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Comparison of β-Glucosidase Activities in Different *Streptomyces* Strains

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Cellobiase (β-glucosidase) production was compared for two streptomycetes: *Streptomyces flavogriseus*, a known producer of cellulase complex, and *Streptomyces* sp. strain CB-12, a strain isolated for its rapid growth on cellobiose. The optimal conditions for enzyme activity were established in relation to pH, temperature, enzyme stability, and substrate affinity. The production of β-glucosidase by the two strains depended on the carbon substrate in the medium. Cellobiose was found to repress the biosynthesis of the enzyme in *S. flavogriseus* and to stimulate its production in strain CB-12. The biosynthesis of the enzyme correlated well with the accumulation of glucose in the culture filtrates. The combined action of the β-glucosidases produced by the two *Streptomyces* strains might allow a better utilization of the reaction products which arise during the biodegradation of cellulose.

The activity of the different enzymes of the cellulase complex during the degradation of cellulose depends on the action of endo- and exo-β-1,4-glucanases and of β-glucosidases. The modes of action of bacterial and fungal cellulases appear to be similar and have been studied by several researchers (1, 2, 8, 19). Models for the regulation of these enzymes have been proposed by Gong and Tsao (5) and others (7, 16). Among these enzymes, cellobiase, a β-glucosidase (EC 3.2.1.21) hydrolyzing the disaccharide cellobiose to two molecules of D-glucose, was found to be of particular importance for the efficient functioning of the cellulase system (15, 17). Any accumulation of cellobiose inhibits the initial enzyme attack on the cellulose substrate by β-1,4-glucan cellobiohydrolase (EC 3.2.1.91) and by β-1,4-glucan-4-glucanohydrolase (EC 3.2.1.4) through end product inhibition (1, 2, 6, 12, 18, 19). It was found that β-glucosidase activity among the many microorganisms that produce cellulolytic enzymes generally occurred intracellularly and in comparatively small quantities. This can be considered as a possible limiting factor in the enzymatic degradation of cellulose (14).

In a research program for improved cellobiase production by *Streptomyces flavogriseus* by genetic recombination through protoplast fusion, we studied the activities and characteristics of β-glucosidase from different streptomyces strains. In the present paper, we wish to report a comparison of cellobiase activities of the cellulolytic *S. flavogriseus* and of a recently isolated, highly active *Streptomyces* sp. unable to produce β-glucanases.

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MATERIALS AND METHODS

Microbial strains. *S. flavogriseus* IAF-45 CD (ATCC 33331), a cellulase-producing strain, was described previously (8, 9). *Streptomyces* sp. strain CB-12 was isolated from composted sheep manure.

Culture media. *S. flavogriseus* was maintained on agar slants as described earlier (8), whereas strain CB-12 was maintained on slants containing the following: oatmeal (Heinz Co.), 20 g; tomato paste (Hunt-Wesson, Canada), 20 g; powdered cellulose (J. T. Baker Chemical Co.), 50 g; agar (Difco Laboratories), 20 g; all dissolved in 1 liter of distilled water. The pH was adjusted to 7.0 with 1 N NaOH. All media were sterilized at 121°C for 20 min.

The basal medium (BM) used in the submerged culture studies had the following composition: $(NH_4)_2SO_4$, 1.4 g; K_2HPO_4, 2.5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; yeast extract (Difco), 1 g; Difco Proteose Peptone, 1 g; trace element solution ($ZnSO_4 \cdot 7H_2O$, 140 mg; $MnSO_4 \cdot H_2O$, 160 mg; $FeSO_4 \cdot 7H_2O$, 500 mg; $CoCl_2$, 200 mg; in 1,000 ml of distilled water adjusted to pH 3 to 3.5), 1 ml; Tween 80, 2 ml; and distilled water to 1,000 ml; the pH was 7.0. After sterilization at 121°C for 20 min, 300 mg of $CaCl_2 \cdot 2H_2O$ was added aseptically. One of the following carbon substrates was used and added aseptically to the BM: D-glucose (J. T. Baker Chemical Co.), 0.25 to 1%; D-cellobiose (Sigma Chemical Co.), 0.25 to 1%; or Avicel pH 105 (F. M. C. Corp), 1%.

Inoculum and preculture. A spore suspension was prepared by adding 5 ml of sterile physiological saline solution containing 1% Tween 80 to a 7-day-old slant culture. A total of 1 ml was inoculated into a 125-ml

Erlenmeyer flask containing 20 ml of Trypticase soy broth (BBL Microbiology Systems). The flasks were incubated at 29°C on a rotary shaker at 240 rpm for 22 h. A 10-ml sample of this culture was centrifuged at 5,000 rpm for 10 min in a clinical centrifuge, washed twice with 10 ml of BM, and resuspended in 10 ml of the same medium. One milliliter of this suspension served as an inoculum for 20 ml of culture medium (BM plus carbon susbstrate).

Culture conditions. Growth studies were carried out in a controlled environment incubator shaker (model G 25; New Brunswick Scientific Co.) at 29°C under rotary agitation at 240 rpm, using 20 or 100 ml of medium contained, respectively, in 125- or 500-ml Erlenmeyer flasks. Some experiments were conducted in a BioFlo chemostat (model C 30; New Brunswick Scientific Co.), using 350 ml of medium at an agitation rate of 400 rpm and an aeration of 0.5 vol/vol per min. The pH was maintained at 6.6 to 7.0 by automatic additions of sterile 1 N KOH.

Enzyme preparations. Samples of culture broth (10 ml) were taken aseptically at regular intervals throughout the growth phase and centrifuged at 2,000 × *g* for 10 min. The supernatant was decanted and served as a standard enzyme preparation. The mycelial pellet was washed twice with 0.05 M sodium phosphate buffer (Sørensen) at either pH 6.5 for strain CB-12 or pH 7.0 for strain IAF-45 CD and resuspended in 10 ml of the corresponding buffer. The mycelia in this suspension were disrupted for 5 min in an ultrasonic sonifier (Biosonik IV; Brownwill Co.) operated at full power with the microtip applying bursts of 60 s at a time, while continuously cooling the sample in an ice bath. The disrupted cells were centrifuged at 17,000 × *g* for 15 min in a Beckman J21-B centrifuge. The supernatant was used as the intracellular enzyme preparation.

Analytical Methods. (i) Protein. The concentration of extra- and intracellular protein was determined by the Lowry method (10), using bovine serum albumin, fraction V (Sigma Chemical Co.), as a standard,

(ii) β-glucosidase (EC 3.2.1.21). The activity of this enzyme was assayed by using cellobiose as the substrate, using the method of Berghem and Petterson (1) modified as follows. The assay mixture, consisting of 2 to 100 mM cellobiose in 50 mM sodium phosphate buffer (pH 6.5 or 7.0), was preincubated for 5 min at 40°C, and the reaction was started by the addition of 100 μl of enzyme to give a final volume of 1 ml. After incubation at 40°C for 10 min, the reaction was stopped by heating the sample in boiling water for 5 min. For the determination of the optimal pH, an additional buffer such as citrate phosphate (McIlvain; 75 mM; pH 4.8 to 6.0) was used. In this case, the reaction mixture was adjusted to pH 7 (just before heating) by adding 1 ml of sodium phosphate buffer (50 mM) of suitable pH. The amount of glucose liberated by the reaction was determined by the method of Bergmeyer (3), using glucose oxidase, peroxidase, and *o*-dianisidine as reagents. The results were expressed as nanomoles of glucose released per minute per milligram of protein at assay conditions. Because of the small concentrations of glucose present in the cellobiose, all results were corrected against a reagent blank.

(iii) Residual glucose determination. Samples 1 ml were removed aseptically from the cultures at regular intervals throughout the growth period. These were heated for 5 min in boiling water, followed by centrifugation. The glucose content was determined (3). One milliliter of diluted inactivated sample with 2 ml of peroxidase reagent (100 ml of phosphate buffer [0.5 M, pH 7] with 10 mg of peroxidase, 10 mg of glucose oxidase, and 0.5 ml of 1% *o*-dianisidine in methanol) was used. After incubation for 30 min at 37°C, the glucose content was determined against a standard curve with glucose.

(iv) Filter paper cellulase. Extracellular cellulase activity was determined by measuring the amount of reducing sugars released from Whatman no. 1 filter paper by the method of Mandels et al. (11), and the enzyme activity was expressed as micromoles of reducing sugars released per minute per milliliter of enzyme preparation.

RESULTS

The data shown in Fig. 1 reveal the influence of pH on enzyme activity. *S. flavogriseus* IAF-45 CD showed maximum activity between pH 6.5 and 7.5. This activity decreased rapidly at pH 8 and 6, with only trace activity at pH 5.5. For *Streptomyces* sp. strain CB-12 the optimal values were found between pH 6 and 6.5. This stain retained much of its activity in the acidic range, even down to pH 5.5.

The influence of heat on cellobiase is shown in Fig. 2. The enzyme activity was determined at pH 6.5, i.e., close to the optimal pH values for both strains. The enzyme preparation was incubated without substrate for 60 min at 0, 25, 30, and 40°C, and residual enzymatic activity was determined every 10 min. A comparison of the enzymes in the two strains at 40°C (optimal reaction temperature [8]) showed that the cello-

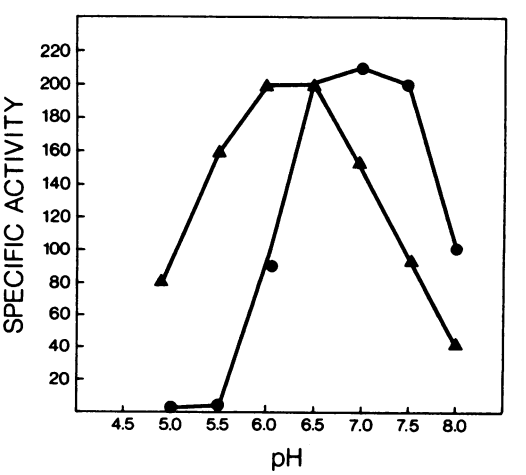

FIG. 1. Cellobiase activity as a function of pH. We used 20 mM cellobiose and 75 mM citrate-phosphate buffer (pH 4.8 to 6) or 50 mM phosphate buffer (pH 6.5 to 8). Symbols: ▲, *Streptomyces* sp. strain CB-12; ●, *S. flavogriseus* IAF-45 CD. Specific activity equals nanomoles of glucose per minute per milligram of protein.

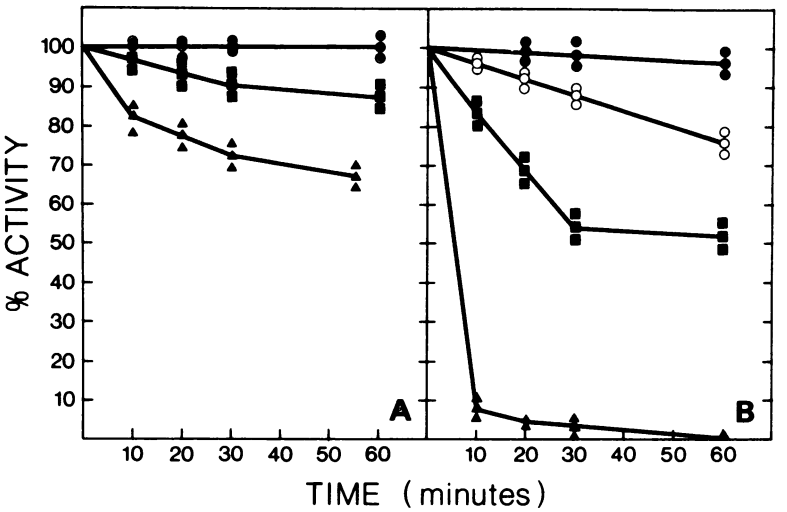

FIG. 2. Heat stability of cellobiase (intracellular enzyme preparation) in 50 mM phosphate buffer, pH 6.5. Protein concentration, 1 mg/ml. Residual activity was assayed with 20 mM cellobiose in 50 mM phosphate buffer (pH 6.5) for 10 min at 40°C. (A) *Streptomyces* sp. strain CB-12; (B) *S. flavogriseus* IAF-45 CD. Symbols: ●, 0°C; ○, 25°C; ■, 30°C; ▲, 40°C.

biase from strain CB-12 was more stable than that from *S. flavogriseus*. The maximal stability of the latter was found to be at pH 7.0, which also corresponds to its pH optimum. In the presence of cellobiose (20 mM) the enzymes from both strains were stable under the standard assay conditions (10 min at 40°C).

The influence of different cellobiose concentrations (between 10 and 100 mM) on the in vitro cellobiase activity of the two strains showed no significant inhibition within these limits. Michaelis-Menten constants (K_m) were determined at cellobiose concentrations of 2, 2.5, 5, 10, and 20 mM to obtain an indication of the substrate affinity of the crude enzymes. The K_m values obtained from Lineweaver-Burk plots were 2.56 mM for strain CB-12 cellobiase and 8.05 mM for *S. flavogriseus* IAF-45 CD cellobiase.

The production of the β-glucosidases of the two strains, when grown on basal medium containing either cellobiose or glucose, is shown in Fig. 3. The enzyme produced by *S. flavogriseus* IAF-45 CD when it was grown on cellobiose was markedly repressed and, without pH control, was totally suppressed at a substrate concentration of 1% (Fig. 3A). On the other hand, strain CB-12 produced significant amounts of enzyme at substrate concentrations of 0.5% and higher. This is also reflected in the growth patterns of the two strains. Whereas cellobiose was well utilized by strain CB-12, only low growth of *S. flavogriseus* could be obtained on this substrate. Cellobiase production by *S. flavogriseus* was induced by glucose (Fig. 3B), reaching optimal levels at 0.5%, whereas strain CB-12 under these conditions produced only trace amounts of enzyme.

Finally, we studied the glucose accumulation in cultures grown on BM with 0.5% cellobiose as the substrate. Significant amounts of glucose accumulated in the culture filtrate of strain CB-12, whereas only small quantities could be detected in that of *S. flavogriseus* (Fig. 4).

DISCUSSION

The relative lability of *S. flavogriseus* enzyme toward acidic pH might explain why the cellobiase activity in cultures declined steadily in the later stages of cellulolysis, when the pH of the culture medium dropped gradually to pH 5.9 or less (Table 1). In cultures grown under pH control (pH 6.8) the cellobiase levels are distinctly higher after 72 h, indicating a better enzyme stability. This also could be the reason for the higher overall extracellular cellulase activity as expressed by the filter paper cellulase values. Over the same growth period, strain CB-12 yielded an increased cellobiase production without apparent influence by the decreasing pH values.

Although the two enzymes show a difference in heat lability (Fig. 2), the presence of cellobiose in the reaction mixture increased the stability of both enzymes. This could perhaps be explained by protection of the active groups of the enzymes by the substrate (4, 13).

The Michaelis-Menten constants indicate a higher cellobiose affinity for the strain CB-12

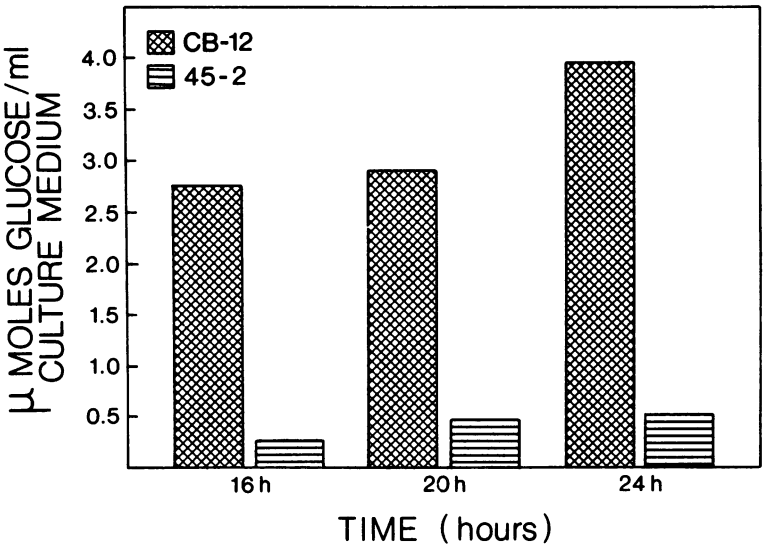

FIG. 3. Comparison of cellobiase biosynthesis in *S. flavogriseus* IAF-45 CD and *Streptomyces* sp. strain CB-12 grown in BM containing various concentrations of cellobiose (A) or glucose (B). Symbols: ■, *Streptomyces* sp. strain CB-12; ●, *S. flavogriseus* IAF-45 CD; ○, *S. flavogriseus* with pH control. Specific activity equals nanomoles per minute per milligram of protein.

enzyme than for that of *S. flavogriseus* IAF-45 CD. The K_m value of 2.56 mM for the former is comparable to the K_m value of 3.3 mM found by Inglin et al. (7) for partially purified β-glucosidase of *Trichoderma reesei*.

Comparing the growth patterns of the two strains, we can conclude that cellobiose represses the biosynthesis of the enzyme in *S. flavogriseus* and enhances it in *Streptomyces* sp. strain CB-12. Under similar conditions in vitro, 30 mM cellobiose (1%) in the reaction mixture does not inhibit cellobiase activity in either strain. The accumulation of glucose in the culture (Fig. 4) correlates well with results regarding the cello-

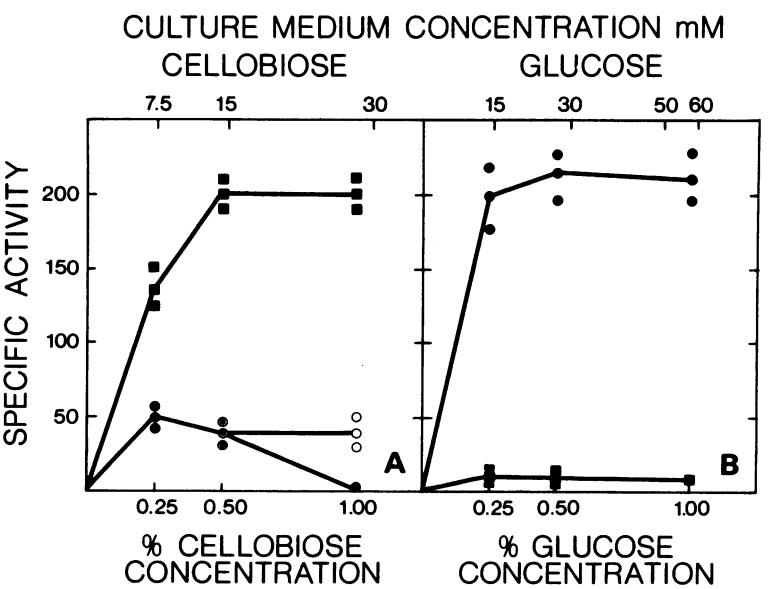

FIG. 4. Comparison between amounts of glucose released in the culture medium by *Streptomyces* sp. strain CB-12 and *S. flavogriseus* IAF-45 CD grown on 0.5% cellobiose.

TABLE 1. Cellobiase and filter paper cellulase activities in *S. flavogriseus* IAF-45 CD and *Streptomyces* sp. strain CB-12 as a function of pH and time of growth

Strain	Culture medium	Time of growth (h)	pH	Enzymatic activity	
				Cellobiase[a]	Filter paper cellulase[b]
IAF-45 CD	BM + 1% Avicel	24	6.98	280	0.083
		48	6.50	178	0.240
		72	5.90	109	0.333
IAF-45 CD	BM + 1% Avicel (with pH Control)	24	6.80	250	0.071
		48	6.80	238	0.383
		72	6.80	200	0.480
CB-12	BM + 2% cellobiose	24	6.60	87	0
		48	5.58	156	0
		72	5.24	329	0

[a] Nanomoles per minute per milligram of protein.
[b] Micromoles per minute per milliliter.

biase synthesis of the two strains grown in cellobiose as the substrate (Fig. 3A).

The results show that *Streptomyces* sp. strain CB-12 produces a cellobiase with relatively good acid and heat stability and is able to utilize cellobiose for the biosynthesis of this enzyme. *S. flavogriseus*, on the other hand, produces not only the endo- and exoglucanases of the cellulase complex (8) but also produces a β-glucosidase that is synthesized even in the presence of large quantities of glucose.

Thus, the combined action of the two *Streptomyces* species could render the microbial degradation of cellulose more efficient, preventing the accumulation of cellobiose in the medium and thus reducing the potential product inhibition on the enzyme complex. Such a combination could be achieved either by having the characteristics of the two enzymes expressed in one strain (obtained possibly by protoplast fusion) or by their coproduction in a mixed culture.

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