APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1984, p. 560–565
00099-2240/84/030560-06$02.00/0
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Vol. 47, No. 3

Production and Characterization of Cellulase and β-Glucosidase from a Mutant of *Alternaria alternata*

BASIL J. MACRIS

Department of Biology, Nuclear Research Center "Demokritos," Aghia Paraskevi, Attiki, Greece

Received 14 September 1983/Accepted 16 December 1983

A mutant of *Alternaria alternata* excreted enhanced levels of carboxymethylcellulase and particularly β-glucosidase when grown in cellulose liquid media. Both enzymes were purified two- to four-fold by ammonium sulfate precipitation and gel filtration, and the kinetic data showed K_m values of 16.64 mg/ml of culture fluid for carboxymethylcellulase and 0.14 mM *p*-nitrophenyl-β-D-glucoside and 0.81 mM cellobiose for β-glucosidase at pH 5. Carboxymethylcellulase and extracellular β-glucosidase functioned optimally at pH 5 to 6 and 4.5 to 5 and at temperatures of 55 to 60 and 70 to 75°C, respectively. Both temperature optima and thermostability of β-glucosidase were among the highest ever reported for the same enzyme excreted from cellulase and β-glucosidase hyperproducing microorganisms.

The hydrolysis of cellulose has been considered as a route for its conversion into food, feeds, fuel, and chemicals. Although enzymic hydrolysis of cellulose compares favorably to acid and alkali hydrolysis because enzymes are recoverable, specific, low in energy requirements, and nonpolluting, the cost of production and the low yield of cellulase are the major problems in the economics of the process and influence its application in the large-scale saccharification of cellulosic materials (24). Therefore, if enzymic hydrolysis of cellulose is to become an economically competitive method, these deficiencies must be overcome by either introducing more suitable microbial species or inducing mutant strains excreting higher levels of cellulases or both.

Among the thousands of fungal species growing on cellulose as a sole carbon source, only a limited number excrete cellulolytic enzymes (27). Members of the genus *Trichoderma* (1, 10, 13, 18, 22, 23, 27, 31, 34), *Penicillium* (4, 25) and the species *Aspergillus terreus* (8), *Fusarium* spp. (L. S. Trivedi and K. K. Rao, VI Int. Ferment. Symp., London, Ontario, Canada, abstr. no. F-13.4, 1980), *Phanerochaete chrysosporium* (5, 30), and *Sclerotium rolfsii* (24, 26) have been reported to elaborate high cellulase activities. *Trichoderma* spp. are the most intensively studied and favored microorganisms in this respect.

The present work was undertaken to investigate certain factors affecting production and activity of carboxymethylcellulase (CMCase) and β-glucosidase excreted at promising levels by a mutant strain of *Alternaria alternata*. The wild type of this fungus has been recently employed for the production of elevated levels of a thermostable β-galactosidase (17).

MATERIALS AND METHODS

Organism. A laboratory strain (wild type) of *A. alternata* (Fr.) Keissler was used. The stock culture was maintained on a sterile growth medium described below supplemented with 1% glucose, 1% cellulose 123, and 0.3% agar.

Chemicals. The chemicals used in this work were microcrystalline cellulose powder (Avicel; Macherey & Nagel, Düren, Federal Republic of Germany), cellulose powder 123 (Schleicher & Schüll, Dassel, Federal Republic of Germany), carboxymethylcellulose sodium salt (low viscosity; Sigma Chemical Co., St. Louis, Mo.), *p*-nitrophenyl-β-D-glucoside (pNPG) (Sigma), cellobiose (Fluka AG, Buchs SG, Switzerland), D-glucose (Riedel-De Haën AG, Hannover, Federal Republic of Germany), bacitracin, cytochrome *c*, egg albumin (Serva Finbiochemica, Heidelberg, Federal Republic of Germany), and pig heart transaminase (kindly provided by A. Evangelopoulos, National Research Institute, Athens, Greece).

Growth. The liquid growth medium contained (grams per liter): NaH_2PO_4, 12; KH_2PO_4, 2; $(NH_4)_2HPO_4$, 7; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.3; urea, 0.3; proteose peptone, 0.25; yeast extract, 0.1; Tween 80, 0.3. Also, the following trace elements were added (milligrams per liter): $FeSO_4 \cdot 7H_2O$, 5; $MnSO_4 \cdot 7H_2O$, 15; $ZnSO_4 \cdot 7H_2O$, 34; $CoCl_2$, 2. Glucose, at a concentration of 1% (wt/vol), was added to the growth medium. Also, wheat bran (2% [wt/vol]) supplemented the growth medium as specified. Before heat sterilization (121°C for 30 min) the medium was adjusted to pH 5 with 0.1 M H_3PO_4. An inoculum of 10^5 conidia (wild type) or mycelia (mutant M7) was transferred from the stock culture to 250-ml Erlenmeyer flasks containing 100 ml of sterile growth medium. Duplicates of flasks were incubated at 30°C in a rotary shaker operating at 200 rpm. At the end of day 2 of cultivation, when fungal growth was completed, dry-oven-sterilized (120°C for 30 min) cellulose 123 was added aseptically to growth medium at a concentration of 1% (wt/vol). After incubation the fungal biomass was harvested by centrifugation (5,000 × *g* for 10 min), and the clarified supernatant was used as the source of the enzymes.

Cell-bound levels of β-glucosidase were detected in the clarified cell-free extract. The extract was prepared by washing the harvested mycelium three times with distilled water and breaking it down in a chilled mortar with the aid of sand in 0.1 M acetate buffer, pH 5. The resulting extract was clarified by centrifugation at 12,000 × *g* for 15 min at 0°C.

Induction and selection of mutants. Mutants were induced by gamma radiation. A suspension of about 10^5 conidia per ml in 0.05 M phosphate buffer, pH 5, were irradiated in a gamma cell-type (Gammacell 220, Atomic Energy of Canada Ltd., Ottawa, Canada) ^{60}Co source with a dose rate of 2.5 krad/min. The amount of radiation given was 70 krad, resulting in a ca. 10% survival level. The irradiated conidia were plated on the growth medium described above supplemented with 0.3% agar and 0.5% cellulose 123 as the carbon source. Sodium dodecyl sulfate (0.08%) added to the growth

medium strongly restricted growth of the fungus. After incubation at 28°C for 6 to 7 days, the mutants were selected on the basis of their colony size. Of ca. 5,000 colonies inspected, 39 were selected. Further screening, based on β-glucosidase excretion, was done by growing the selected mutants for 5 days at 28°C in the liquid medium described above supplemented with 1% cellulose 123 as the sole carbon source. During the second screening, the mutants M7, M24, M32, and M36 excreted 4.9, 3.1, 3.6, and 2.8 times higher β-glucosidase than the wild type, respectively. Based on these results, the nonspore-forming mutant M7 was selected for further studies.

Enzyme purification. Solid $(NH_4)_2SO_4$ was added to clarified culture fluid to obtain 20% saturation. After centrifugation (10,000 × *g* for 10 min) the sediment was discarded, and ammonium sulfate was added to the supernatant to 80% saturation. The mixture was centrifuged for 10 min at 10,000 × *g*, and the precipitate was suspended in distilled water and freeze-dried.

Freeze-dried enzyme preparation was dissolved in 0.1 M acetate buffer, pH 5, and subjected to gel filtration for further purification and molecular weight determination of enzymes. The filtration was carried out at room temperature by using a column (2.5 by 90 cm) of Sephadex G-75 equilibrated with 0.1 M acetate buffer, pH 5. For determination of the molecular weight of β-glucosidase and CMCase, the method of Andrews (2) was employed with bacitracin, cytochrome *c*, egg albumin, and pig heart transaminase as molecular weight markers. The elution volumes of the markers were determined spectrophotometrically at 280 nm or at 410 nm for cytochrome *c*.

Enzyme assays and analytical methods. Avicelase (exo-1,4-β-glucanase, EC 3.2.1.74) and CMCase (endo-1,4-β-D-glucanase, EC 3.2.1.4) were assayed by measuring the reducing sugars as glucose by the dinitrosalicylic acid method (32) as described by Herr (13). The reaction mixture in the case of Avicelase contained 0.1 ml of clarified culture liquid and 0.4 ml of 1% (wt/vol) Avicel suspension in 0.1 M sodium acetate buffer, pH 5.0. In the case of CMCase, the reaction mixture contained 0.01 to 0.1 ml of clarified culture liquid made up to 0.5 ml with a 2.5% (wt/vol) low viscosity carboxymethylcellulose solution in 0.1 M sodium acetate buffer (pH 5.0). After incubation at 40°C for 60 and 10 min for Avicelase and CMCase, respectively, the unhydrolyzed Avicel was filtered off, and the reaction was stopped by the addition of 0.5 ml dinitrosalicylic acid. The tubes were then heated for 5 min in a boiling water bath and cooled for 5 min under running tap water; 2 ml of water was added, and the absorbance was measured at 546 nm. The sugar values were read from a glucose calibration curve. One unit of Avicelase or CMCase activity was defined as the amount of enzyme required to liberate 1 microequivalent of reducing sugars per min.

β-Glucosidase (EC 3.2.1.21) was measured by using pNPG as the substrate as described by Herr (13). The reaction mixture contained 0.01 to 0.1 ml of clarified culture liquid made up to 1 ml with 2 mM pNPG solution in 0.1 M acetate buffer, pH 5.0. The tubes were incubated at 40°C for 5 min, and the reaction mixture was stopped by the addition of 2 ml of 1 M Na_2CO_3 solution. One unit of β-glucosidase activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per min. When cellobiose was used as the substrate, conditions were as described above except that the reaction was stopped by boiling for 5 min. On cooling, the amount of glucose was determined with a glucose-oxidase-chromogen reagent (ISTV Sclavo, Divisione Diagnostici, Siena, Italy). One unit of β-glucosidase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of cellobiose per min.

Protein was determined by the method of Lowry et al. (15).

RESULTS AND DISCUSSION

In the present work CMCase and extracellular β-glucosidase, but not Avicelase, were produced by both wild-type and mutant strains of *A. alternata* grown on glucose as a sole carbon source (Fig. 1A). The pattern of excretion of both enzymes was similar to that observed in *Trichoderma viride* ITCC-1433 grown on glucose as the carbon source, but the reported yields were considerably lower than those found in the present experiment (13). Also, the parent and the mutant strains of the cellulase-hyperproducing fungus *S. rolfsii* excreted little or no cellulases when cultivated on glucose as a sole carbon source (24).

Supplementation of growth medium with 2% wheat bran resulted in the induction of Avicelase formation and in the

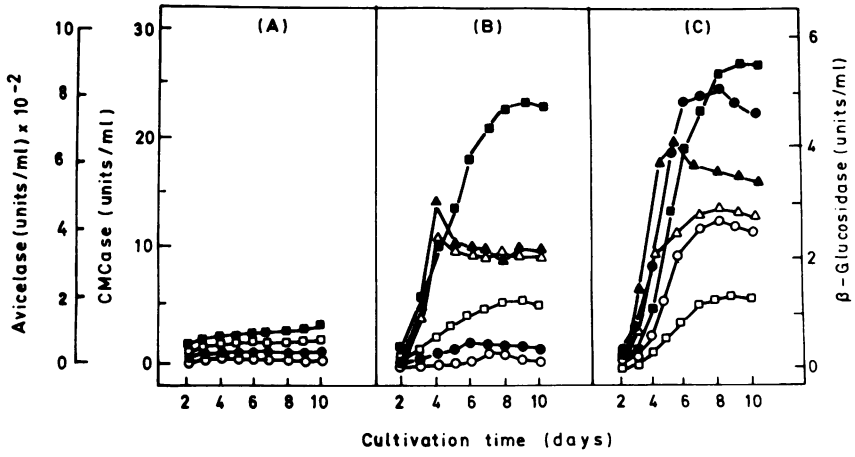

FIG. 1. Growth of wild-type and mutant (M7) strains of *A. alternata* on mineral medium supplemented with: (A) 1% (wt/vol) glucose, (B) 1% (wt/vol) glucose and 2% (wt/vol) wheat bran, and (C) 1% (wt/vol) glucose, 2% (wt/vol) wheat bran, and 1% (wt/vol) cellulose 123. Symbols: ○, wild-type CMCase; ●, M7 CMCase; □ wild-type β-glucosidase; ■, M7 β-glucosidase; △, wild-type Avicelase; ▲, M7 Avicelase.

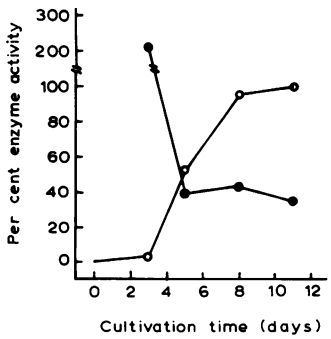

FIG. 2. Location of β-glucosidase during growth of *A. alternata* on mineral medium supplemented with 1% (wt/vol) glucose, 2% (wt/vol) wheat bran, and 1% (wt/vol) cellulose 123. The cell-bound enzyme activity was expressed as a percentage of that of extracellular β-glucosidase at each time interval measured. Symbols: ●, cell bound; ○, extracellular.

significant enhancement of β-glucosidase excretion (Fig. 1B). The latter enzyme was produced by the wild-type and mutant strains at levels which were three to six times higher than the respective values observed in the same medium without wheat bran (Fig. 1A). Significant improvement of cellulase yields with the addition of wheat or rice bran has been reported previously (26).

Maximum enhancement of all enzymes was obtained when cellulose 123 was added to growth medium (Fig. 1C). The latter compound is an alkali-treated cellulose, and it has been used for cellulase induction in *T. viride* (13) and growth enhancement of *Cellulomonas* bacteria (11). The yields of CMCase (25.5 U/ml) and β-glucosidase (5.4 U/ml) were ca. 20 and 280% higher, respectively, than the respective maximum yields of *T. viride* ITCC-1433 and higher than mutant strains QM 9123 and QM 9414 (from Natick Laboratories) of the same fungus (13, 19, 28).

β-Glucosidase has been reported to be a cell-bound enzyme in certain microorganisms (12, 16, 29). The proportion of cell-bound to extracellular β-glucosidase was studied in the mutant strain (Fig. 2). Enzyme levels in the culture fluid were lower than those of cell bound at the beginning of growth and increased thereafter. The pattern of β-glucosidase production by *A. alternata* is different from that reported for *Aspergillus niger* and *Aspergillus phoenicis* (29). It is worth mentioning that cell-bound β-glucosidase levels represent a significant proportion (35 to 40%) of the maximum yield of the extracellular enzyme.

Production of CMCase and β-glucosidase by *A. alternata* seems to be promising if one takes into consideration that relatively high yields were obtained from the beginning and that mutant selection in microorganisms may result in a very large enhancement of enzyme yield (6). Induction and selection of cellulase-hyperproducing mutants in microorganisms required many years of mutagenesis, and mutants excreting cellulases at levels even lower than those reported in this work continue to appear in the literature (7). The most improved mutant, Rut-C30 of *Trichoderma resei*, produced ca. 50% higher β-glucosidase and three to nine times higher CMCase than the mutant strain of the present work assayed at optimum conditions, but Rut-C30 was grown in a fermentor under strictly controlled conditions which greatly influenced enzyme production (31). We are improving enzyme yields through mutation and selection as well as optimization of growth conditions in the fermentor.

Table 1 presents the recovery of CMCase and β-glucosidase from the mutant strain in the course of purification studies. The protein precipitated in the range of 20 to 80% ammonium sulfate saturation contained most of the activity of both enzymes. Similar results were reported for the cellulase system of *A. terreus* (8). Subsequent gel filtration of the freeze-dried preparation of enzymes resulted in a distinct separation of the two enzymes (Fig. 3) with profiles which were quite different from those of *Talaromyces emersonii* cellulase complex submitted to gel filtration through Sephadex G-75 (20). The loss of enzyme activity during purification could be attributed to the fact that all purification steps were carried out at room temperature.

The apparent molecular weight of β-glucosidase was found to be 45,000 and was compared to those reported for other fungal species (35). CMCase had an apparent molecular weight of 13,000, which was significantly lower than that reported for other fungi (33).

The effect of pH on enzyme activity appears in Fig. 4. The optimum values of CMCase and β-glucosidase activities were at pH 5 to 6 and 4.5 to 5, respectively. Acidic pH optima were reported for both CMCase and β-glucosidase excreted from other fungi (8, 21). At optimum pH, the K_m and V_{max} values (correlation coefficient, 0.998) of CMCase were 16.64 mg/ml of culture fluid and 15.81 microequivalent per min per mg of protein, respectively. For β-glucosidase, the respective values with pNPG as the substrate were 0.14 mM and 4.41 μmol/min per mg of protein, whereas with cellobiose as the substrate these values were 0.81 mM and 2.44 μmol/min per mg of protein. For comparative purposes it is noted that *A. alternata* extracellular β-glucosidase exhibited about the same high affinity for pNPG as *T. viride* (14, 28), *A. phoenicis* (29), and *T. emersonii* (21) enzymes and a higher affinity for cellobiose than two of the three β-glucosidase forms of the latter fungus. Sternberg (28) has reported two β-glucosidase forms of *T. viride* QM 9414, one with a high (K_m, 1.5 mM) and one with a low (K_m, 50 mM) affinity for cellobiose.

CMCase and extracellular β-glucosidase-from *A. alternata* functioned optimally at 55 to 60 and 70 to 75°C, respectively (Fig. 5). Although the temperature optima of the former enzyme were within the range reported for mesophil-

TABLE 1. Flow sheet for purification of *A. alternata* M7 CMCase and β-glucosidase

Fraction	Volume (ml)		Activity (U/ml)		Sp. act.[a]		Yield (%)		Purification factor	
	CMCase	β-Glucosidase	CMCase	β-Glucosidase	CMCase	β-Glucosidase	CMCase	β-Glucosidase	CMCase	β-Glucosidase
Crude	145	145	19.3	2.5	21.4	2.8	100	100	1.0	1.0
$(NH_4)SO_4$ precipitation (20 to 80%)	30	30	74.9	8.7	44.0	5.1	80	72	2.0	1.8
Freeze-drying	66	66	29.6	3.5	23.3	2.7	47	63	1.1	1.0
Gel filtration (Sephadex)	92.7	49	3	1	46.8	11.9	10	13	2.2	4.2

[a] Expressed as units per milligram of protein (15).

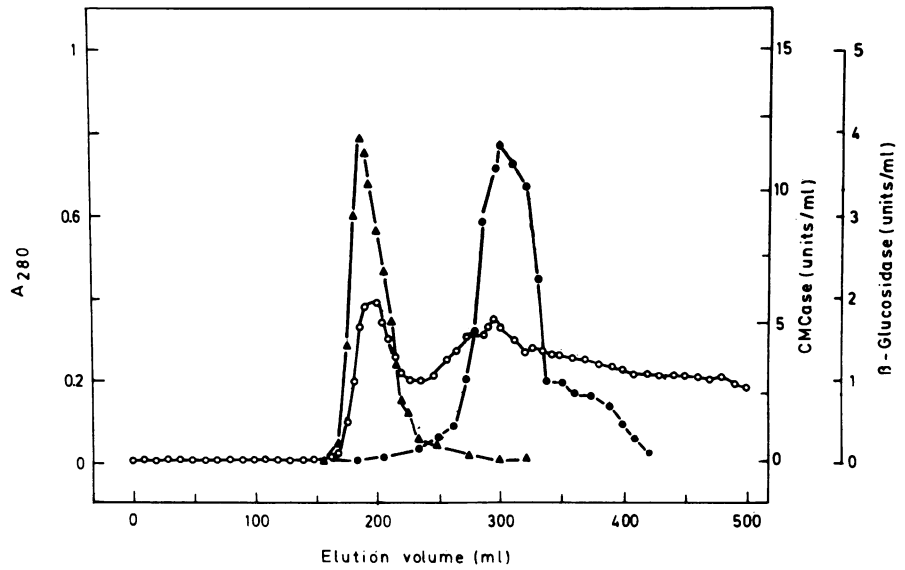

FIG. 3. Elution pattern of *A. alternata* M7 CMCase and β-glucosidase from a Sephadex G-75 column. The column was eluted with 0.1 M acetate buffer, pH 5, at room temperature. Flow rate, 20 ml/h; fraction volume, 5 ml. Symbols: ○, absorbance at 280 nm; ●, CMCase; ▲, β-glucosidase.

ic and thermophilic microorganisms (8, 9), the optima of β-glucosidase competed favorably with those reported for certain thermophilic cellulolytic microorganisms (9, 21). Arrhenius plot (correlation coefficient, 0.978) for the hydrolysis of pNPG by β-glucosidase over the range 30 to 75°C gave an energy of activation of 7,824 cal/mol (ca. 32,835 J/mol), which was about 1.5 to 4.5 times lower than those reported for the three β-glucosidase forms of *T. emersonii* (21). Apart from the high temperature optima of both enzymes and particularly of β-glucosidase, the thermostability of an enzyme is a very important property. Cellulolytic enzymes that are stable at high temperatures can be used in cellulose saccharification processes at elevated temperatures to protect both substrate and products of the enzymic reaction from microbial contamination and deterioration (9). The results reported in this work (Fig. 6) show that the extracellular β-glucosidase of *A. alternata* exhibited high thermostability properties. The half-life activities of the crude enzyme were 3.5 days, 1.8 h, and 10 min at 60, 65, and 70°C, respectively, whereas at the same temperatures the respective half-lives of the purified enzyme were 19.2 h, 1.1 h, and 7.8 min. Although the observed differences between the crude and the purified enzyme could be attributed to the different pH used (crude, pH 6.5; purified, pH 5) and to the presence of protective substances in the crude preparation, the above thermostability figures compare favorably with most reported in the literature. Except for the *T. emersonii* β-glucosidase forms, which had half-lives from 2 to 410 min at 70°C and pH 5 (21), the β-glucosidase of the present work was more thermostable than that of the thermophilus bacterium *Thermomonospora* sp., in which a maximum half-life of less than 1 h at 60°C was observed for the crude enzyme preparation (9). Also, *A. alternata* β-glucosidase exhibited considerably higher thermostability proper-

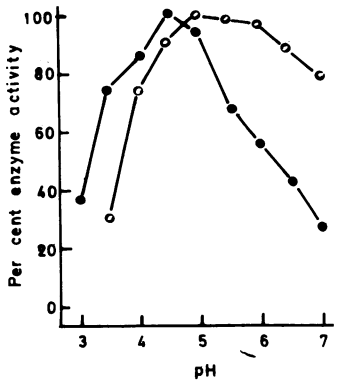

FIG. 4. Effect of pH on *A. alternata* M7 CMCase and β-glucosidase activities. The enzymes were assayed in 0.1 M acetate buffer in the pH range of 3 to 5 and in 0.1 M phosphate buffer in the pH range of 5.5 to 7, using pNPG as the substrate for β-glucosidase and conditions described in the text. Symbols: ○, CMCase; ●, β-glucosidase.

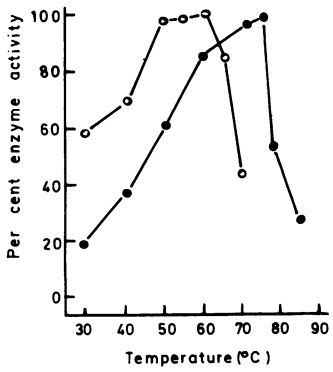

FIG. 5. Effect of temperature on *A. alternata* M7 CMCase and β-glucosidase activities. The reaction mixture was prepared by using pNPG as the substrate for β-glucosidase and heated at different temperatures for the periods of time described in the text. Symbols: ○, CMCase; ●, β-glucosidase.

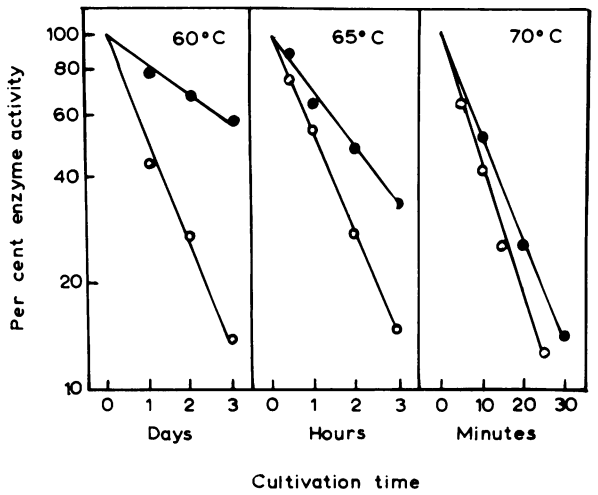

FIG. 6. Thermal stability of β-glucosidase excreted from *A. alternata*. The enzyme solution was heated at different temperatures, and the activity was assayed as described in the text and expressed as percentage of the appropriate zero time control. Symbols: ●, crude enzyme (pH 6.5); ○, purified enzyme in 0.1 M acetate buffer (pH 5).

ties than the same enzyme from *A. phoenicis* (3) and competed favorably with β-glucosidase excreted from *A. niger* (36).

In conclusion, *A. alternata* proved to be a potential candidate for the production of certain extracellular enzymes involved in the saccharification of cellulose with yields and properties which make this fungus worthy of further investigation.

ACKNOWLEDGMENT

This work was supported in part by grant no. 2933/Rb from the International Atomic Energy Agency.

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