



**This is an by copyright after embargo allowed publisher's PDF of an article
published in**

Rapp, P., Wagner, F.

**Production and properties of xylan-degrading enzymes
from Cellulomonas uda**

**(1986) Applied and Environmental Microbiology, 51 (4),
pp. 746-752.**

Production and Properties of Xylan-Degrading Enzymes from *Cellulomonas uda*

PETER RAPP¹* AND FRITZ WAGNER²

Gesellschaft für Biotechnologische Forschung mbH,¹ and Institut für Biochemie und Biotechnologie der Universität Braunschweig,² D-3300 Brunswick, Federal Republic of Germany

Received 15 October 1985/Accepted 30 December 1985

Xylan degradation and production of β -xylanase and β -xylosidase activities were studied in cultures of *Cellulomonas uda* grown on purified xylan from birchwood. β -Xylanase activity was found to be associated with the cells, although in various degrees. The formation of β -xylanase activity was induced by xylotri-ose and repressed by xylose. β -Xylosidase activity was cell bound. Both constitutive and inducible β -xylosidase activities were suggested. β -Xylanase and β -xylosidase activities were inhibited competitively by xylose. β -Xylanase activity had a pronounced optimum pH of 5.8, whereas the optimum pH of β -xylosidase activity ranged from 5.4 to 6.1. The major products of xylan degradation by a crude preparation of β -xylanase activity, in decreasing order of amount, were xylobiose, xylotri-ose, xylose, and small amounts of xylo-tetra-ose. This pattern suggests that β -xylanase activity secreted by *C. uda* is of the endosplitting type. Supernatants of cultures grown on cellulose showed not only β -glucanase but also β -xylanase activity. The latter could be attributed to an endo-1,4- β -glucanase activity which had a low β -xylanase activity.

Xylans are constituents of many plant cell walls. They are composed of a backbone of 1,4- β -linked anhydro-D-xylose units, variously substituted by L-arabinose and 4-O-methyl-D-glucuronic acid (1, 5, 8, 12). The degree of polymerization ranges from 50 to 200. Xylans can be hydrolyzed by β -xylanase (endo-1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) and by β -xylosidase (exo-1,4- β -D-xylan xylohydrolase; EC 3.2.1.37). Investigations of xylan degradation and the enzymes involved have mainly focused on fungi (9-11, 17, 18, 21, 23, 33, 44; M. Meagher, B. Y. Tao, J. M. Chow, and P. J. Reilly, Abstr. VII Int. Biotechnol. Symp. 1984, vol. 2, p. 622-623). Among hemicellulose-degrading bacteria, data have been reported for the genera *Bacillus* (12-15, 19, 25, 26, 32, 34, 35, 45, 47, 48, 51, 52, 54), *Ruminococcus* (3, 8, 37, 38), *Streptomyces* (29-31), *Bacteroides* (20, 46), and *Cellulomonas* (6, 16, 36, 41). In this paper we describe the xylan degradation and the production, localization, regulation, and several other properties of β -xylanase and β -xylosidase activities of *Cellulomonas uda*.

MATERIALS AND METHODS

Chemicals. Xylan (stated origin, birchwood; average molecular weight, 25,000) was purchased from Roth (Karlsruhe, Federal Republic of Germany). It contained approximately 0.5% L-arabinose and glucose as well as small traces of glucuronic acid residues. Sodium carboxymethyl xylan was synthesized by the procedure used for preparation of sodium carboxymethyl cellulose (2). Microcrystalline cellulose (no. 2331) was from E. Merck AG (Darmstadt, Federal Republic of Germany). Bio-Gel P-2 (400 mesh) was supplied by Bio-Rad Laboratories (Munich, Federal Republic of Germany). *p*-Hydroxymercuribenzoate and ethyl mercu-rithiosalicylate were purchased from Serva (Heidelberg, Federal Republic of Germany).

Xylobiose, xylotri-ose, and xylo-tetra-ose were produced by enzymatic hydrolysis of birchwood xylan by using crude

β -xylanase activity-containing preparations (culture super-natants) of *C. uda*. The reaction was performed at 30°C for 3 h and was stopped by heating (5 min at 95°C). After centrifugation, the resultant supernatant was concentrated under reduced pressure and chromatographed on a Bio-Gel P-2 column (1.8 by 185 cm). It was eluted with water at a flow rate of 24 ml/h. Carbohydrates in the effluent were detected and quantitatively measured by the 3,5-dinitrosalicylic acid procedure (4). Fractions still consisting of two or three sugars were separated by preparative thin-layer chromatography.

Microorganism growth conditions. After growth of *C. uda* on an agar slant (50), the bacteria were washed off into 500-ml shake flasks containing 100 ml of basal medium (50) with 0.5% (wt/vol) xylan or another carbohydrate and cultured for 24 h on a rotary shaker (100 rpm) at 30°C. A total of 5 ml of this first subculture was used to inoculate 250 ml of medium (described above) and the mixture was cultivated by shaking as before. In shake-flask cultures, the pH value was adjusted daily to 7.0 by adding 1 N sterile NaOH. For 15-liter batch cultivations, 250 ml of the second subculture, grown as described above for 36 h with 1% (wt/vol) xylan, was inoculated into 14.75 liters of basal medium (50) supplemented with 2% (wt/vol) xylan. Cultivations were performed in a 20-liter bioreactor (Biola-fitte, Poissy, France) equipped with three turbine impellers (each with six flat blades). The cultures were agitated at 300 rpm and aerated at 0.8 m³/h. The pH value was automatically adjusted to 6.8 by titration with 6.25% (vol/vol) NH₄OH. *C. uda* was grown in basal medium with 1% (wt/vol) microcrystalline cellulose as the carbon source as described previously (50).

Thin-layer chromatography. Thin-layer chromatography was performed on Merck Silica Gel 60 plates (2 and 0.5 mm thick) with the solvent system ethyl acetate-propan-2-ol-water (20:13:4, vol/vol/vol). Sugars were detected by spraying with aniline phthalate reagent.

Analysis. Bacterial growth was monitored by determining the nitrogen content of the cells by Kjeldahl analysis. Bacterial protein was calculated as N \times 6.25, and bacterial

* Corresponding author.

dry weight was estimated by assuming an average protein content of bacterial cells of 60%. For soluble carbon sources, cell growth was determined by measuring the optical density at 546 nm (OD_{546}).

Soluble protein was measured by the method of Lowry et al. (27). A standard curve was prepared from determinations with bovine serum albumin. Concentration of insoluble xylan was calculated by using the difference between the dry weight of the solids, made up of cells and insoluble xylan, and the amount of bacterial dry weight. Dry weight of the solids was determined as described previously (50). Concentration of soluble xylan was calculated from the difference between the dry weight of the polymers, which were precipitated with 3 volumes of ethanol, and the concentration of soluble protein in the supernatant of the culture. Composition of xylan was analyzed by acid hydrolysis (42) and subsequent thin-layer chromatography, as well as gas-liquid chromatography (43). Determination of cellulose was carried out as described previously (50).

Enzyme assays. The xylan solution used in the β -xylanase assay was prepared by suspending 1% (wt/vol) ball-milled xylan in 0.07 M phosphate buffer (pH 5.8). About 0.7% (wt/vol) of the xylan was dissolved after heating to 80°C for approximately 15 min, and then cooling and centrifuging. For the determination of β -xylanase activity, 1 ml of 0.7% (wt/vol) xylan dissolved in 0.07 M phosphate buffer (pH 5.8) and 1 ml of enzyme solution were mixed and incubated at 50°C for 5 min. The reaction was stopped in an ice bath, 3,5-dinitrosalicylic acid reagent (4) was added, and the solution was well mixed and then heated in a boiling water bath for 12 min. As a control, 1 ml of xylan solution was incubated and cooled, and 1 ml of enzyme solution and 3 ml of 3,5-dinitrosalicylic acid reagent were added to correct for reducing sugars in the substrate and enzyme solution. Reducing sugar equivalents were measured in both the original and the control solutions by the colorimetric method of Miller et al. (28) with D-xylose as the standard. One unit of β -xylanase activity was defined as the amount of enzyme that produced 1 μ mol of reducing sugar per min under the given conditions. β -Xylanase activity of the culture broth was assayed in the same way as described above, except that culture broth, instead of enzyme solution, was used. The activity of β -xylanase adsorbed on insoluble xylan and associated with the cells was then calculated from the difference between the activity measured in the culture broth and the activity determined in the supernatant of the culture. For measurement of cell-associated β -xylanase activity of cultures grown on nonxylan substrates, cells harvested from 250-ml shake-flask cultures were washed with 0.07 M phosphate buffer (pH 5.8) and suspended in 10 ml of the buffer. β -Xylanase activity of this cell suspension was assayed as described above. Cell-associated β -xylanase activity of cultures grown on xylan was determined by a procedure modified from that described above. Cells were grown in basal medium (250 ml per 1-liter shake flask) with 1% (wt/vol) glucose for 24 h, harvested aseptically by centrifugation, and washed with 0.07 M phosphate buffer (pH 5.8). Cells, free of glucose, were aseptically suspended in basal medium without yeast extract but with 0.06% dissolved xylan (250 ml in each of two 1-liter shake flasks). Before suspending the cells, the xylan-containing medium was centrifuged again at 12,000 $\times g$ for 1 h to ensure that no insoluble xylan was left in the culture medium. Cell suspensions were incubated on a rotary shaker (100 rpm) at 30°C. Samples (250 ml) were taken at intervals and centrifuged. The pellet was washed with 0.07 M phosphate buffer (pH 5.8) and suspended in 30 ml of the

buffer. β -Xylanase activity of the cell suspension was measured as described above.

The decrease in viscosity of sodium carboxymethyl xylan dissolved in 0.07 M phosphate buffer (pH 5.8) was monitored by using a Rotovisco RV3 viscometer with an MK 50 measuring head and an MV II/MV St rotor/stator system (Haake, Karlsruhe, Federal Republic of Germany).

β -Xylosidase activity was determined with *p*-nitrophenyl- β -D-xylopyranoside as the substrate. The reaction mixture contained 0.5 ml of 20 mM *p*-nitrophenyl- β -D-xyloside in 0.07 M phosphate buffer (pH 6.0) and 0.5 ml of either enzyme solution or culture broth. After incubation at 45°C for 10 min, 1 ml of 1 M sodium carbonate solution was added, and the mixture was chilled to about 4°C and centrifuged. The subsequent procedure and calculation of the enzyme activity were the same as those used for the determination of β -glucosidase activity as described previously (50).

Ultrasonic disruption of the cells. Cells grown for 24 h in basal medium with 1% (wt/vol) glucose were harvested by centrifugation, washed with 0.07 M phosphate buffer (pH 5.8), and suspended in the same buffer to an OD_{546} of 0.6 (1:50 [vol/vol]; diluted with water). The suspension was ultrasonically disrupted for 45 min by cooling in an ice bath with a Sonifier (B-30; Branson Sonic Power Co., Danbury, Conn.). The 0.5-in. (1.27 cm) horn was used, and the output control was set at 10 and pulsed for 20 min with a duty cycle of 40% and then pulsed for 25 min with a duty cycle of 90%. Samples were examined by microscope to confirm that most of the cells had been disrupted. For measurement of β -xylanase and β -xylosidase activities released after sonication, the sonicated suspension was centrifuged and the supernatant was removed. For the determination of both enzyme activities still adhering to the cell debris, the pellet obtained was suspended in 0.07 M phosphate buffer (pH 5.8) (the same amount that was used to suspend the cells before sonication).

Induction and repression of β -xylanase formation. Cells were grown in basal medium (250 ml in each of two 1-liter shake flasks) with 2% (wt/vol) glucose for 34 h, harvested aseptically by centrifugation, and washed twice with 0.85% (wt/vol) NaCl solution. The cells were then aseptically suspended in basal medium without yeast extract and with 0.1% (wt/vol) xylan. The mono-, di-, and trisaccharides were dissolved in small amounts of basal medium (also without yeast extract), sterilized by membrane filtration, and aseptically transferred into 100-ml shake flasks containing 50 ml of the cell suspension. They were incubated on a rotary shaker at 30°C. Samples (6 ml) were taken at intervals and centrifuged, and the β -xylanase activity in the supernatant was determined.

RESULTS

Xylan degradation and production and localization of β -xylanase activity. Growth and xylan degradation during a 15-liter batch cultivation on 2% (wt/vol) purified xylan from birchwood were measured (Fig. 1). After 80 h of cultivation, a bacterial dry weight of 8.4 g/liter was reached, and the xylan content was reduced by 82%. This corresponded to a growth yield of 0.45 g of bacterial dry weight per g of xylan consumed. At the beginning of cultivation, 63% of the total amount of xylan was found to be dissolved in the basal medium. Within the first 12 h, when the xylanase activity was still low (Fig. 2), 90% of this soluble portion was hydrolyzed. After about 35 h no soluble xylan could be

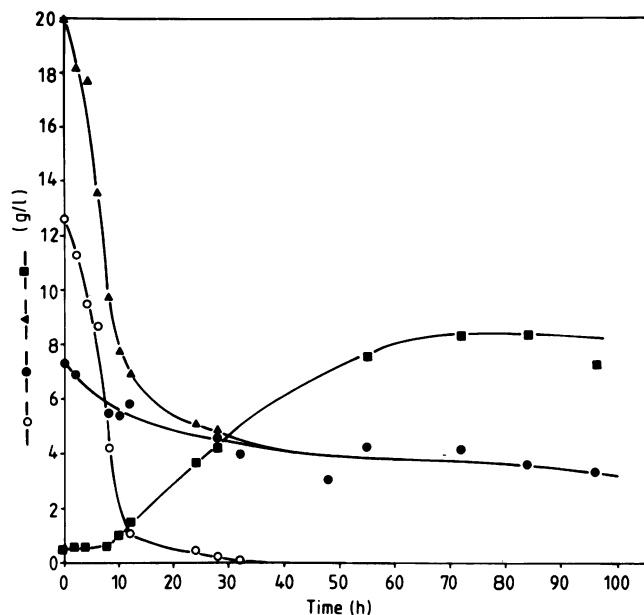


FIG. 1. Growth and xylan degradation during a 15-liter batch cultivation in basal medium with 2% (wt/vol) xylan as the carbon source. Cultivation was carried out at 30°C and pH 6.8 in a 20-liter bioreactor. It was aerated at 0.8 m³/h and agitated with three turbine impellers (six flat blades each) at 300 rpm. Symbols: ▲, total xylan; ○, soluble xylan; ●, insoluble xylan; ■, bacterial dry weight.

detected in the culture, whereas within the same period only 43% of the insoluble xylan was degraded.

During growth on mono- and disaccharides (Table 1), very low β -xylanase activity was produced. After 24 h of cultivation in shake flasks, β -xylanase activities of approximately 0.01 U/ml (extracellular) and 0.004 U/ml (cell associated)

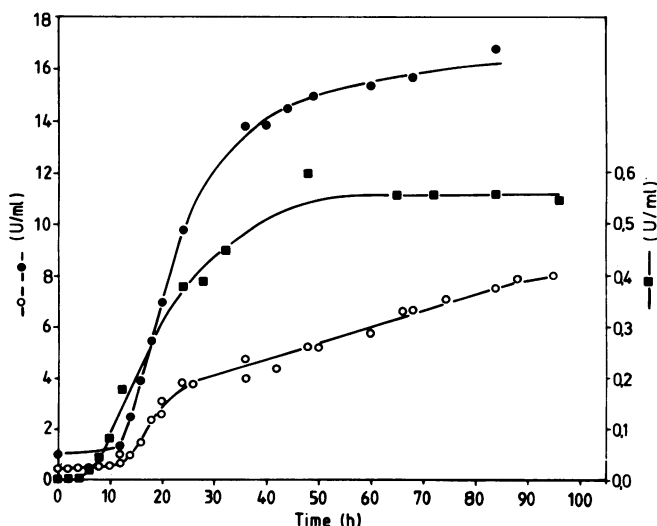


FIG. 2. Formation of β -xylanase and β -xylosidase during a 15-liter batch cultivation in basal medium with 2% (wt/vol) xylan as the carbon source. Cultivation conditions are described in the legend to Fig. 1. Symbols: ●, β -xylanase activity of the culture broth; ○, β -xylanase activity of the supernatant; ■, cell-bound β -xylosidase activity.

TABLE 1. Formation of β -xylosidase activity

Carbon source (1% [wt/vol]) ^a	Growth (OD ₅₄₆)	Cell-bound β -xylosidase activity (U/ml)
D-Glucose	10.0	0.066
D-Galactose	16.9	0.046
D-Mannose	12.0	0.035
D-Xylose	10.0	0.065
L-Arabinose	10.0	0.061
Cellobiose	12.7	0.080
Maltose	15.0	0.049
Starch	14.2	0.037
Cellulose	4.63 ^b	0.070
Xylan	3.43 ^b	0.210

^a Cultivation was carried out in shake flasks (250 ml per 1-liter flask) for 60 h or (for cellulose and xylan) for 120 h.

^b Grams of bacterial dry weight per liter.

were detected. The difference between the β -xylanase activity measured in the culture broth and that determined in the culture supernatant varied from 50 to 67.5% during 90 h of a 15-liter batch cultivation on 2% (wt/vol) xylan (Fig. 2). The adsorption of β -xylanase activity to insoluble xylan made it difficult to determine the cell-associated β -xylanase activity. Therefore, cells grown for 24 h on 1% (wt/vol) glucose were incubated for 17 h in basal medium with 0.06% (wt/vol) dissolved xylan. The percentage of β -xylanase activity associated with the cells varied between 1% (initially) and 45% (after 17 h of incubation) under these conditions (Fig. 3). When this incubation was extended to 66 h, the portion of cell-associated β -xylanase activity declined to 15 to 20% of the total β -xylanase activity. The latter value was never exceeded during incubation of glucose-grown cells for 33 h on 0.025% (wt/vol) xylan. To determine whether the β -xylanase activity associated with the cells was located on the cell surface, glucose-grown cells incubated for 6.5 h in basal medium with 0.06% (wt/vol) xylan were disrupted by sonifi-

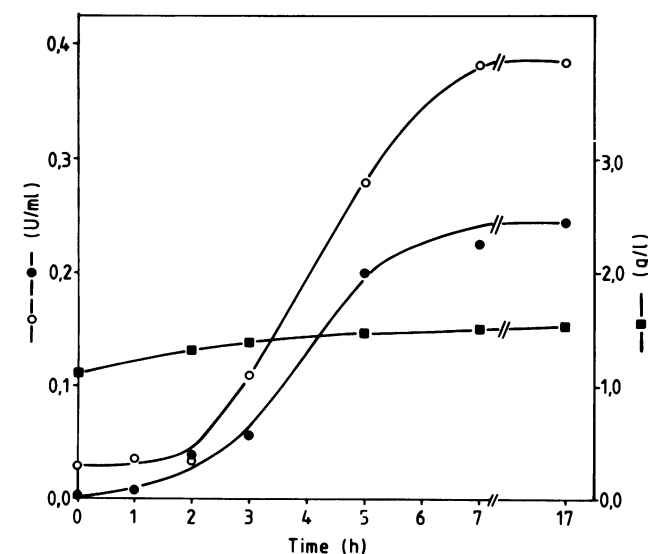


FIG. 3. Formation of extracellular and cell-associated β -xylanase activity by washed glucose-grown cells transferred to basal medium with 0.06% (wt/vol) dissolved xylan. Symbols: ○, extracellular β -xylanase activity; ●, cell-associated β -xylanase activity; ■, bacterial dry weight.

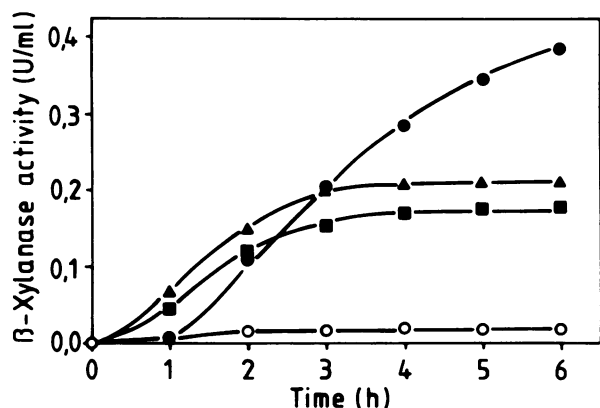


FIG. 4. Formation of extracellular β -xylanase activity by washed glucose-grown cells transferred to basal medium and supplied with 0.5 mM xylotriose (■), 1.0 mM xylotriose (▲), 0.1% (wt/vol) xylan (●), and no addition (○). OD_{546} of the cells suspended in basal medium was 0.12.

cation. The suspension of intact bacteria had a β -xylanase activity of 0.305 U/ml. After sonification, an activity of 0.545 U/ml was measured. The supernatant of this sonicated cell suspension exhibited a β -xylanase activity of 0.375 U/ml, and the cell debris, suspended in the same amount of buffer as were the cells before sonification, showed a β -xylanase activity of 0.15 U/ml. β -Xylosidase activity, which was also present in the disrupted cell preparation, probably caused the level of β -xylanase activity to appear higher than it actually was.

Formation and localization of β -xylosidase activity. β -Xylosidase activity was always found to be cell bound, regardless of the carbon source used (Table 1 and Fig. 2). No β -xylosidase activity could be measured in the supernatants of cultures grown on xylan or on the other carbohydrates listed in Table 1. The amount of β -xylosidase activity bound to the cell increased about fourfold, from 0.14 and 0.15 U (when grown on glucose or microcrystalline cellulose, respectively) to 0.61 U of β -xylosidase activity per mg of bacterial dry weight (when the bacterium was grown on xylan). Sonication of glucose-grown cells incubated for 6.5 h in basal medium with 0.06% (wt/vol) xylan led to a release of 76% of the β -xylosidase activity originally bound to the cells. Finally, we do not know whether this enzyme activity cleaved xylobiose, as it hydrolyzed the artificial substrate *p*-nitrophenyl- β -D-xyloside.

Induction and repression of β -xylanase formation. During growth on nonxylan substrates, very small amounts of β -xylanase activity were measured (Table 1). These small amounts represented the basal level necessary for the formation in the cells of a low-molecular-weight compound from the exogenous substrate xylan. To determine the small xylan fragment responsible for induction of further β -xylanase synthesis, washed, glucose-grown cells with repressed β -xylanase formation were incubated in basal medium with potentially inducing compounds such as xylose, xylobiose, and xylotriose. The formation of extracellular β -xylanase activity was used as a parameter of induction and repression. Xylotriose showed a distinct inducing effect at concentrations of 0.5 and 1.0 mM (Fig. 4). The extracellular β -xylanase formation stopped after 3 to 4 h because of the exhaustion of the inducing xylotriose. Although xylobiose, ranging in concentration from 0.1 to 1.0 mM, was taken up by the cells, it barely induced the formation of extracellular

β -xylanase activity. On the other hand, 1.0 and 5.0 mM xylose and glucose, respectively, distinctly repressed this enzyme formation.

Inhibition of β -xylanase and β -xylosidase activities. Initial reaction velocities of xylan degradation by supernatants of cultures grown on xylan were determined with and without the addition of xylose, xylobiose, glucose, and cellobiose. The β -xylanase activity was inhibited competitively only by xylose, with a K_i value of 50 mM. For kinetic studies of the cell-bound β -xylosidase activity, the initial rates of hydrolysis of *p*-nitrophenyl- β -D-xyloside by suspensions of washed, glucose-grown cells were measured with and without the addition of xylose, glucose, and cellobiose. The β -xylosidase activity was inhibited competitively by xylose, with a K_i of 650 mM. The influence of sulfhydryl reagents and EDTA on the activities of β -xylanase and β -xylosidase was also studied. The addition of 1 mM *p*-hydroxymercuribenzoate reduced the β -xylosidase activity by 26%. More pronounced was the influence of 1 mM ethyl mercurithiosalicylate, which inhibited about 80% of the β -xylosidase activity. On the other hand, the β -xylanase activity was not affected by the two sulfhydryl reagents.

Effect of pH and temperature on β -xylanase and β -xylosidase activities. The optimum pH value of 5.8 for β -xylanase activity was rather pronounced when compared with that for β -xylosidase activity, which ranged from 5.4 to 6.1. Maximum β -xylanase activity was determined at 58°C. β -Xylosidase activity was highest at 43 to 45°C. The thermostability of β -xylanase activity was determined by incubating a β -xylanase-activity-containing culture supernatant at various temperatures. After incubation at 45 and 50°C for 1 h, 5 and 24%, respectively, of the enzyme activity was lost. Half-lives of 60 min at 55°C and 10 min at 60°C were measured. For determination of the thermostability of cell-bound β -xylosidase activity, cells were harvested aseptically, suspended in sterile 0.07 M phosphate buffer (pH 6.0), and incubated at different temperatures. When they were incubated at 40°C for 1 h, β -xylosidase activity was reduced by 10%. At 50 and 60°C, half-lives of 6 and 2 min, respectively, were measured.

Substrate specificity of β -xylanase activity and pattern of xylan hydrolysis. Xylan from birchwood, used throughout this study, was almost devoid of constituents other than xylose. Only L-arabinose and glucose (about 0.5%), as well as traces of glucuronic acid residues, could be detected. The low concentration of L-arabinose and glucose indicated that the xylan that was used contained a small amount of L-arabinofuranose side chains and probably small amounts of cellulose or another type of a β -linked glucan as well. β -Xylanase-activity-containing supernatants of xylan-grown cultures degraded not only this purified xylan but also another lot of birchwood xylan which contained approximately 12% L-arabinose and about 2% glucose. Both sugars were detected as hydrolysis products of this arabinoxylan by β -xylanase-containing culture supernatants. These preparations were also slightly active against sodium carboxymethyl cellulose, but were unable to degrade microcrystalline cellulose. Chromatographic product analysis of xylan hydrolysis for 3 h at 30°C by a β -xylanase-activity-containing culture supernatant which was free of β -xylosidase activity showed that xylobiose, xylotriose, and xylose were mainly produced. Small amounts of xylotetraose were also detected. The oligomeric pattern of xylan hydrolysis suggested that an endo- β -xylanase activity was primarily responsible for the cleavage of xylan by *C. uda* culture fluids. This conclusion was supported by the finding that a supernatant of a culture

grown on xylan efficiently decreased the viscosity of a sodium carboxymethyl xylan solution.

1,4- β -Glucanase activity with reference to a side activity towards xylan. Supernatants of cultures grown on microcrystalline cellulose showed not only β -glucanase but also β -xylanase activity. This raised the question as to whether this xylanase activity was due to β -xylanase activity, possibly the same produced during cultivation on xylan, or to β -glucanase activity, which is also able to hydrolyze xylan to some extent. This xylan-hydrolyzing enzyme activity from cultures grown on microcrystalline cellulose had a rather broad range of optimum pH values, ranging from 6.1 to 6.8. On the other hand, the β -xylanase activity produced during growth on xylan had a pronounced optimum at pH 5.8. Both enzyme activities also differed somewhat in their temperature optima. The xylanase activity originating from cultures grown on xylan had a broader optimum range at 58°C than did microcrystalline cellulose-grown cultures, which had a well-pronounced optimum at 60°C. Furthermore, both enzyme activities were inhibited by xylose. The xylan-hydrolyzing enzyme activity from cultures grown on microcrystalline cellulose was inhibited noncompetitively, with a K_i value of 72 mM, and the true β -xylanase activity was inhibited competitively, with a K_i value of 50 mM. Finally, the true β -xylanase activity was not inhibited by cellobiose, xylobiose, or glucose, whereas the xylan-hydrolyzing activity determined in cultures grown on microcrystalline cellulose was inhibited by cellobiose. All these results suggest that the xylan-hydrolyzing enzyme activity, measured in cultures grown on cellulose, is different from the xylanase activity produced during cultivation on xylan. In a previous study (50), it was reported that endo-1,4- β -glucanase activity had an optimum range from pH 6.2 to 6.8, thus corresponding to that of xylan-hydrolyzing enzyme activity determined in cultures grown on microcrystalline cellulose. Moreover, this endoglucanase activity was also found to be inhibited by cellobiose, similar to xylan-hydrolyzing enzyme activity. This comparison led to the conclusion that endo-1,4- β -glucanase activity also had some activity towards xylan.

DISCUSSION

During a 15-liter batch cultivation of *C. uda*, 84% of the 2% (wt/vol) xylan initially present in the culture was degraded within 80 h of incubation. Besides the dissolved xylan, which made up 63% of the total initial amount, about half of the insoluble portion was hydrolyzed. The incomplete degradation of the insoluble xylan was probably not due to 4-*O*-methyl-D-glucuronic acid branches, since they could be detected only in traces in the xylan used. Although a crude β -xylanase preparation from *C. uda* was capable of liberating L-arabinose from arabinoxylan, 1,3- α -arabinofuranosyl branches probably impeded the xylan hydrolysis to some extent. Daly et al. (6) found that the removal of L-arabinose side chains from an arabinoxylan by a *Cellulomonas* strain was a relatively slow process, compared with the cleavage of the xylan backbone. On the other hand, it also seems likely that the residual xylan was more tightly bound in aggregates than the xylan that was utilized, and was thus less accessible to the hydrolyzing enzymes (46).

The data describing the association of the β -xylanase activity with the cells are by no means extensive enough to permit a final conclusion, but they do demonstrate that the extent of association of β -xylanase activity with the cells depends on both their age and the conditions under which the cells were cultivated. Growing cells seem to associate

more readily with β -xylanase activity than do those that are in a nongrowth stage. This changing degree of association prevents the β -xylanase activity of *C. uda* from being regarded as a true surface-bound enzyme activity (39), in contrast to the β -xylosidase activity, which was never detected in the culture supernatant. It seems more likely that the cell-associated β -xylanase activity was an enzyme activity en route to the exterior, as has been suggested for the α -amylase of *Bacillus* spp. (40).

Although a varying amount of β -xylanase activity was found to be associated with the cells, extracellular β -xylanase activity was always present to some degree. Therefore, the appearance of this extracellular β -xylanase activity could be used as at least a qualitative measure for the inducible formation of β -xylanase activity by small xylan fragments. These fragments are produced by the action of small amounts of constitutively formed β -xylanase activity. However, these sugars are probably degraded or transformed either extra- or intracellularly to the nonmetabolizable, gratuitous inducer of the β -xylanase synthesis. It was shown that xylotri-ose exhibited a distinct inducing effect on the production of extracellular β -xylanase activity, in contrast to xylobiose, which elicited only a very weak response (data not shown).

β -Xylosidase activity was formed during growth on many mono- and disaccharides. It was located in the periplasmic space or in the cell wall, and the activity bound to the cell was increased when cells were grown on xylan. This suggested the existence of an inducible and constitutive β -xylosidase activity. Although *p*-nitrophenyl- β -D-xyloside was a convenient substrate for assaying β -xylosidase activity, its detection was not necessarily proof of xylobiose activity. β -Xylosidase is listed in *Enzyme Nomenclature* as an exo-1,4- β -D-xylosidase (EC 3.2.1.37) which removes successive D-xylose residues from the nonreducing termini of xylan and hydrolyzes xylobiose. The β -xylosidase activity of *C. uda*, if assumed to be inducible, may fulfill these requirements, but it is questionable to assume that this is also valid for constitutive activity, which is more probably a xylosidase activity comparable to glucosidase activity (EC 3.2.1.21). Extracellular β -xylanase activity was inhibited competitively by xylose and not by xylobiose, cellobiose, and glucose. It was not affected by sulfhydryl-binding reagents. On the contrary, β -xylosidase activity responded to sulfhydryl-binding reagents, thus indicating that a cysteine residue was most probably essential for its activity.

The hydrolysis pattern of xylan during its incubation with a crude preparation of β -xylanase activity indicated that the enzyme activity mainly responsible for the xylan degradation was an endo- β -xylanase activity. The assumption was supported by the fact that this crude preparation of β -xylanase activity rapidly decreased the viscosity of a sodium carboxymethyl xylan solution. This finding was not surprising, since most bacterial xylanases are endoxylanases (13, 15, 19, 22, 35, 52, 54). The occurrence of L-arabinose as a product of hydrolysis of arabinoxylan by a supernatant of a culture grown on xylan suggests that a β -xylanase or an α -arabinofuranosidase activity cleaves the 1,3- α -L-arabinofuranosyl side chain linkages.

Numerous fungal and bacterial 1,4- β -glucanases and xylanases show some degree of cross-specificity (21, 23, 24, 36, 37, 49, 53, 54). A crude β -xylanase preparation from *C. uda* also degraded sodium carboxymethyl cellulose to some extent, but did not degrade microcrystalline cellulose. On the other hand, supernatants of cultures of *C. uda* grown on microcrystalline cellulose exhibited 1,4- β -glucanase activity

and also xylanase activity. Comparison of the physicochemical properties of the xylan-hydrolyzing enzyme activity from cultures grown on microcrystalline cellulose with those of the β -xylanase activity from xylan-grown cultures shows that their optimum pH values and inhibition characteristics obviously differ. It was shown that the pH optimum values and kinetic properties of the xylan-hydrolyzing enzyme activity from microcrystalline cellulose-grown cultures corresponded to those of an endo-1,4- β -glucanase activity (50).

ACKNOWLEDGMENT

We thank B. Gosch and U. Kiersten for their skillful technical assistance.

LITERATURE CITED

- Aspinall, G. O., and J. E. McKay. 1958. The hemicelluloses of European larch (*Larix decidua*). Part I. The constitution of a xylan. *J. Chem. Soc.* 1:1059-1066.
- Balser, K., and M. Iseringhausen. 1975. Celluloseäther, p. 192-212. In E. Bartholomé, E. Bickert, H. Hellmann, H. Ley, and W. Weigert (ed.), *Ullmanns Enzyklopädie der technischen Chemie*, vol. 9. Verlag Chemie, Weinheim, Federal Republic of Germany.
- Brice, R. E., and J. M. Morrison. 1982. The degradation of isolated hemicelluloses and lignin-hemicellulose complexes by cell-free, rumen hemicellulases. *Carbohydr. Res.* 101:93-100.
- Bruner, R. L. 1984. Determination of reducing value. 3,5-Dinitrosalicylic acid method. *Methods Carbohydr. Chem.* 4:67-71.
- Comtat, J., J.-P. Joseleau, C. Bosso, and F. Barnoud. 1974. Characterization of structurally similar neutral and acidic tetrasaccharides obtained from the enzymic hydrolyzate of a 4-O-methyl-D-glucurono-D-xylan. *Carbohydr. Res.* 38:217-224.
- Daly, J. M., M. Švejkar, and P. A. D. Rickard. 1983. The hydrolysis of xylan by *Cellulomonas* preparations. *J. Chem. Tech. Biotechnol. B Chem. Technol.* 33:216-220.
- De Gussem, R. L., G. M. Aerts, M. Claeysens, and C. K. de Bruyne. 1978. Purification and properties of an induced β -D-glucosidase from *Stachybotrys atra*. *Biochim. Biophys. Acta* 525:142-153.
- Dehority, B. A. 1973. Hemicellulose degradation by rumen bacteria. *Fed. Proc.* 32:1819-1825.
- Dekker, R. F. H. 1983. Bioconversion of hemicellulose: aspects of hemicellulase production by *Trichoderma reesei* QM 9414 and enzymic saccharification of hemicellulose. *Biotechnol. Bioeng.* 25:1127-1146.
- Dekker, R. F. H., and G. N. Richards. 1976. Hemicellulases: their occurrence, purification, properties, and mode of action. *Adv. Carbohydr. Chem. Biochem.* 32:277-352.
- Deleyn, F., M. Claeysens, J. van Beeumen, and C. K. de Bruyne. 1978. Purification and properties of β -xylosidase from *Penicillium wortmanni*. *Can. J. Biochem.* 56:43-50.
- Deschamps, A. M., J. Comtat, N. Nouvion, and J. M. Lebeault. 1982. Degradation of purified birch-wood xylan and production of xylanase by wood-decaying bacteria. *J. Gen. Appl. Microbiol.* 28:275-280.
- Esteban, R., A. Chordi, and T. G. Villa. 1983. Some aspects of a 1,4- β -D-xylanase and a β -D-xylosidase secreted by *Bacillus coagulans* strain 26. *FEMS Microbiol. Lett.* 17:163-166.
- Esteban, R., A. R. Nebreda, J. R. Villanueva, and T. G. Villa. 1984. Possible role of cAMP in the synthesis of β -glucanases and β -xylanases of *Bacillus circulans* WL-12. *FEMS Microbiol. Lett.* 23:91-94.
- Esteban, R., J. R. Villanueva, and T. G. Villa. 1982. β -D-Xylanases of *Bacillus circulans* WL-12. *Can. J. Microbiol.* 28:733-739.
- Gilkes, N. R., D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren. 1984. A mutant of *Escherichia coli* that leaks cellulase activity encoded by cloned cellulase genes from *Cellulomonas fimi*. *Bio/Technology* 2:259-263.
- Gorbacheva, L. V., and N. A. Rodionova. 1977. Studies on xylan-degrading enzymes. I. Purification and characterization of endo-1,4- β -xylanase from *Aspergillus niger* str. 14 on xylan and xylooligosaccharides. *Biochim. Biophys. Acta* 484:79-93.
- Gorbacheva, L. V., and N. A. Rodionova. 1977. Studies on xylan-degrading enzymes. II. Action pattern of endo-1,4- β -xylanase from *Aspergillus niger* str. 14 on xylan and xylooligosaccharides. *Biochim. Biophys. Acta* 484:94-102.
- Horikoshi, K., and Y. Atsukawa. 1973. Xylanase produced by alkalophilic *Bacillus* no. C-59-2. *Agric. Biol. Chem.* 37:2097-2103.
- Howard, B. H., G. Jones, and M. R. Purdom. 1960. The pentosanases of some rumen bacteria. *Biochem. J.* 74:173-180.
- Hurst, P. L., P. A. Sullivan, and M. G. Shepherd. 1978. Substrate specificity and mode of action of a cellulase from *Aspergillus niger*. *Biochem. J.* 169:389-395.
- Inaoka, M., and H. Soda. 1956. Crystalline xylanase. *Nature (London)* 178:202-203.
- John, M., B. Schmidt, and J. Schmidt. 1979. Purification and some properties of five endo-1,4- β -D-xylanases and a β -D-xylosidase produced by a strain of *Aspergillus niger*. *Can. J. Biochem.* 57:125-134.
- Kanda, T., K. Wakabayashi, and K. Nisizawa. 1976. Xylanase activity of an endo-cellulase of carboxymethyl-cellulase type from *Irpex lacteus* (Polyporus tulipiferae). *J. Biochem. (Tokyo)* 79:989-995.
- Kerstens-Hilderson, H., M. Claeysens, E. van Doorslaer, E. Saman, and C. K. de Bruyne. 1982. β -D-Xylosidase from *Bacillus pumilus*. *Methods Enzymol.* 83:631-639.
- Kerstens-Hilderson, H., F. G. Loontjens, M. Claeysens, and C. K. de Bruyne. 1969. Partial purification and properties of an induced β -D-xylosidase of *Bacillus pumilus* 12. *Eur. J. Biochem.* 7:434-441.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Miller, G. L., R. Blum, W. E. Glennon, and A. L. Burton. 1960. Measurement of carboxymethylcellulase activity. *Anal. Biochem.* 2:127-132.
- Nakanishi, K., and T. Yasui. 1980. Kinetic studies on xylanase induction by β -xyloside in *Streptomyces* sp. *Agric. Biol. Chem.* 44:1885-1889.
- Nakanishi, K., and T. Yasui. 1980. Production of xylanase by *Streptomyces* sp., using non-metabolizable inducer. *Agric. Biol. Chem.* 44:2729-2730.
- Nakanishi, K., T. Yasui, and T. Kobayashi. 1976. A preliminary experiment on the xylanase production by *Streptomyces* sp. *J. Ferment. Technol.* 54:813-817.
- Okazaki, W., T. Akiba, K. Horikoshi, and R. Akahoshi. 1984. Production and properties of two types of xylanases from alkalophilic thermophilic *Bacillus* sp. *Appl. Microbiol. Biotechnol.* 19:335-340.
- Paice, M. G., L. Jurasek, M. R. Carpenter, and L. B. Smillie. 1978. Production, characterization, and partial amino acid sequence of xylanase A from *Schizophyllum commune*. *Appl. Environ. Microbiol.* 36:802-808.
- Panbangred, W., O. Kawaguchi, T. Tomita, A. Shinmyo, and H. Okada. 1984. Isolation of two β -xylosidase genes of *Bacillus pumilus* and comparison of their gene products. *Eur. J. Biochem.* 138:267-273.
- Panbangred, W., A. Shinmyo, S. Kinoshita, and H. Okada. 1983. Purification and properties of endoxylanase produced by *Bacillus pumilus*. *Agric. Biol. Chem.* 47:957-963.
- Peiris, S. P., P. A. D. Rickard, and N. W. Dunn. 1982. Comparison of the xylanolytic and cellulolytic activities of *Cellulomonas*. *Eur. J. Appl. Microbiol. Biotechnol.* 14:169-173.
- Pettipher, G. L., and M. J. Latham. 1979. Characteristics of enzymes produced by *Ruminococcus flavefaciens* which degrade plant cell walls. *J. Gen. Microbiol.* 110:21-27.
- Pettipher, G. L., and M. J. Latham. 1979. Production of enzymes degrading plant cell walls and fermentation of cellobiose by *Ruminococcus flavefaciens* in batch and continuous culture. *J. Gen. Microbiol.* 110:29-38.

39. Pollock, M. R. 1962. Exoenzymes, p. 121-178. In I. C. Gunsalus and R. Y. Stanier (ed.), *The bacteria*, vol. 4. Academic Press, Inc., New York.
40. Priest, F. G. 1977. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol. Rev.* **41**:711-753.
41. Rajoka, M. I., and K. A. Malik. 1984. Cellulase and hemicellulase production by *Cellulomonas flavigena* NIAB 441. *Biotechnol. Lett.* **6**:597-600.
42. Rapp, P., C. H. Beck, and F. Wagner. 1979. Formation of exopolysaccharides by *Rhodococcus erythropolis* and partial characterization of a heteropolysaccharide of high molecular weight. *Eur. J. Appl. Microbiol. Biotechnol.* **7**:67-78.
43. Rapp, P., H. Bock, V. Wray, and F. Wagner. 1979. Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erythropolis* grown on *n*-alkanes. *J. Gen. Microbiol.* **115**:491-503.
44. Rodionova, N. A., I. M. Tavabilov, and A. M. Bezborodov. 1983. β -Xylosidase from *Aspergillus niger* 15: purification and properties. *J. Appl. Biochem.* **5**:300-312.
45. Roncero, M. I. G. 1983. Genes controlling xylan utilization by *Bacillus subtilis*. *J. Bacteriol.* **156**:257-263.
46. Salyers, A. A., F. Gherardini, and M. O'Brien. 1981. Utilization of xylan by two species of human colonic *Bacteroides*. *Appl. Environ. Microbiol.* **41**:1065-1068.
47. Saman, E., M. Claeysens, and C. K. de Bruyne. 1978. Study of the sulfhydryl groups of β -D-xylosidase from *Bacillus pumilus*. *Eur. J. Biochem.* **85**:301-307.
48. Sandhu, J. S., and J. F. Kennedy. 1984. Molecular cloning of *Bacillus polymyxa* 1,4- β -D-xylanase gene in *Escherichia coli*. *Enzyme Microb. Technol.* **6**:271-274.
49. Shikata, S., and K. Nisizawa. 1975. Purification and properties of an exo-cellulase component of novel type from *Trichoderma viride*. *J. Biochem. (Tokyo)* **78**:499-512.
50. Stoppok, W., P. Rapp, and F. Wagner. 1982. Formation, location, and regulation of endo-1,4- β -glucanases and β -glucosidases from *Cellulomonas uda*. *Appl. Environ. Microbiol.* **44**:44-53.
51. Takahashi, M., and Y. Hashimoto. 1963. Studies on bacterial xylanase (III). Crystallization and some enzymatic properties of bacterial xylanase. *J. Ferment. Technol.* **41**:181-186.
52. Takahashi, M., and Y. Hashimoto. 1963. Studies on bacterial xylanase (IV). On the nature of the crystalline xylanase of *Bac. subtilis* G-2. *J. Ferment. Technol.* **41**:186-189.
53. Toda, S., H. Suzuki, and K. Nisizawa. 1971. Some enzymic properties and the substrate specificities of *Trichoderma* cellulases with special reference to their activity toward xylan. *J. Ferment. Technol.* **49**:499-521.
54. Uchino, F., and T. Nakane. 1981. A thermostable xylanase from a thermophilic acidophilic *Bacillus* sp. *Agric. Biol. Chem.* **45**:1121-1127.