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*(From the Quartermaster Research and Development Laboratories, Philadelphia)*

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## A CELLULOLYTIC ENZYME PREPARATION FROM MYROTHECIUM VERRUCARIA

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Cellulolytic enzyme preparations active against various forms of cellulose have been reported by a number of investigators who have used a variety of different sources (8). Fungi which have been employed for the preparation of such extracts are *Sporotrichum carnis* (15), *Aspergillus oryzae* (3, 2), *Merulius lachrymans* (9), and *Coniophora cerebella* (7), while bacteria have included *Bacterium protozoides* (5), *Bacterium bosporum* (5), *Cellulobacillus myxogenes* (14), *Cellulobacillus mucosus* (14), *Plectridium cellulolyticum* (10), several species of *Cytophaga* (1), and various thermophilic cellulose-decomposing bacteria (11). In addition, active cellulolytic extracts have been prepared from the intestinal juice of the snail *Helix pomatia* (6), from green malt (12), and from the protozoon *Eudiplodinium neglectum* which was isolated from cattle rumen (4). Although the data presented by several of these investigators are certainly suggestive of true cellulolytic enzyme activity, nevertheless these workers in general have not conclusively established the cell-free nature of their preparations. This is of considerable importance in view of failure of other investigators to obtain cell-free cellulolytic enzymatic preparation from fungi. Questions were raised as to the possibility of bacterial or fungal contamination during the period (usually several days or more) of testing for cellulolytic activity.

The present paper describes the preparation and some of the properties of a cell-free cellulolytic enzyme from *Myrothecium verrucaria*, which has been shown to be one of the most active of the cellulose-destroying fungi isolated from deteriorated fabrics (16).

### EXPERIMENTAL

*Preparation of Enzymatic Extract*—300 gm. of filter paper (E and D No. 615) were ground through a 40 mesh sieve in a Wiley mill and autoclaved in a 5 gallon Pyrex bottle, to which were added 10 liters of a sterile mineral solution of the composition shown in Table I. After cooling, the flask was inoculated with 100 ml. of a spore suspension of *Myrothecium verrucaria*, United States Department of Agriculture No. 1334.2, containing about

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80,000 spores per ml., from a 3 week culture of the fungus grown on filter paper. Sterile aeration was provided by passing the air through a water tower and a cotton filter at a rate of about 500 ml. per minute. At the same time, the bottle was shaken at a rate of about fifteen rotary cycles per minute in order to keep the ground filter paper in suspension. The entire set-up was kept in a room maintained at 30°. After 10 days incubation the cellulose-mycelium mixture was filtered on a Büchner funnel, and the yellow filtrate containing the bulk of the cellulolytic activity was stored at 5° in the presence of toluene as an antiseptic. No appreciable loss of activity was observed over a period of several months.

#### Testing Method for Cellulolytic Activity

An index of cellulolytic activity of the different preparations was obtained by the amount of reducing substances formed from cellulose under

TABLE I  
Composition of Mineral Solution

	gm.		mg.
KH <sub>2</sub> PO <sub>4</sub>	0.20	Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> ·6H <sub>2</sub> O	0.054
K <sub>2</sub> HPO <sub>4</sub>	0.15	(NH <sub>4</sub> )P(Mo <sub>3</sub> O <sub>10</sub> ) <sub>4</sub>	0.024
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	2.00	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.050
Na <sub>2</sub> HPO <sub>4</sub>	1.50	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.0025
NH <sub>4</sub> NO <sub>3</sub>	0.60	MnSO <sub>4</sub>	0.0055
NaNO <sub>3</sub>	3.80	H <sub>3</sub> BO <sub>3</sub>	0.057
			ml.
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.30	H <sub>2</sub> O	1000

standard conditions. In the test, 5 ml. of the dialyzed test solution were added to 5 ml. of a 0.05 M phosphate buffer (pH 5.0) and 100 mg. of de-waxed cotton, which had been pulverized in a ball mill for 24 hours. 5 drops of toluene were added as an antiseptic to the mixture in a 125 ml. Erlenmeyer flask. After incubation for 4 days at 30°, the mixture was filtered and the amount of reducing sugars in the filtrate was determined by titration (13).

In order to insure that the action of the cellulolytic enzymes and not of contaminating microorganisms is determined by the method, aliquot samples of the enzyme solution were tested with and without sterilization by Berkefeld filtration, as well as with and without the addition of fungal spores to the test flasks. The results given in Table II clearly show that the conversion of cellulose into reducing sugars is not brought about by contaminating organisms.

The percentage of cellulose breakdown, when cellophane and pulverized

filter paper and cotton were used as substrates, was also determined in several experiments by loss in weight of cellulose, and the values so obtained checked within 20 per cent with those obtained by titration, assuming glucose to be the product of the enzymatic hydrolysis.

#### Properties of Enzyme Preparation

*Extracellular Nature*—Experiments were conducted to determine whether the cellulolytic enzyme (or enzymes) was intra- or extracellular in nature.

TABLE II  
Cell-Free Nature of Cellulolytic Preparations

Treatment of enzyme preparation	Spores added	Toluene added	Cellulose breakdown*
		ml.	per cent
Untreated	0	1.0	11.8
	+	1.0	12.8
Berkefeld filter	0	0	9.0
	0	1.0	10.5
Control		0	0
		1.0	0

\* Calculated from the reducing sugar formed, assuming glucose to be the only sugar present.

TABLE III  
Cellulolytic Activity of Metabolic Filtrate and Mycelial Extract of *Myrothecium verrucaria*

Substrate	Per cent cellulose breakdown		
	Metabolic filtrate (a)	Mycelial extract (b)	(a) + (b)
Ground cellophane	28.0	6.0	23.0
Pulverized filter paper	8.0	3.0	9.0
Xylan	14.5	6.0	

*Myrothecium verrucaria* was cultured for 5 days on ground filter paper in the manner described above. The metabolic mixture was filtered, giving a clear yellowish filtrate and a residue of mycelium and undigested cellulose. The residue was ground with sand in a volume of culture medium equivalent to the filtrate originally present. A third test solution was made through a combination of the first two. The three solutions were dialyzed against distilled water for 24 hours in cellophane tubing and tested for cellulolytic activity with different substrates. Results of the 5 day test are given in Table III. The figures obtained strongly suggest that the

cellulolytic activity is present in the filtrate and not in the mycelium. The low activity in the so called "mycelial extract" may be due to the filtrate absorbed by the residue of mycelium plus undigested cellulose.

*Temperature Stability and Optimum*—In testing for heat stability, aliquot portions of the same preparation at pH 6.0 were kept for 10 minutes at different temperatures and then tested for activity. Results are given in Table IV, which indicate that 50° is the threshold value at which inactivation begins.

TABLE IV  
*Heat Stability of Cellulolytic Preparation*

Temperature (10 min. standing)	Cellulose breakdown
°C.	per cent
22	13.1
30	13.2
40	13.5
50	8.4
60	2.7
70	0.0

TABLE V  
*Temperature Optimum of Cellulolytic Preparation*

Incubating temperature	Cellulose breakdown
°C.	per cent
0	4.7
3	6.2
25	13.9
31	13.9
40	16.0
50	4.2

The optimum temperature for enzymatic action was studied by incubating the enzyme-buffer-pulverized filter paper mixture for 4 days and determining the percentage conversion of cellulose into reducing sugars. The results show an optimum around 40° (Table V).

*pH Stability and Optimum*—The stability of the crude enzymatic solution was also studied. Different solutions were adjusted to their respective pH values with dilute hydrochloric acid and sodium hydroxide, allowed to stand for 48 hours, readjusted to pH 5.0, then assayed for cellulolytic activity in the usual manner. The results of the experiment, given in Table VI, show rapid inactivation beyond the limits of pH 5.0 and 9.0.

A series of 0.05 M phosphate buffers of pH 4.0, 5.0, 6.0, and 7.0 was prepared to determine the pH at which the greatest rate of cellulolytic action takes place. Various enzymatic aliquot solutions were adjusted with phosphoric acid and sodium hydroxide to the same respective values. The cellulolytic activity at these various pH values was tested against the pulverized dewaxed cotton in the usual way. The results in Table VII indicate a pH optimum of 5.5.

TABLE VI  
*Stability of Enzymatic Preparation at Different pH Values*

pH (48 hrs. standing)	Cellulose breakdown
	per cent
1.0	0.0
3.0	2.7
5.0	8.5
7.0	11.6
9.0	8.4
11.0	0.0
13.0	0.0

TABLE VII  
*Optimum pH of Cellulolytic Enzymes*

pH	Cellulose breakdown
	per cent
4.0	8.5
5.0	14.3
6.0	14.2
7.0	6.9

*Concentration Curve*—Dialyzed metabolic filtrate from *Myrothecium verrucaria* grown on ground filter paper was concentrated at 20 mm. mercury vacuum at a bath temperature of 40–50° for 6 hours to one-fiftieth of its original volume. Different dilutions of this concentrate were tested for their cellulolytic rates against pulverized filter paper. The results are plotted in Fig. 1.

*Product of Enzymatic Breakdown*—250 ml. of the enzymatic preparation and 250 ml. of 0.05 M phosphate buffer, pH 5.0, were added to 5.0 gm. of pulverized dewaxed cotton. After the addition of 10 ml. of toluene as an antiseptic the stoppered Erlenmeyer flask was incubated for 14 days at 30° with daily gentle shaking. Shaffer-Somogyi titration of the metabolic filtrate at the end of the incubation period showed a conversion of 22.9 per

cent of the cellulose into reducing sugars, calculated as glucose. 100 ml. of the filtrate, containing 250 mg. of glucose according to the titration, were concentrated *in vacuo* to 7 ml. The osazone was prepared with a yield of 215 mg., and after two recrystallizations from 50 per cent ethanol, melted at 201–203°.<sup>1</sup> A simultaneously prepared glucosazone showed a melting point of 200–202° with a mixed melting point of 197–201°. Cellobiosazone gave a value of 196–198° and a mixed melting point of 180–185°. These data clearly indicate that glucose is one of the main products from the enzymatic breakdown of cellulose.

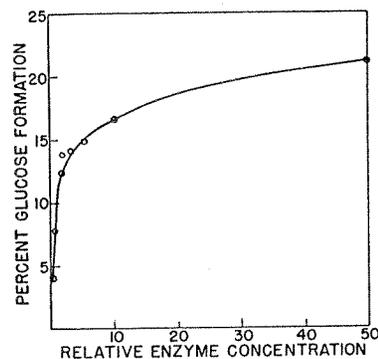


FIG. 1. Effect of enzyme concentration on rate of cellulolytic action

#### SUMMARY

A method has been described for the preparation of a cell-free enzymatic preparation from the fungus, *Myrothecium verrucaria*, capable of degrading cellulose. The cellulolytic enzyme (or enzymes) was extracellular, with an optimum temperature of 40° and an optimum pH of 5.5 when acting on cotton. It is rapidly inactivated at temperatures above 50° and at values beyond the limits of pH 5.0 and 9.0. The main breakdown product from cellulose was shown to be glucose.

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<sup>1</sup> All melting points were taken in capillary tubes and are uncorrected.