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ISOLATION, PURIFICATION, AND ENZYMATIC ACTIVITY OF CELLULASE COMPONENTS OF THE FUNGUS *Aspergillus terreus*

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The accumulation dynamics of cellulolytic enzymes in culture media of the basidiomycete fungi Panus tigrinus, Pleurotus ostreatus, Fomes fomentarus, *and the micromycete* Aspergillus terreus *were studied during a long incubation period. It was found that* A. terreus *was the most active producer of cellulolytic enzymes among the studied fungi. Two protein fractions with cellulase activity were isolated using gel filtration and ion-exchange chromatography. PAAG electrophoresis showed that fraction-I consisted of four components; fraction-II, an electrophoretically homogeneous protein.*

Key words: cellulase, endoglucanase activity, chromatography, *Aspergillus terreus*, electrophoresis.

It is known that cellulolytic enzymes are produced by various microorganisms belonging to various taxonomic groups [1, 2]. Cellulases have broad applications and are used mainly in food, textile, chemical, medical, pharmaceutical, paper, and wood processing industries. They are also added to animal feed and to agricultural process wastes [3-6].

The goal of our work was to study the ability of wood-destroying fungi to produce highly active cellulases and to produce active preparations of cellulolytic enzymes.

The accumulation dynamics of cellulolytic enzymes in culture media of the fungi *Panus tigrinus*, *Pleurotus ostreatus*, *Fomes fomentarus*, and *Aspergillus terreus* were determined during different periods of the growth and development, from 7 to 14 days.

The results show that *A. terreus* was the best producer of cellulase compared with the other fungi. The maximum endoglucanase activity was observed 10 days from the start of growth. Therefore, 10-day culture liquid of *A. terreus* was first filtered through glass wool and then through a millipore filter (0.2 μm pore size) to separate the supernatant from the cell culture in order to isolate enzyme components of the cellulase complex. Cellulase activity in solution was determined by the Wood and Bhat method [7] using Na-CMC (1%) as substrate. The solution (220 mL) contained 106.1 units of total cellulase activity (Table 1).

The protein concentration determined by the Lowry method [8] indicated 154 mg of protein in the collected culture medium from *A. terreus*. The specific activity of the cellulolytic enzyme in the culture medium was 0.688 U/mg of protein. Then the culture medium was concentrated in a rotary evaporator to 17 mL. The total cellulase activity and protein concentration in the concentrated enzyme solution was 83.38 U and 108.56 mg, respectively. Analysis of the proteins by SDS-PAAG electrophoresis showed nine protein bands with molecular weights from 17 to 120 kDa in the concentrated enzyme solution.

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TABLE 1. Stages of Cellulase Isolation from Culture Medium of *A. terreus*

Purification stage	Total activity, U/mL	Total protein, mg	Specific activity, U/mg protein	Purification degree	Reduction activity, %
Culture medium	106.1/220	154	0.688	-	100
Rotary evaporation	83.38/17	108.56	0.768	1.12	78.59
Sephadex G-10 (desalting)	66.47/150	63.38	1.048	1.52	62.64
Drying	64.54/456.2	63.38	1.018	1.48	60.83
TSK HW-55 (gel filtration)	59.82/340	35.12	1.70	2.47	56.38
Drying	56.86/	35.12	1.62	2.35	53.59
DEAE TSK HW 650 (ion-exchange chromatography)					
Cellulase-I	3.01/10	3.41	0.88	1.28	2.83
Cellulase-II	24.03/80	0.96	25.03	36.38	22.65

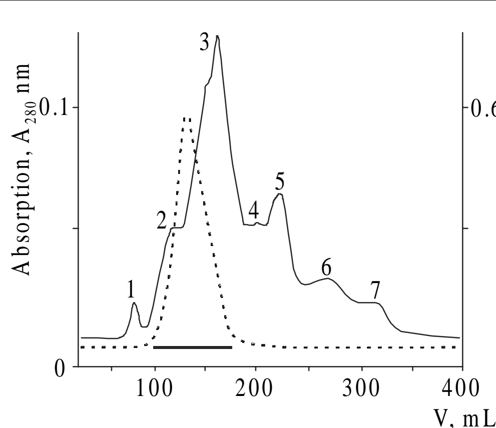

Fig. 1

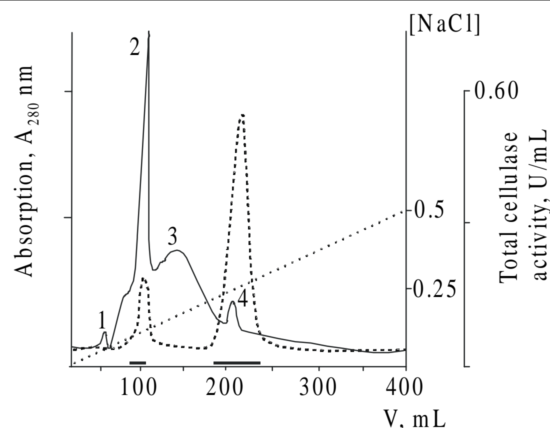

Fig. 2

Fig. 1. Gel filtration of a solution of cellulolytic enzymes over a column of TSK HW-55f gel.

Fig. 2. Ion-exchange chromatography of the active fraction over a column of DEAE-TSK HW-650S gel.

The cellulolytic enzymes of the concentrated enzyme solution were isolated and purified first by desalting over a column of Sephadex G-10. Fractions containing cellulolytic activity (150 mL) were collected and dried. It was observed that the protein solution (63.38 mg) contained 64.54 units of total cellulase activity. Thus, the specific activity of the resulting enzymatic preparation was 1.018 U/mg of protein after desalting. The desalted enzyme mass had 60% of the initial endoglucanase activity (Table 1).

Lyophilized powder was dissolved in sodium acetate buffer (0.01 M, pH 4.9) to isolate the enzyme. Gel filtration over a column of TSK HW-55f gave the elution profile shown in Fig. 1.

The separation produced seven protein fractions. Protein fractions containing cellulase activity were combined (59.82 U/340 mL). The combined fraction contained 35.12 mg of protein with specific cellulase activity 1.70 U/mg. The degree of purification was 2.47 times.

Further purification used ion-exchange chromatography over a column of DEAE-TSK HW-650S gel that was equilibrated beforehand with sodium acetate buffer (0.01 M, pH 4.9) with elution by a linear gradient of NaCl (0-0.5 M) at a flow rate of 30 mL/h. Figure 2 shows the elution profile of the proteins.

Four protein fractions were eluted by the NaCl gradient. Of these, only two had cellulase activity. Fraction-I contained 3.41 mg of protein in 10 mL and had 3.01 U of cellulase activity. Active fraction-II had 24.03 U of cellulase activity (in 80 mL) and contained 0.96 mg of protein. Thus, the specific activity of this fraction was 25.03 U/mg of protein.

Electrophoresis of the proteins in PAAG (7.5%) showed that fraction-I contained four protein components with molecular weights 17, 30, 35, and 37 kDa. Fraction-II was an electrophoretically homogeneous protein with molecular weight 30 kDa (Fig. 3).

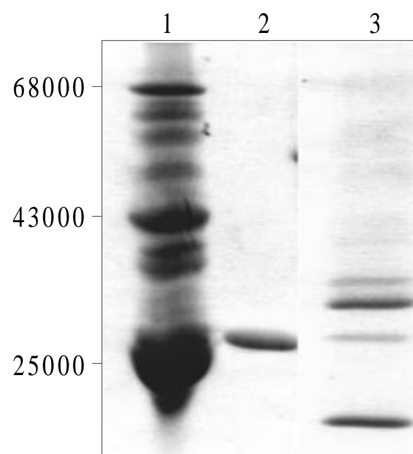

Fig. 3. Electrophoresis of active fractions after ion-exchange chromatography: standard markers (1), cellulase-II (2), cellulase-I (3).

Thus, the most active producer of cellulolytic enzymes among the fungi *P. tigrinus*, *P. ostreatus*, *F. fomentarus*, and *A. terreus* was the last. The maximum content of cellulolytic enzymes was observed after 10 days of growth in culture medium with birch chips. The optimum method for isolating cellulase enzymes from *A. terreus* was developed. Two highly active enzymes were isolated. These were celulase-I and cellulase-II with specific activities of 0.88 and 25.03 U/mg of protein, respectively.

EXPERIMENTAL

Sephadex G-10 (Pharmacia, Sweden), TSK HW-55f, DEAE-TSK HW-650S (Toyo Soda Company, Japan), and PAAG were prepared by the literature method [9]. The fungi *P. tigrinus*, *P. ostreatus*, *F. fomentarus*, and *A. terreus* were taken from the culture collection of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan. All other reagents were analytically pure and obtained from Sigma Chemical Company.

These fungi were kept on slanted malt-agar gel. Analytical samples were taken every 12 h. Fungi were cultivated using medium containing plant wastes such as pulp from cotton plants and birch chips of various (2-5%) concentration and particle sizes with added 7% malt (5%). Fungi were cultivated in Ehrlenmeyer flasks (750 mL) with growth medium (200 mL) and grown on a rocker at 240 rpm at 30°C for 3-21 d.

Endoglucanase activity was determined by the Wood and Bhat method [7] as follows. Culture medium (0.1 mL) or enzyme samples was treated with Na-CMC (1%, 1 mL) and incubated for 30 min at 40°C. After incubation, the mixture was treated with Samoji reagent (2 mL), boiled on a water bath for 15 min at 100°C, cooled, and treated with Nelson reagent (2 mL) and distilled water (4 mL). The extinction coefficient of the mixture was measured spectrophotometrically at 520 nm in UV light. The standard was glucose. Enzyme activity was calculated from a calibration curve constructed using glucose.

Protein was determined by the Lowry method [8] using bovine serum albumin as a standard.

Culture medium with high endoglucanase acivity was filtered through a millipore filter (220 mL) and concentrated in a rotary evaporator at 25°C to a volume of 17 mL.

Desalting. Concentrated culture medium (17 mL) containing protein (108.56 mg) was placed on a column of Sephadex G-10 (2.6 × 75 cm) and eluted with distilled water at a flow rate of 30 mL/h. The optical density of the eluted proteins was measured at 280 nm with a sensitivity of 0.1. Active fractions were combined and lyophilized.

Gel Filtration. Lyophilized enzyme was dissolved in sodium acetate buffer (5 mL, 0.01 M, pH 4.9), placed on a column (1.6 × 125 cm) of TSK HW-55f gel equilibrated with the same buffer, and eluted at flow rate 45 mL/h. The optical density of the eluted proteins was measured at 280 nm with a sensitivity of 0.1.

Ion-exchange Chromatography. The active fraction (340 mL) obtained after gel filtration was placed without drying on a column of DEAE-TSK HW-650S (1.6 × 15 cm) equilibrated with sodium acetate buffer (0.01 M, pH 4.9). The column

was eluted first with the aforementioned buffer and then with a linearly increasing gradient of NaCl (0-0.5 M) at 30 mL/h. The optical density of the eluted proteins was measured at 280 nm with a sensitivity of 0.1.

Electrophoresis. Electrophoresis was performed by the literature method [9].

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