[14].

In contrast with these observations, 4-thioxylobiose (**4**), a close substrate analog whose structural modification lies in the presence of an interglycosidic sulfur linkage expected to provide an enhanced stability to hydrolysis, elicited an immediate response of D-xylanase synthesis without being appreciably consumed by the cells (Fig. 4). β-D-Xylosidase and L-arabinofuranosidase activities were also induced, but with a 6-h lag phase. With increasing amounts of **4**, a nearly linear increase in xylanase activity was found, whereas for xylobiose a different curve was obtained (Fig. 5). An increase in xylanase activity was observed, up to a concentration of 1mM of the latter disaccharide in the culture medium, but concentrations higher than 1mM had no further significant effect on enzyme induction, which is probably attributable to a catabolite retro-inhibition process[3].

The xylanase system in *T. lignorum* was also induced by methyl β-D-xylopyranoside, which was apparently not metabolized (Fig. 6), and the extent of induction was similar to that obtained with **4**. Such inducing ability had previously been demonstrated for methyl β-D-xylopyranoside with *C. albidus*[3] and a streptomyces strain[2]. In the latter example, the rate of xylanase synthesis was found to be dependent on the concentration of the inducer in the culture medium[15], and it was suggested that the inducer may enter the cell by a diffusion process[16]. The same explanation may be proposed for the action of **4** in view of the similarity in induction pattern between this inducer and methyl β-D-xylopyranoside (Figs. 4 and 6).

TABLE II

COMPARATIVE EFFECTS OF XYLOBIOSE AND 4-THIOXYLOBIOSE (**4**) ON THE PRODUCTION OF D-XYLAN-DEGRADING ENZYMES AFTER 72 h OF CULTURE

Inducers	*Cell dry-weight (g/L)*	*Extracellular protein (mg/mL)*	D-*Xylanase activity (U/mL)*	D-*Xylosidase activity (mU/mL)*	α-L-*Arabino-furanosidase activity (mU/mL)*
Xylobiose (0.5%)	7.2	0.18	21	180	42
4-Thioxylobiose (**4**)[a]	4.7	0.09	8	27	60

[a]In 2mM concentration and 0.5% glycerol added as carbon source.

However, the possibility that xylobiose may not be the actual natural inducer for xylanases has to be considered in view of the considerably lower inductive effect of **4** as compared to this disaccharide (Table II). Such a molecule may be subjected to *trans*-glycosidation during transport across the cell membrane, leading to alternative interglycosidic linkages. This has been demonstrated for the induction of β-D-galactosidase in *E. coli* where *trans*-glycosidation oligosaccharides arising from the action of β-D-galactosidase on lactose were found to be the actual inducers of the lac operon[17]. A similar hypothesis was provided[18] in order to explain the inducing ability of sophorose for cellulases in *Trichoderma* sp. As *trans*-glycosidation processes would not be expected to occur with **4**, the lower induction properties of this substrate analog for xylanase might thus be explained. However, other positional isomers of **4** will have to be synthetized, and tested as inducers, in order to confirm this proposition.

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