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Formation, Location, and Regulation of Endo-1,4-β-Glucanases and β-Glucosidases from *Cellulomonas uda*

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The formation and location of endo-1,4-β-glucanases and β-glucosidases were studied in cultures of *Cellulomonas uda* grown on microcrystalline cellulose, carboxymethyl cellulose, printed newspaper, and some mono- or disaccharides. Endo-1,4-Glucanases were found to be extracellular, but a very small amount of cell-bound endo-1,4-β-glucanase was considered to be the basal endoglucanase level of the cells. The formation of extracellular endo-1,4-β-glucanases was induced by cellobiose and repressed by glucose. Extracellular endoglucanase activity was inhibited by cellobiose but not by glucose. β-Glucosidases, on the other hand, were formed constitutively and found to be cell bound. β-Glucosidase activity was inhibited noncompetitively by glucose. Some characteristics such as the optimal pH for and the thermostability of the endoglucanases and β-glucosidases and the end products of cellulose degradation were determined.

The principal enzymes involved in cellulose degradation are the endo- and exo-1,4-β-glucanases and the β-glucosidases. The mechanism of bacterial cellulose degradation is possibly similar to that in fungi (32). Regions of low crystallinity in the cellulose fiber are attacked in a random fashion by endo-1,4-β-glucanase (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4), and free chain ends are created. Exo-1,4-β-glucanase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91) starts the degradation from the nonreducing chain ends by removing cellobiose and, to a smaller extent, glucose. Cellobiose can be hydrolyzed to glucose by the action of β-glucosidase (EC 3.2.1.21) or cellobiose phosphorylase (EC 2.4.1.20). The present paper is concerned only with the endo- and not with the exoglucanases, despite the fact that *Cellulomonas uda* rather rapidly degrades microcrystalline cellulose and printed newspaper. The reason for this is that at present no simple, exclusive method exists for measuring exo-1,4-β-glucanase activity.

The endo-1,4-β-glucanases of bacteria were found to be either exclusively cell bound (10) or extracellular (15, 26, 33) or both cell bound and extracellular (6, 31, 34, 44). The bacterial 1,4-β-glucosidases, however, were always found to be cell bound (1, 16, 17, 24).

The synthesis of 1,4-β-glucanases in most bacteria is regulated by induction and catabolite repression mechanisms like those which regulate cellulase formation in fungi. The inducers include cellulose, cellobiose, and sophorose, whereas glucose and cellobiose at higher concentrations act as catabolite repressors of 1,4-β-glucanase formation (5, 6, 8, 26, 40); but cellobiose and glucose can act also as inhibitors of 1,4-β-glucanases and β-glucosidases (10, 26, 40).

The genus *Cellulomonas* is among the best characterized cellulolytic bacteria (4, 12, 18–21, 40). In the present work, a bacterium identified as *C. uda* was found to produce 1,4-β-glucanases and β-glucosidases. The production, location, and regulation of the endo-1,4-β-glucanases and β-glucosidases were studied.

MATERIALS AND METHODS

Chemicals. All chemicals used were analytical grade. Carboxymethyl cellulose (CM-cellulose; Schleicher & Schüll, Dassel, FRG) had an exchange capacity of 1.37 mV/liter per gram. Microcrystalline cellulose (Avicel, no. 2331) was from E. Merck AG, Darmstadt, FRG; and sodium carboxymethyl cellulose (Na-CM-cellulose) type 16110 was obtained from Serva, Heidelberg, FRG, with a degree of polymerization of 500 to 520 and a molecular weight of about 100,000. Printed newspaper was obtained from Braunschweiger Zeitung, with a cellulose content of 52.7% (wt/vol) and 4.1% (wt/vol) water. The paper was cut into small pieces and ball milled to a fine powder.

Organism and growth. *C. uda* was isolated from Indian sugar cane and identified by N. Weiß, Munich. The bacterium is gram positive but decolorizes readily. The peptidoglycan is of the Orn-Gly type. The guanine plus cytosine content of the DNA is 72%. It is nonmotile and produces slime from glucose and acid from arabinose, maltose, sucrose, and trehalose but not from ribose. It does not reduce nitrate to nitrite.

The bacterium was grown at 30°C in basal medium consisting of (per liter of deionized water): K_2HPO_4, 4.35 g; KH_2PO_4, 1.70 g; NH_4Cl, 2.12 g; $MgSO_4 \cdot 7H_2O$,

0.38 g; $MnSO_4 \cdot H_2O$, 0.05 g; $FeSO_4 \cdot 7H_2O$, 0.05 g; yeast extract, 1.0 g. $CaCl_2 \cdot 2H_2O$, 0.03 g, and ascorbic acid, 0.01 g, were added aseptically, as were the following trace metals: $ZnSO_4 \cdot 7H_2O$, 1.0×10^{-3} g; $CoCl_2 \cdot 6H_2O$, 1.5×10^{-3} g; $Na_2MoO_4 \cdot 2H_2O$, 0.2×10^{-3} g; $CuSo_4 \cdot 5H_2O$, 0.035×10^{-3} g; KI, 0.15×10^{-3} g. The pH value before autoclaving was set at 7.2.

A vitamin solution (3 ml) was added to the basal medium (1,000 ml) without yeast extract to study the nutritional requirements for the growth of *C. uda*. It contained (per liter of deionized water): Biotin, 2 mg; nicotinic acid, 20 mg; thiamine, 10 mg; *p*-aminobenzoic acid, 10 mg; panthothenic acid, 5 mg; pyridoxal hydrochloride, 50 mg; cyanocobalamine, 20 mg.

The organism was grown for 4 days on an agar slant of basal medium supplemented with 20 g of agar (Difco Laboratories, Detroit, Mich.) and 20 g of CM-cellulose per liter. The bacteria were washed off into 1-liter shake flasks containing 250 ml of basal medium with 0.5% (wt/vol) glucose and cultured for 24 h, or with 1% (wt/vol) of a cellulose substrate for 48 h on a rotary shaker (100 rpm). This culture (5 ml) was used to inoculate 250 ml of basal medium with noncellulose or cellulose substrates as carbon sources and cultivated with shaking as above. In shake flask cultures the pH value was adjusted daily to 6.8 by adding 1 N sterile NaOH.

For 50- and 70-liter batch cultivations, 1.4 and 2.0 liters, respectively, of a shake flask culture, grown as described above for 48 h on basal medium with 1% (wt/vol) cellulose substrate, were inoculated into 48.6 and 68 liters, respectively, of basal medium supplemented with 2% (wt/vol) of the same cellulose substrate. The cultivations were performed in an 80-liter bioreactor (type 50b; Giovanola Frères S.A., Monthey, Switzerland) equipped with a draught tube and driven by a specially constructed propeller (intensor system; Giovanola Frères S.A.) at 1,500 rpm. The cultures were aerated at 0.119 vol/vol per min, and the pH was automatically adjusted to 6.8 by titration with 14.7 M NH_4OH.

Bacterial growth on cellulose was followed by determination of the nitrogen content of the cells by Kjeldahl analysis. Bacterial protein was calculated as N × 6.25 and bacterial dry weight was calculated by assuming an average protein content in bacterial cells of 60%. The dry weight of the solids was also used to determine the bacterial dry weight of cultures with Avicel or CM-cellulose as the carbon source. It was calculated from the difference between the dry weight of the solids, comprising the cells and residual cellulose, and the amount of residual cellulose, determined as described previously (36). The dry weight of the solids was determined by centrifuging the culture (20 ml at 9,600 × *g* for 20 min), washing the pellet two times with water (10 ml), and drying it at 40°C under reduced pressure to constant weight.

For the soluble carbon sources, cell growth was determined by measuring the optical density at 546 nm. The cell concentration in cultures with cellulose substrates was determined sometimes also by the Lowry method as modified by Huang et al. (22). The culture suspension (5 ml) was allowed to stand for 30 min at room temperature to settle the insoluble cellulosic materials. A sample (2 ml) was withdrawn from the supernatant and centrifuged for 10 min at 3,000 × *g*. The supernatant was discarded, and the pellet was washed two times with 0.85% (wt/vol) NaCl solution (2.0 ml) to remove soluble proteins still adsorbed to the cells. The pellet was suspended in 0.85% (wt/vol) NaCl solution (2.0 ml), 2 N NaOH (2 ml) was added to the cell suspension (1 ml), and the suspension was heated for 10 min in a boiling water bath. After being cooled in an ice bath, the sample was centrifuged for 15 min at 3,000 × *g*. The clear supernatant (1 ml) was assayed by the method of Lowry et al. (28). A standard curve was prepared from determinations with bovine serum albumin. By this method only about 40% of the total bacterial protein could be measured. Neither elevation of the NaOH concentration nor an increase in the boiling time increased the amount of dissolved protein. As the course of bacterial growth determined by this method was in agreement with that obtained by determining the bacterial nitrogen content, optical density, or the difference between dried solids and residual cellulose, this method could be applied to follow the bacterial growth (Fig. 1).

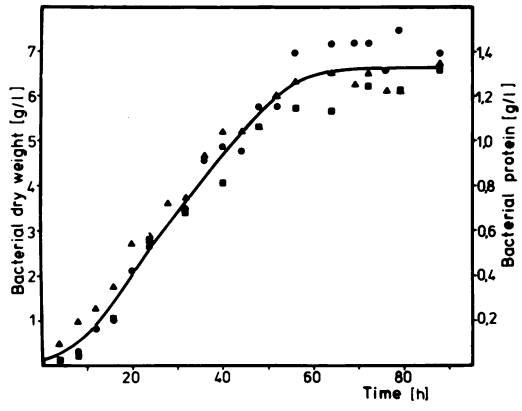

FIG. 1. Comparison of three different methods for determination of bacterial growth during a 70-liter batch cultivation on basal medium with 2% (wt/vol) Avicel as the carbon source. (●) Bacterial protein (grams per liter) determined by the method of Huang et al. (22). This method released only 40% of the total bacterial protein. (■) Bacterial dry weight (grams per liter) determined by the Kjeldahl method. (▲) Bacterial dry weight (grams per liter) calculated from the difference between the dried solids and the residual cellulose. Cultivation was carried out at 30°C and pH 6.8 in an 80-liter bioreactor. It was aerated at 0.119 vol/vol per min and agitated with the intensor system (Giovanola Fréres S.A.) at 1,500 rpm.

Determination of cellulose content. The cellulose content of cultures was determined as described previously (36).

Enzyme assays. The viscosimetric measurement of endo-1,4-β-glucanase activity was carried out as follows. Na-CM-cellulose solution was prepared by dissolving Na-CM-cellulose (0.5 g) and sodium azide (0.03 g) in 0.005 M Tris-hydrochloride–NaOH buffer, pH 6.8 (100 ml), overnight on a rotary shaker (100 rpm) at 27°C. The solution was stored at 4°C and shaken as described above for 30 min before use. Stable solutions were thus obtained with efflux times of about 86.5 s at 37°C by using a Micro-Ubbelohde viscometer

TABLE 1. Influence of casamino acids, vitamins, and yeast extract on growth of and cellulose degradation by *C. uda*[a]

Addition (% [wt/vol])	Residual CM-cellulose (% [wt/vol])	Bacterial dry weight[b] (g/liter)	Endo-1,4-β-Glucanase activity in supernatant (relative U/ml)
None	90	0.35	
Casamino acids[c] (0.1)	85	0.80	
Vitamins[d]	72	1.85	
Casamino acids plus vitamins (0.1)	67	2.75	
Yeast extract			
0.05	53	3.00	0.899
0.10	51	3.10	0.960
0.20	52	3.20	0.900
0.50	57	3.45	0.673

[a] Cultivation was carried out in shake flasks (250 ml of medium per 1-liter flask) for 72 h on 2% (wt/vol) CM-cellulose.

[b] Bacterial dry weight was calculated from the bacterial protein determined by the method of Huang et al. (22); see the text.

[c] Casamino acids were vitamin free.

[d] Composition of the vitamin additions is given in the text.

no. 2. Enzyme samples were obtained either as culture supernatants or cell suspensions. The latter were obtained from cultures grown on soluble carbon sources by centrifugation (2 ml at 3,000 × *g* for 20 min), washing the pellet with 0.85% (wt/vol) NaCl solution (2 ml), and suspending the cells in 0.85% (wt/vol) NaCl solution (2 ml). For measurement of the cell-bound endo-1,4-β-glucanase activity in the cultures grown on insoluble cellulose, culture suspensions (5 ml) were allowed to stand for 30 min at room temperature to permit settling of the insoluble cellulose. A sample (2 ml) was withdrawn from the cellulose-free supernatant and centrifuged (3,000 × *g* for 20 min), and the cells obtained were washed and suspended as described above. The following method was routinely used for the viscometric assay. Na-CM-cellulose solution (2.5 ml) was poured into the viscometer and warmed for 10 min at 37°C. The enzyme solution or cell suspension (0.1 ml) was pipetted into the Na-CM-cellulose solution and thoroughly mixed, and the efflux time was recorded two or three times within 5 min with a built-in quartz clock (Schott, Mainz, FRG). Relative units of endo-1,4-β-glucanase activity were calculated from the increase of reciprocal, specific viscosity (η_{sp}) by the following formula (23): $(d/dt)\cdot(1/\eta_{sp}) = [(\eta_{sp1} - \eta_{sp2})/(\eta_{sp1}\cdot\eta_{sp2})]\cdot 1/t$.

β-Glucosidase activity was determined with *p*-nitrophenyl-β-D-glucoside as the substrate (7). The reaction mixture contained 50 mM *p*-nitrophenyl-β-D-glucoside in 0.05 M Tris-hydrochloride–NaOH buffer, pH 6.8 (1 ml), and 1 ml of the cell suspension. After incubation at 50°C for 10 min, 1 M sodium carbonate solution was added, and the mixture was chilled to about 4°C and centrifuged (3,000 × *g* for 15 min). The absorbance of the clear supernatant was measured at 400 nm. The β-glucosidase activity was calculated as follows: micromoles of *p*-nitrophenol formed per minute = $(EV)/(tv\epsilon d)$, where E is the extinction at 400 nm, V is the total volume of the reaction mixture in cubic centimeters, t is the reaction time in minutes, v is the volume of the sample in cubic centimeters, $\epsilon = 18.8$ (cm^2/μmol), and d is the light path of the cuvette in centimeters.

Viscometric method for studies of induction and repression of endo-1,4-β-glucanases. Investigations of the induction and repression of endo-1,4-β-glucanases were carried out by the method of Eriksson and Hamp (13). Cells used for the experiments were obtained by growing *C. uda* in basal medium (250 ml) with 0.5% (wt/vol) glucose for 15 h. The cells were harvested aseptically by centrifugation and washed with sterile 0.85% (wt/vol) NaCl solution. Wet cells corresponding to a bacterial dry weight of 0.05 to 0.074 g were aseptically transferred into 500-ml shake flasks containing basal medium (100 ml) with 1% (wt/vol) Na-CM-cellulose (pH 6.8) either alone or in combination with the inducer or repressor sugars being studied. The mono- and disaccharides were dissolved in small amounts of basal medium and sterilized by membrane filtration (average pore size, 0.45 μm) to avoid degradation. The Na-CM-cellulose induction cultures with and without sugars were incubated on a shaker at 30°C. Samples (4 ml) were taken at intervals and centrifuged to remove the cells, and the viscosity was measured at 30°C as described above to determine the endoglucanase activity. The percent decrease from the initial viscosity was plotted as a function of the incubation time, and the incubation time required to obtain a 50% reduction in viscosity was taken as a measure for the induction or repression of endo-1,4-β-glucanase formation.

Ultrasonic disruption of the cells. Cells grown for 24 h on basal medium with 0.5% (wt/vol) cellobiose were harvested by centrifugation, washed with 0.85% (wt/vol) sterile NaCl solution, suspended in 0.05 M Tris-hydrochloride–NaOH buffer (pH 6.8), and centrifuged again (30,000 × *g* for 20 min). The supernatant, which was free of β-glucosidase activity, was discarded. The cells were suspended again in Tris-hydrochloride–NaOH buffer (pH 6.8) to an optical density at 546 nm of 2.1 and ultrasonically disrupted for 30 min under cooling in an ice bath with a Sonifier (J-17-A; Branson Sonic Power Co., Danbury, Conn.). A microtip was used, and the output control was set at 4. Samples were examined microscopically to confirm that most of the cells had been disrupted. For measurement of

the β-glucosidases released after sonication, the sonicated suspension was centrifuged (30,000 × *g* at 4°C for 30 min), and the supernatant was removed. For the determination of β-glucosidases still adhering to the cell debris, the pellet obtained was suspended in the same amount of Tris-hydrochloride–NaOH buffer (pH 6.8) as was used to suspend the cells before sonication.

Mass spectrometry of TMS. Trimethylsilyl derivatives of sugars were prepared as described by Brobst (9). They were purified by thin-layer chromatography on Merck silica gel plates (60 F_{254}) with the solvent system propan-2-ol-water (85:15, vol/vol). The mass spectra of trimethylsilylated sugars (TMS) were recorded with an A.E.I. MS-9 instrument operated at 8 kV accelerating voltage and 70 eV ionizing voltage. Samples were inserted directly into the ion source at the minimum temperature.

Gas-liquid chromatography of TMS. Gas-liquid chromatography of TMS was carried out with a Perkin Elmer gas chromatograph (M 900) equipped with a flame ionization detector. A stainless-steel column (200 by 0.27 cm diameter) packed with 1% SE 52 on Chromosorb G was used. The column was operated for the chromatography of TMS-glucose at 120°C and for analysis of TMS-cellobiose at 190°C with an N_2 flow rate of 29 ml/min. Peak area measurements were made with an autolab system IV computing integrator (Spectra Physics).

High-pressure liquid chromatography. Fast separation of sugars was done with a high-pressure, size-exclusion chromatograph (Knauer, West Berlin, Germany). The column (25 cm by 0.46 cm diameter) was packed with LiChrosorb NH_2 (10 μm). As the mobile phase, CH_3CN-water (70:30, vol/vol) was used at a flow rate of 2.0 ml/min. Sugars were detected with a refractometer (Knauer).

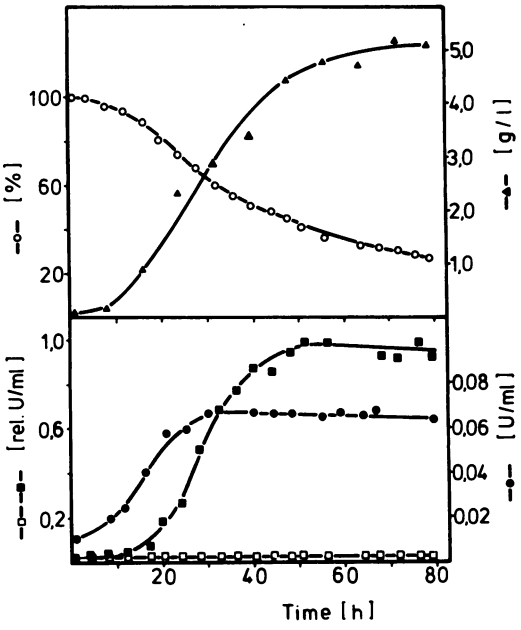

FIG. 2. Growth, cellulose degradation, and formation of endo-1,4-β-glucanases and β-glucosidases during a 70-liter batch cultivation in basal medium with 2% (wt/vol) Avicel as the carbon source. (○) Cellulose concentration (percent); (▲) bacterial dry weight (grams per liter); (■) endo-1,4-β-glucanase activity in the supernatant (relative units per milliliter); (●) cell-bound β-glucosidase activity (units per milliliter); (□) cell-bound endo-1,4-β-glucanase activity (relative units per milliliter). Cultivation conditions are described in the legend to Fig. 1.

RESULTS

Growth, cellulose degradation, and formation of endo-1,4-β-glucanases and β-glucosidases during growth on cellulose. The addition of 0.1% (wt/vol) yeast extract to the culture medium significantly stimulated growth, cellulose degradation, and endoglucanase formation of *C. uda* during cultivation on 2% (wt/vol) CM-cellulose. Higher concentrations of yeast extract enhanced the bacterial growth but lowered endoglucanase formation and cellulose degradation. To find out whether amino acids or vitamins were responsible for these enhancements, we replaced the 0.1% (wt/vol) yeast extract with vitamin-free casamino acids and vitamins. Neither of these nor their combination increased cellulose degradation or growth to the same extent as did the yeast extract (Table 1).

The growth and cellulose degradation of cultures grown on 2% (wt/vol) Avicel, CM-cellulose, and ball-milled printed newspaper can be compared only to a certain extent, as the newspaper used also contained hemicelluloses and lignin. During a 70-liter batch cultivation of 80 h on 2% (wt/vol) Avicel, the cellulose content was reduced by 75% (Fig. 2). Within the same period the cellulose concentration during a 50-liter batch cultivation on 2% (wt/vol) CM-cellulose was reduced by only 60% (Fig. 3), and during cultivation on 2% (wt/vol) ball-milled printed newspaper it was reduced by half (Fig. 4). The highest bacterial dry weight of 5.0 g/liter was reached on 2% (wt/vol) Avicel. This corresponded to a growth yield of 0.33 (grams of dry biomass per gram of cellulose consumed). In cultures with 2% (wt/vol) CM-cellulose or ball-milled printed newspaper as the carbon source, a bacterial dry weight of 3.0 and 2.8 g/liter, respectively, was obtained, corresponding to growth yields of 0.25 and 0.53. The latter value is certainly too high and is not the real growth yield, as *C. uda* also utilizes hemicelluloses as a carbon source when grown on printed newspaper (unpublished data).

Endoglucanase formation in these cultures could not be readily compared either, because the total endoglucanase activity, including that adsorbed on residual cellulose, was not measured. We determined the endoglucanase activity in the supernatant, and, when Avicel and CM-cellulose were used as carbon sources, we also

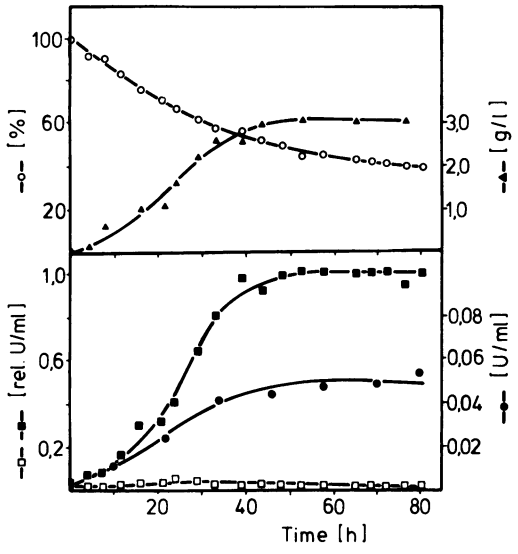

FIG. 3. Growth, cellulose degradation, and formation of endo-1,4-β-glucanases and β-glucosidases during a 50-liter batch cultivation in basal medium with 2% (wt/vol) CM-cellulose as the carbon source. (○) Cellulose concentration (percent); (▲) bacterial dry weight (grams per liter) calculated from the bacterial protein as determined by the method of Huang et al. (22); (■) endo-1,4-β-glucanase activity in the supernatant (relative units per milliliter); (●) cell-bound β-glucosidase activity (units per milliliter); (□) cell-bound endo-1,4-β-glucanase activity (relative units per milliliter). Cultivation conditions are described in the legend to Fig. 1.

determined the cell-bound activity. Yields ranged from 0.55 relative U/ml on printed newspaper to 1.0 relative U/ml on Avicel.

The β-glucosidase activity was always found to be associated with the cells and could not be detected in the supernatant of any of the three cultures throughout the period of cultivation. The yields, ranging between 0.03 and 0.065 U/ml, were relatively low (Fig. 2–4).

Growth and formation of β-glucosidases during cultivation on noncellulose substrates. Growth and formation of β-glucosidases after 56 h of cultivation with mono- and disaccharides, starch, or peptone is shown in Table 2. Cell-bound β-glucosidase activity was always found and formed constitutively (Fig. 2–4; Table 2), but no β-glucosidase activity could be measured in the supernatants of those cultures grown with glucose, mannose, cellobiose, maltose, lactose, starch, or peptone.

Location of endo-1,4-β-glucanases. To study whether the endo-1,4-β-glucanases were partly cell bound, we measured the endoglucanase activity of the cells during 70- and 50-liter batch cultivations on 2% (wt/vol) Avicel and CM-cellulose, respectively (Fig. 2 and 3). In both cultivations, only about 1% or less of the endoglucanase activity of the culture supernatant could be detected at the cells. A similarly small amount of cell-bound, but no extracellular, endoglucanase activity was found when the cells were grown with mono- and disaccharides, starch, or peptone (Table 2). This very low cell-bound activity was considered to be the basal endo-1,4-β-glucanase level of the cells.

Location of β-glucosidases. The β-glucosidases were always found to be associated with the cells, as already mentioned, regardless of the carbon source. They must be located outside the cytoplasmic membrane, either in the periplasmic space or in the cell wall, because otherwise they would not be detected by the assay with *p*-nitrophenyl-β-D-glucoside as the substrate. Even in the late stationary growth phase, when cell lysis takes place, no β-glucosidase activity was measured in the supernatant of the cultures. This indicates again that although the β-glucosidases are associated with the cells, the enzymes may not be cytoplasmic. The cells were disrupted by sonication to confirm the absence of cytoplasmic β-glucosidase activity. No cytoplasmic β-glucosidase was released by sonication, or the total β-glucosidase activity would have increased in the suspension after this treatment (Table 3). On the other hand, about 75% of the β-glucosidases originally associated with the cells were released (Table 3).

Induction and repression of endo-1,4-β-glucan-

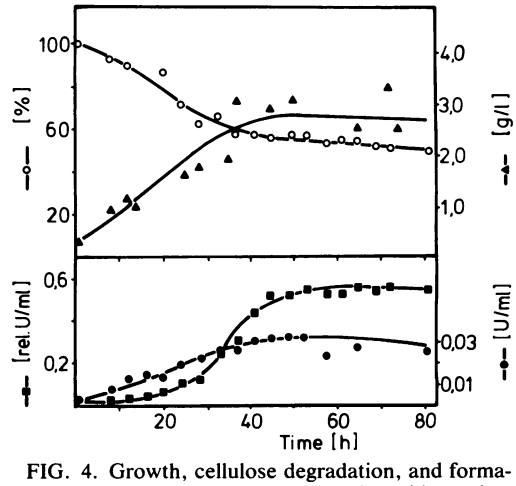

FIG. 4. Growth, cellulose degradation, and formation of endo-1,4-β-glucanases and β-glucosidases during a 50-liter batch cultivation in basal medium with 2% (wt/vol) ball-milled printed newspaper as the carbon source. (○) Cellulose concentration (percent); (▲) bacterial dry weight (grams per liter); (■) endo-1,4-β-glucanase activity in the supernatant (relative units per milliliter); (●) cell-bound β-glucosidase activity (units per milliliter). Cultivation conditions are described in the legend to Fig. 1.

TABLE 2. Growth and formation of endo-1,4-β-glucanase and β-glucosidase activity on noncellulose substrates[a]

Carbon source (0.5%, wt/vol)	Growth (OD_{546})[b]	Endo-1,4-β-Glucanase activity in supernatant (relative U/ml)	Endo-1,4-β-Glucanase activity of twice-washed cells[c] (relative U/ml)	β-Glucosidase activity in total culture (U/ml)
Glucose	2.20	0.00	≤0.01	0.045
Mannose	2.22	0.00	≤0.01	0.019
Cellobiose	2.20	0.00	≤0.01	0.059
Maltose	2.22	0.00	≤0.01	0.030
Lactose	1.76	0.00	≤0.01	
Starch	2.25	0.00	≤0.01	0.039
Peptone	0.80	0.00	≤0.01	0.010

[a] Cultivation was carried out in shake flasks (250 ml of medium per 1-liter flask) for 56 h.
[b] OD_{546}, Optical density at 546 nm.
[c] The cells, washed twice with 0.85% (wt/vol) NaCl solution, were suspended in 0.005 M Tris-hydrochloride–NaOH buffer (pH 6.8) to the same optical density as the original culture.

ase formation. To study the induction and repression of endo-1,4-β-glucanase formation in *C. uda*, we applied the method of Eriksson and Hamp (13). Cells in 1% (wt/vol) Na-CM-cellulose medium were incubated alone or together with the inducing or repressing sugars being studied. The incubation time required to obtain a 50% reduction in viscosity was taken as a measure of the ability of the sugars to induce or repress enzyme synthesis. The endoglucanases were induced when the drop in viscosity was more rapid than in the control assay and repressed when this decrease was slower. Cellobiose (0.05 and 0.1 mM) had a distinct inducing effect, whereas higher cellobiose concentrations of 1.0 and 2.0 mM repressed endoglucanase formation. Sophorose, a powerful inducer of the endoglucanases in *Trichoderma reesei* (13, 27, 39), neither induced nor repressed endo-1,4-β-glucanase formation in *C. uda* (Table 4). Moreover, a glucose concentration of 1 mM delayed the decrease of the viscosity as compared with the control. More distinct was the catabolite repression caused by 3 mM glucose (Table 4). The repressing effect of glucose on endoglucanase production and cellulose degradation was examined further by adding it daily in concentrations of 0.56 to 55.67 mM to cultures grown on 2% (wt/vol) CM-cellulose. The ratio of endoglucanase activity to bacterial dry weight and the degraded cellulose measured in these cultures after 80 h of cultivation are shown in Table 5. The daily addition of 0.56 mM glucose did not remarkably lower either the ratio of endoglucanase activity to bacterial dry weight or the percentage of degraded cellulose. Only when 2.78 mM was added was endoglucanase production of the cells diminished, but the amount of residual cellulose was the same as in the control assay. This lower endoglucanase productivity was counterbalanced, in this case, by a higher pro-

TABLE 3. Release of β-glucosidases from the cells by sonication

Prepn	β-Glucosidase activity (mU/ml)
Cell suspension before sonication[a]	20
Suspension after sonication	19
Supernatant after sonication	15
Cell debris after sonication[b]	5

[a] The optical density of the cells suspended in 0.05 M Tris-hydrochloride–NaOH buffer (pH 6.8) was 2.1 at 546 nm.
[b] The pellet obtained after centrifugation was suspended in the same amount of buffer as were the cells before sonication.

TABLE 4. Induction and repression of endo-1,4-β-glucanase formation[a]

Inducing or repressing compound added	Concn (mM)	Time required for 50% reduction in viscosity (min)
Cellobiose	0.00	78
	0.05	69
	0.10	63
	1.00	114
	2.00	120
Sophorose	0.00	78
	0.33	78
	1.00	78
Glucose[b]	0.00	66
	1.00	75
	3.00	81

[a] Induction or repression of endoglucanase formation was measured by the capillary viscosimetric method of Eriksson and Hamp (13) and expressed as the incubation time required to obtain a 50% reduction in viscosity of the 1% (wt/vol) Na-CM-cellulose medium. The cell concentration was 0.5 g/liter (determined as bacterial dry weight).
[b] Another batch of cells was used for these measurements.

TABLE 5. Influence of glucose on endo-1,4,-β-glucanase formation and cellulose degradation[a]

Glucose (mM)	Relative units of endo-1,4-β-glucanase activity per mg (bacterial dry weight)	Cellulose degraded (%)
0.00	0.247	46
0.56	0.242	46
2.78	0.164	46
5.56	0.162	46
16.67	0.150	46
27.78	0.022	30
55.56	0.008	5

[a] Glucose at the indicated concentrations was added daily to cultures grown on basal medium with 2% (wt/vol) CM-cellulose. Determinations were carried out after 80 h of cultivation in shake flasks (250 ml of medium per 1-liter flask) at 30°C and pH 6.8 on a rotary shaker (100 rpm).

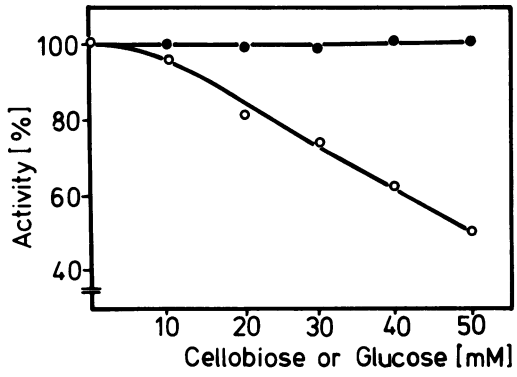

FIG. 5. Influence of glucose (●) and cellobiose (○) on endo-1,4-β-glucanase activity. The activity was determined viscosimetrically in a 0.5% (wt/vol) Na-CM-cellulose solution. The initial endo-1,4-β-glucanase activity was 0.014 relative U/ml.

duction of cell mass due to the additional glucose. However, a remarkable decrease in cellulose degradation from 46 to 30% was caused by the daily addition of 27.78 mM glucose, whereas the ratio of endoglucanase activity to bacterial dry weight was lowered to 0.022 relative U/mg. The addition of 55.56 mM glucose finally reduced this ratio to 0.008, and only 5% of the CM-cellulose was degraded.

The repressing effect of higher cellobiose concentrations on endoglucanase formation and cellulose degradation was studied as described above (Table 6). A daily addition of 8.77 mM cellobiose lowered the ratio of endoglucanase activity to bacterial dry weight from 0.247 to 0.016 relative U/mg and reduced cellulose degradation from 46 to 33%. When 14.62 mM cellobiose was added, the endoglucanase production of the cells was diminished to 0.008 relative U/mg, and cellulose degradation was reduced to 20%. Daily addition of 29.24 mM cellobiose reduced these two parameters to the same extent as did 55.56 mM glucose (Tables 5 and 6).

TABLE 6. Influence of cellobiose on endo-1,4-β-glucanase formation and cellulose degradation[a]

Cellobiose (mM)	Relative units of endo-1,4-β-glucanase activity per mg (bacterial dry weight)	Cellulose degraded (%)
0.00	0.247	46
8.77	0.016	33
14.62	0.008	20
29.24	0.008	5

[a] Cellobiose at the indicated concentrations was added daily to cultures grown on basal medium with 2% (wt/vol) CM-cellulose. Determinations were carried out after 80 h of cultivation in shake flasks (250 ml of medium per 1-liter flask) at 30°C and pH 6.8 on a rotary shaker (100 rpm).

Product inhibition of endo-1,4-β-glucanases and β-glucosidases. The effect of glucose and cellobiose on endo-1,4-β-glucanase activity is shown in Fig. 5. Glucose did not affect this enzyme activity in concentrations up to 50 mM, whereas cellobiose at this concentration reduced endoglucanase activity by 50%. β-Glucosidase activity when *p*-nitrophenyl-β-D-glucoside was used as substrate was inhibited noncompetitively by glucose, with a K_i value of 0.667 mM. The K_m value for *p*-nitrophenyl-β-D-glucoside was 103.5 mM.

Characteristics of endo-1,4-β-glucanases and β-glucosidases. The optimum pH of 6.8 for extracellular endo-1,4-β-glucanase activity was found to be very pronounced, whereas that of the cell-bound β-glucosidase activity ranged from 6.0 to 7.0 (Fig. 6).

To study the thermostability of the endo-1,4-β-glucanases, we incubated samples of endoglucanase-containing culture supernatant at different temperatures, and the half-life of the activity was determined. Half-lives of 90 min at 50°C, 39 min at 55°C, and 11 min at 60°C were measured. For determination of the thermostability of the cell-bound β-glucosidases, cells suspended in 0.85% (wt/vol) NaCl solution were incubated also. β-Glucosidase activity half-lives of 19 min at 50°C, 3 min at 55°C, and only 1 min at 60°C were measured.

End products of cellulose degradation. By means of gas-liquid chromatography, mass spectrometry, and high-pressure liquid chromatography an attempt was made to determine whether glucose, cellobiose, or both were end products of cellulose degradation by the extracellular enzymes. In the supernatant of a culture grown on Avicel, cellobiose and glucose were found in a proportion of 6:1. After acid hydrolysis of the lyophilized culture supernatant (35), no sugar

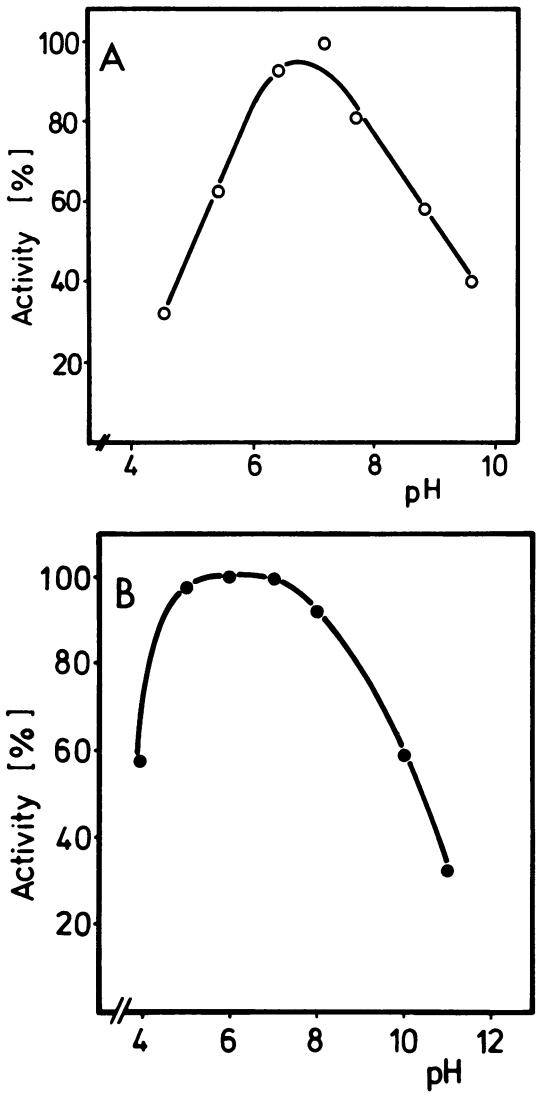

FIG. 6. Influence of pH on extracellular endo-1,4-β-glucanase activity (○) and on cell-bound β-glucosidase activity (●).

other than glucose could be detected by gas-liquid chromatography or mass spectrometry.

DISCUSSION

C. uda grew well on cellulose when the culture medium contained 0.1% (wt/vol) yeast extract, which could only partly be substituted for by vitamins and amino acids. A similar growth requirement was found for *Cellulomonas flavigena* (19) and another *Cellulomonas* strain isolated by Choi et al. (11).

C. uda can rapidly degrade not only Avicel and CM-cellulose but also lignocellulosic materials, e.g., printed newspaper (Fig. 2 through 4). The growth yield of cultivation with Avicel as the carbon source was higher than it was with CM-cellulose. The growth yield calculated from the biomass produced and cellulose consumed during cultivation on printed newspaper cannot be compared with the others, as *C. uda* also utilizes the hemicelluloses of the newspaper.

It is now generally accepted that a low level of constitutive enzymes degrades their exogenous substrates, and the resultant low-molecular-weight products enter the cell and induce further synthesis of extracellular enzymes (33). Such a basal level of constitutive cellulases was confirmed for *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*) (13) and *T. reesei* (14). The capillary viscometric method developed by Eriksson and Hamp (13) for the measurement of endoglucanase activity is very sensitive and was therefore used to determine the basal level of endo-1,4-β-glucanases at *C. uda*. During cultivation with mono- and disaccharides or peptone, no extracellular, but very small amounts of cell-bound, endo-1,4-β-glucanase activity, representing the basal endoglucanase level, could always be measured (Table 2). Similarly small amounts of cell-bound endoglucanase activity, 1% or less of the endoglucanase activity in the supernatant, were determined also during growth on Avicel and CM-cellulose (Fig. 2 and 3).

The induction of endo-1,4-β-glucanase formation in *C. uda* was studied also by the method of Eriksson and Hamp (13). Endoglucanase synthesis in *C. uda* can be induced by small amounts of cellobiose (Table 4), as is the case for another *Cellulomonas* species described by Stewart and Leatherwood (40). However, higher cellobiose concentrations repressed the endoglucanase synthesis (Tables 4 and 6). Sophorose, which induces cellulase synthesis in *T. reesei* (13, 27, 39) and in *Pseudomonas fluorescens* subsp. *cellulosa* (43) was not effective at all as either an inducer or repressor of endoglucanase formation in *C. uda* (Table 4). Although it is established that cellobiose and sophorose act as inducers in a large number of cellulose-degrading microorganisms, the mechanism of induction remains unknown, and in no instance has a nonmetabolizable, gratuitous inducer been described.

Another essential role in the regulation of extracellular enzyme formation is played by catabolite repression of inducible or constitutive enzyme synthesis. Such a repression of endoglucanase formation in *C. uda* occurred in the presence of glucose (Tables 4 and 5) and, as mentioned above, also occurred in the presence of higher concentrations of cellobiose (Tables 4 and 6). Catabolite repression of 1,4-β-glucanase formation by glucose and cellobiose was also

established in other cellulose-degrading bacteria (5, 11, 26, 40–42). As with the mechanism of induction, that of catabolite repression of the 1,4-β-glucanase synthesis is not completely known. Glucose and other readily metabolizable substrates may alter the level of cyclic AMP and GMP (33) or affect the translation of 1,4-β-glucanase RNA (29, 30).

The β-glucosidases from *C. uda* were formed constitutively and found to be cell bound regardless of the carbon source. However, the cultures grown on mono- and disaccharides with about the same cell density in the stationary growth phase had different β-glucosidase activities (Table 2), but these differences did not appear to be the result of inhibition. The initial concentration of the sugars (0.5% [wt/vol]) was such that they were rapidly used up, and no inhibition could take place in the stationary growth phase. These results indicate that there may be two or more β-glucosidases and that the synthesis of one or more of them may be regulated by catabolite repression. β-Glucosidases in other *Cellulomonas* species seem to be similar to those in *C. uda* in that they are also cell bound and have different specific activities during growth on glucose and cellobiose (17). In contrast to *Pseudomonas* species (24, 34, 44) in which both cyto- and periplasmic β-glucosidases were found, no cytoplasmic glucosidase activity could be measured in *C. uda*. The release of 75% of the β-glucosidases into the supernatant by sonication of a cell suspension of *C. uda* provides evidence for the location of β-glucosidases in the periplasmic space of the cells, as was reported for *Clostridium thermocellum* (1). In addition to these periplasmic β-glucosidases, *C. uda* probably also possesses a cellobiose phosphorylase, and possibly possesses a cellodextrin phosphorylase (S. Kretschmer, Ph.D. thesis, Technische Universität Braunschweig, Braunschweig, FRG) as *Clostridium thermocellum* does (2, 3, 37).

The products of 1,4-β-glucanase and β-glucosidase action not only repressed enzyme synthesis in *C. uda* but also inhibited these enzymes in a sequential manner. When the extracellular glucose level rose during cellulose degradation, the β-glucosidase activity was inhibited noncompetitively and the cellobiose concentration increased. High cellobiose concentrations, on the other hand, inhibited the extracellular endo-1,4-β-glucanase activity, thus slowing down the hydrolysis of cellulose. Inhibition of carboxymethyl cellulase activity by cellobiose was also observed in other *Cellulomonas* species (11) and in *Cytophaga* species (10), whereas the endoglucanases from *Clostridium thermocellum* were not inhibited by either cellobiose or glucose (38). As end products of cellulose hydrolysis by *C. uda*, cellobiose and glucose were found in a proportion of 6:1. The small amount of glucose most probably arises from the action of exoglucanases, but the participation of endoglucanases cannot be ruled out, as an endoglucanase from *T. reesei* acts on cellobiose (25).

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