THE EFFECT OF TEMPERATURE, HYDROGEN ION AND CERTAIN GROWTH SUBSTRATES ON THE ACTIVITY OF THE CELLULOLYTIC ENZYME COMPLEX IN SELECTED MARINE FUNGI

JAMES R. JENSEN

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by

James Robert Jensen

A Thesis Submitted to the Faculty of the
College of Science
In Partial Fulfillment of the Requirements for the
Degree of Master of Science

Florida Atlantic University
Boca Raton, Florida
June 1971

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. P. L. Sguros, Department of Biological Sciences. It was submitted to the Faculty of the College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Halosphaeria mediosetigera and Culcitalna achraspora have been studied to elucidate the mechanism by which they degrade cellulosic materials in the sea. H. mediosetigera (700 LC_1), a C_1-less variant, was also employed in this work. Standardized shake-cultures were grown on cellulose and cellulose derivatives supplemented with NH_4NO_3, tris(hydroxymethyl)aminomethane and yeast extract in artificial sea water (Lyman and Fleming), pH 7.5, at 25 C. Induced C_1 and C_x enzyme and cellobiase activities were determined colorimetrically by correlation with cotton fiber weight loss, by the formation of reducing sugars from carboxymethylcellulose and by Glucostat, respectively. Optimum pH values for both C_x enzyme and cellobiase of C. achraspora were 6.0 at 37 C while those for H. mediosetigera (700 LC_1) were 5.0 and 6.0, respectively, at 37 C. In both cultures, C_x enzymes had a variable optimum temperature (37-50 C) at pH 6.0, depending on the length of the incubation period, while cellobiases had the same optimum temperature (50 C) at pH 6.0. Thermostability observations on C_x enzyme and cellobiase of C. achraspora showed almost complete deactivation at 100 C in 15 min for the former and 50 C in 15 min for the latter. Thermostability observations on C_x enzyme and cellobiase of H. mediosetigera (700 LC_1) showed almost complete deactivation at 45 C in 15 min for the former and at 50 C in 15 min for the latter. Data indicate that at least three enzymatic functions are involved in cellulose breakdown by these cultures.

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INTRODUCTION

Cellulolytic microorganisms are involved in the destruction of cellulose resulting in extensive damage to raw materials and endproducts of wood, cotton and other natural textile fiber manufacture. However, the harmful effects produced by these organisms is small in comparison to their beneficial and, in fact, vital role as regulators of the carbon dioxide equilibrium in nature. Approximately 3×10^{10} tons of carbon in the form of carbon dioxide are transformed yearly into plant materials over the earth and about one third of the organic material produced is cellulose. If the microbial degradation of cellulose did not continuously occur, atmospheric carbon dioxide would not be consumed by new plant growth and the accumulation of dead vegetation would suppress new production (Norkrans, 1966). The earth's biota would suffer drastically from the effects of increased temperature and disrupted gas ratios in the atmosphere, as well as from decreased plant production.

While bacteria are also involved, fungal genera appear to dominate in the process of cellulose biodegradation. Until recently, no comprehensive study concerning fungal decomposition of cellulose had been carried out. An early study by White et al. (1948) measured the cellulose decomposition capacities of a large number of fungal strains. Later, Marsh and Bollenbacher (1949) examined the cellulolytic activity of more than 400 fungal isolates and showed that cellulolytic activity was widely distributed, but not universal, among the fungi in general. Reese, Siu and Levinson (1950) were the first to determine the cellulolytic activities of culture filtrates obtained from selected fungi grown on media containing carboxymethylcellulose (CMC) and other cellulose derivatives. All of these studies were focused on terrestrial fungal forms.

In recent years, increasing interest has been shown in the role of filamentous marine fungi in cellulolytic degradative processes. Barghoorn and Linder (1944) first recognized the presence of an abundant fungal mycota on submerged wood. Subsequently, Meyers and Scott (1968) demonstrated the infestation of submerged wood and other cellulosic substrates by a diverse biota of ascomycetous and deuteromycetous marine fungi including the Deuteromycete, Culcitalna achraspora, and the Ascomycete, Halosphaeria mediosetigera. The former has been found in temperate marine areas and the latter is prevalent in northern-to-temperate waters (Jones, 1968). Meyers and Reynolds (1959) suggested that marine fungal infestation of wood begins in winter producing a weakening of the wood which then allows a spring invasion by marine wood-boring worms and clams. Since these fungi can utilize large cellulose molecules as a carbon source, extracellular enzymes capable of converting cellulose to soluble sugars should be expected in the culture medium (Cochrane, 1958). Culture filtrates from cellulolytic marine fungi containing such "cellulases" have been prepared (Meyers and Scott, 1968).

Previous studies on terrestrial fungi have shown that a specific pattern of attack was usually involved in the hydrolysis of cellulose to soluble sugars (Fig. 1). At least three different types of enzymes were believed to be involved and were regarded as a complex. The first, called "C_1 enzyme" by Reese et al. (1950), was suggested as responsible for the initial step in the breakdown of native cellulose. This mechanism provided the capacity to initiate the attack on insoluble, highly-ordered native cellulose altering it to a form susceptible to the next enzyme in the complex.

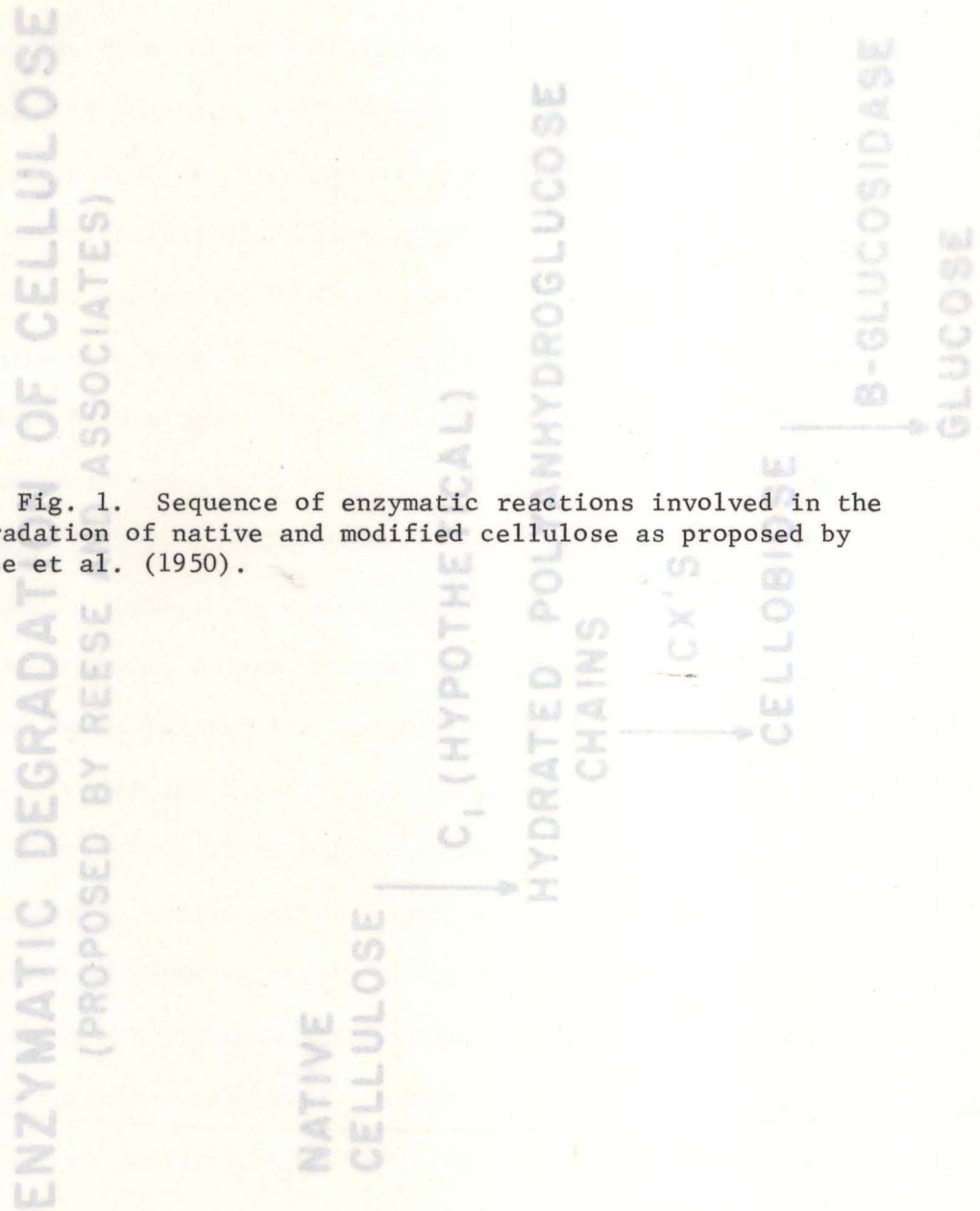
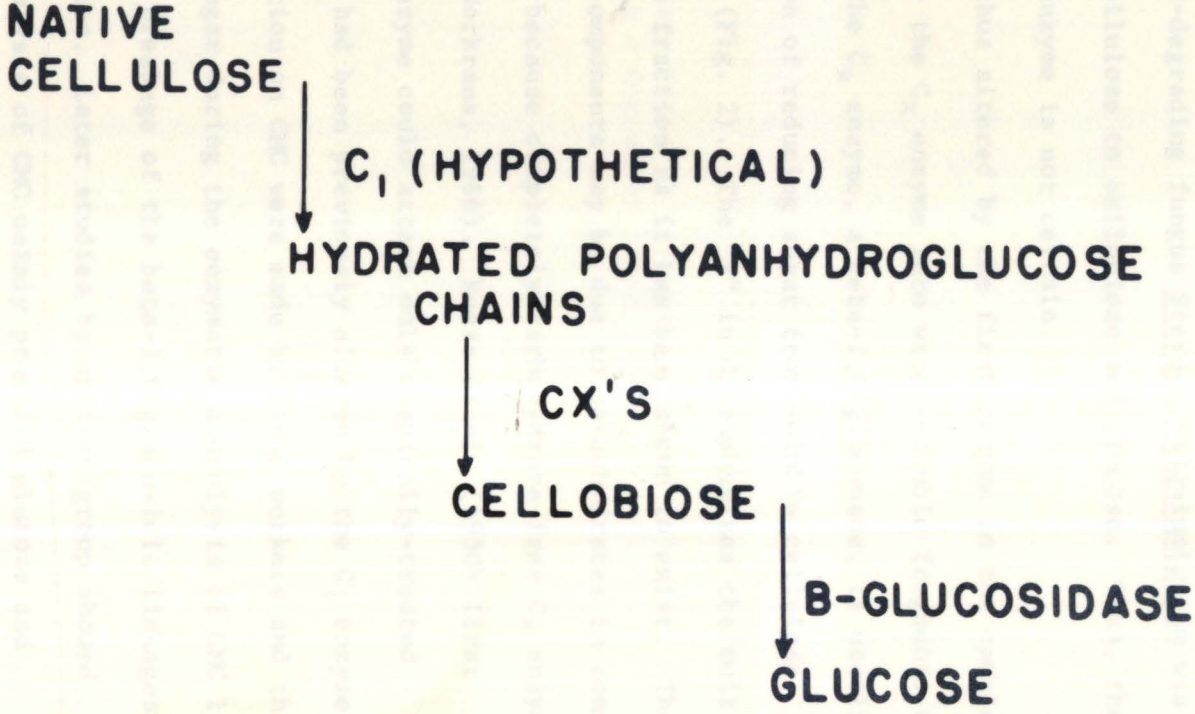


Fig. 1. Sequence of enzymatic reactions involved in the degradation of native and modified cellulose as proposed by Reese et al. (1950).

ENZYMATIC DEGRADATION OF CELLULOSE

(PROPOSED BY REESE AND ASSOCIATES)



Many studies have shown the existence of a C_1 enzyme. Selby and Maitland (1965, 1967), studying Trichoderma viride and Myrothecium verrucaria, and Wood (1968), using Trichoderma koningii, claimed to have completely separated a "C_1 factor" from the hydrolytic fraction of the complex. Bjoerndal and Eriksson (1968), however, claimed that only one enzyme from the wood-degrading fungus Stereum sanguinolentum was employed in degrading cellulose to cellobiose and glucose. Thus, the universality of the C_1 enzyme is not certain.

Native cellulose, thus altered by the first enzyme in the complex, can now be hydrolyzed by the C_x enzyme into water-soluble fragments of low molecular weight. The C_x enzyme, a beta-1,4-glucanase, is usually measured by the formation of reducing sugar from soluble cellulose derivatives such as CMC (Fig. 2). The "x" in C_x emphasizes the multi-component nature of this fraction as it has been shown to exist. These physically distinct C_x components may be due to carbohydrates in complex with the C_x enzyme because completely carbohydrate-free C_x enzyme has not been isolated (Norkrans, 1966). Reese et al. (1950) first suggested that the C_x enzyme could attack only chemically-treated cellulose or that which had been previously altered by the C_1 enzyme. Studies of C_x enzyme action on CMC were made by these workers and the formation of reducing sugar during the enzymatic hydrolysis of CMC led to the idea of a random breakage of the beta-1,4-glucosidic linkages in the cellulose molecules. Later studies by another group showed that the enzymatic hydrolysis of CMC mainly produced glucose and cellobiose along with some higher saccharides (Sison, Schubert and Nord, 1958).

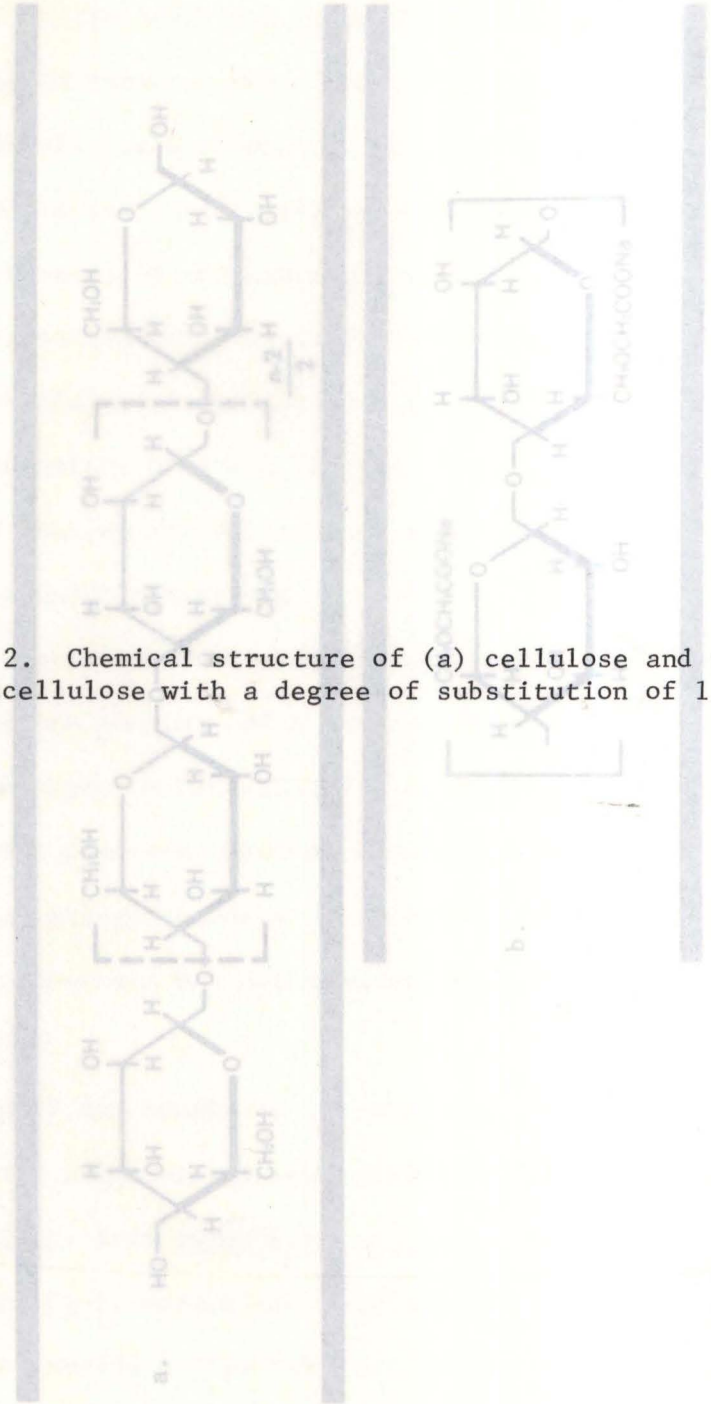
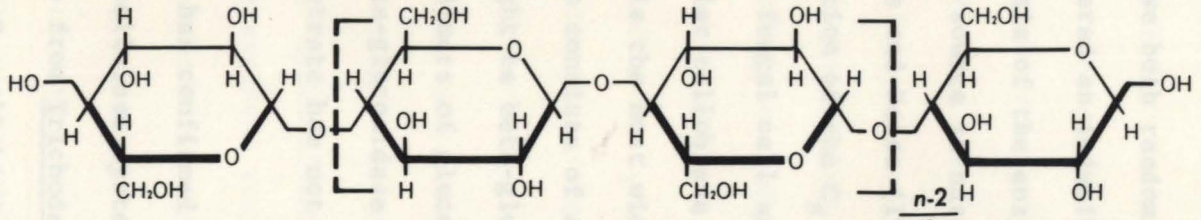
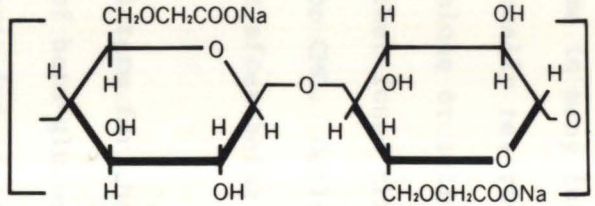


Fig. 2. Chemical structure of (a) cellulose and (b) carboxymethylcellulose with a degree of substitution of 1.0.

a.



b.



Recently, King and Vessel (1969) reported that the C_x fraction is composed of two types of beta-1,4-glucanases, "exo" and "endo." The exo-beta-1,4-glucanase removes single glucose units from the non-reducing end of the cellulose polymer while the endo-beta-1,4-glucanase acts in a random manner. Thus the C_x enzyme component of the cellulase system may involve both random and terminal types of action. The C_x enzyme is considered an inducible enzyme in many fungi (Basu and Ghose, 1960) and synthesis of the enzyme is usually restricted to situations where the carbon source is native cellulose or cellulose derivatives. Levinson, Mandels and Reese (1951) further found that cellobiose was formed by the action of the C_x enzyme on CMC. Cellobiose could then either enter the fungal cell as such or after being converted to glucose by an extracellular cellobiase.

Cellobiase is the most widely used term for the final member of the complex which consists of a group of beta-glucosidases. A more accurate name might be beta-glucodimerase, indicating the ability to act on all beta dimers of glucose (King and Vessel, 1969). The preparation of a beta-glucosidase of such specificity that only cellobiose can act as a substrate has not been achieved (Gascoigne and Gascoigne, 1960).

Recent work has confirmed the importance of these enzymes functioning together in the cellulase system. Selby and Maitland (1967) fractionated culture filtrates from Trichoderma viride, a terrestrial fungus, and found that the C_1-C_x-cellobiase complex was essential for the complete degradation of native cotton fibers. These components, which together

converted cotton into water-soluble products, lost this ability when separated, but regained it quantitatively when recombined in their original proportions. The C_1 component of the cellulase enzyme complex was reported to be a heat-labile glycoprotein with a carbohydrate: protein ratio of approximately one! It was homogeneous on the Sephadex G-75 and DEAE-Sephadex chromatograph columns and gave a single protein band by disc electrophoresis at pH 6.7. The molecular weight was about 61,000. The most significant feature of these results was that the C_1 component, when freed of C_x enzyme, had little or no action on cotton or cellobiose; although inactive alone, it was essential for the extensive solubilization of cotton by the whole complex. Recently, Wood (1968) studied the cellulolytic enzyme system of Trichoderma koningii, another terrestrial species, and separated the components which again proved to be C_1 and C_x enzymes and cellobiase.

No studies have been made on the actual mechanism of cellulose degradation by marine fungal species to our knowledge. Meyers, Prindle and Reynolds (1960) made preliminary studies on the gross aspects of cellulolytic activity in selected marine Ascomycetes and Deuteromycetes which included H. mediosetigera and C. achraspora. Their results indicated that the residual tensile strength of Manila twine was significantly reduced after 23-78 days with either species in 2-liter Fernbach flasks containing yeast extract-sea water broth. These cultures reduced the strength of the twine by more than 50 pounds indicating considerable degradation of the fiber. Observations were made on the cellulolytic activity of the filtrates on a CMC test substrate after the twine was removed. In general, there was greater total reducing sugar production

by the deuteromycetous species than by the ascomycetous isolates. No quantitative comparison of enzymatic activity and residual tensile strength could be made because the cellulolytic activity of the fungal filtrates excluded the role of enzymes entrapped with the fungal mycelia within the infested twine.

Subsequently, Meyers and Scott (1968) made gravimetric analyses of cellulose utilization by representative marine Ascomycetes including H. mediosetigera and, within the limits of this approach, showed significant activity based on cellulose weight loss. Intensive degradative activity in the filtrates occured when the pH of the filtrate was in the range of 6-8. Negligible amounts of C_x enzyme were found in the filtrates due to the adsorption of the enzyme to the mycelia or to the cellulose particles in the medium.

As part of an overall study of carbon metabolism in marine fungi, the investigation reported herein was initiated to elucidate the actual mechanism by which ascomycetous and deuteromycetous marine fungi degrade cellulosic materials in the sea.

MATERIAL AND METHODS

Cultures. The filamentous marine fungi used were the Deuteromycete, Culcitalna achraspora (230) (Meyers and Moore, 1960), the Ascomycete, Halosphaeria mediosetigera (700) (Johnson, 1958) and a "C_1-less" strain of H. mediosetigera designated (700 LC_1).

Reagents. Analytical grade reagents were used to prepare media, analytical solutions and buffers. Biochemical reagents were obtained from Sigma Chemical Co., Worthington Biochemical Co. and Eastman Organic Chemical Co. The alkali-washed, ground form of cellulose, Alphacel, was obtained from Nutritional Biochemical Corporation and carboxymethylcellulose (CMC 4M6F) from Hercules Incorporated. CMC (4M6F) has a degree of substitution range of 0.38 to 0.48 indicating that an average of 3.8 to 4.8 carboxymethyl groups are substituted per 10 anhydroglucose units. The M stands for medium viscosity, F designates the food grade standard and the number 6 indicates a 6000 centipoise maximum viscosity in a 1.0% solution. Native cotton fibers were kindly supplied by Brentex Mills, Incorporated. Artificial sea water (ASW) was prepared according to Lyman and Fleming (1940) as shown in Table 1.

Growth media. Growth was carried out in liquid media containing various carbon sources (glucose, cellobiose, CMC, Alphacel or native cotton fibers) 5.0-10.0, tris(hydroxymethyl)aminomethane buffer 1.21, NH_4NO_3 2.40 and yeast extract 1.0 g/liter in ASW, adjusted to pH 7.5 with 12 N HCl.

Equipment and conditions. Fungal cultures were grown in 250, 500 and 1000-ml Pyrex conical flasks on a New Brunswick shaker reciprocating at 66-8 cm strokes per min (spm) and 25 C.

TABLE 1

Composition of Sea Water

(Lyman and Fleming, 1940)

Ingredients	g/liter
Sodium chloride	23.500
Magnesium chloride	10.700
Sodium sulfate	3.900
Calcium chloride	1.100
Potassium chloride	0.700
Sodium bicarbonate	0.200
Potassium bromide	0.100
Boric acid	0.030
Strontium chloride	0.020
Sodium fluoride	0.003

Quantitation and transfer of standard inocula employed Bellco wide-bore (1 mm minimum) volumetric pipettes and homogenization in Waring stainless steel semi-micro blendors.

Culture media were sterilized in flasks by autoclaving at 15 psi for 20 min.

Centrifuging was done in an International HR-1 unit at 4 C and 12,000 X g.

For cell extraction work, mycelia were disintegrated in a 40-ml Rosett cooling cell, immersed in an ice-salt mixture at -15 C, at 20 kHz with a Branson Sonifier for 20 min. Maximum extract temperatures were 4-10 C.

The Beckman Model DU Spectrophotometer was used for photometric determinations of protein by the "280-260 method" (Layne, 1957).

A Bausch and Lomb "Spectronic 20" was used for colorimetric determinations of C_x enzyme and cellobiase activity. Folin-Wu tubes were used in assays for the former enzyme.

A 4-liter Soxhlet extraction apparatus was used for cotton purification.

Sintered-glass filter sticks of medium porosity were used for the C_1 enzyme assay.

Mycelial quantitation. Quantitative procedures for inoculum production and growth (Sguros, Meyers and Simms, 1962) were accomplished by filtering mycelial samples on tared Whatman GF/A glass filter discs previously dried in vacuo at 60 C. The discs were then redried in the same manner and weighed to the nearest 0.1 mg.

Chemical methods. Purification of native cotton fibers was accomplished by the method of Corbett (1963). The fibers, consisting of 94% cellulose and certain impurities such as protein, lignin, pectin and lipid, were cut into short lengths and extracted in the Soxhlet apparatus for 18 hr with chloroform, followed by 95% ethanol for an additional 18 hr. The residue was then boiled for 8 hr in an atmosphere of nitrogen with 50 ml of 1.0% NaOH per g of cotton. The purified fibers were washed with water until alkali-free and then washed sequentially with acetone, ethanol and ether and dried at room temperature.

Enzymological methods. C_1 enzyme assay. The "loosening" of cotton fiber bundles by the C_1 enzyme was measured by the colorimetric procedure of Halliwell (1958). The extent of enzymatic activity was determined by the loss of insoluble substrate using the following technique: the liquid remaining after enzyme action on the cotton fibers was removed by a sintered-glass filter stick (Fig. 3) and the residue washed with distilled water. The loss was measured colorimetrically by acid hydrolysis of the residual cellulose to glucose and oxidation of the latter with $K_2Cr_2O_7$.

To a 16 X 150 mm test tube was added: 0.2-1.2 mg of purified cotton fibers, 1.3 ml acetate buffer (0.2 M, pH 5.5), 1.7 ml distilled water and 1.0 ml of cellulase solution (cell-free filtrate). The control tube lacked only cell-free filtrate and the volume was replaced with distilled water. The tubes were incubated at 37 C in a water bath for 7 days, with occasional shaking, and the soluble contents of each removed by filter sticks as before. The residues were washed gently with 20.0 ml of distilled water and dried with maximum water aspirator vacuum. To each of the above tubes, plus two empty tubes (blanks), was added 0.9 ml distilled

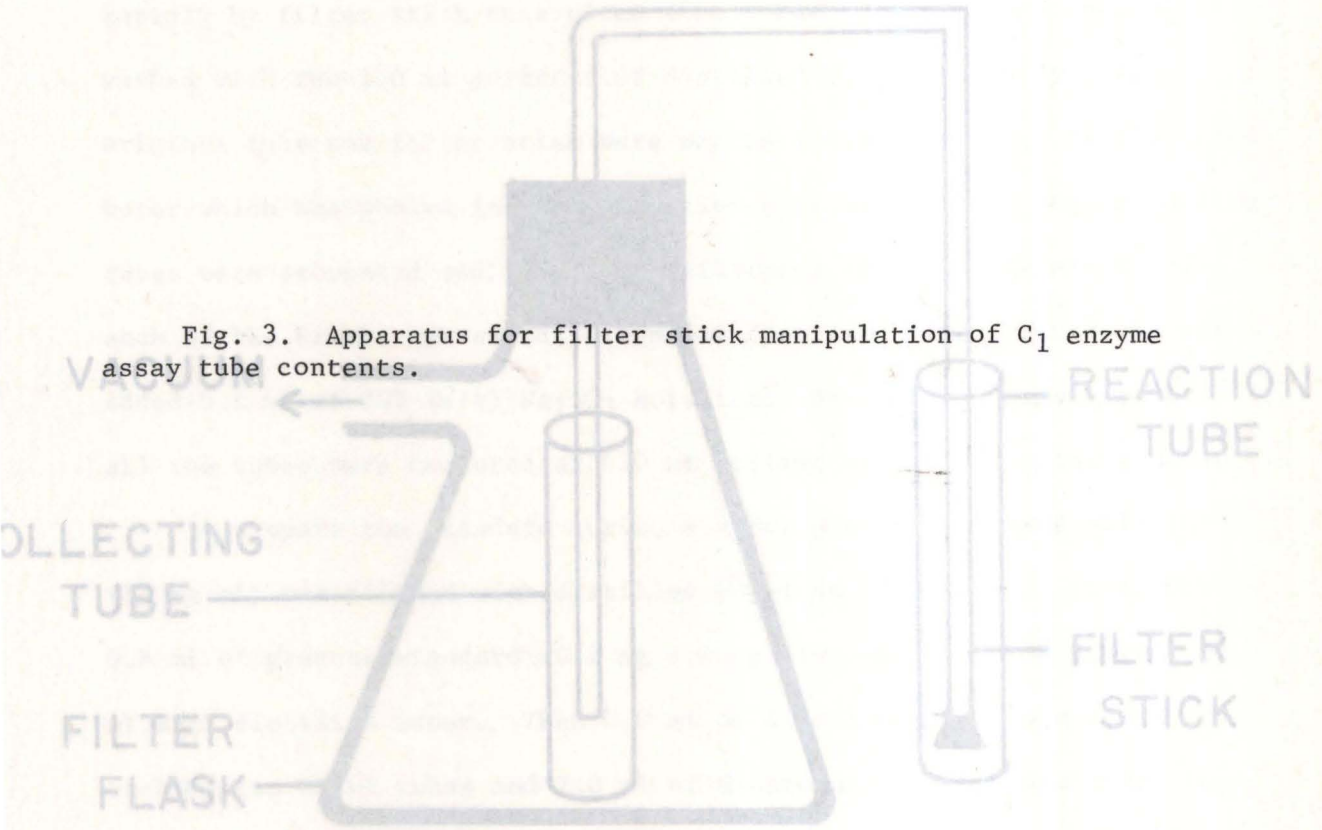
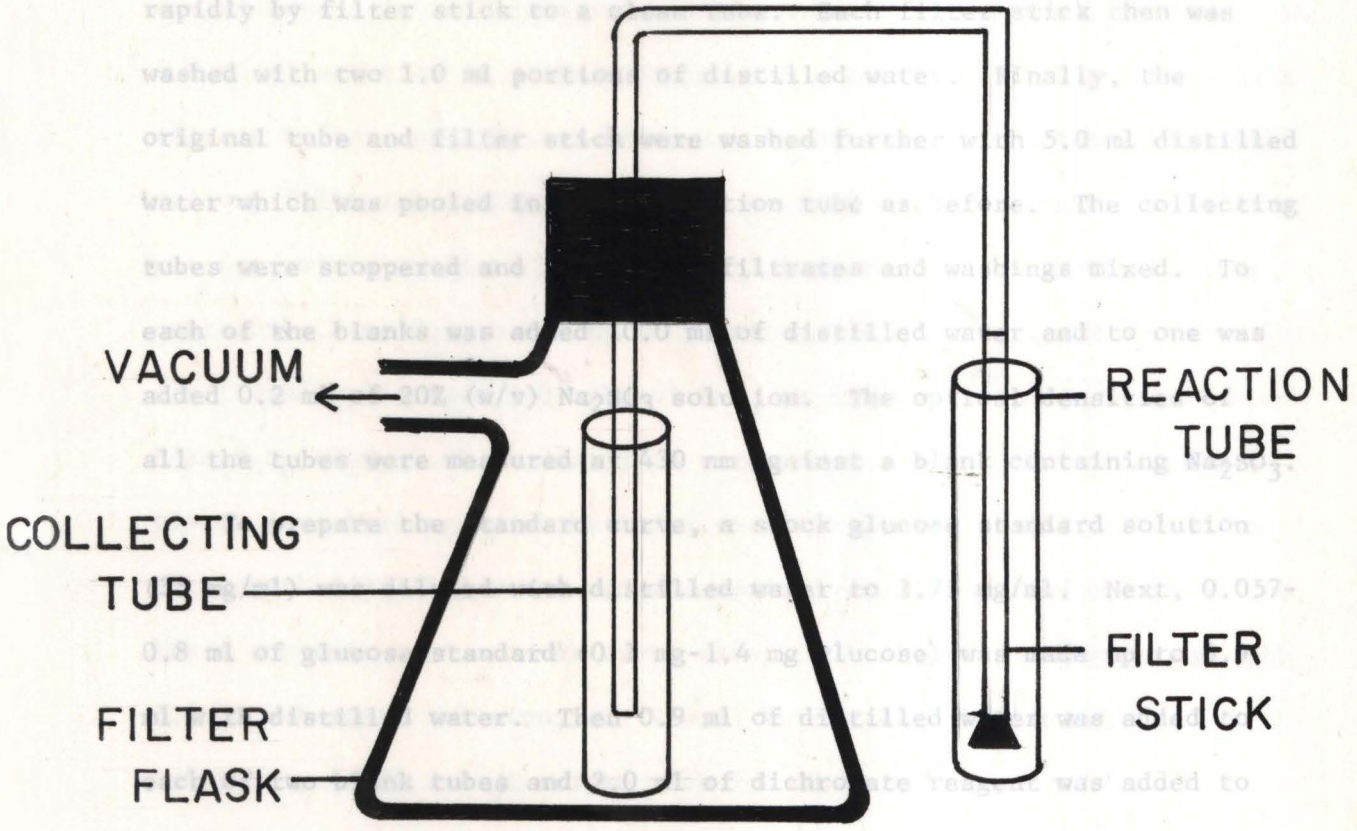


Fig. 3. Apparatus for filter stick manipulation of C_1 enzyme assay tube contents.


VACUUM
REACTION TUBE
COLLECTING TUBE
FILTER STICK
FILTER FLASK

water and 2.0 ml of dichromate solution made by dissolving 5 g $K_2Cr_2O_7$ in 20 ml of hot distilled water, cooling and diluting to 1 liter with 36 N H_2SO_4. The sintered-glass filter stick discs used for the experimental and control tubes were rinsed several times with reagent mixture and left in the tubes. The tubes were stoppered, heated for 1.5 hr in a boiling water bath, and cooled for 5 min at room temperature. To the experimental and control tubes were added 3.0 ml of distilled water. After cooling completely, the liquid portion of each tube was removed rapidly by filter stick to a clean tube. Each filter stick then was washed with two 1.0 ml portions of distilled water. Finally, the original tube and filter stick were washed further with 5.0 ml distilled water which was pooled in the collection tube as before. The collecting tubes were stoppered and the pooled filtrates and washings mixed. To each of the blanks was added 10.0 ml of distilled water and to one was added 0.2 ml of 20% (w/v) Na_2SO_3 solution. The optical densities of all the tubes were measured at 430 nm against a blank containing Na_2SO_3.

To prepare the standard curve, a stock glucose standard solution (35 mg/ml) was diluted with distilled water to 1.75 mg/ml. Next, 0.057-0.8 ml of glucose standard (0.1 mg-1.4 mg glucose) was made up to 0.9 ml with distilled water. Then 0.9 ml of distilled water was added to each of two blank tubes and 2.0 ml of dichromate reagent was added to all tubes, the contents mixed carefully, and the tubes stoppered and heated in a boiling water bath for 30 min. After cooling under the tap, 10.0 ml of distilled water was added to each tube and to one of the blank tubes was added 0.2 ml Na_2SO_3 solution. The optical densities of all solutions were read at 430 nm against the blank containing sulfite.

Optical density was plotted against the mg glucose added. The difference in optical density between the experimental and control tubes, corresponding to this value of glucose from the standard curve, was multiplied by 0.9 to obtain the mg cellulose per tube.

C_x enzyme assay. The index of C_x enzyme activity employed for cells and for culture filtrates was the amount of reducing sugar produced from CMC (4M6F) under specified conditions. The C_x enzyme has the capacity to hydrolyze the beta-1,4-glucosidic linkages of cellulose. The C_x enzyme assays in cellulolytic response studies were made as follows: to 9.0 ml of solution in a 18 X 150 mm tube containing 0.55% CMC in 0.05 M citrate buffer, pH 5.0, was added 1.0 ml of enzyme solution (cell-free culture filtrate). After incubation at 37 C for 1 hr, reducing sugar was determined (Miller, 1959). To a 1.0 ml sample in a Folin-Wu tube, containing from 0.1 to 1.0 mg of glucose, 3.0 ml of reagent (3,5-dinitrosalicylic acid 1.0, phenol 0.2, Na_2SO_3 0.05, NaOH 1.0 and $NaKC_4H_4O_6 \cdot 4H_2O$ 20.0% in distilled water) was added. The solution was placed in a boiling water bath for exactly 15 min, cooled, diluted to 25 ml with distilled water and the optical density determined at 550 nm. Three controls were used in every assay: (1) 1.0 ml boiled enzyme with 9.0 ml substrate solution, (2) 1.0 ml buffer with 9.0 ml substrate solution and (3) 1.0 ml enzyme with 9.0 ml citrate buffer.

In the pH-reaction rate studies, 0.05 M citrate-phosphate buffer was used while the temperature-reaction rate, temperature stability and pH stability studies employed 0.05 M, pH 6.0 citrate buffer.

Cellobiase assay. The index of cellobiase activity in cells and in culture filtrates was the amount of glucose produced from cellobiose under specified conditions. Cellobiase, the enzyme capable of hydrolyzing the beta-1,4-glucosidic linkages of cellulose, was measured by the amount of glucose produced by the hydrolysis of cellobiose.

Cellobiase assays in the cellulolytic response studies were made as follows: to 9.0 ml of solution in a 18 X 150 mm tube containing 0.5% cellobiose in 0.02 M phosphage buffer, pH 7.0, was added 1.0 ml of enzyme solution (cell-free culture filtrate). After incubation for 1 hr at 37 C, the reaction was terminated by boiling a 1.0 ml sample. To this was added 9.0 ml of Glucostat reagent (a coupled-enzyme system suitable for the specific, colorimetric determination of glucose). After 10 min at 25 C, 0.05 ml of 4 N HCl was added to stop the reaction and stabilize the color. The reaction time was the same for all samples which were allowed to stand for 5 min after stopping the reaction, and then read on a colorimeter at 400 nm, setting a reagent blank at zero absorbancy. A stock standard solution was prepared to contain 0.05-0.30 mg/ml glucose. The controls consisted of (1) 1.0 ml boiled enzyme plus 9.0 ml of 0.5% cellobiose, (2) 1.0 ml enzyme plus 9.0 ml of buffer and (3) 1.0 ml distilled water plus 9.0 ml of 0.5% cellobiose.

In pH-reaction rate studies, 0.5% cellobiose solution was prepared in 0.05 M citrate-phosphate buffer. In temperature-reaction rate, temperature stability and pH stability studies, 0.5% cellobiose solution was prepared in 0.05 M, pH 6.0 phosphate buffer.

Microbiology. Growth and standardized inocula preparation described by Sguros, Meyers and Reynolds (1961) and Sguros et al. (1962)

was employed. Typically, inocula were started by transferring mycelial growth from a 6 day old stock culture, maintained on medium 1410 in 500-ml flasks stored at 4 C, to 500-ml flasks containing 100 ml of fresh medium 1410. Cultures were incubated on the shaker until the late linear phase. Heavy growth was obtained in approximately 6 days, at which time one flask was removed, the contents transferred to a sterile semi-microblendor and homogenized for 35 seconds. Finally, 100 ml of fresh medium 1410 in a 500-ml flask was inoculated with 4 ml (8 mg) of mycelial homogenate.

After 6 days shaker growth, the culture was filtered aseptically on Whatman #1 paper, using a Millipore funnel and support, and washed four times with 50 ml portions of distilled water. The washed mycelial pad was transferred to a sterile semi-microblendor and homogenized in 50.0 ml distilled water for 30 seconds. Three 2.0 ml samples were drawn for quantitation and the remainder stored in the blendor overnight at 4 C. After the weight of mycelium (mg/ml) was established, the stored suspension was diluted with distilled water to bring the mycelial weight to approximately 2 mg/ml and homogenized for 5 seconds to ensure uniform dispersion. The number and size of flasks and media volumes were determined by the total crop required for the experimental program. The amount of inoculum required for each volume of medium was determined by previous data of Sguros and Meyers (1962) (Table 2). Those cultures designated for future experimental standard inocula were grown for 6 days to the late linear phase in the same manner.

Culture response procedures. In determining the response of these marine fungi to Alphacel, CMC, cellobiose and glucose, the following method was used for each carbon source: two sets of 27-250 ml shake-flasks were

TABLE 2

Effect of Scale - Up Procedures on the Mycelial Yields of *H. mediosetigera* (700), *C. achraspora* (230), and *H. alopallonella* (710) in Medium 1410 (Sguros and Meyers, 1962).

	Designations	Conditions and Results				
A	Flask[1] Volume (ml)	125	250	500	1000	2000
B	Medium Volume (ml)	25	50	100	200	400
C	Inoculum Volume (ml)	1	2	4	8	16
D	Inoculum Weight (mg)	2	4	8	16	32
E	Medium Surface Area (cm^2)	32.3	45.2	75.4	113.0	154.0
F	Medium Depth (cm)	0.9	1.4	1.6	2.0	2.4
G	Surface-Volume Ratio E/B	1.29	0.91	0.75	0.57	0.39
H	Surface-Depth Ratio E/F	33.9	32.3	47.0	56.5	64.0
	Culture 700					
	Total Yield (mg)	54	113	225	454	957
	Yield (mg/ml)	2.2	2.3	2.3	2.3	2.4
	Fold Increase	27	28	28	28	30
	Culture 230					
	Total Yield (mg)	53	105	213	440	872
	Yield (mg/ml)	2.1	2.1	2.1	2.2	2.2
	Fold Increase	27	26	26	28	27
	Culture 710					
	Total Yield (mg)	50	101	210	492	952
	Yield (mg/ml)	2.0	2.0	2.1	2.5	2.4
	Fold Increase	25	25	26	31	30

[1]Erlenmeyer shake-flasks

prepared, each flask containing 50 ml of medium 1410-less-glucose in addition to 0.5% of the carbon source to be studied. For each carbon source three uninoculated controls were used to establish base-lines for pH and protein concentration after sterilization. Each flask, except controls, were inoculated with 2.0 ml of standard inoculum (2mg/ml) from a 6 day, medium 1410-grown culture in linear phase and all flasks were placed on the shaker to be harvested at predetermined intervals.

Cell-free filtrates were assayed for C_x enzyme and cellobiase activity in triplicate at 1, 3, 5, 7, 9, 11, 13 and 15 days after growth on each carbon source. Culture sets were removed and the clear medium filtrates collected through Whatman #1 paper. The filtrates were stored at 4 C overnight and final pH, protein determinations and enzyme assays performed on the following day.

To obtain filtrates from C. achraspora with high C_1 and C_x enzyme and cellobiase activities, 1-liter flasks, containing 200 ml of medium 1410-less-glucose perfused with 0.5-1.0% native cotton fibers, were inoculated with 8.0 ml (16 mg) of standard 6 day inoculum. After 12 and 20 days growth under standard conditions, cultures were filtered through Whatman #1 paper and stored at 10 C after the addition of sodium azide (0.005 moles/liter) to prevent bacterial contamination. These filtrates served as sources for extracellular C_x enzyme and cellobiase used for temperature and pH studies, as well as for the C_1 enzyme assays. The filtered cells, to be used later for intracellular enzyme studies, were washed with 100 ml of distilled water and the wet pads stored at -10 C. Filtrates from 7 day old cultures, grown in 1-liter flasks under standard conditions, on medium 1410-less-glucose with 1.0% Alphacel and with 1.0% CMC carbon sources, also were obtained for the C_1 enzyme assays.

H. mediosetigera (700 LC_1) was also assayed for C_1 and C_x enzymes and cellobiase activities by an identical procedure except that Alphacel was substituted for native cotton fibers in 7 and 10 day old cultures owing to the apparent inability of this strain to initiate growth on native cotton. Mycelia for intracellular enzyme studies and filtrates for C_1 enzyme assays were obtained in the same manner as for C. achraspora.

A culture of H. mediosetigera (700), carried since isolation on cellulosic media, was obtained to determine the species' true ability to initiate growth on native cotton fibers. The culture was started by inoculating a 500-ml flask, containing medium 1410, with mycelia obtained from a balsa wood sample on which the culture was maintained. After 3 weeks of shaker growth, the culture was homogenized in a semi-micro blendor for 30 seconds to prepare crude inoculum (4 ml) for another 500-ml flask containing medium 1410. After growing the culture 6 days on the shaker, the culture was homogenized as before to prepare crude inoculum (4 ml) for a 1-liter flask containing medium 1410-less-glucose with 1.0% cotton fibers. This flask was placed on the shaker after inoculation.

Specific procedures employed for all pH and temperature studies are detailed in the descriptive text for each figure to follow.

RESULTS

Time-course studies demonstrated that in both H. mediosetigera (700 LC_1) and C. achraspora, C_x enzyme and cellobiase were induced by growth on CMC and on Alphacel (Fig. 4-7). In all cases, the activity of the C_x enzyme was predominant under the experimental conditions, but the extent and time of occurrence of maximum activity varied with filtrate history and growth substrate. Although growth conditions were identical, Alphacel-grown C. achraspora showed maximum C_x enzyme activity much earlier than when grown on CMC, but the duration of maximum activity was more pronounced with the latter carbon source (Fig. 4 and 6). The pH remained constant during the action of CMC media filtrates while Alphacel filtrates showed a distinct pH drop coinciding with maximum C_x enzyme activity. The same pH trend was exhibited in the case of filtrates from Alphacel- and CMC-grown H. mediosetigera (700 LC_1) (Fig. 5 and 7). In both species, however, pH decreases were followed by a return to initial levels as C_x enzyme activity declined from maximum values. Filtrates of H. mediosetigera (700 LC_1) showed a far greater uniformity in the time of occurrence of maximum C_x enzyme activity (9 days), but contrary to the data on C. achraspora, the C_x enzyme maximum persisted much longer in Alphacel media filtrates.

In all culture filtrates, regardless of substrate, cellobiase activity rose with C_x enzymatic activity, but tapered off long before reaching comparable levels. Only in filtrates from CMC-grown C. achraspora did the time of cellobiase maximum activity coincide with that of the C_x enzyme, but the activity level averaged only about 20% of that for the latter.

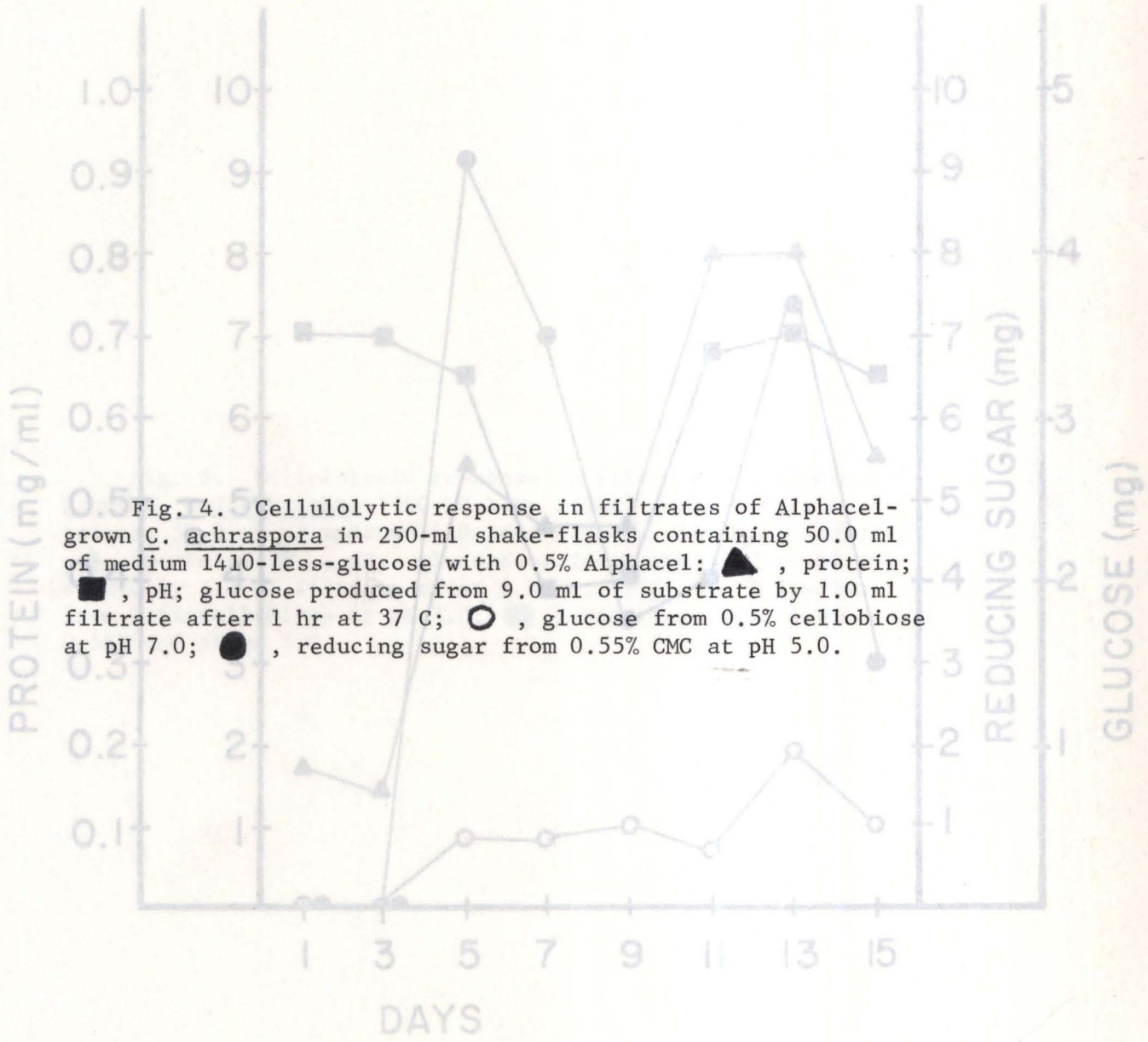
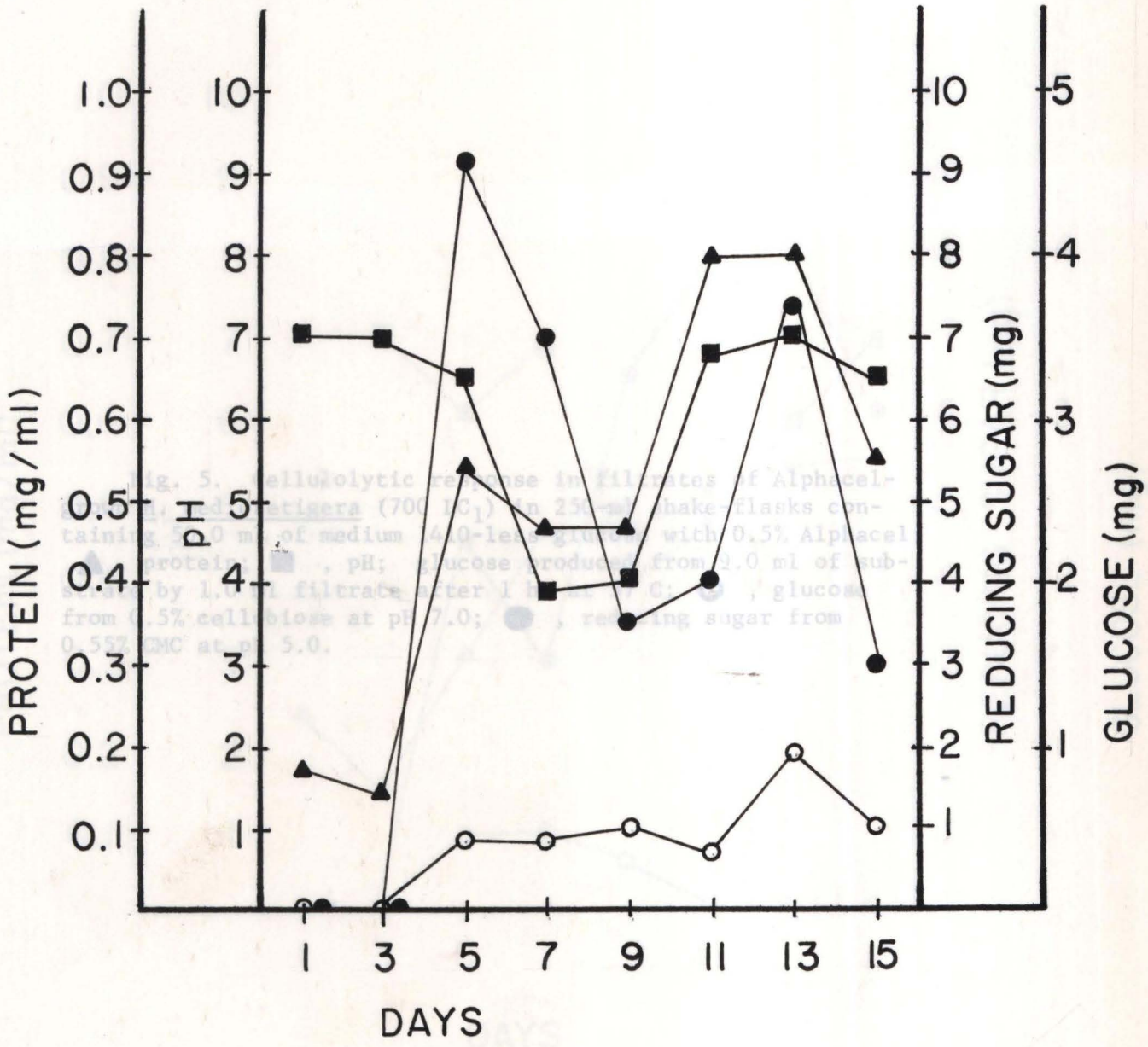


Fig. 4. Cellulolytic response in filtrates of Alphacel-grown C. achraspora in 250-ml shake-flasks containing 50.0 ml of medium 1410-less-glucose with 0.5% Alphacel: ▲, protein; ■, pH; glucose produced from 9.0 ml of substrate by 1.0 ml filtrate after 1 hr at 37 C; ○, glucose from 0.5% cellobiose at pH 7.0; ●, reducing sugar from 0.55% CMC at pH 5.0.


PROTEIN (mg/ml)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
pH
10
9
8
7
6
5
4
3
2
1
REDUCING SUGAR (mg)
10
9
8
7
6
5
4
3
2
1
GLUCOSE (mg)
5
4
3
2
1
1 3 5 7 9 11 13 15
DAYS

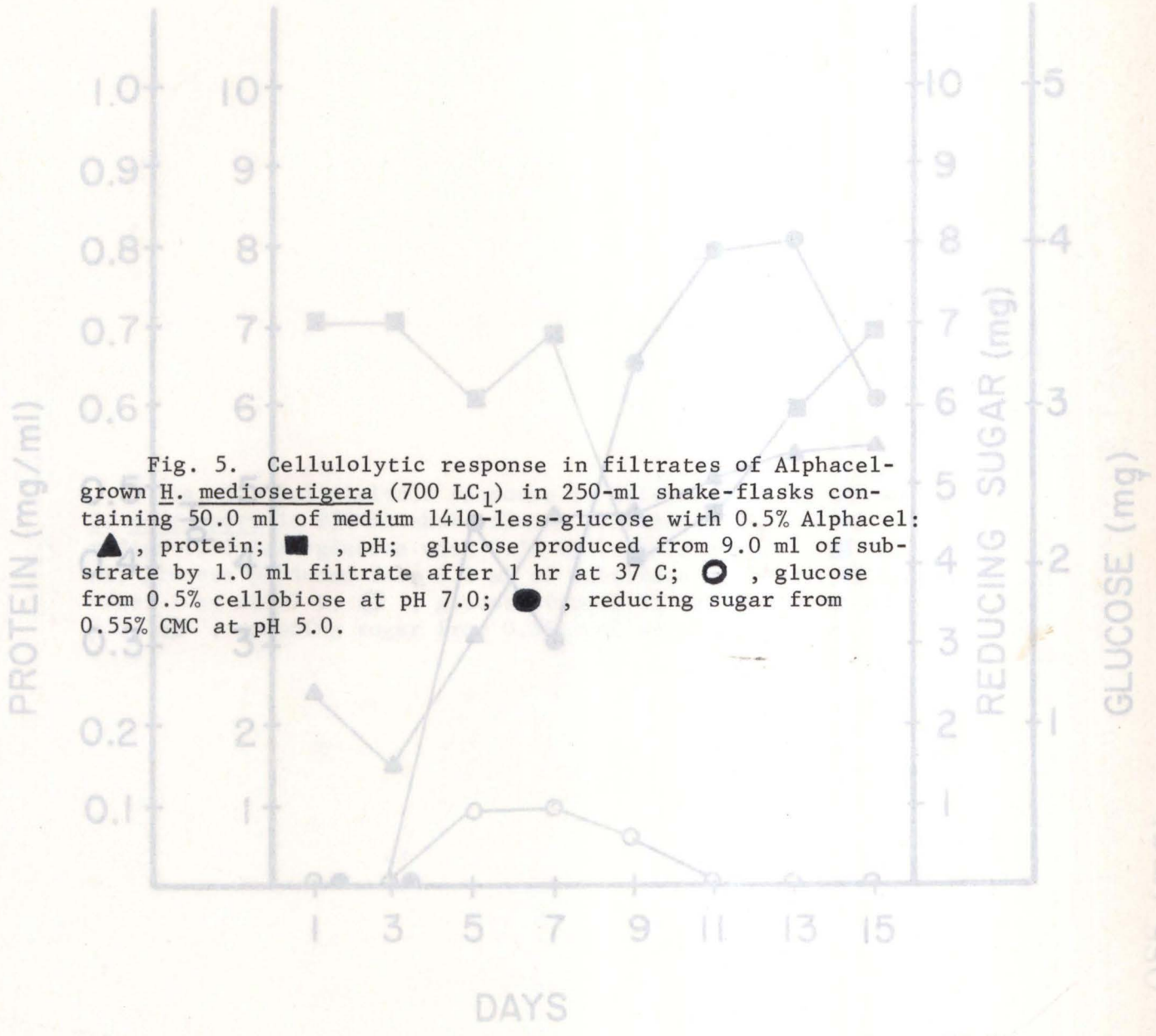
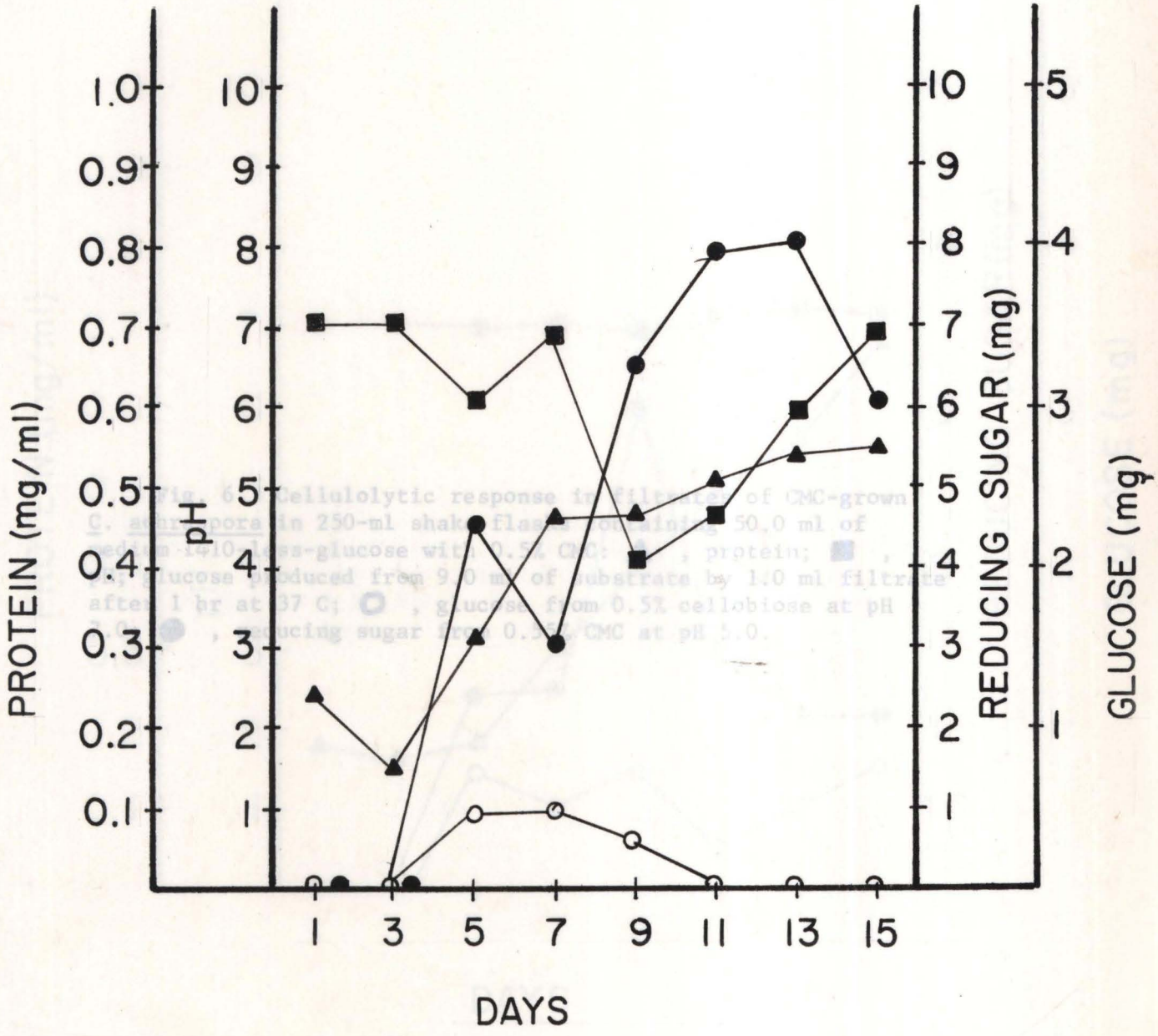


Fig. 5. Cellulolytic response in filtrates of Alphacel-grown H. mediosetigera (700 LC_1) in 250-ml shake-flasks containing 50.0 ml of medium 1410-less-glucose with 0.5% Alphacel: ▲, protein; ■, pH; glucose produced from 9.0 ml of substrate by 1.0 ml filtrate after 1 hr at 37 C; ○, glucose from 0.5% cellobiose at pH 7.0; ●, reducing sugar from 0.55% CMC at pH 5.0.


PROTEIN (mg/ml)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
pH
10
9
8
7
6
5
4
3
2
1
REDUCING SUGAR (mg)
10
9
8
7
6
5
4
3
2
1
GLUCOSE (mg)
5
4
3
2
1
1
3
5
7
9
11
13
15
DAYS

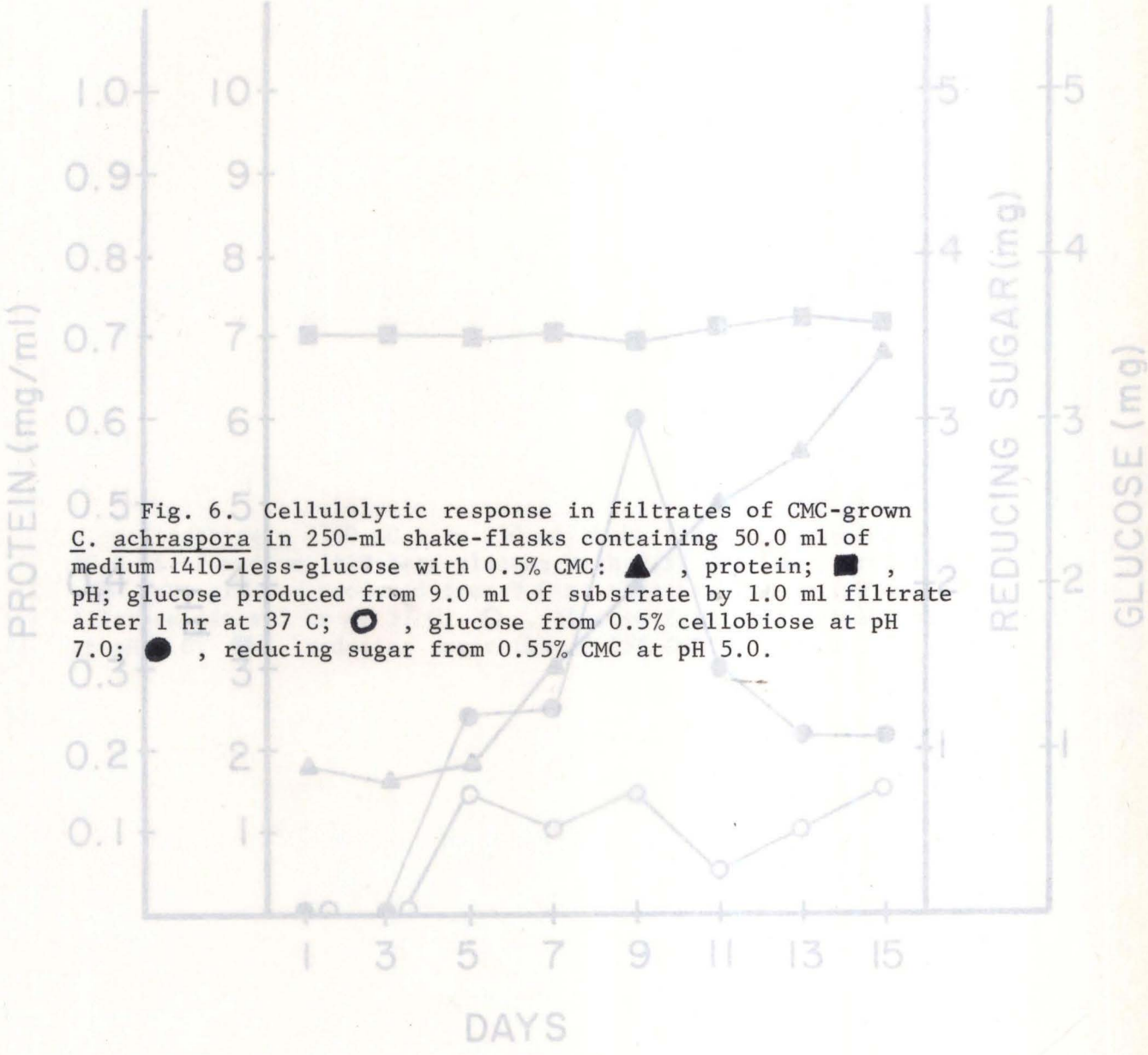
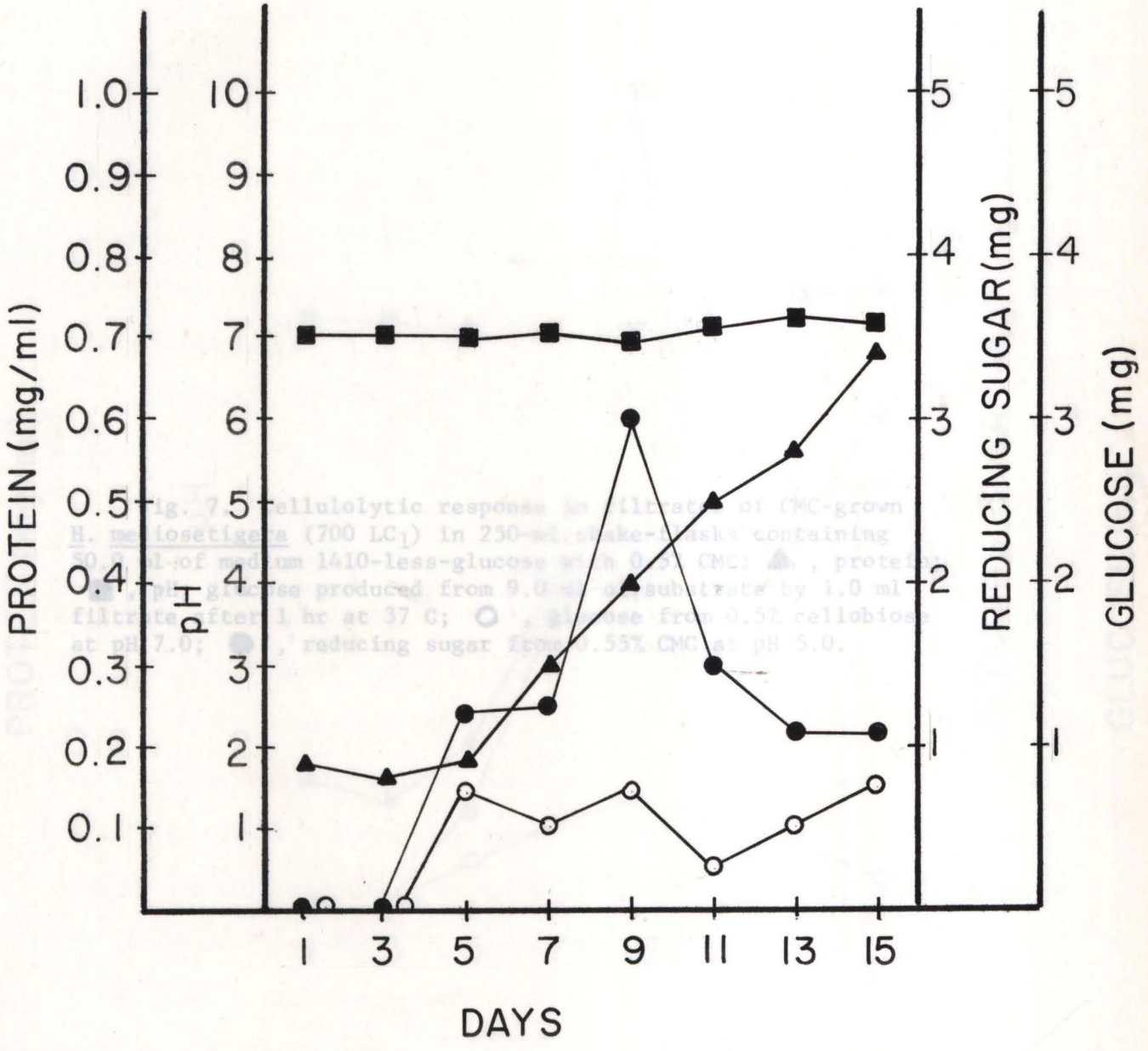


Fig. 6. Cellulolytic response in filtrates of CMC-grown C. achraspora in 250-ml shake-flasks containing 50.0 ml of medium 1410-less-glucose with 0.5% CMC: ▲ , protein; ■ , pH; glucose produced from 9.0 ml of substrate by 1.0 ml filtrate after 1 hr at 37 C; ○ , glucose from 0.5% cellobiose at pH 7.0; ● , reducing sugar from 0.55% CMC at pH 5.0.


PROTEIN (mg/ml)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
pH
10
9
8
7
6
5
4
3
2
1
REDUCING SUGAR(mg)
5
4
3
2
1
GLUCOSE (mg)
5
4
3
2
1
1 3 5 7 9 11 13 15
DAYS

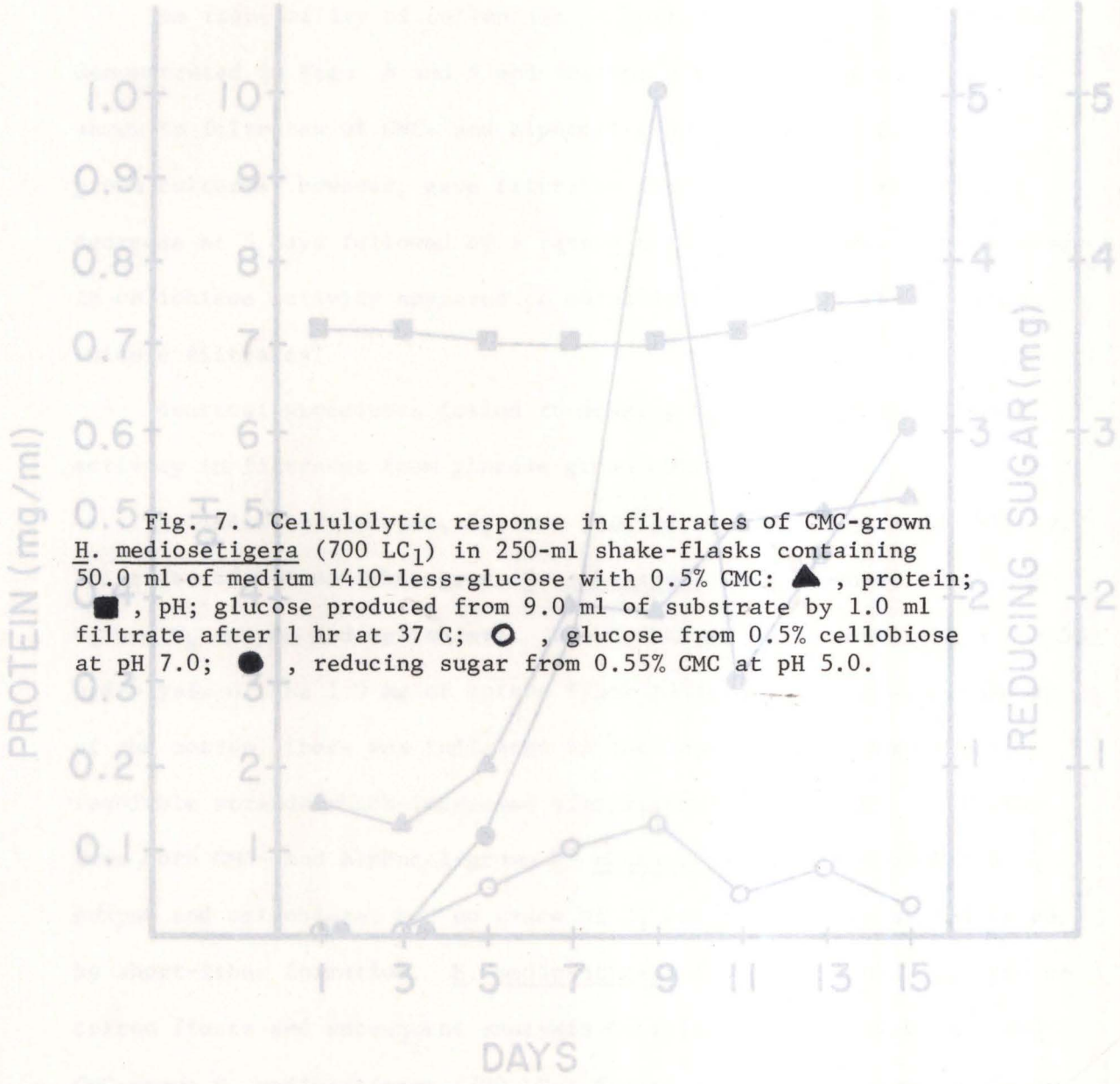
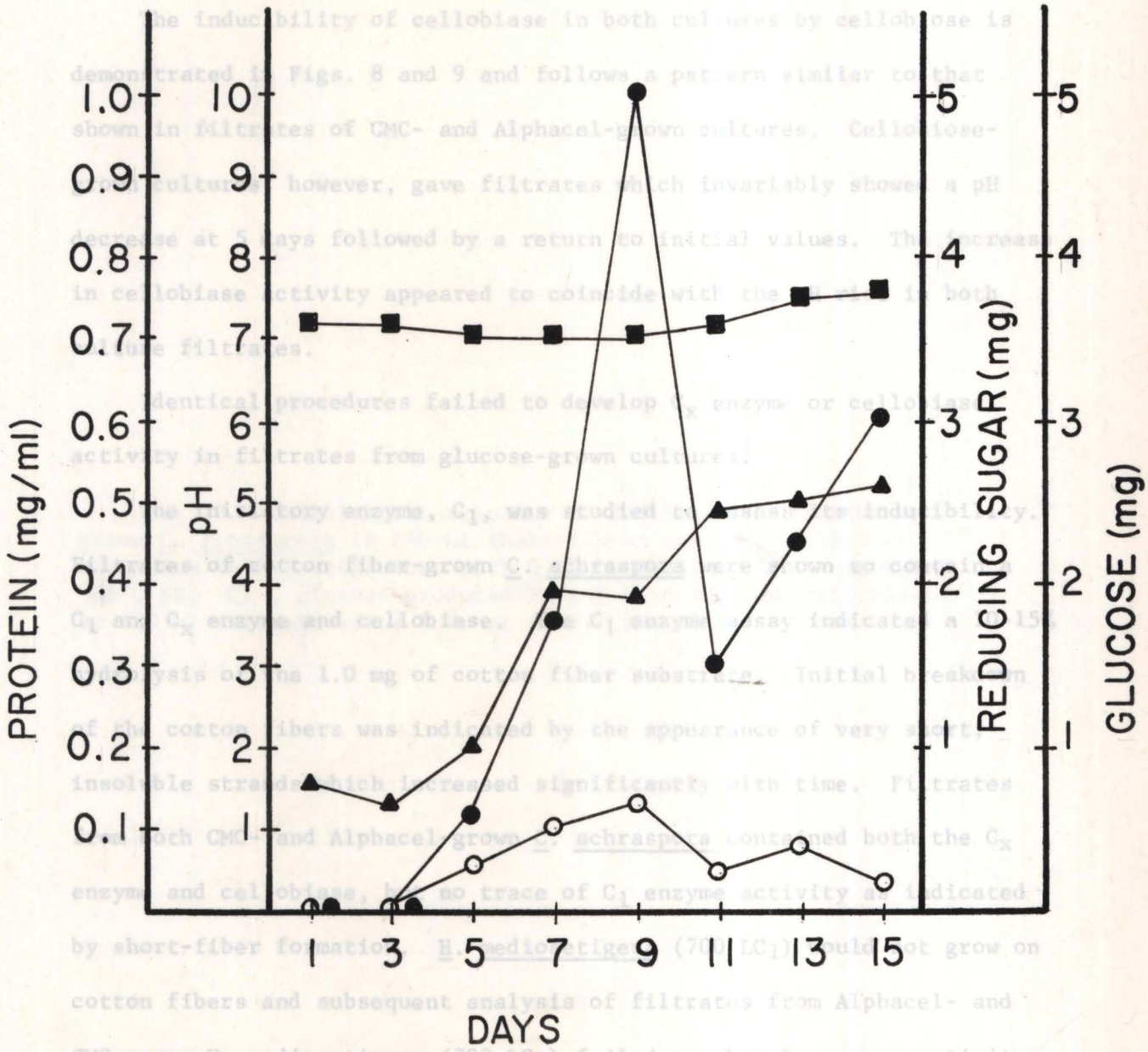


Fig. 7. Cellulolytic response in filtrates of CMC-grown H. mediosetigera (700 LC_1) in 250-ml shake-flasks containing 50.0 ml of medium 1410-less-glucose with 0.5% CMC: ▲ , protein; ■ , pH; glucose produced from 9.0 ml of substrate by 1.0 ml filtrate after 1 hr at 37 C; ○ , glucose from 0.5% cellobiose at pH 7.0; ● , reducing sugar from 0.55% CMC at pH 5.0.


PROTEIN (mg/ml)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
pH
10
9
8
7
6
5
4
3
2
1
REDUCING SUGAR (mg)
5
4
3
2
1
GLUCOSE (mg)
5
4
3
2
1
1
3
5
7
9
11
13
15
DAYS

Average protein levels in all filtrates rose steadily reaching highest levels at the end of the 15 day period. There appeared to be no direct correlation with enzymatic activity during the entire sequence.

The inducibility of cellobiase in both cultures by cellobiose is demonstrated in Figs. 8 and 9 and follows a pattern similar to that shown in filtrates of CMC- and Alphacel-grown cultures. Cellobiose-grown cultures, however, gave filtrates which invariably showed a pH decrease at 5 days followed by a return to initial values. The increase in cellobiase activity appeared to coincide with the pH rise in both culture filtrates.

Identical procedures failed to develop C_x enzyme or cellobiase activity in filtrates from glucose-grown cultures.

The initiatory enzyme, C_1, was studied to assess its inducibility. Filtrates of cotton fiber-grown C. achraspora were shown to contain a C_1 and C_x enzyme and cellobiase. The C_1 enzyme assay indicated a 10-15% hydrolysis of the 1.0 mg of cotton fiber substrate. Initial breakdown of the cotton fibers was indicated by the appearance of very short, insoluble strands which increased significantly with time. Filtrates from both CMC- and Alphacel-grown C. achraspora contained both the C_x enzyme and cellobiase, but no trace of C_1 enzyme activity as indicated by short-fiber formation. H. mediosetigera (700 LC_1) would not grow on cotton fibers and subsequent analysis of filtrates from Alphacel- and CMC-grown H. mediosetigera (700 LC_1) failed to show C_1 enzyme activity although both C_x enzyme and cellobiase were present.

Temperature and pH studies on cellobiase and C_x enzyme activity from filtrates of Alphacel-grown H. mediosetigera (700 LC_1) revealed distinct

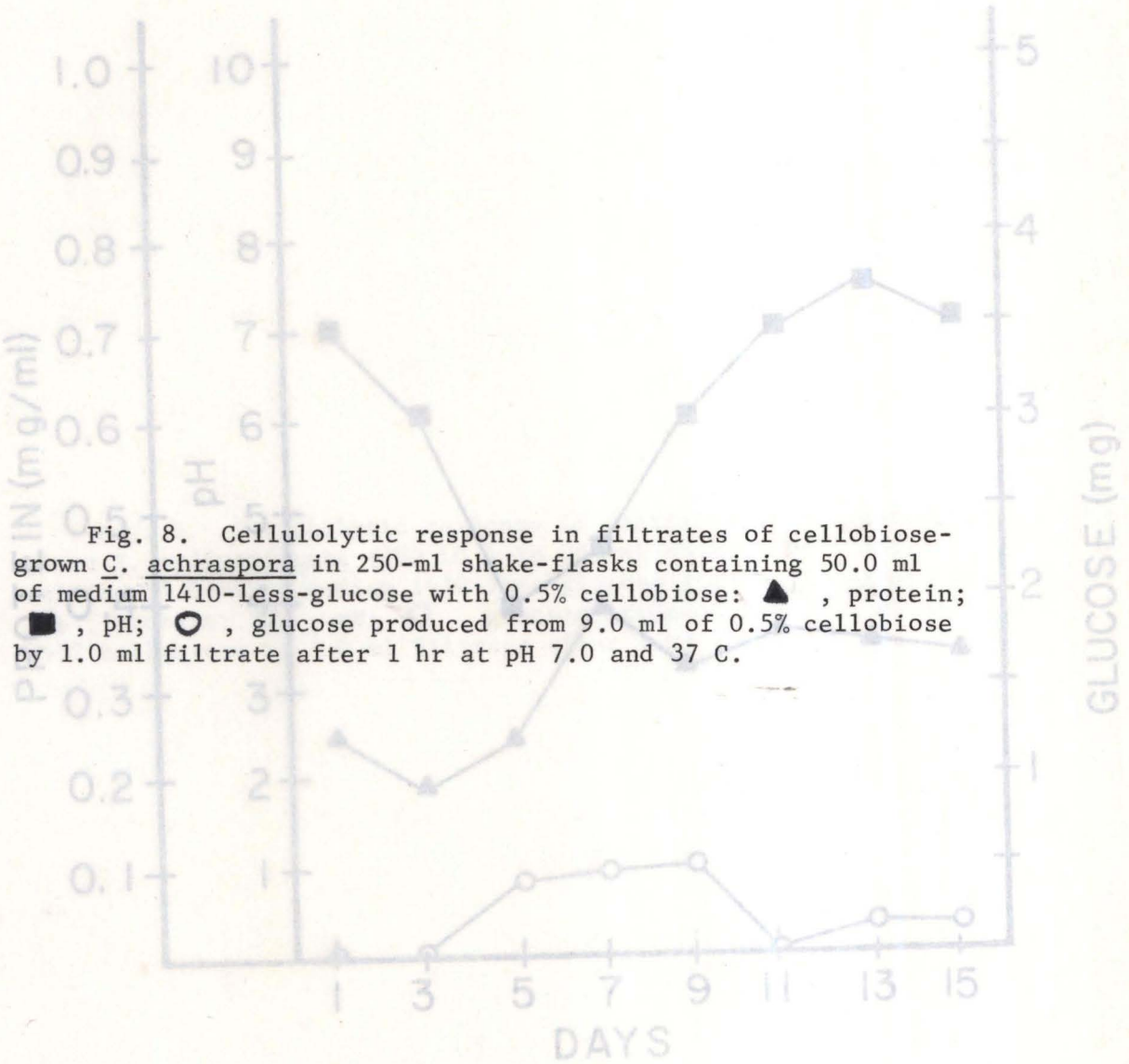
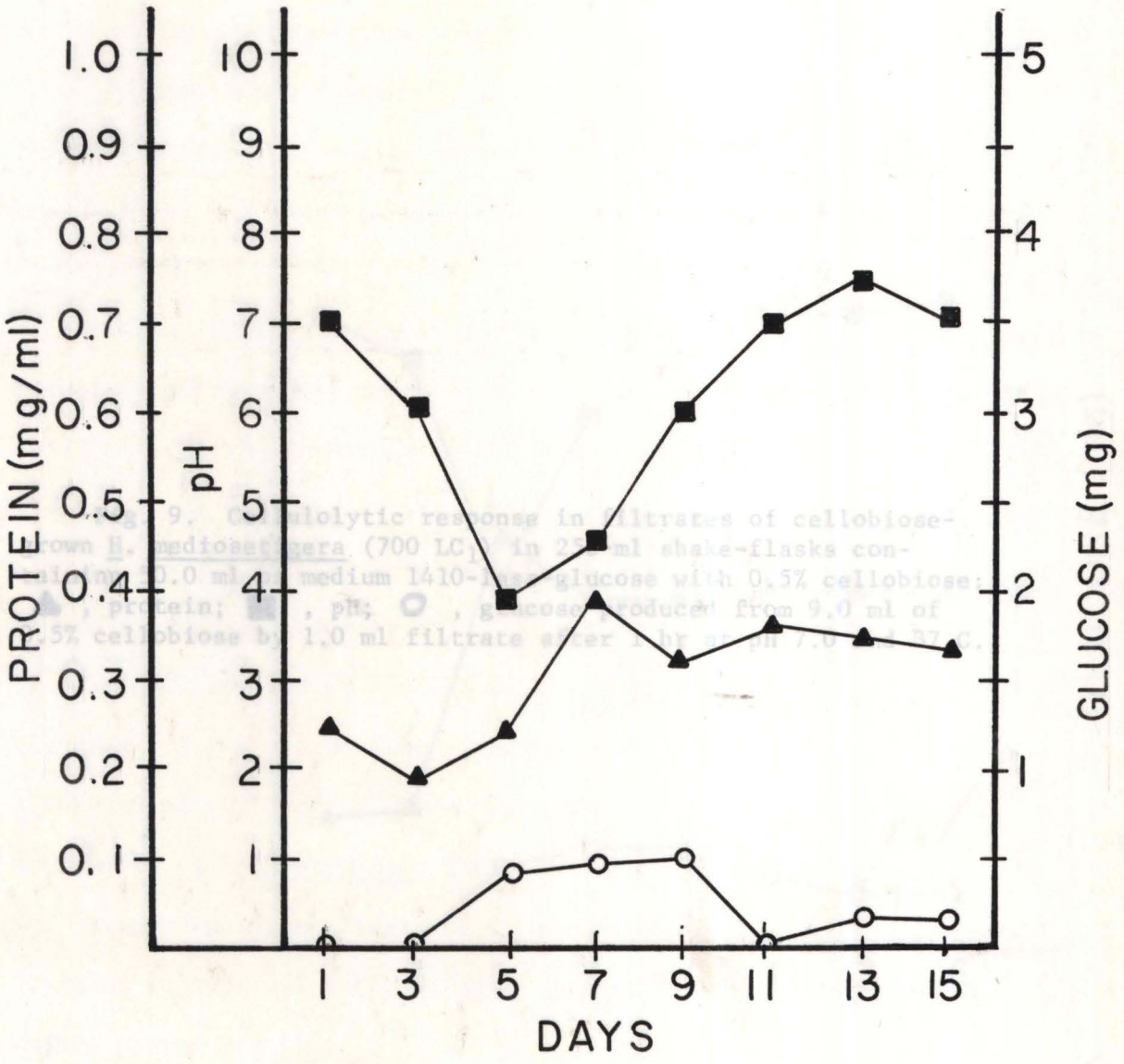


Fig. 8. Cellulolytic response in filtrates of cellobiose-grown C. achraspora in 250-ml shake-flasks containing 50.0 ml of medium 1410-less-glucose with 0.5% cellobiose: ▲ , protein; ■ , pH; ○ , glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml filtrate after 1 hr at pH 7.0 and 37 C.


PROTEIN (mg/ml)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
pH
10
9
8
7
6
5
4
3
2
1
GLUCOSE (mg)
5
4
3
2
1
1
3
5
7
9
11
13
15
DAYS

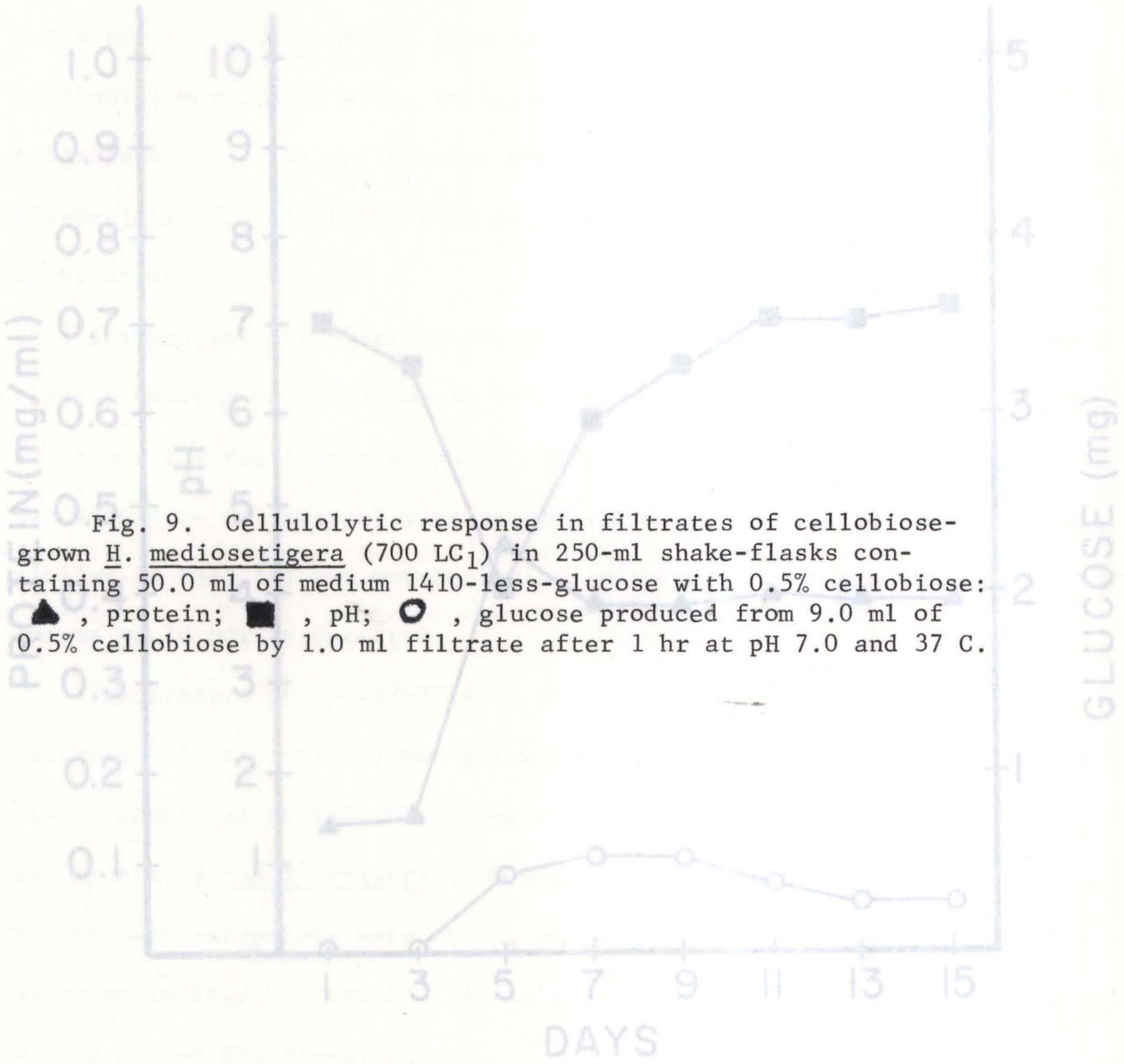
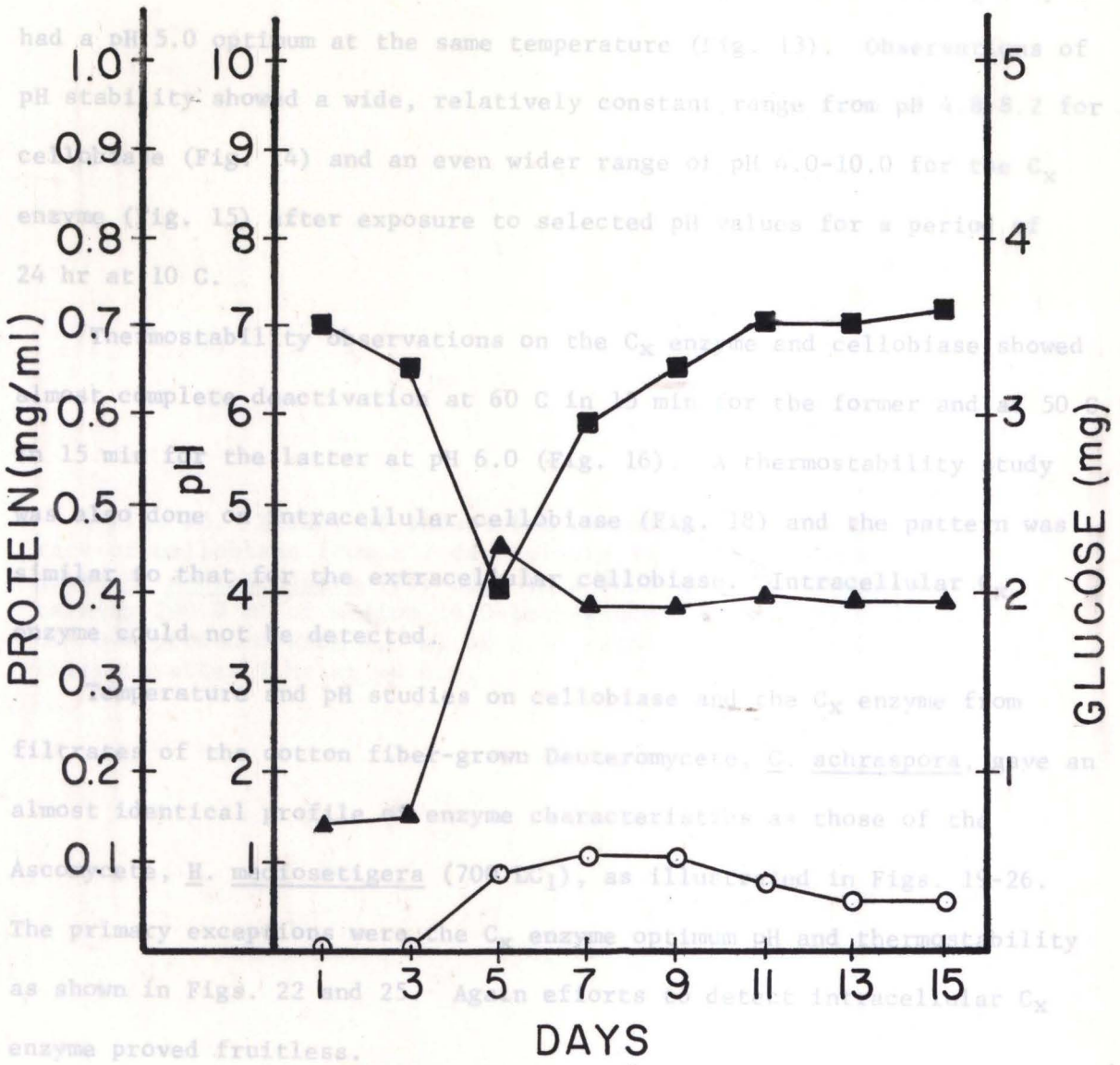


Fig. 9. Cellulolytic response in filtrates of cellobiose-grown H. mediosetigera (700 LC_1) in 250-ml shake-flasks containing 50.0 ml of medium 1410-less-glucose with 0.5% cellobiose: ▲, protein; ■, pH; O, glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml filtrate after 1 hr at pH 7.0 and 37 C.


PROTEIN (mg/ml)
pH
GLUCOSE (mg)
DAYS
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
9
8
7
6
5
4
3
2
1
5
4
3
2
1
1
3
5
7
9
11
13
15

characteristics. The optimum temperature of cellobiase at pH 6.0 was 50 C (Fig. 10) while the C_x enzyme had a variable optimum temperature at the same pH depending on the time of incubation (Fig. 11). The optimum pH of cellobiase at 37 C was pH 6.0 (Fig. 12), but the C_x enzyme had a pH 5.0 optimum at the same temperature (Fig. 13). Observations of pH stability showed a wide, relatively constant range from pH 4.8-8.2 for cellobiase (Fig. 14) and an even wider range of pH 4.0-10.0 for the C_x enzyme (Fig. 15) after exposure to selected pH values for a period of 24 hr at 10 C.

Thermostability observations on the C_x enzyme and cellobiase showed almost complete deactivation at 60 C in 15 min for the former and at 50 C in 15 min for the latter at pH 6.0 (Fig. 16). A thermostability study was also done on intracellular cellobiase (Fig. 18) and the pattern was similar to that for the extracellular cellobiase. Intracellular C_x enzyme could not be detected.

Temperature and pH studies on cellobiase and the C_x enzyme from filtrates of the cotton fiber-grown Deuteromycete, C. achraspora, gave an almost identical profile of enzyme characteristics as those of the Ascomycete, H. mediosetigera (700 LC_1), as illustrated in Figs. 19-26. The primary exceptions were the C_x enzyme optimum pH and thermostability as shown in Figs. 22 and 25. Again efforts to detect intracellular C_x enzyme proved fruitless.

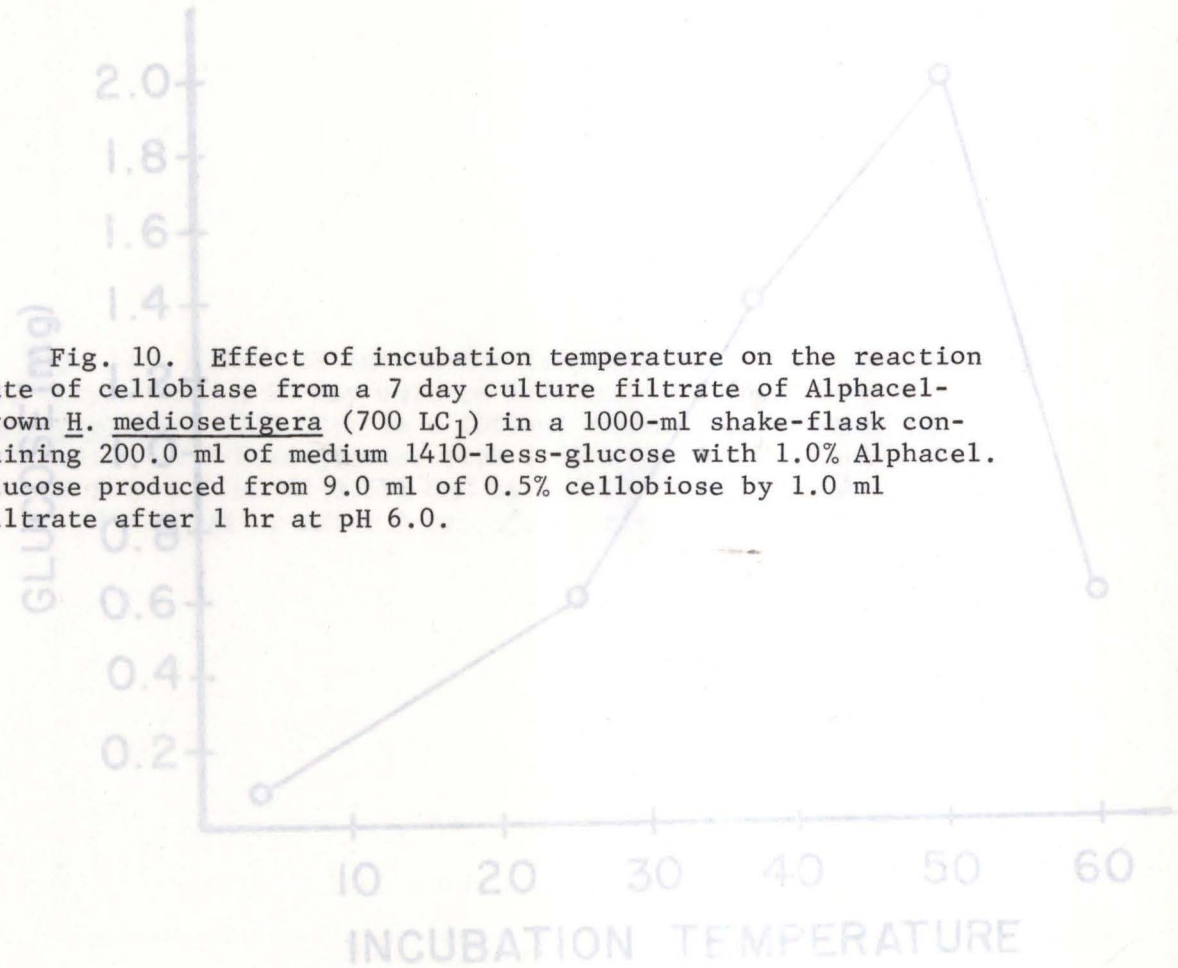
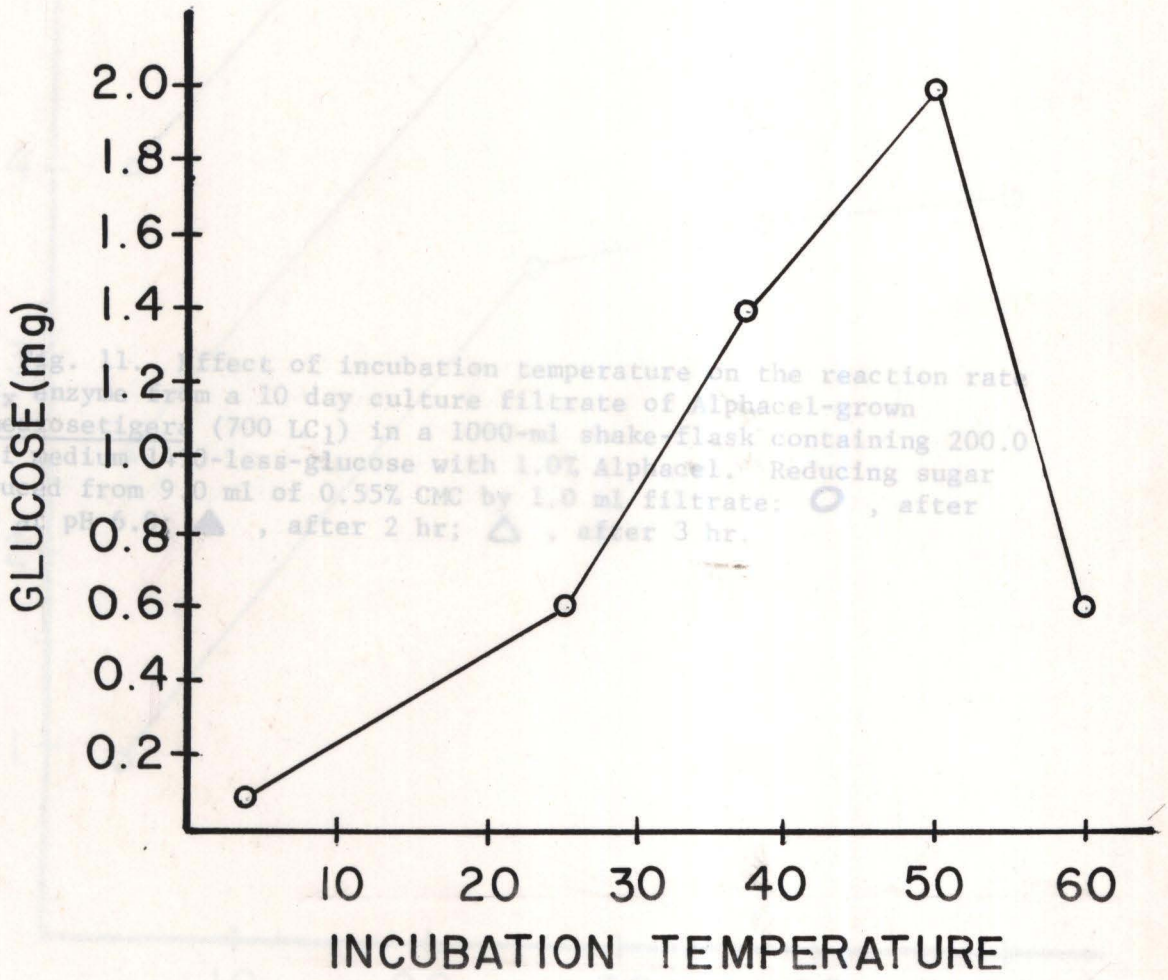


Fig. 10. Effect of incubation temperature on the reaction rate of cellobiase from a 7 day culture filtrate of Alphacel-grown H. mediosetigera (700 LC_1) in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% Alphacel. Glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml filtrate after 1 hr at pH 6.0.


GLUCOSE (mg)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
INCUBATION TEMPERATURE

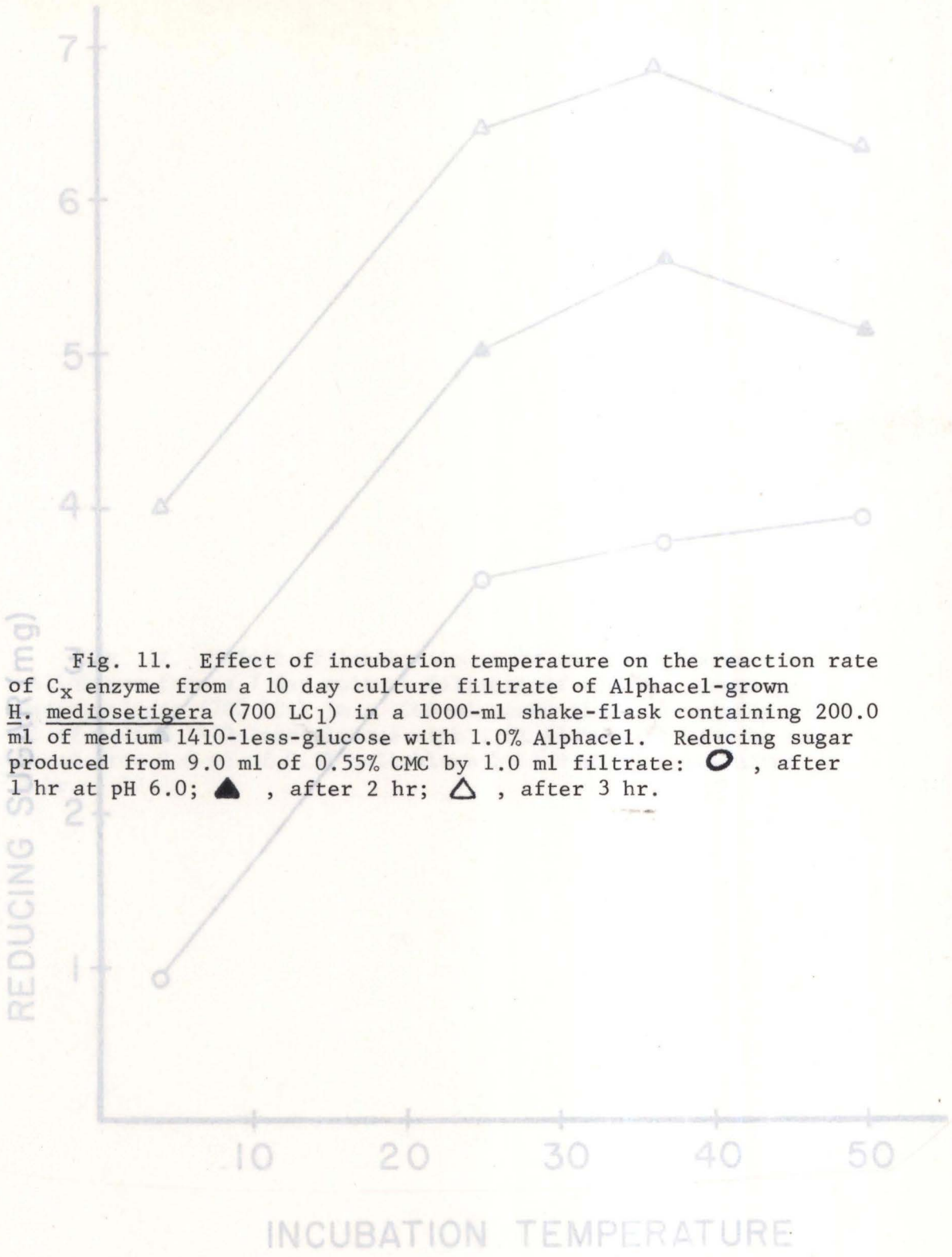
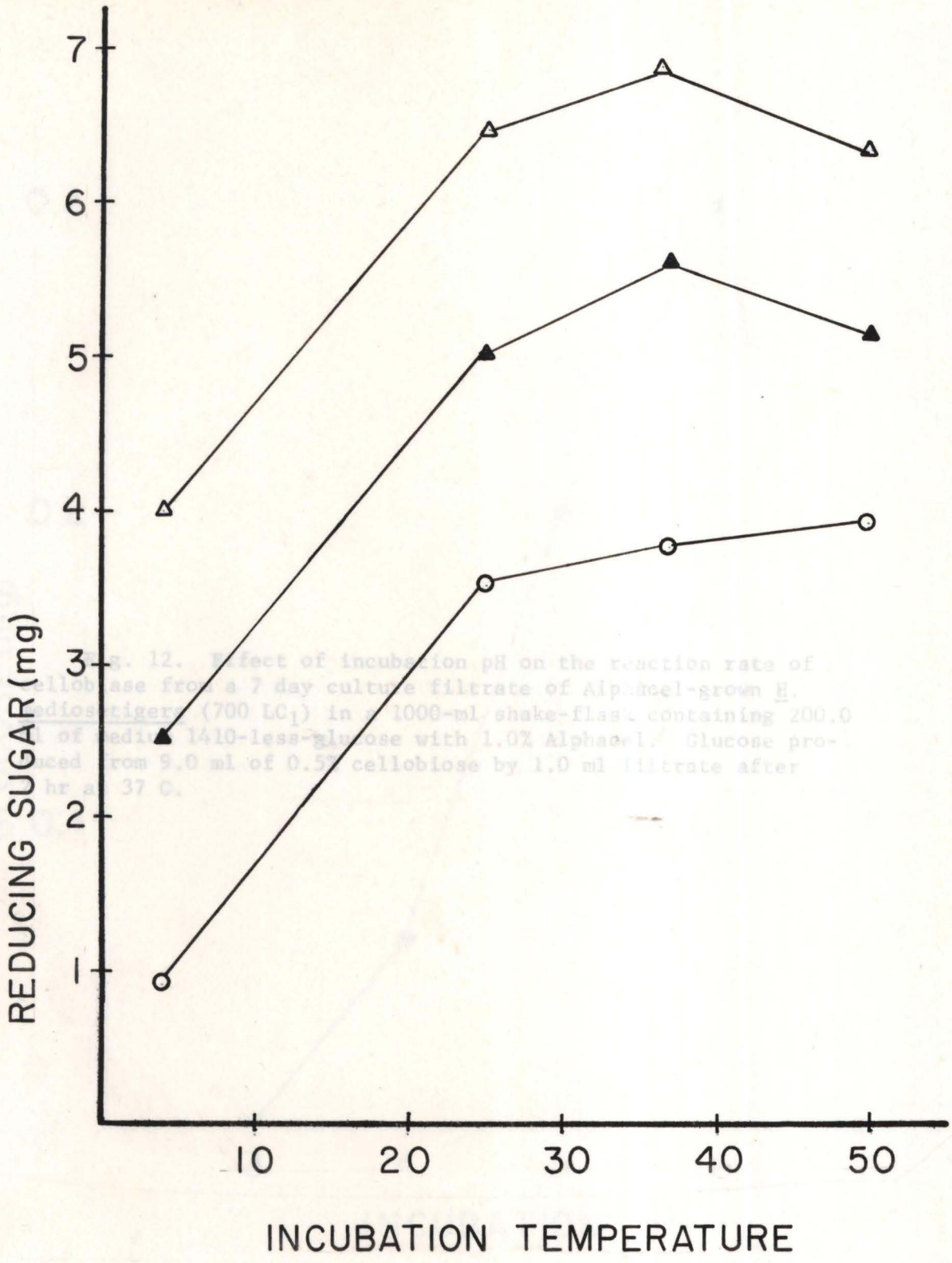


Fig. 11. Effect of incubation temperature on the reaction rate of C_x enzyme from a 10 day culture filtrate of Alphacel-grown H. mediosetigera (700 LC_1) in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% Alphacel. Reducing sugar produced from 9.0 ml of 0.55% CMC by 1.0 ml filtrate: O , after 1 hr at pH 6.0; ▲ , after 2 hr; △ , after 3 hr.


7
6
5
4
3
2
1
REDUCING SUGAR (mg)
10
20
30
40
50
INCUBATION TEMPERATURE

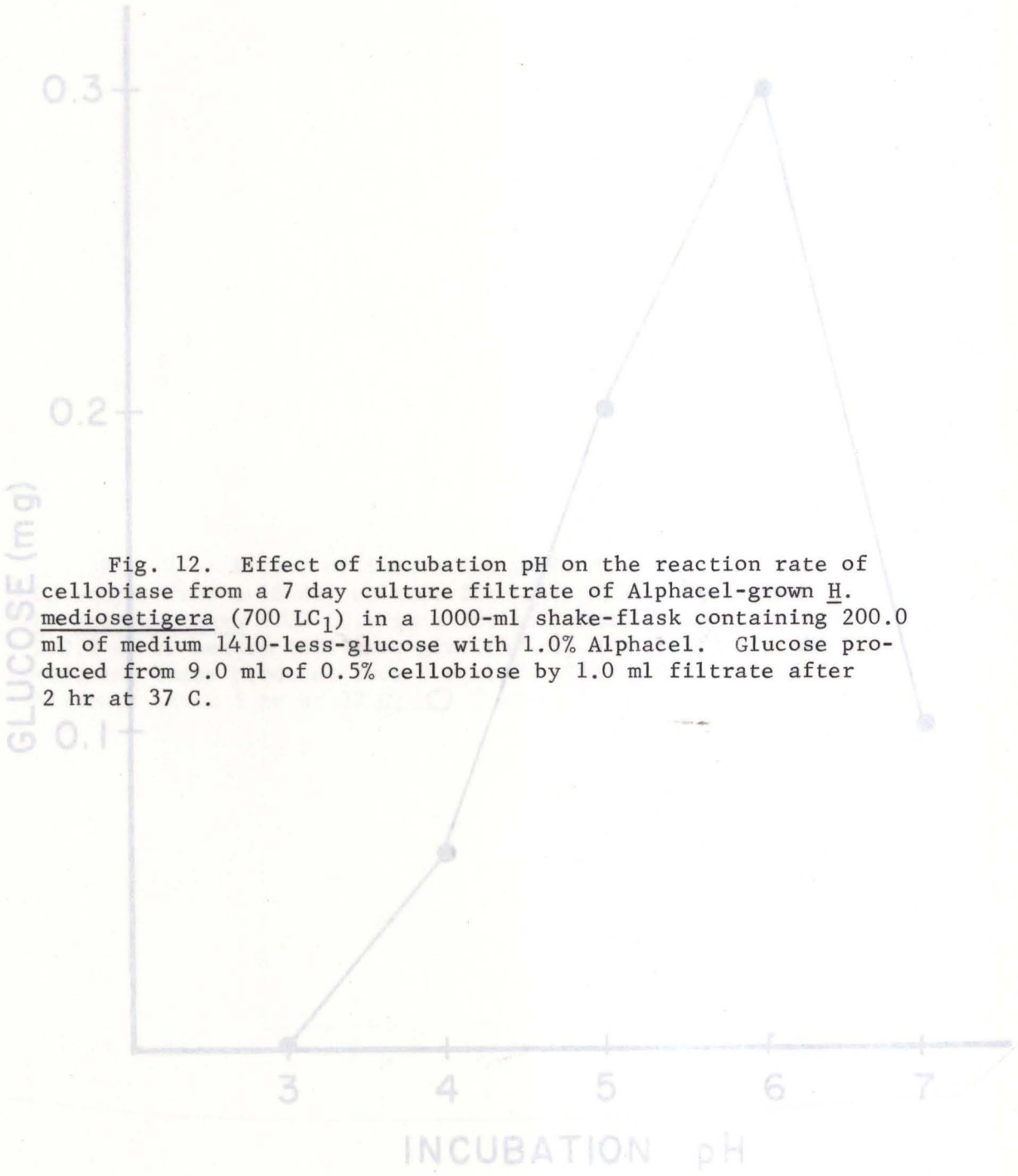
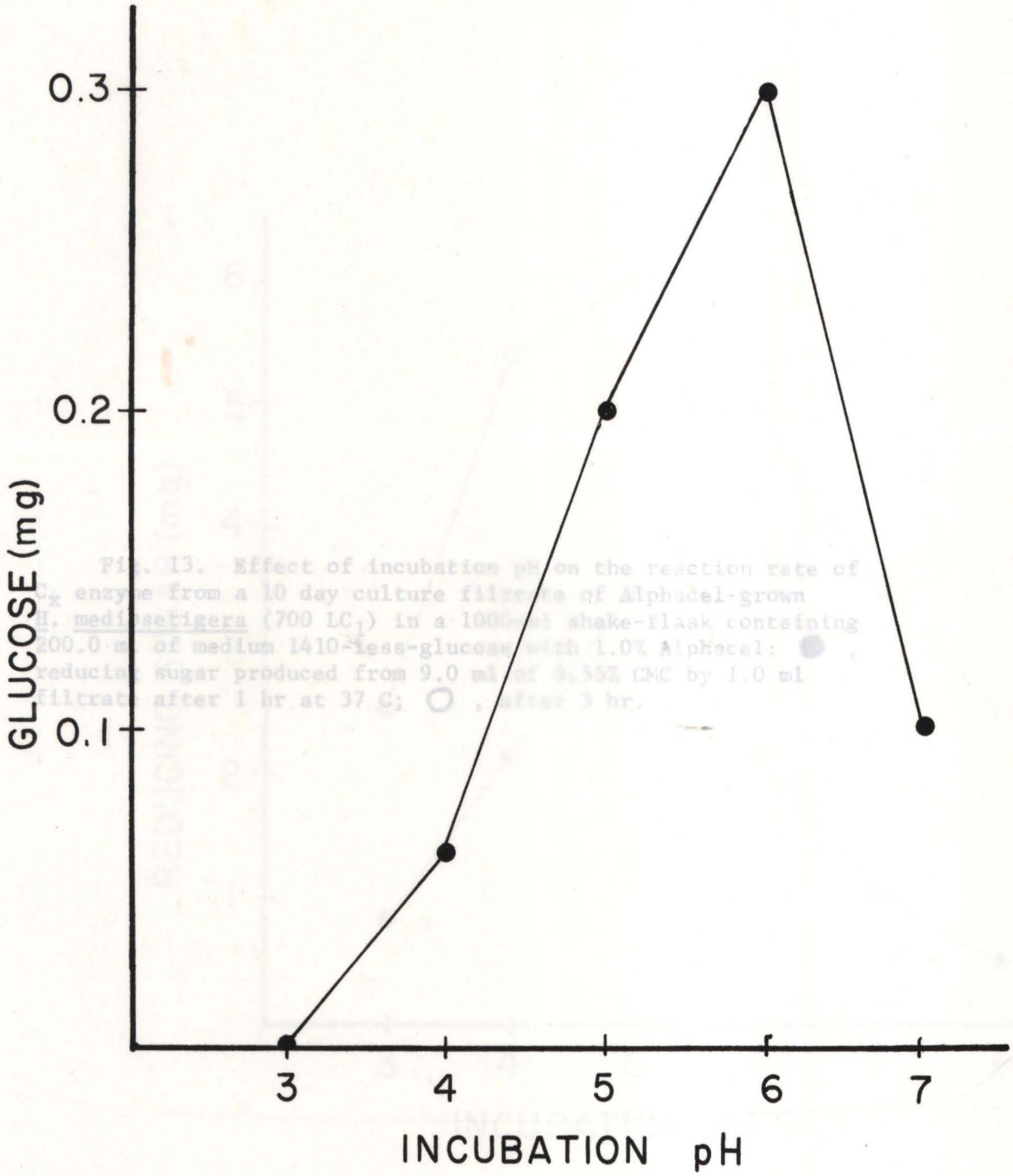


Fig. 12. Effect of incubation pH on the reaction rate of cellobiase from a 7 day culture filtrate of Alphacel-grown H. mediosetigera (700 LC_1) in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% Alphacel. Glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml filtrate after 2 hr at 37 C.


0.3
0.2
0.1
GLUCOSE (mg)
3
4
5
6
7
INCUBATION pH

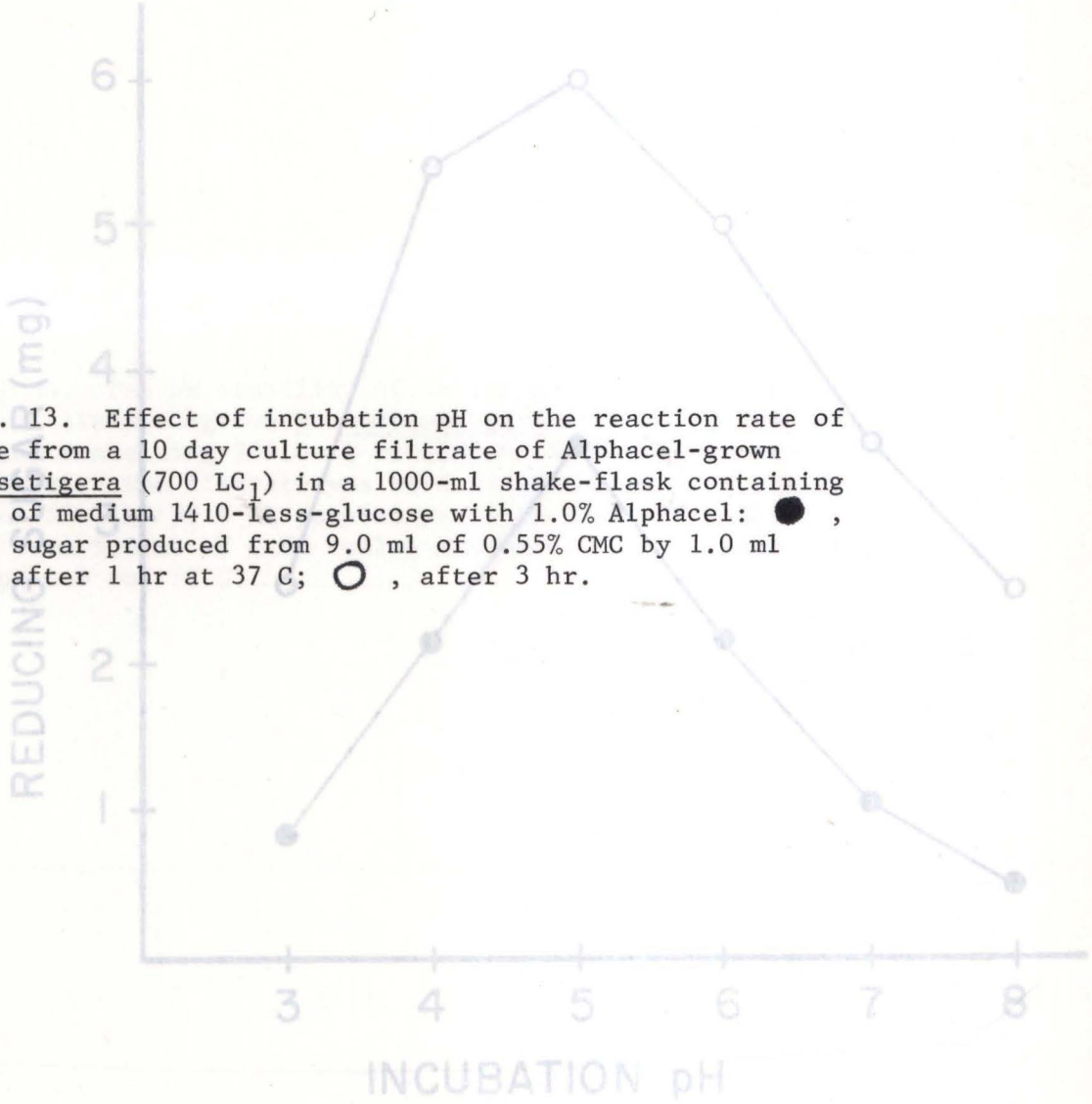
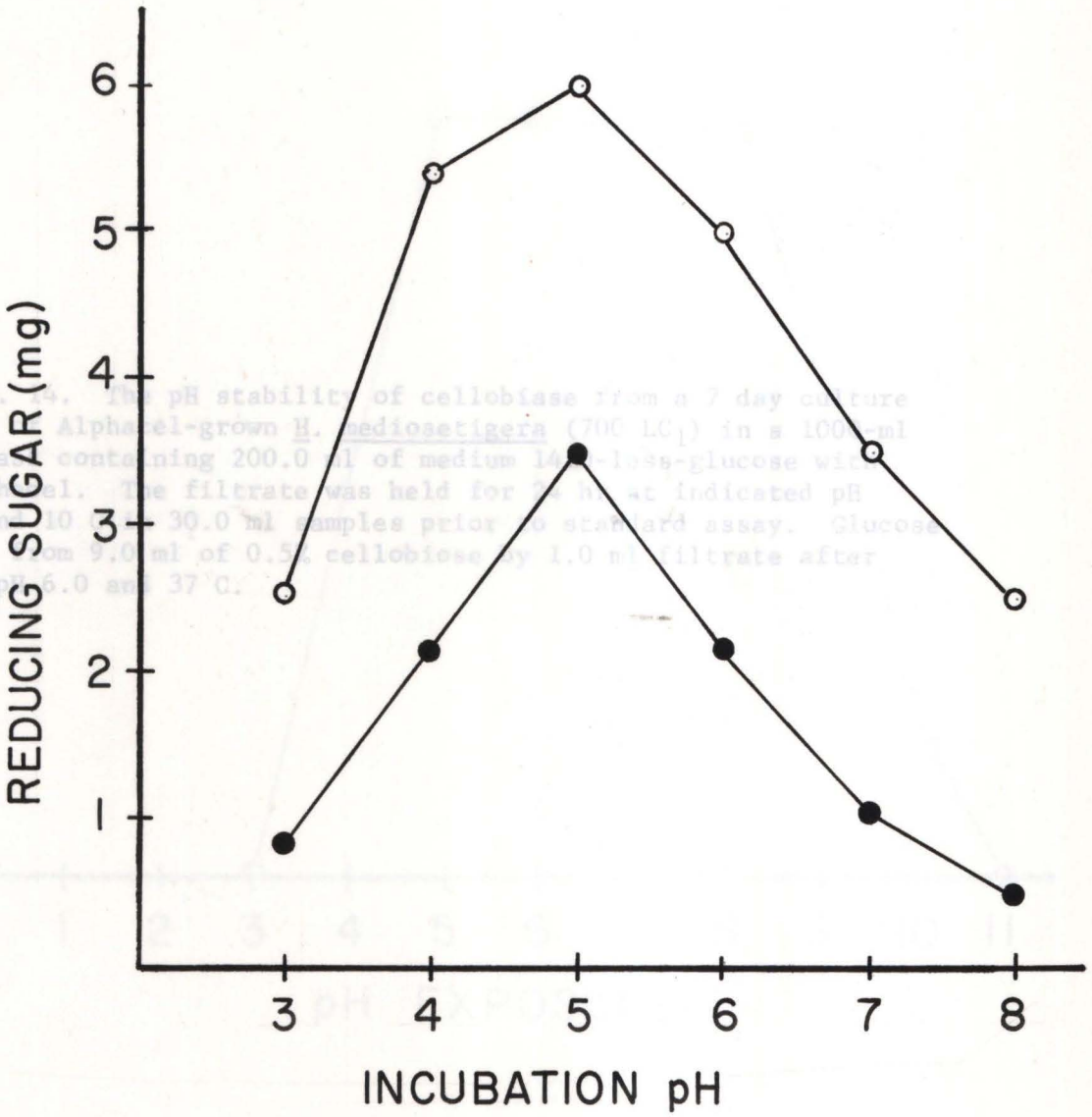


Fig. 13. Effect of incubation pH on the reaction rate of C_x enzyme from a 10 day culture filtrate of Alphacel-grown H. mediosetigera (700 LC_1) in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% Alphacel: ● , reducing sugar produced from 9.0 ml of 0.55% CMC by 1.0 ml filtrate after 1 hr at 37 C; ○ , after 3 hr.


REDUCING SUGAR (mg)
6
5
4
3
2
1
3
4
5
6
7
8
INCUBATION pH

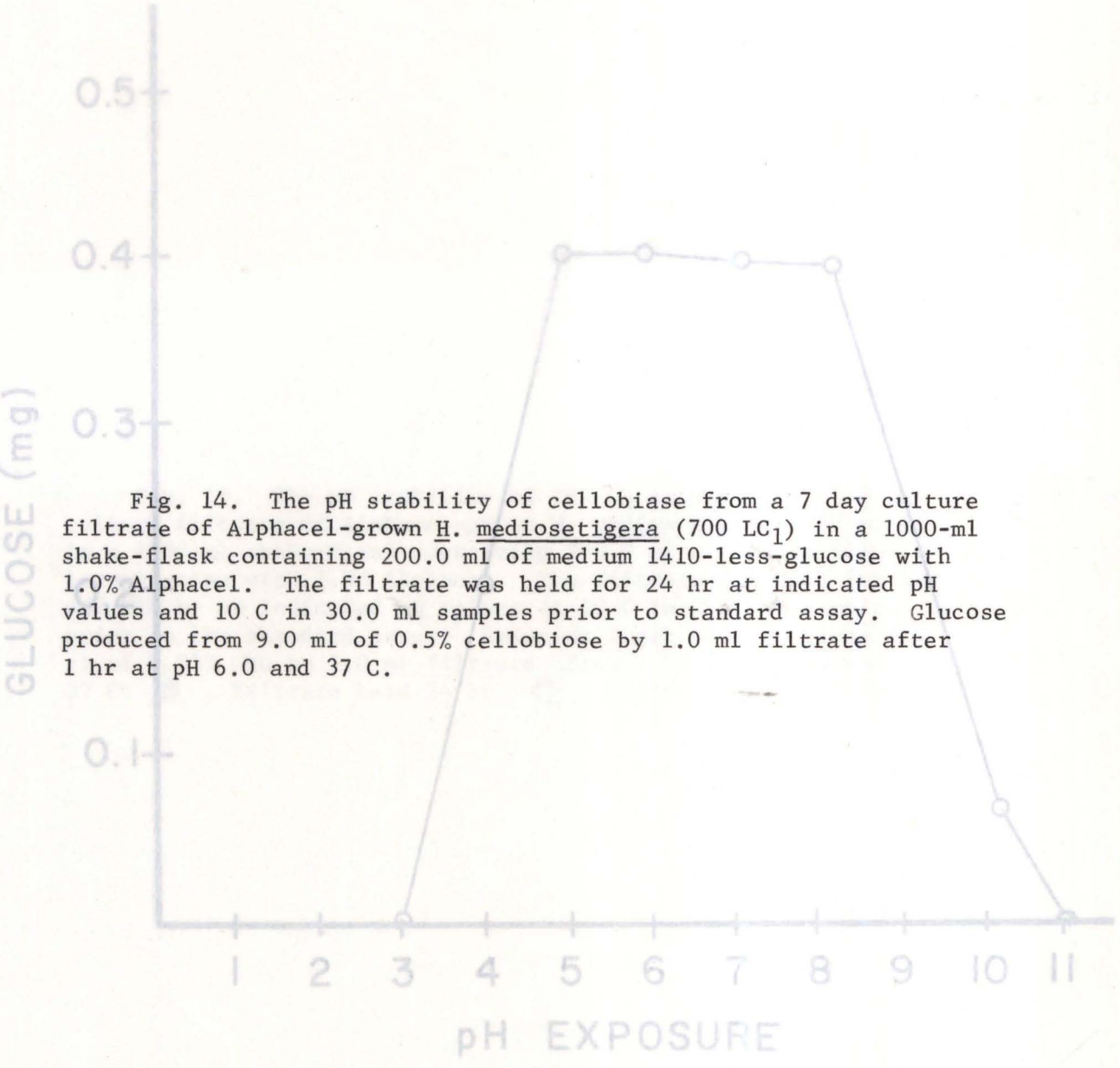
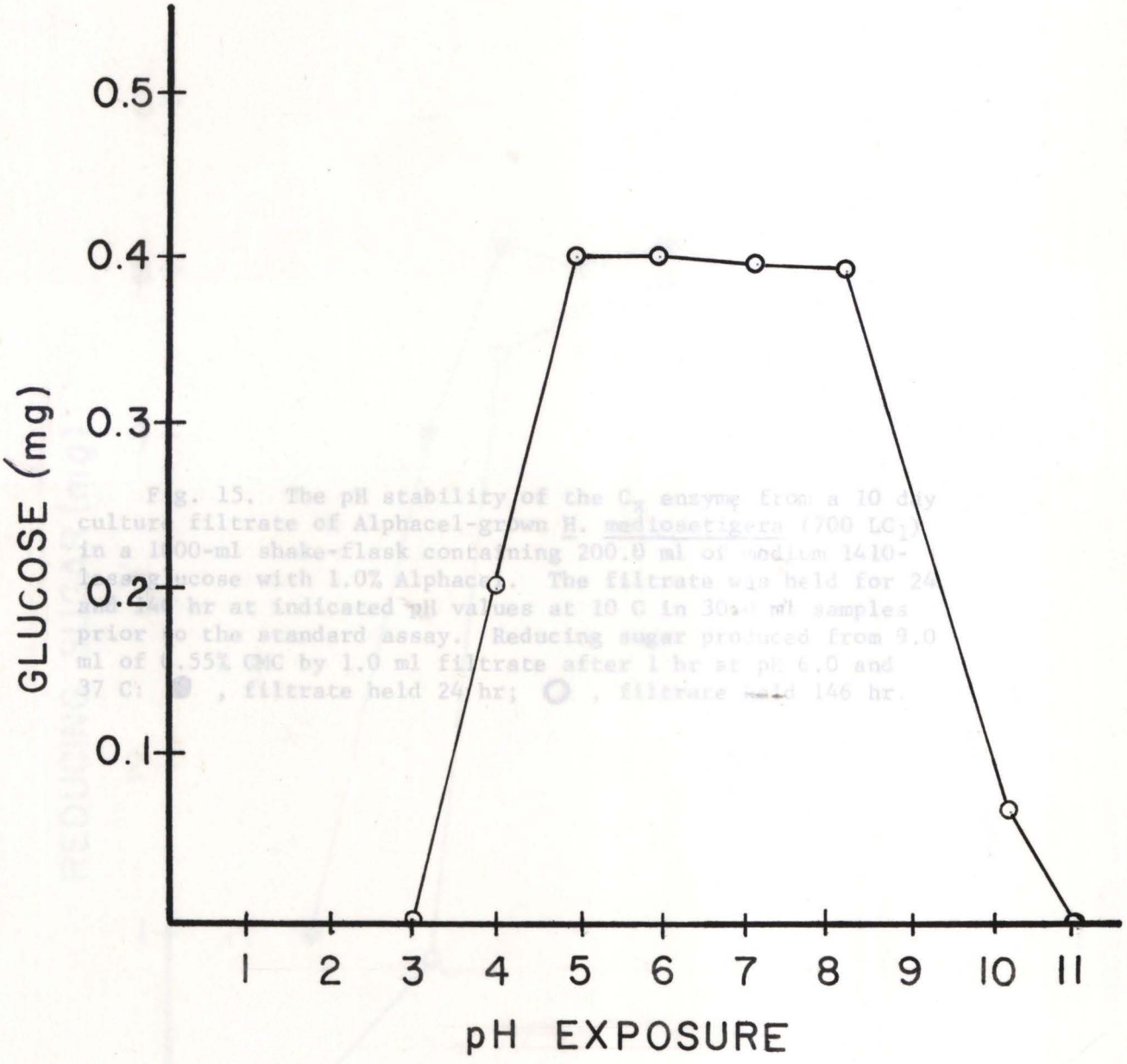


Fig. 14. The pH stability of cellobiase from a 7 day culture filtrate of Alphacel-grown H. mediosetigera (700 LC_1) in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% Alphacel. The filtrate was held for 24 hr at indicated pH values and 10 C in 30.0 ml samples prior to standard assay. Glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml filtrate after 1 hr at pH 6.0 and 37 C.


0.5
0.4
0.3
0.2
0.1
GLUCOSE (mg)
1
2
3
4
5
6
7
8
9
10
11
pH EXPOSURE

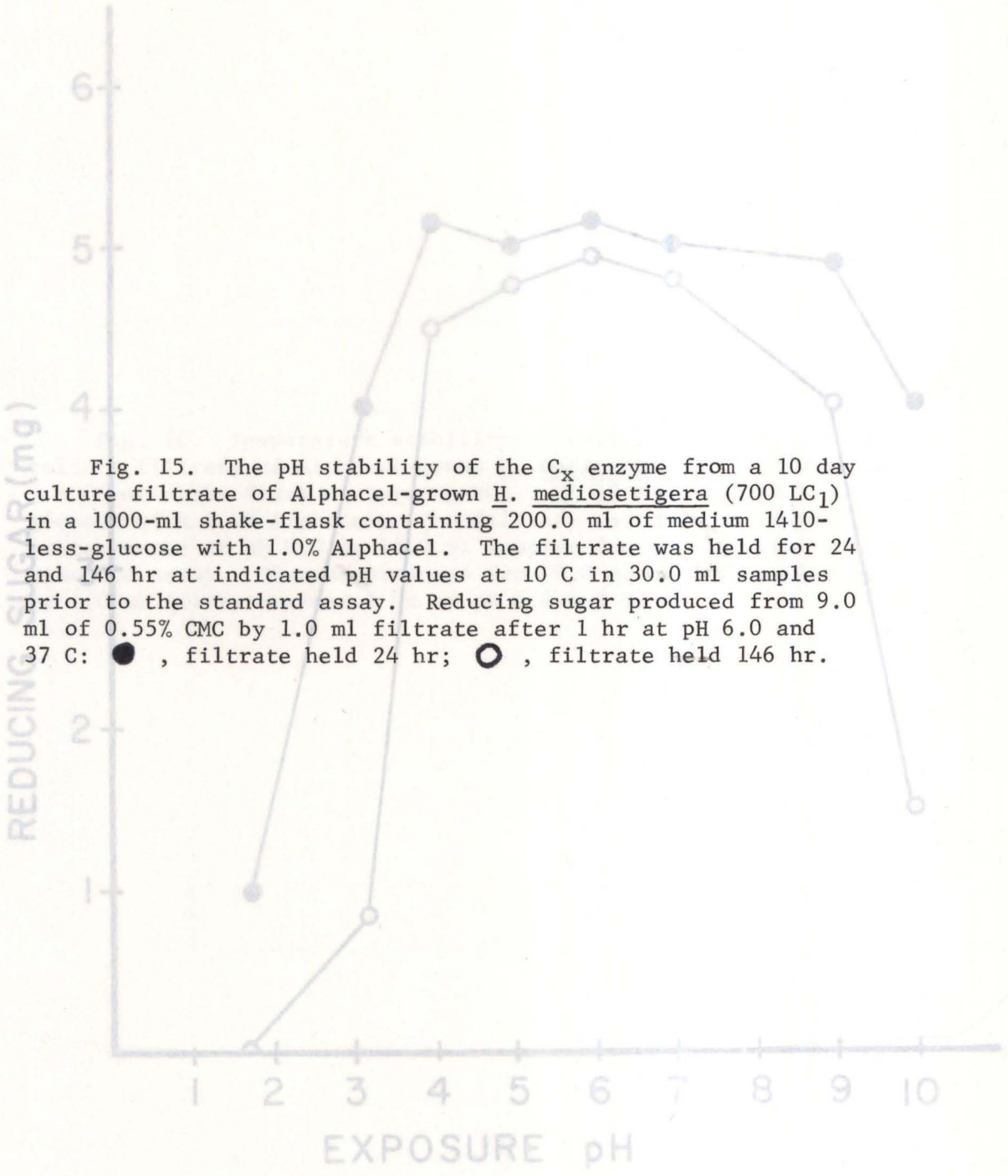
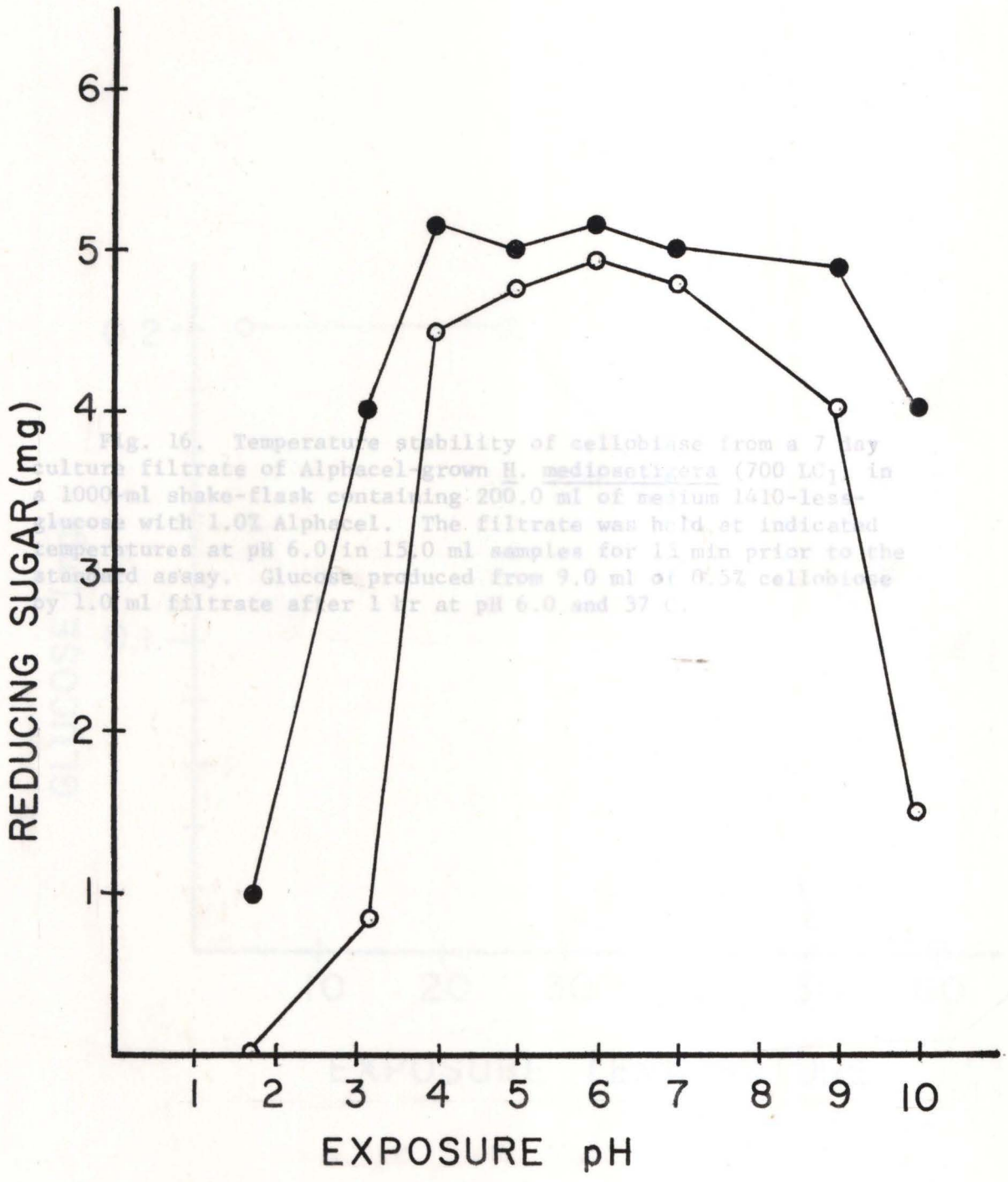


Fig. 15. The pH stability of the C_x enzyme from a 10 day culture filtrate of Alphacel-grown H. mediosetigera (700 LC_1) in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% Alphacel. The filtrate was held for 24 and 146 hr at indicated pH values at 10 C in 30.0 ml samples prior to the standard assay. Reducing sugar produced from 9.0 ml of 0.55% CMC by 1.0 ml filtrate after 1 hr at pH 6.0 and 37 C: ● , filtrate held 24 hr; ○ , filtrate held 146 hr.


REDUCING SUGAR (mg)
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10
EXPOSURE pH

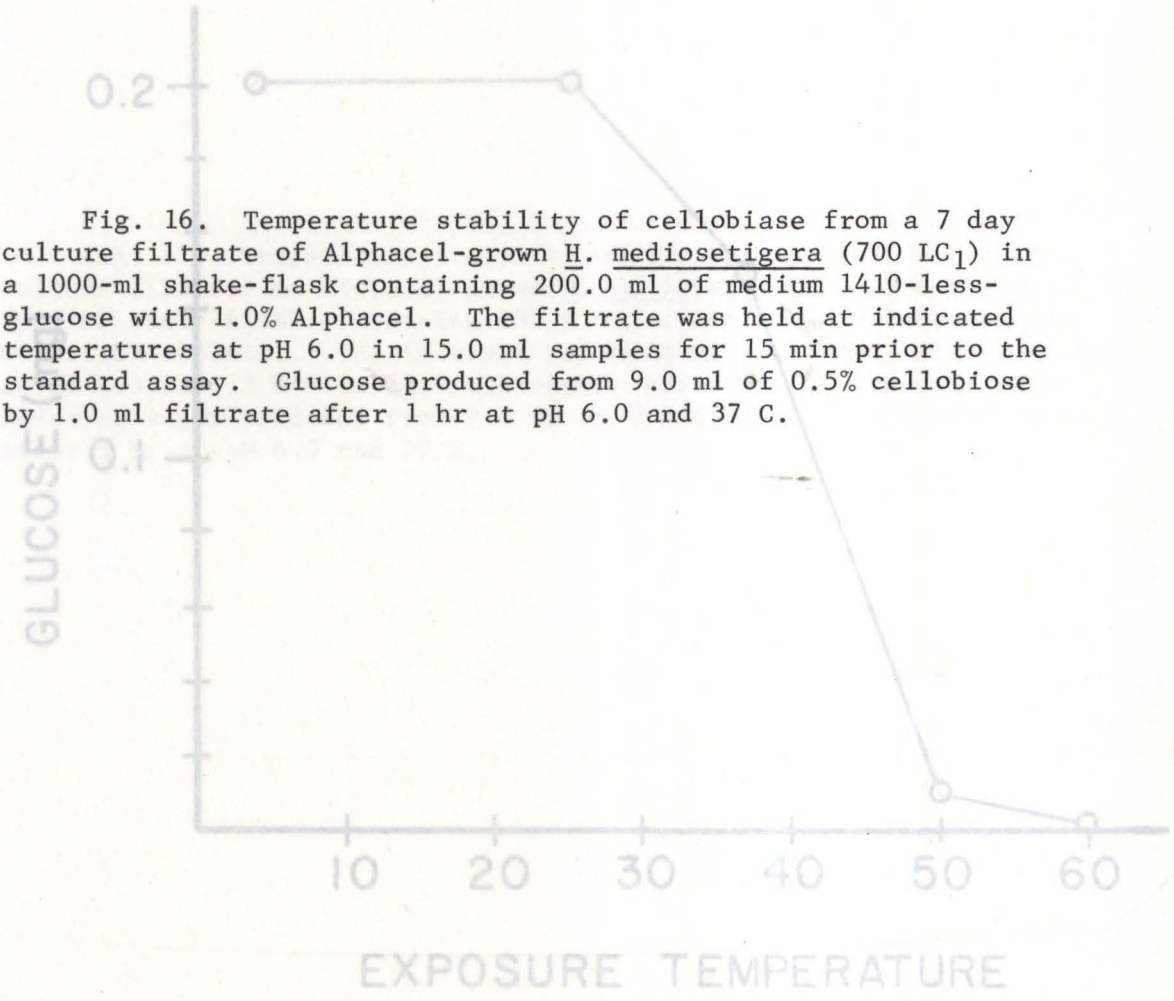
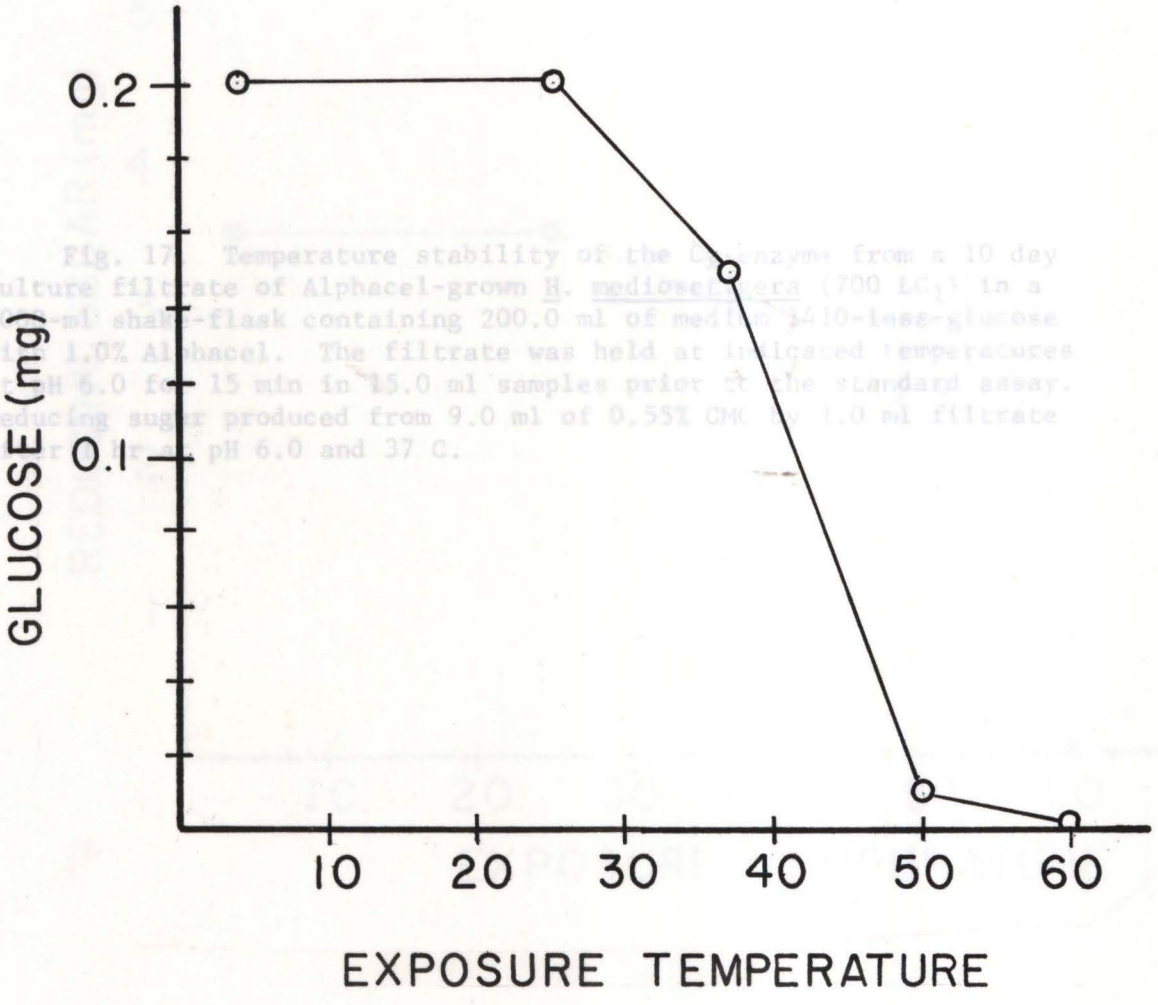


Fig. 16. Temperature stability of cellobiase from a 7 day culture filtrate of Alphacel-grown H. mediosetigera (700 LC_1) in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% Alphacel. The filtrate was held at indicated temperatures at pH 6.0 in 15.0 ml samples for 15 min prior to the standard assay. Glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml filtrate after 1 hr at pH 6.0 and 37 C.


0.2
0.1
GLUCOSE (mg)
10
20
30
40
50
60
EXPOSURE TEMPERATURE

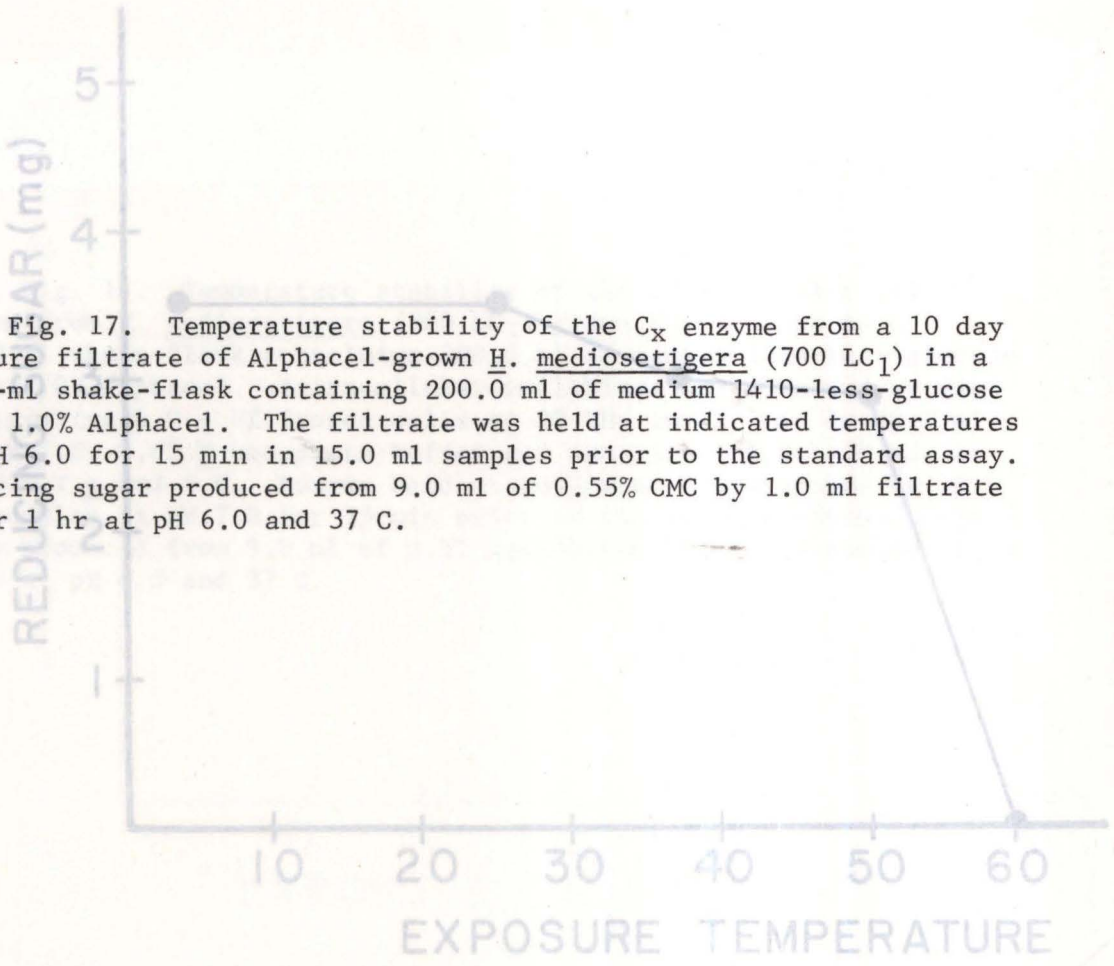
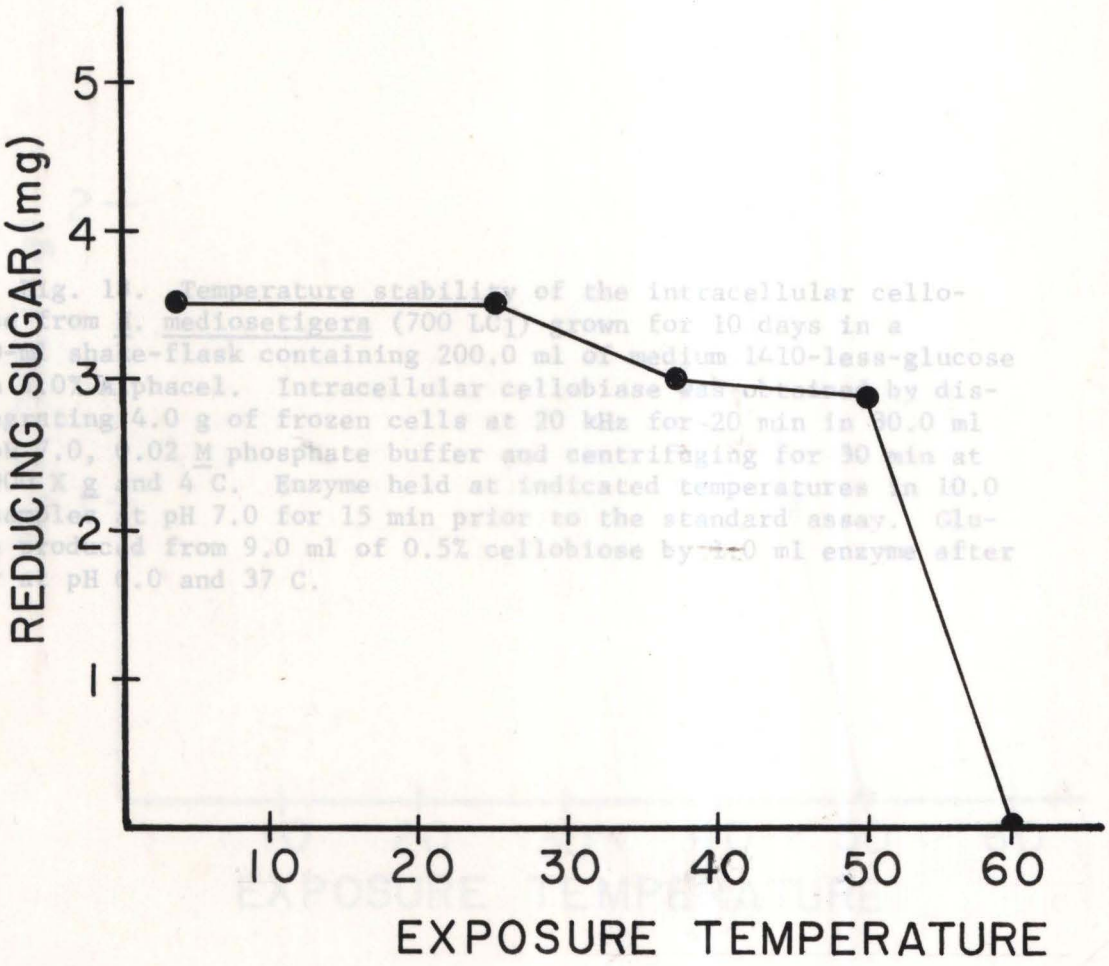


Fig. 17. Temperature stability of the C_x enzyme from a 10 day culture filtrate of Alphacel-grown H. mediosetigera (700 LC_1) in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% Alphacel. The filtrate was held at indicated temperatures at pH 6.0 for 15 min in 15.0 ml samples prior to the standard assay. Reducing sugar produced from 9.0 ml of 0.55% CMC by 1.0 ml filtrate after 1 hr at pH 6.0 and 37 C.


REDUCING SUGAR (mg)
5
4
3
2
1
10
20
30
40
50
60
EXPOSURE TEMPERATURE

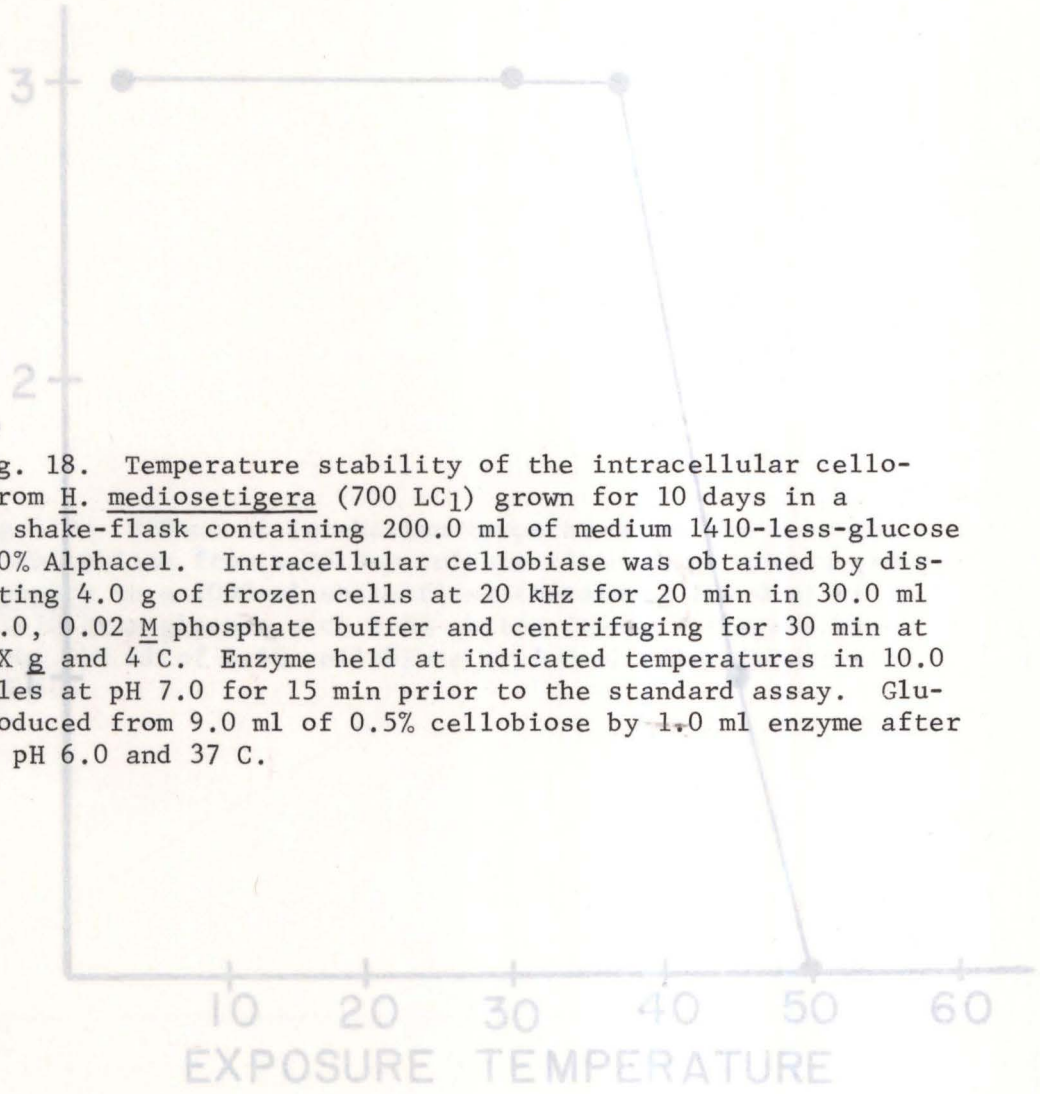
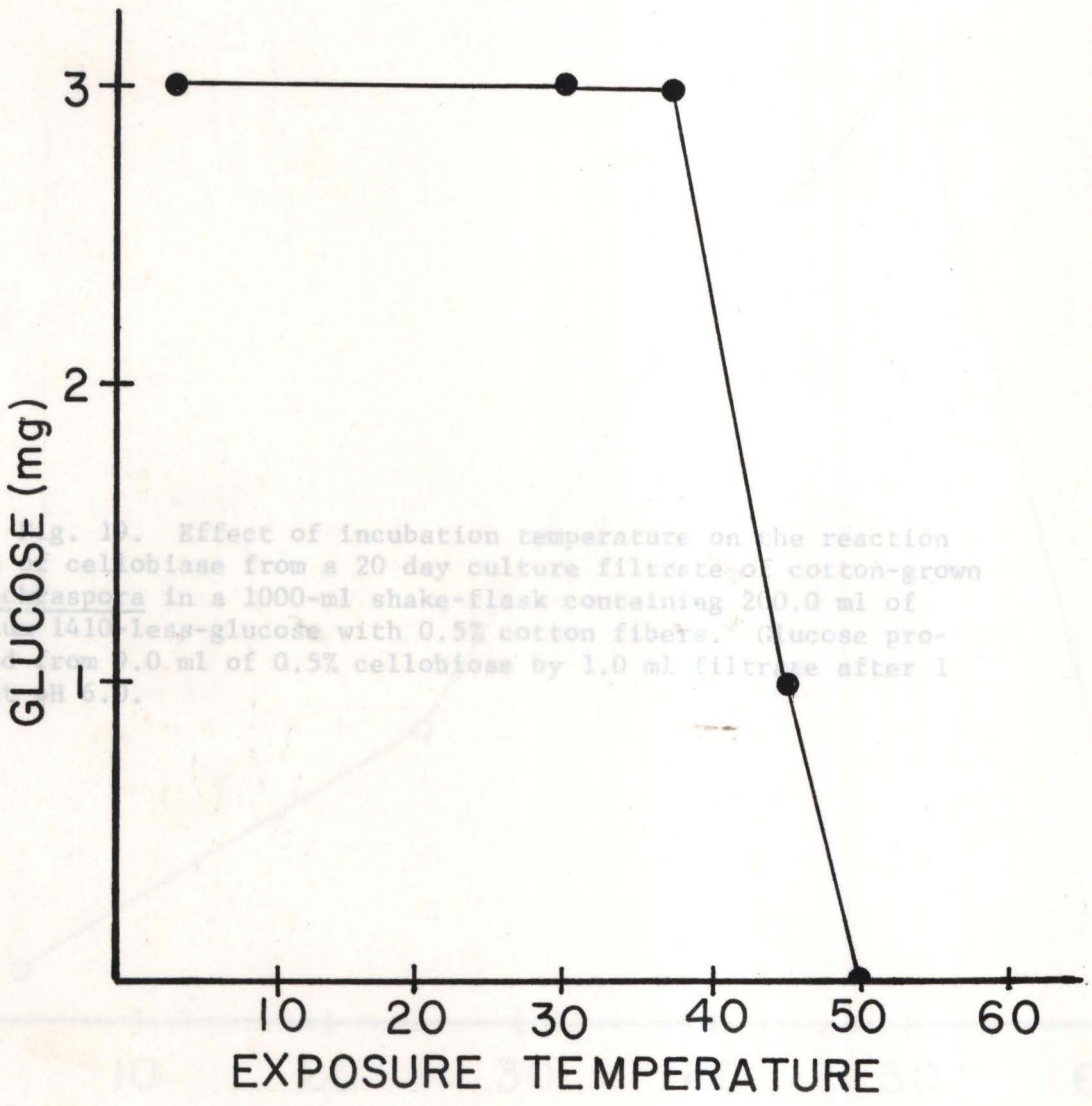


Fig. 18. Temperature stability of the intracellular cellobiase from H. mediosetigera (700 LC_1) grown for 10 days in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% Alphacel. Intracellular cellobiase was obtained by disintegrating 4.0 g of frozen cells at 20 kHz for 20 min in 30.0 ml of pH 7.0, 0.02 M phosphate buffer and centrifuging for 30 min at 12,000 X g and 4 C. Enzyme held at indicated temperatures in 10.0 ml samples at pH 7.0 for 15 min prior to the standard assay. Glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml enzyme after 1 hr at pH 6.0 and 37 C.


3
2
1
GLUCOSE (mg)
10
20
30
40
50
60
EXPOSURE TEMPERATURE

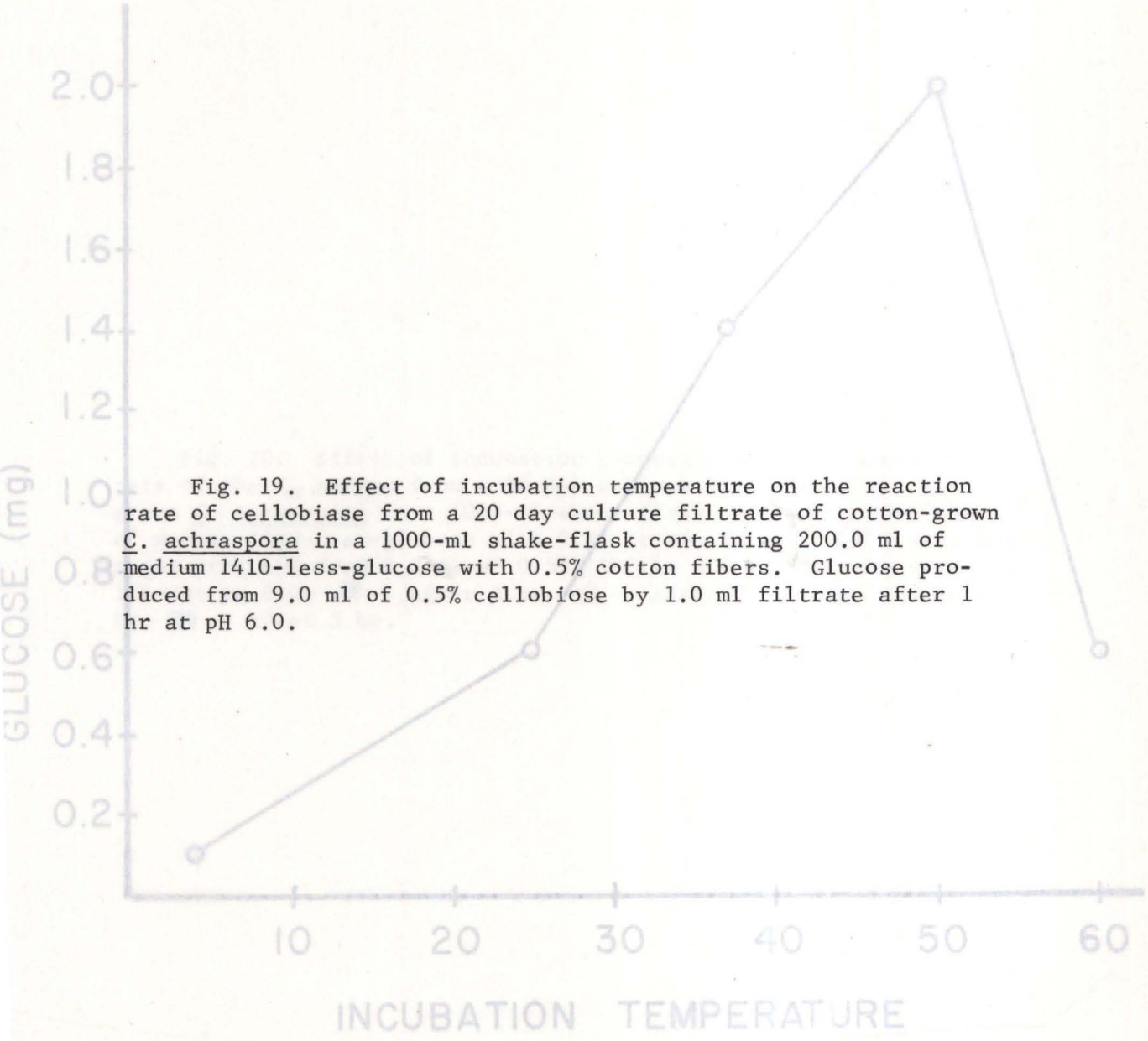
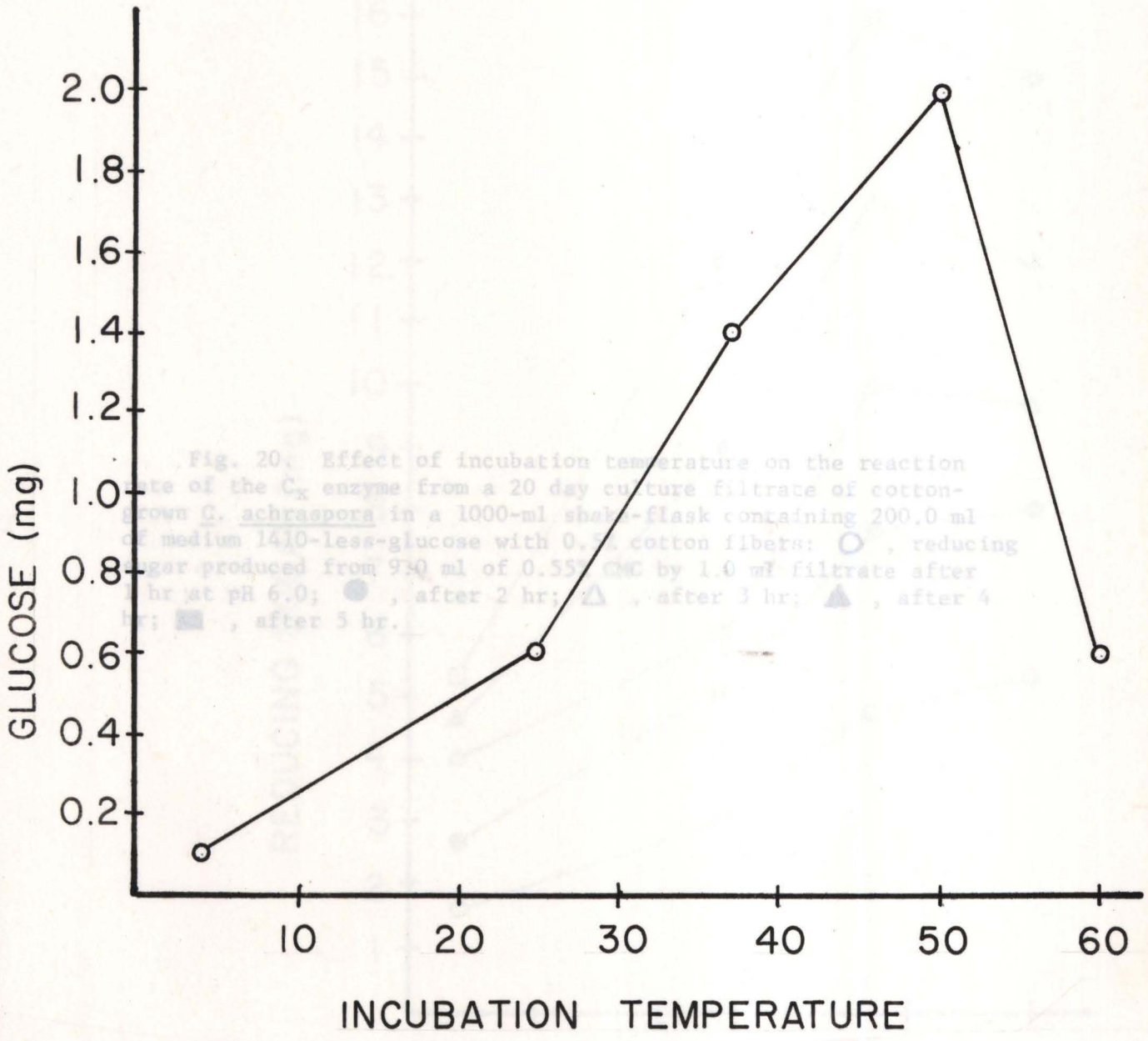


Fig. 19. Effect of incubation temperature on the reaction rate of cellobiase from a 20 day culture filtrate of cotton-grown C. achraspora in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 0.5% cotton fibers. Glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml filtrate after 1 hr at pH 6.0.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
GLUCOSE (mg)
10
20
30
40
50
60
INCUBATION TEMPERATURE

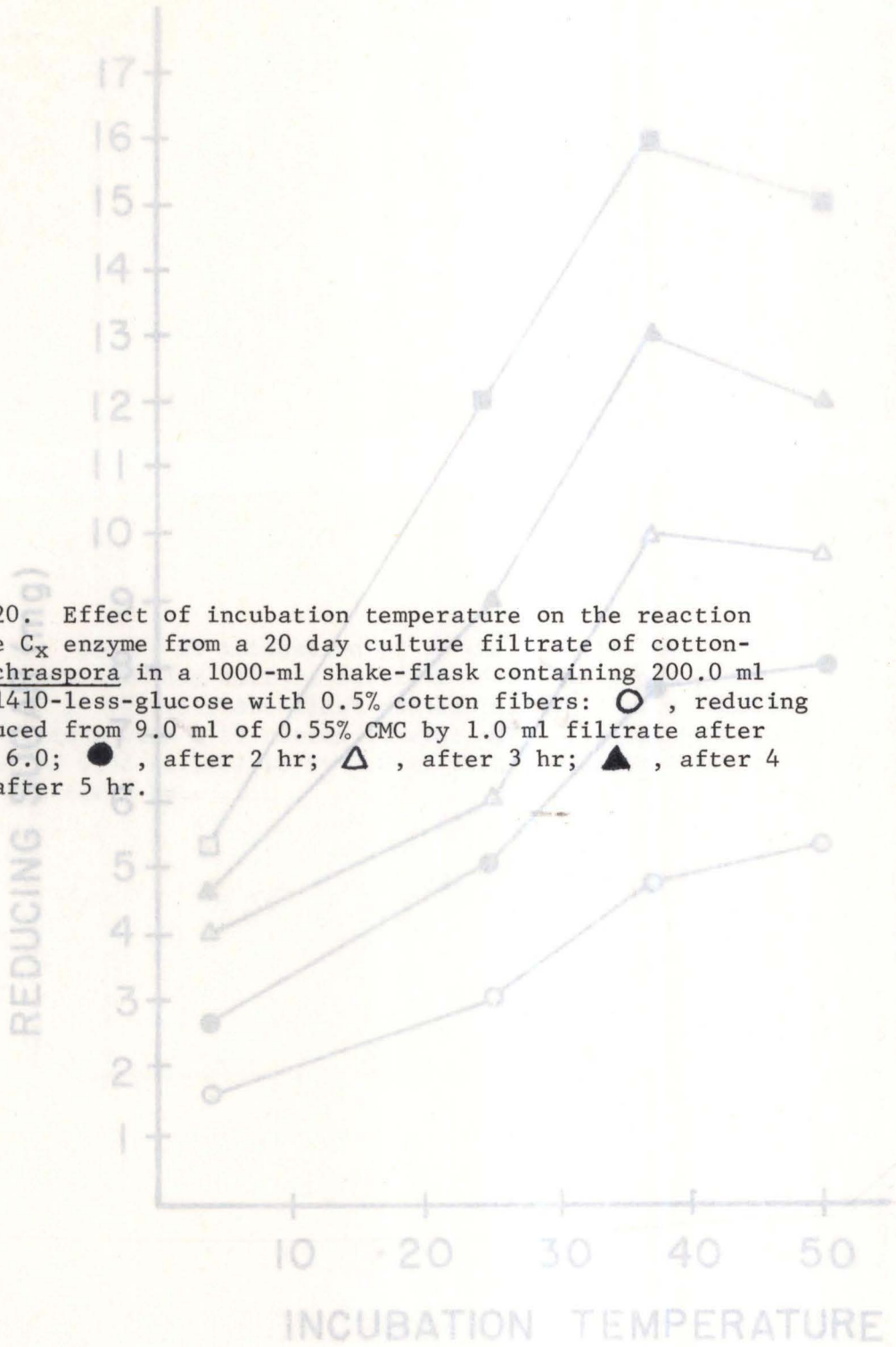
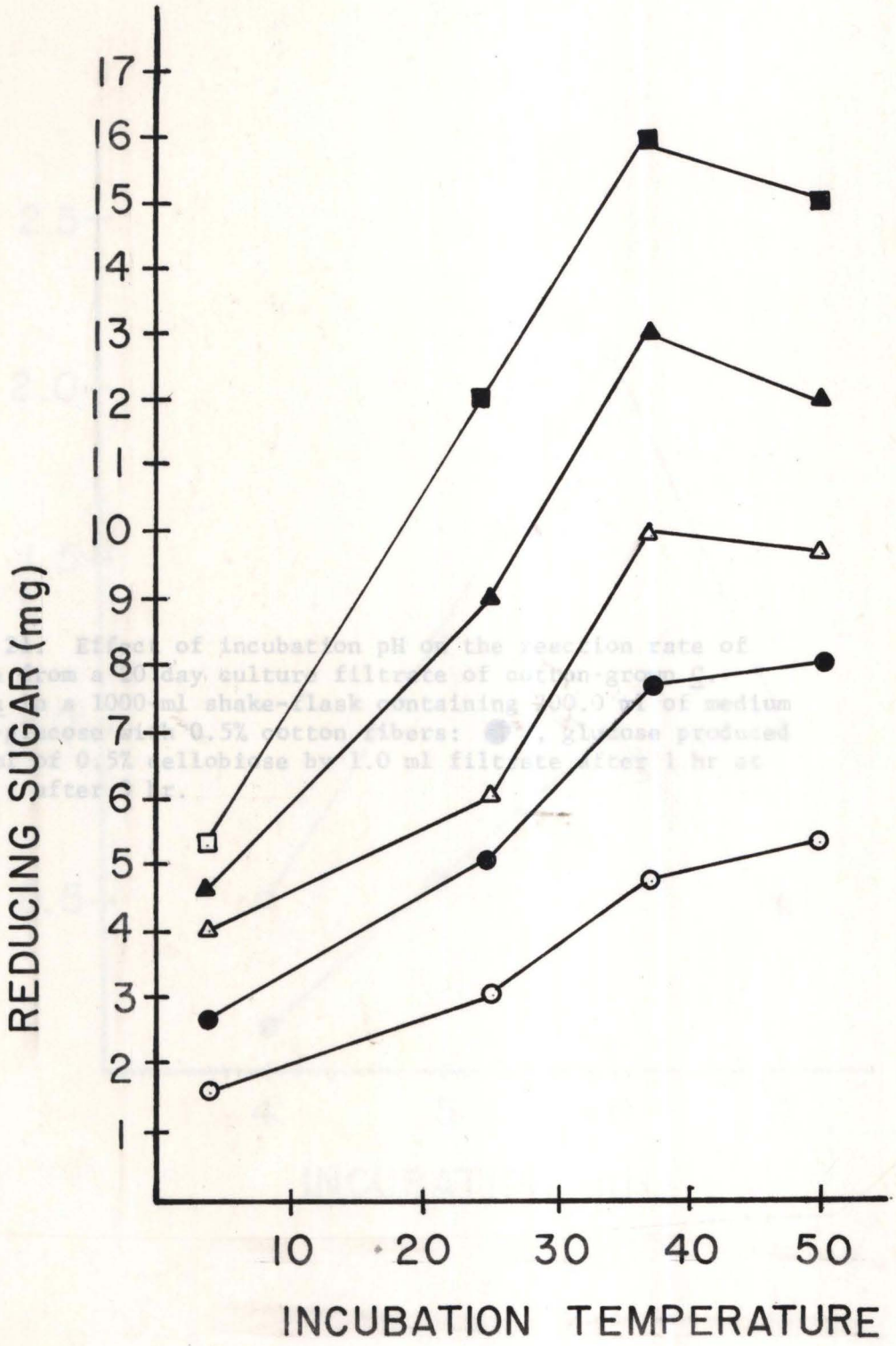


Fig. 20. Effect of incubation temperature on the reaction rate of the C_x enzyme from a 20 day culture filtrate of cotton-grown C. achraspora in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 0.5% cotton fibers: ○ , reducing sugar produced from 9.0 ml of 0.55% CMC by 1.0 ml filtrate after 1 hr at pH 6.0; ● , after 2 hr; △ , after 3 hr; ▲ , after 4 hr; ■ , after 5 hr.


REDUCING SUGAR (mg)
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
INCUBATION TEMPERATURE

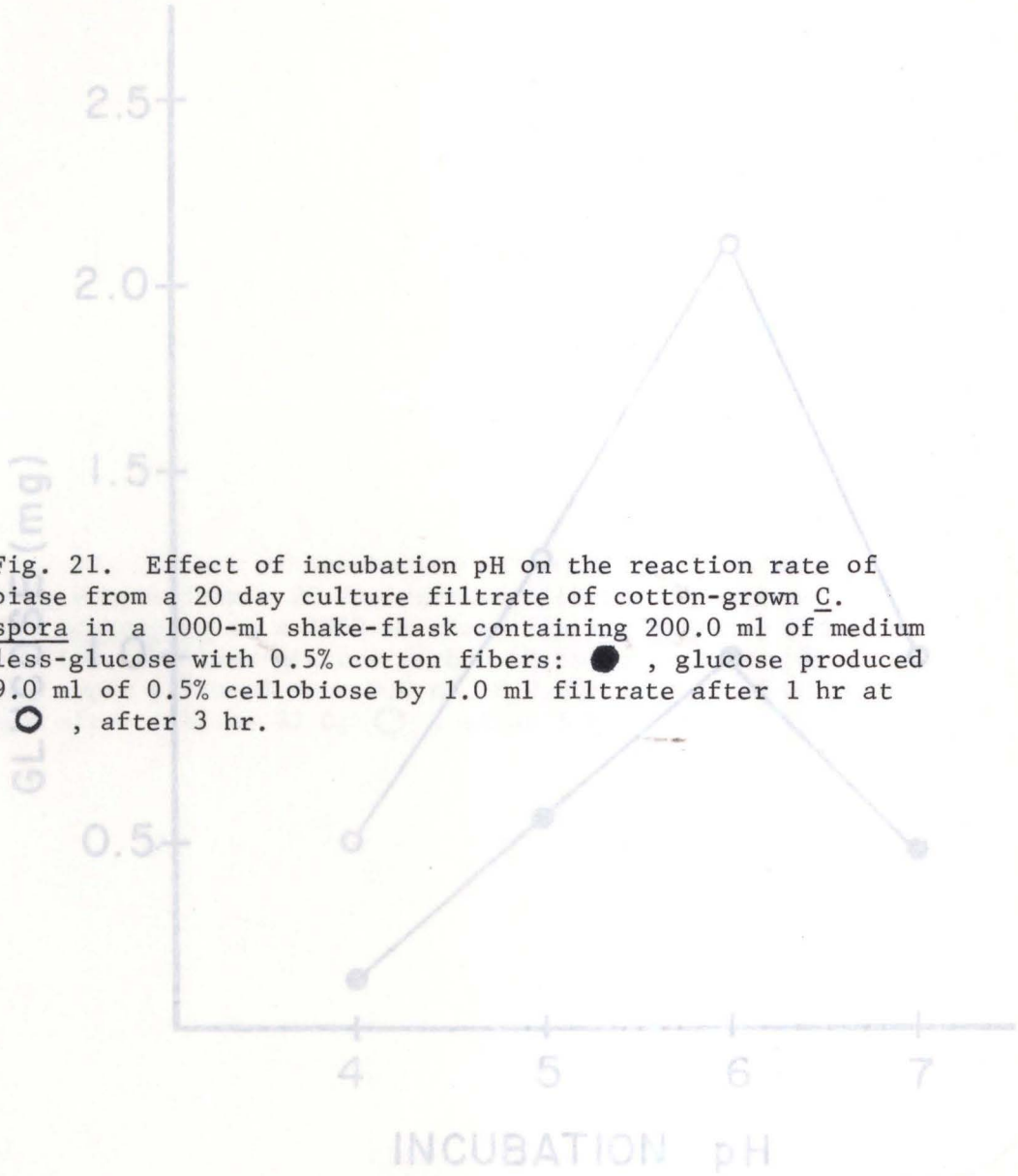
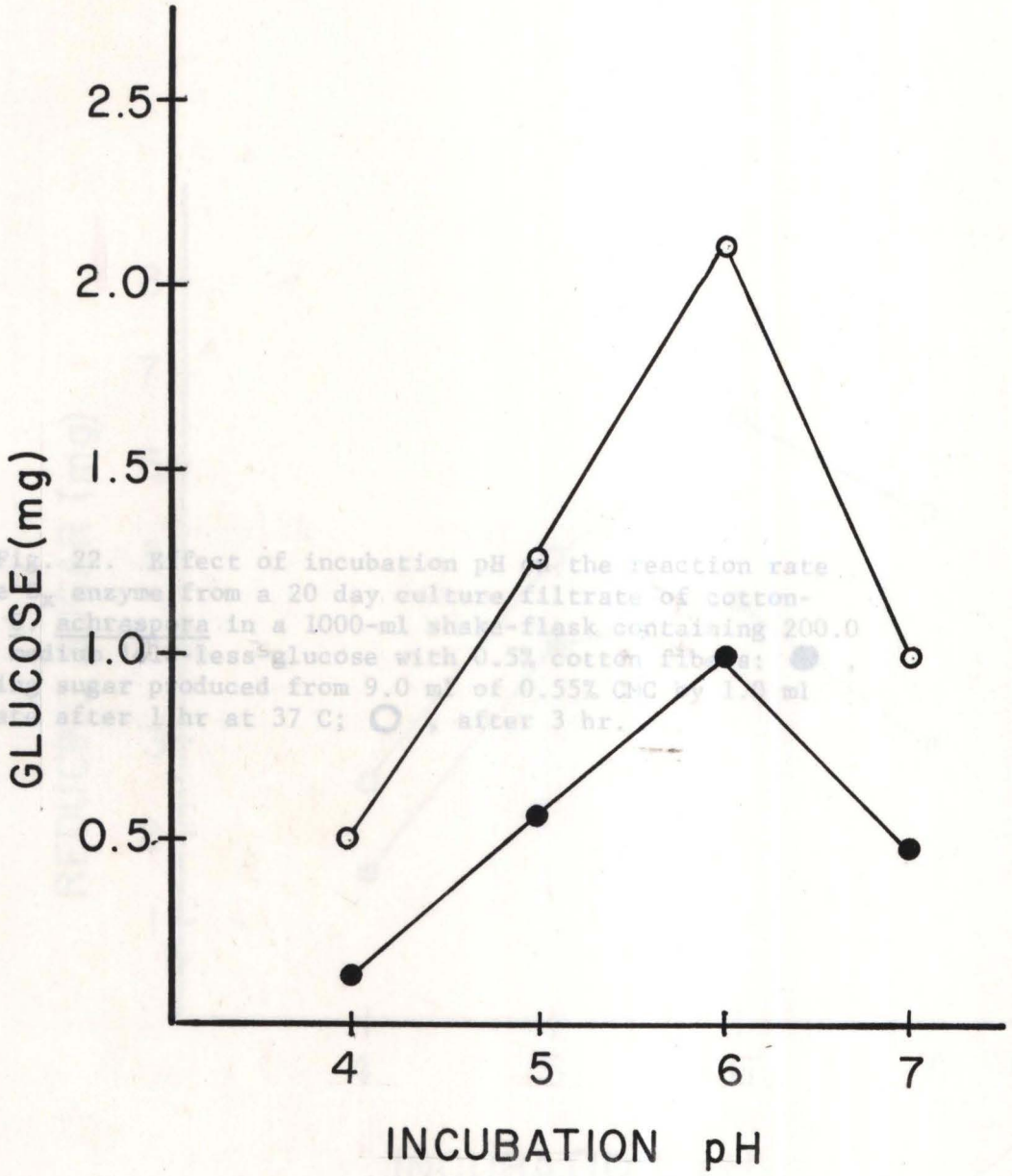


Fig. 21. Effect of incubation pH on the reaction rate of cellobiase from a 20 day culture filtrate of cotton-grown C. achraspora in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 0.5% cotton fibers: ● , glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml filtrate after 1 hr at 37 C; ○ , after 3 hr.


2.5
2.0
1.5
1.0
0.5
GLUCOSE (mg)
4
5
6
7
INCUBATION pH

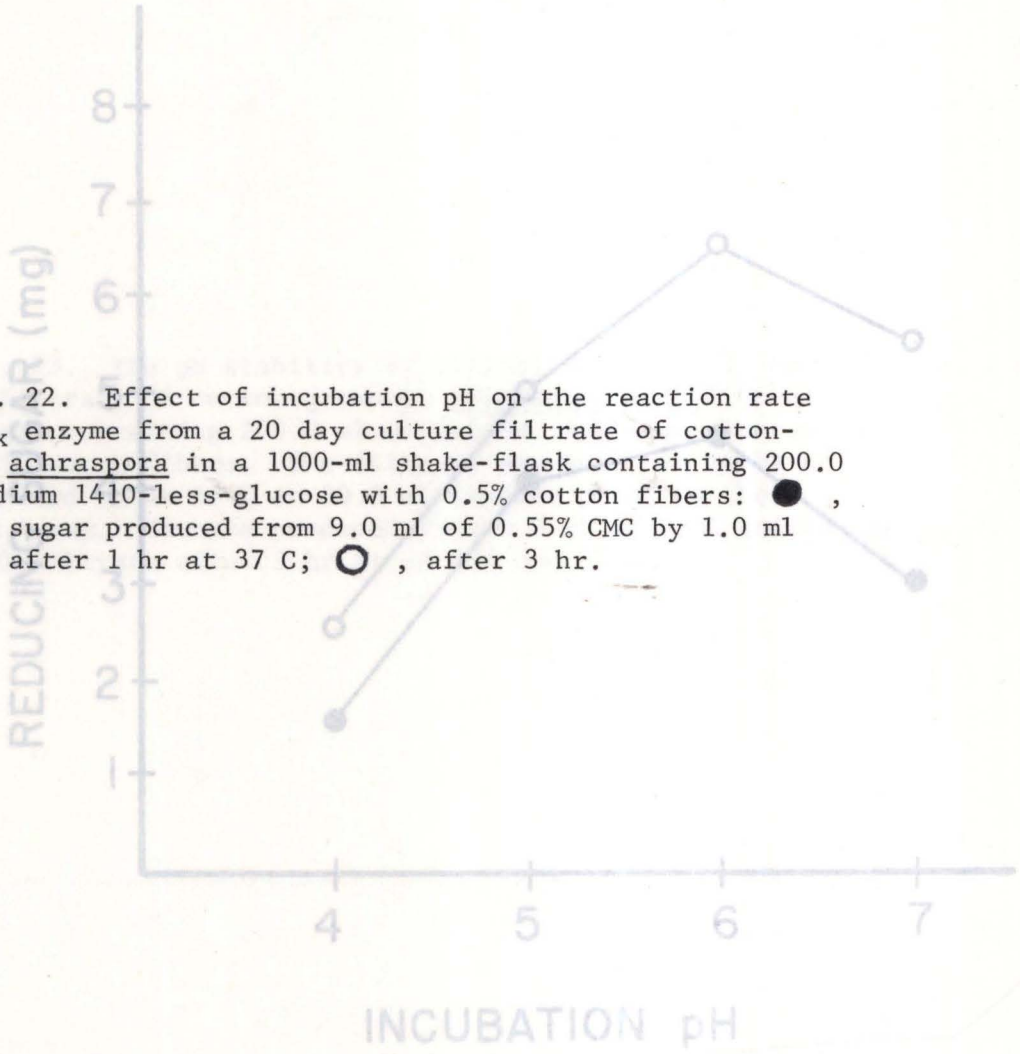
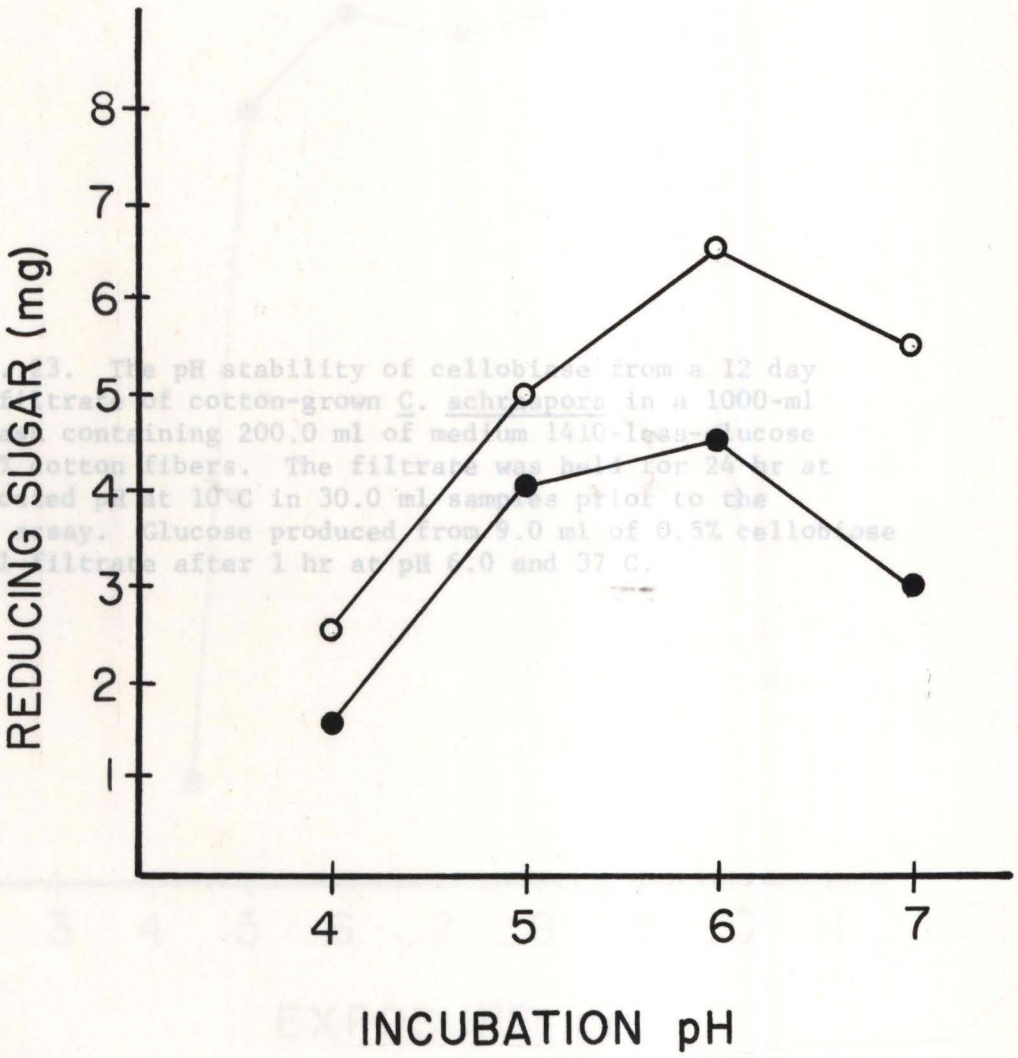


Fig. 22. Effect of incubation pH on the reaction rate of the C_x enzyme from a 20 day culture filtrate of cotton-grown C. achraspora in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 0.5% cotton fibers: ● , reducing sugar produced from 9.0 ml of 0.55% CMC by 1.0 ml filtrate after 1 hr at 37 C; ○ , after 3 hr.


REDUCING SUGAR (mg)
8
7
6
5
4
3
2
1
4
5
6
7
INCUBATION pH

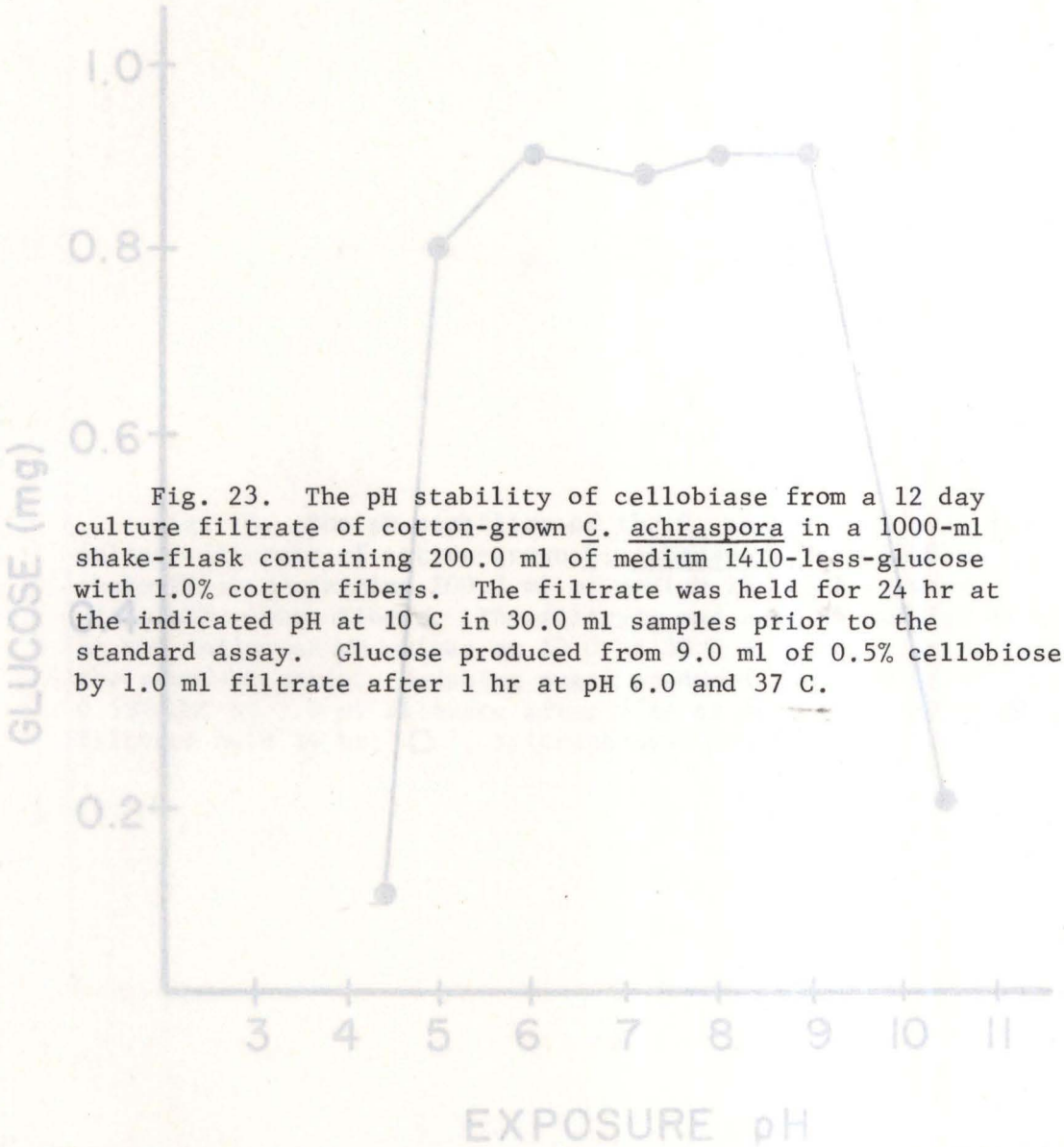
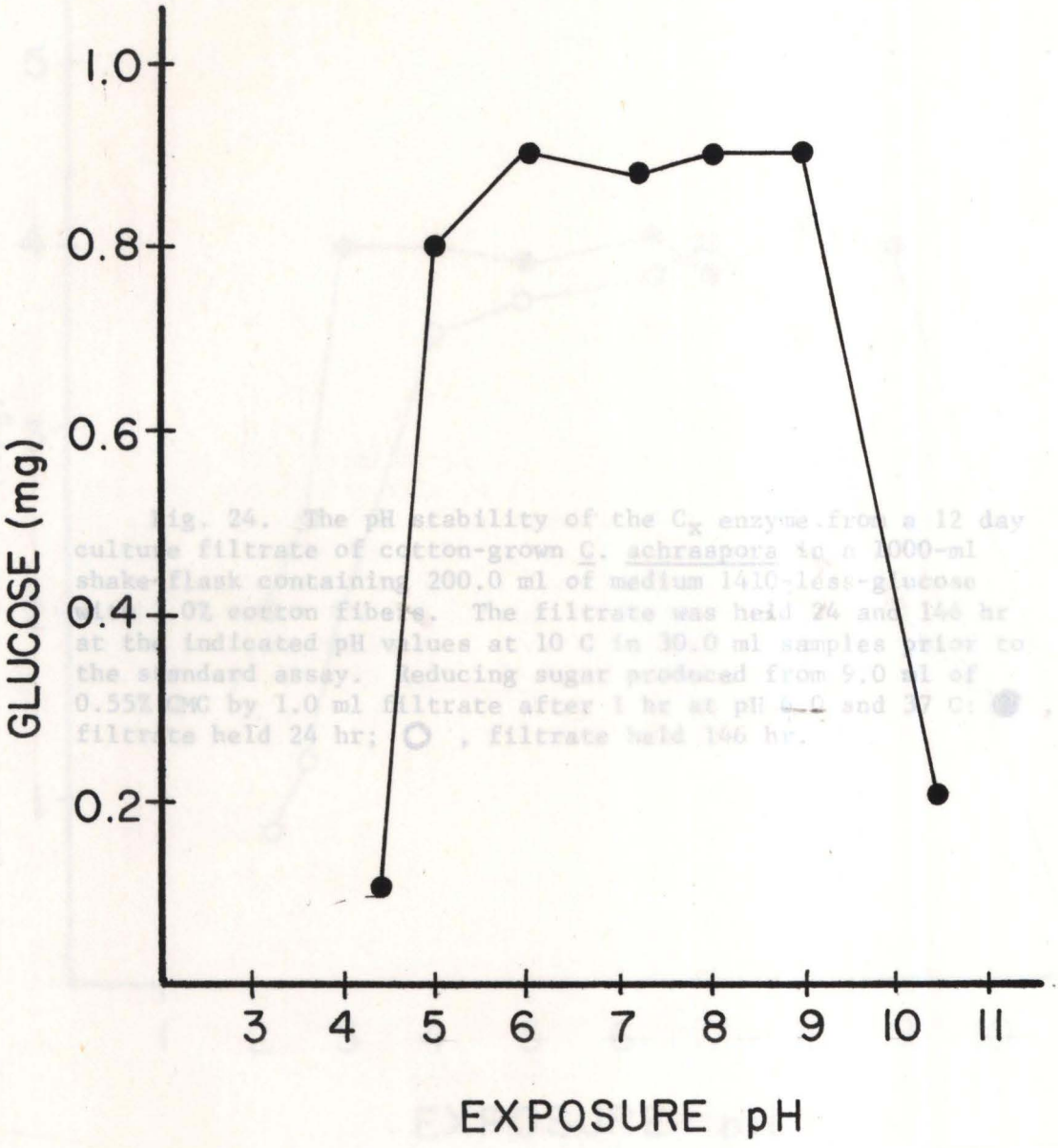


Fig. 23. The pH stability of cellobiase from a 12 day culture filtrate of cotton-grown C. achraspora in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% cotton fibers. The filtrate was held for 24 hr at the indicated pH at 10 C in 30.0 ml samples prior to the standard assay. Glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml filtrate after 1 hr at pH 6.0 and 37 C.


1.0
0.8
0.6
0.4
0.2
GLUCOSE (mg)
3
4
5
6
7
8
9
10
11
EXPOSURE pH

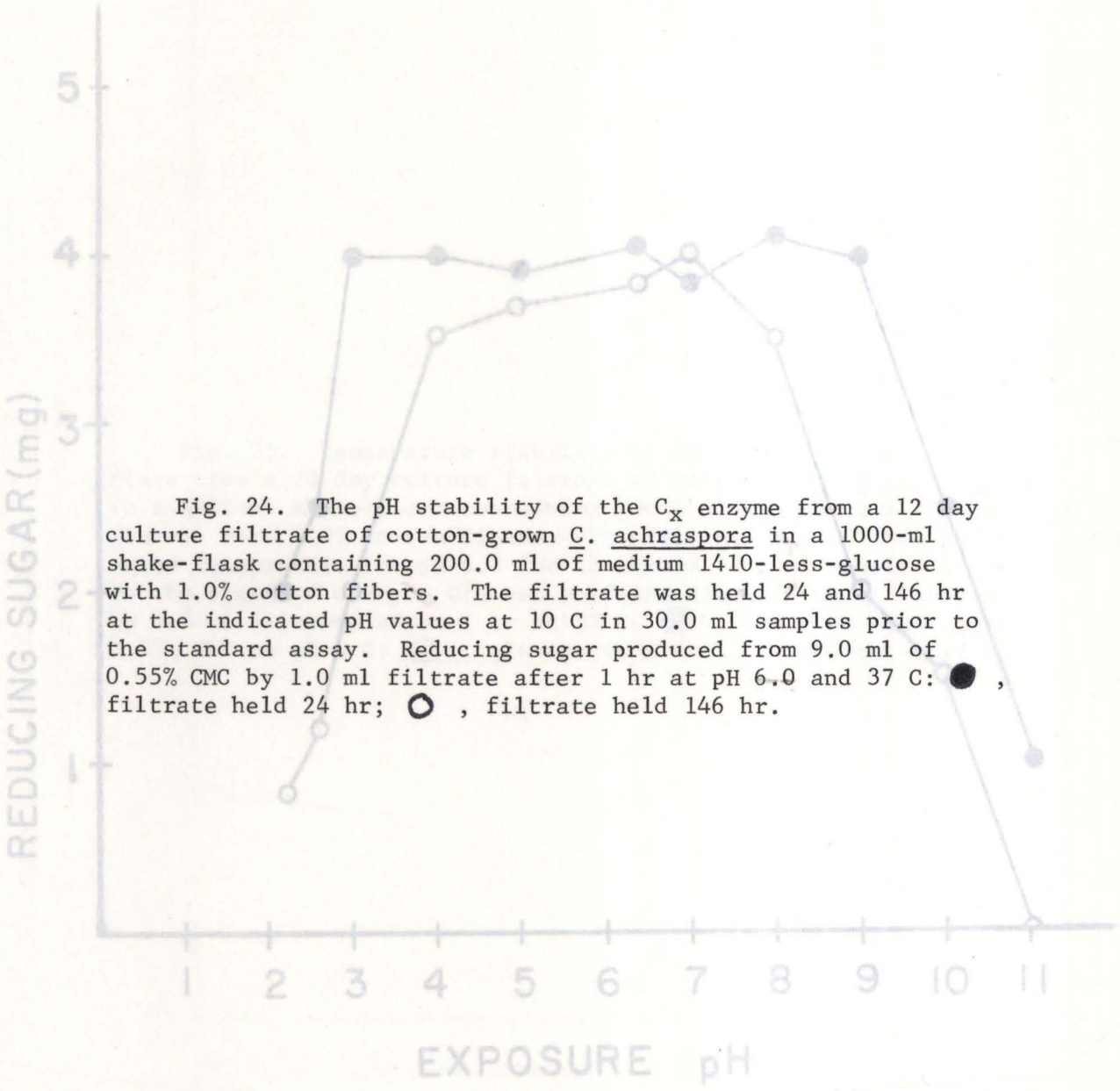
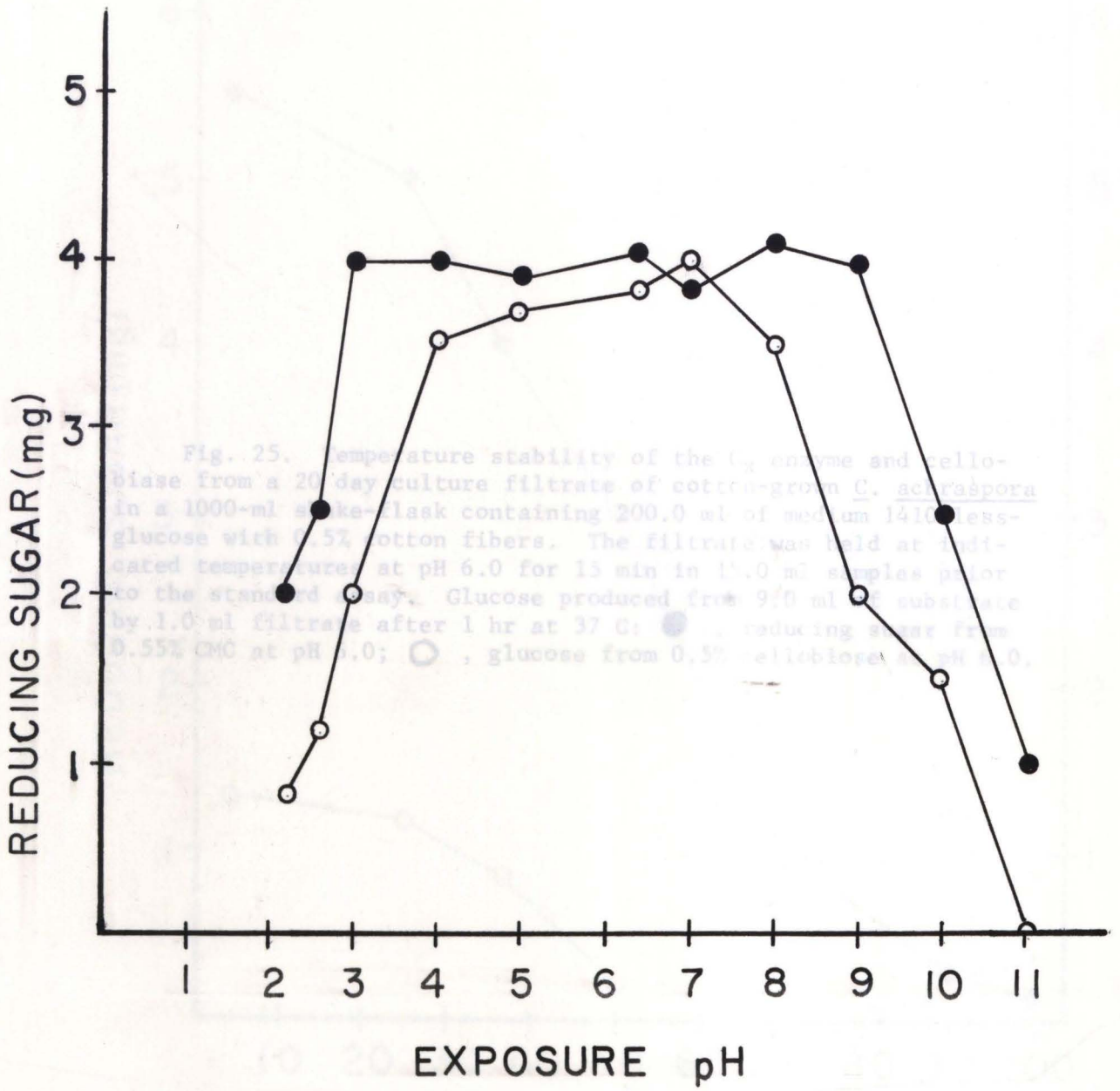


Fig. 24. The pH stability of the C_x enzyme from a 12 day culture filtrate of cotton-grown C. achraspora in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 1.0% cotton fibers. The filtrate was held 24 and 146 hr at the indicated pH values at 10 C in 30.0 ml samples prior to the standard assay. Reducing sugar produced from 9.0 ml of 0.55% CMC by 1.0 ml filtrate after 1 hr at pH 6.0 and 37 C: ● , filtrate held 24 hr; ○ , filtrate held 146 hr.


REDUCING SUGAR (mg)
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
EXPOSURE pH

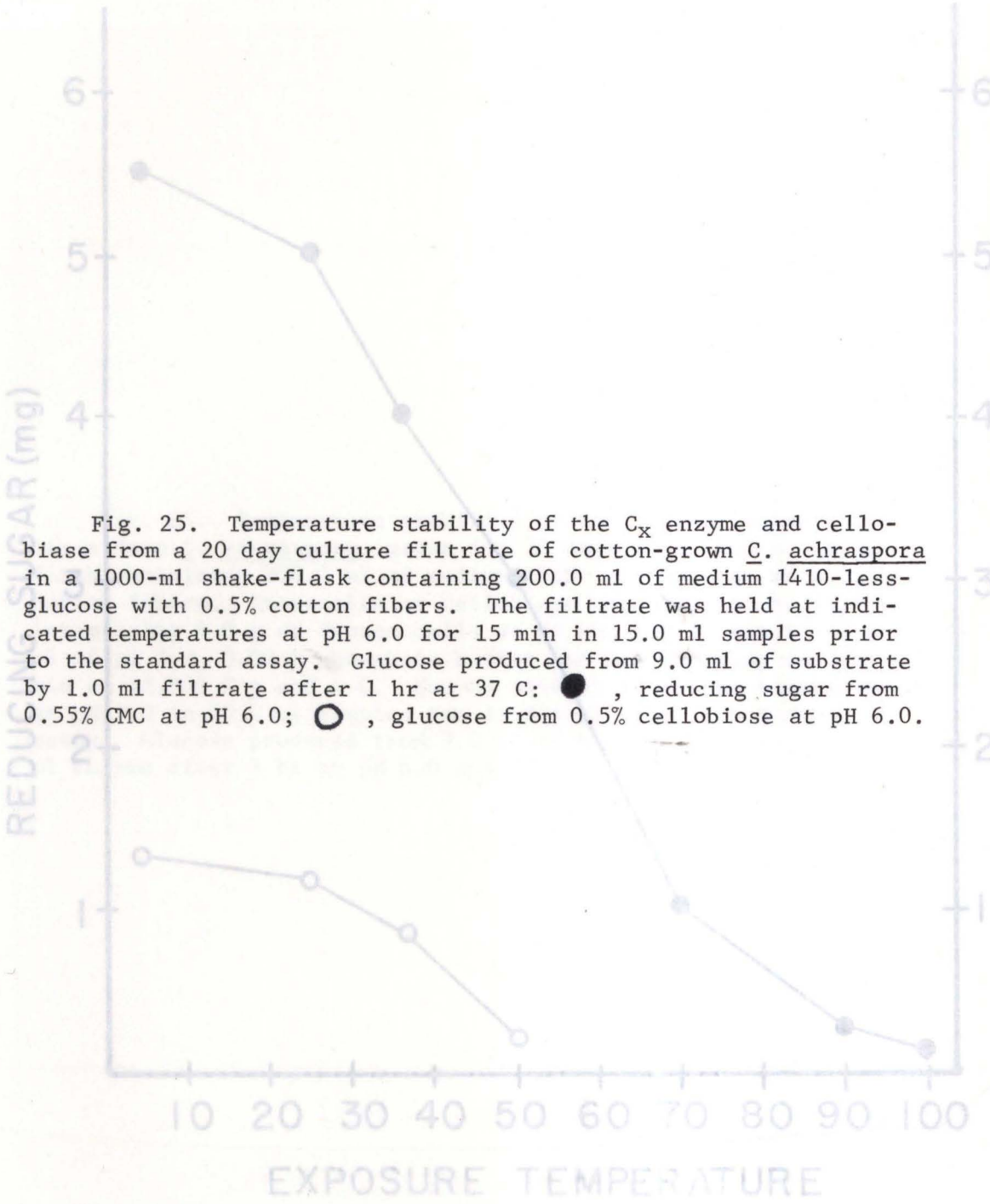
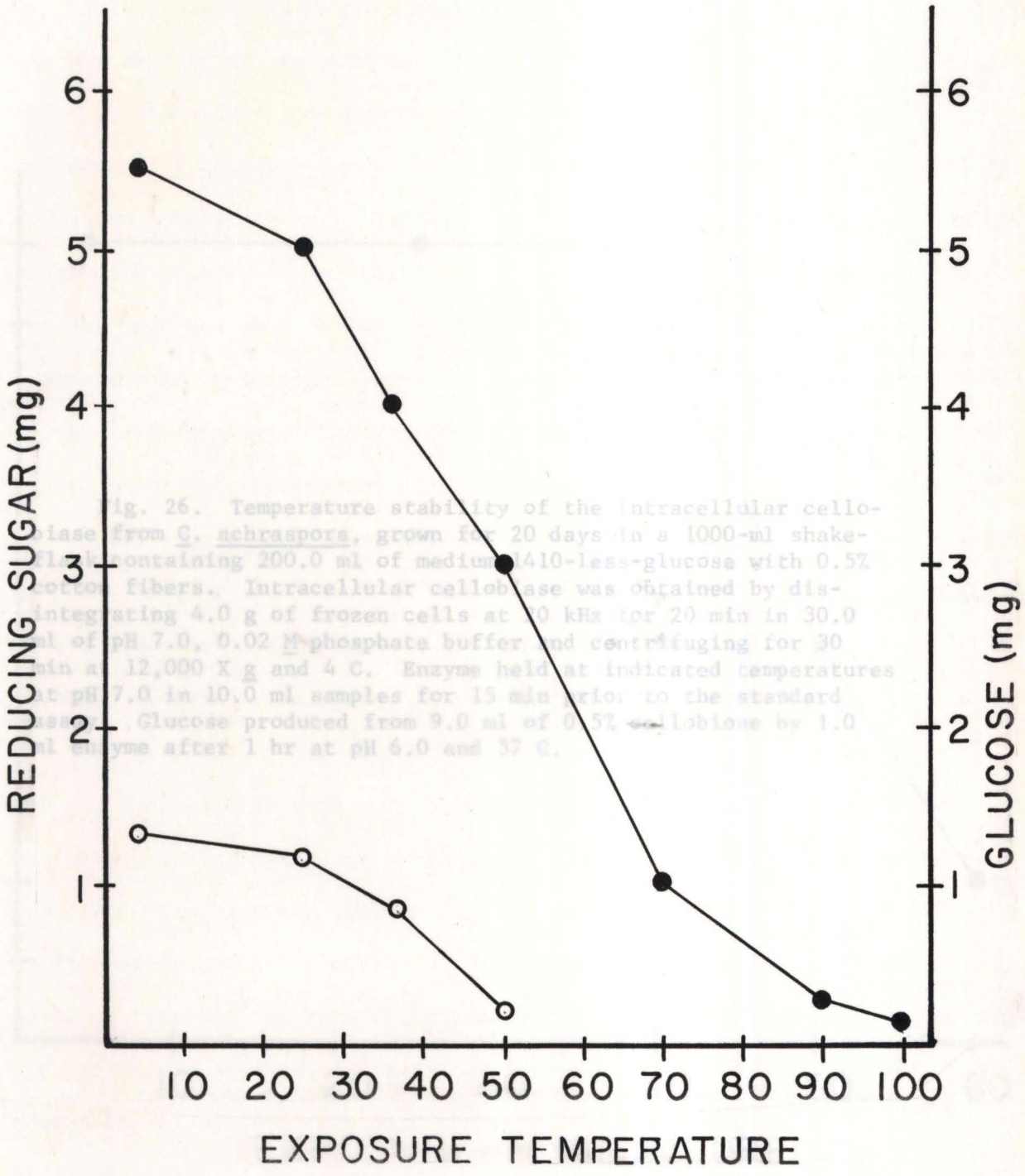


Fig. 25. Temperature stability of the C_X enzyme and cellobiase from a 20 day culture filtrate of cotton-grown C. achraspora in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 0.5% cotton fibers. The filtrate was held at indicated temperatures at pH 6.0 for 15 min in 15.0 ml samples prior to the standard assay. Glucose produced from 9.0 ml of substrate by 1.0 ml filtrate after 1 hr at 37 C: ● , reducing sugar from 0.55% CMC at pH 6.0; ○ , glucose from 0.5% cellobiose at pH 6.0.


REDUCING SUGAR (mg)
GLUCOSE (mg)
EXPOSURE TEMPERATURE
1
2
3
4
5
6
10 20 30 40 50 60 70 80 90 100

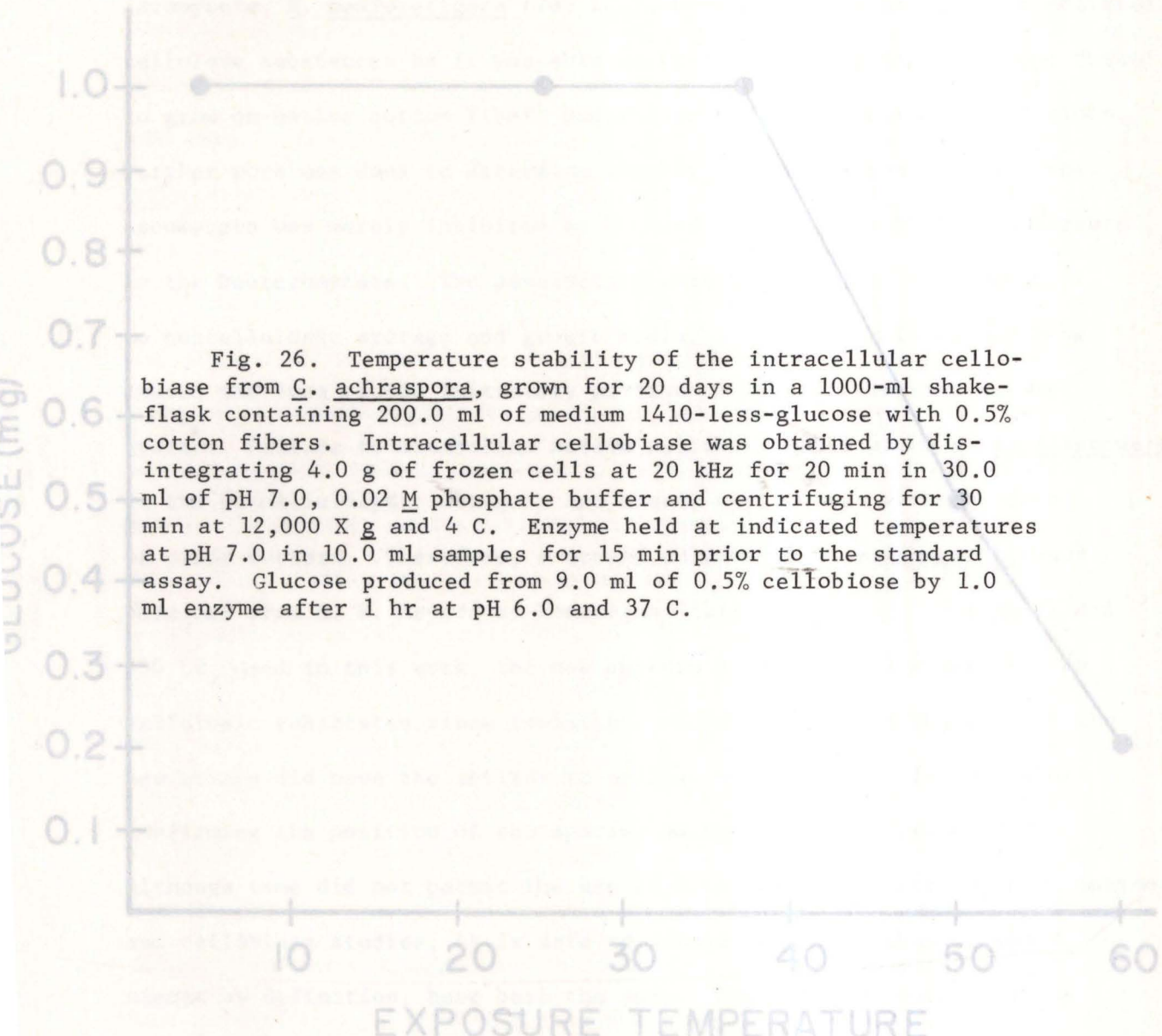
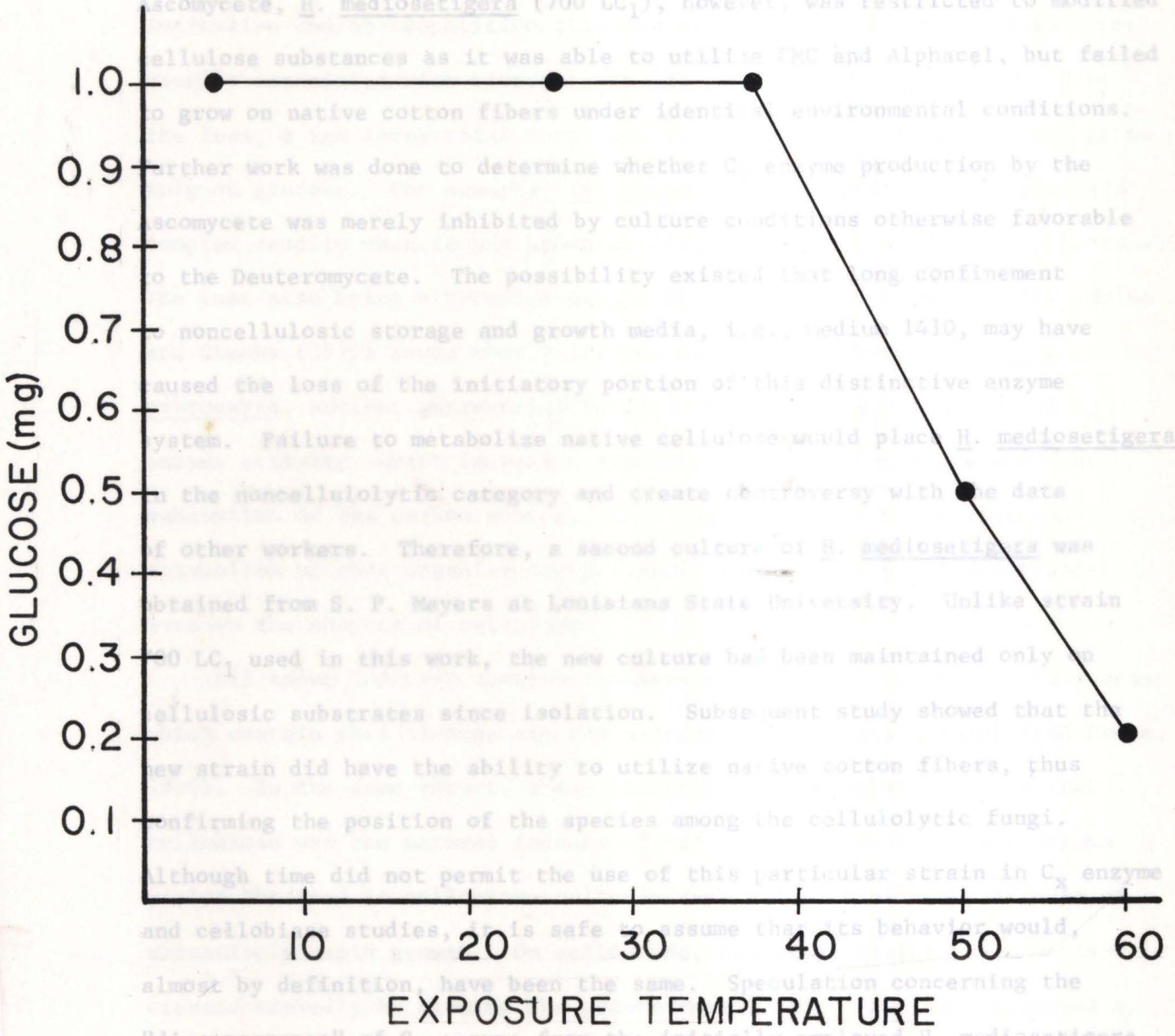


Fig. 26. Temperature stability of the intracellular cellobiase from C. achraspora, grown for 20 days in a 1000-ml shake-flask containing 200.0 ml of medium 1410-less-glucose with 0.5% cotton fibers. Intracellular cellobiase was obtained by disintegrating 4.0 g of frozen cells at 20 kHz for 20 min in 30.0 ml of pH 7.0, 0.02 M phosphate buffer and centrifuging for 30 min at 12,000 X g and 4 C. Enzyme held at indicated temperatures at pH 7.0 in 10.0 ml samples for 15 min prior to the standard assay. Glucose produced from 9.0 ml of 0.5% cellobiose by 1.0 ml enzyme after 1 hr at pH 6.0 and 37 C.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
GLUCOSE (mg)
10
20
30
40
50
60
EXPOSURE TEMPERATURE

DISCUSSION

This research has indicated that the marine Deuteromycete, C. achraspora, has the ability to generate an inducible enzyme system which hydrolyzes the beta-1,4-glucosidic linkages of cellulose and its derivatives. The marine Ascomycete, H. mediosetigera (700 LC_1), however, was restricted to modified cellulose substances as it was able to utilize CMC and Alphacel, but failed to grow on native cotton fibers under identical environmental conditions. Further work was done to determine whether C_1 enzyme production by the Ascomycete was merely inhibited by culture conditions otherwise favorable to the Deuteromycete. The possibility existed that long confinement to noncellulosic storage and growth media, i.e., medium 1410, may have caused the loss of the initiatory portion of this distinctive enzyme system. Failure to metabolize native cellulose would place H. mediosetigera in the noncellulolytic category and create controversy with the data of other workers. Therefore, a second culture of H. mediosetigera was obtained from S. P. Meyers at Louisiana State University. Unlike strain 700 LC_1 used in this work, the new culture had been maintained only on cellulosic substrates since isolation. Subsequent study showed that the new strain did have the ability to utilize native cotton fibers, thus confirming the position of the species among the cellulolytic fungi. Although time did not permit the use of this particular strain in C_x enzyme and cellobiase studies, it is safe to assume that its behavior would, almost by definition, have been the same. Speculation concerning the "disappearance" of C_1 enzyme from the initially employed H. mediosetigera culture would be premature at this time, but it has been considered appropriate to designate it separately as "700 LC_1" throughout this work. Such a variant well may prove a boon to future studies on the cellulase system.

Failure of these marine fungi to develop C_x enzyme or cellobiase in detectable amounts during growth on glucose emphasized the decreasing influence of inducers "low" in a given degradation sequence on the production of precursor enzymes and also suggested a high degree of specificity involving the beta-1,4-glucosidic bond in the induction process. Product inhibition and/or competition also may produce such effects, but such are usually associated with biosynthesis rather than with catabolism. None the less, a few terrestrial fungi are able to produce cellulase when grown only on glucose. For example, Trichoderma viride produced the cellulase complex readily when it was grown on either glucose, cellobiose or lactose, the last also being a beta-1,4-glycoside (Mandels and Reese, 1957). Hulme and Stanks (1970) found that filtrates of glucose-grown Myrothecium verrucaria, another terrestrial fungus, contained a small amount of C_x enzyme activity, which increased significantly in coincidence with the exhaustion of the carbon source. This suggested that the endogenous metabolism of this organism could support the production of cellulases even in the absence of cellulose.

All known inducers contain the beta-glycosidic linkage, but compounds which contain this linkage are not necessarily inducers (Mandels and Reese, 1960). In the same report, these investigators attempted to show that cellobiose was the natural inducer of cellulase and that the low enzyme yields obtained in cellobiose cultures were due to inhibitory effects of excessively rapid growth. On cellobiose, cellulase yields could be increased markedly by slowing the growth rate of the organism. Eventually, it was shown that high concentrations of cellobiose (0.5-1.0%) or other rapidly metabolized carbon sources such as glucose, repress cellulase

formation in such media until the sugar has been consumed. A high concentration of cellobiose, when added to a culture producing cellulase on cellulose, will inactivate the enzyme already formed (Mandels and Reese, 1957). Since cellobiose induced only cellobiase and not C_x enzyme in our marine cultures, it was considered probable that the presence of the glucosidic bond was only one of several factors involved in the induction of the cellulase system.

Because modified cellulose induced C_x enzyme and cellobiase, it was assumed, as a working principle for this present study, that the former enzyme, which hydrolyzes the glucosidic linkage in CMC and Alphacel, is the same catalyst which attacks native cellulose and/or closely related cellodextrans. This assumption was supported by the detection of C_x enzyme in filtrates of C. achraspora grown on native cotton fibers. Studies of enzyme development in culture filtrates showed that cellobiase appeared only after C_x enzyme activity became significant indicating that cellobiase had no substrate until C_x enzyme attacked cellulose.

Environmental factors of significance in determining the cellulolytic response of these fungal cultures proved to be the initial pH of the growth medium and the incubation temperature. The importance of cultural conditions for microbial cellulase production has been examined elsewhere in the past for terrestrial fungi, in particular Trichoderma viride. Shake-cultures always showed greater activity than stationary cultures implying a considerable oxygen requirement (Wood, 1968).

The most suitable cellulose substrate in the C_1 enzyme assay is native cotton fiber since it contains very little noncellulosic material and remains in a truly "native" state after "cleaning." The outermost layer

of the fiber, the cuticle, has a thin primary wall that consists of a loose, random fibrillar network which surrounds the relatively thick secondary wall. Almost all of the extraneous materials (waxes, pectins and certain nitrogenous substances) are contained in the cuticle and primary wall layers. The secondary wall consists of three sub-layers designated S_1, S_2 and S_3 which are formed almost entirely of highly crystalline cellulose (Cowling and Brown, 1969). The S_1 and S_3 layers are normally thin; the S_2 layer is of variable thickness, but usually forms the bulk of the cell wall substance. Cellulose molecules in the S_1 and S_3 layers are deposited in flat helical planes perpendicular to the fiber axis while those nonhelical chains in the S_2 layer are deposited nearly parallel to the fiber axis.

The C_1 enzyme assay on filtrates from Alphacel- and CMC-grown H. mediosetigera (700 LC_1) and Alphacel-, CMC- and cotton-grown C. achraspora indicated that the sequential induction principle appeared to be functioning well in these cultures. Native cotton fibers were necessary for the induction of C_1 enzyme in C. achraspora, while modified cellulose induced only cellobiase and C_x enzyme in both cultures. Because the C_1 enzyme assay was dependent on the presence of both C_1 and C_x enzyme activities, assay conditions unfavorable to either could give negative results.

The exact manner in which these enzymes function together in the solubilization of cotton fibers is still unknown. Selby (1969) has formulated the following hypothesis concerning this interrelationship based on the studies of C_1 and C_x enzymes of Trichoderma viride:

> A further explanation hinges on new theories concerning the supramolecular structure of cotton, in which the fiber is considered to consist of completely crystalline elementary microfibrils, each containing about 100 cellulose chains.

> It is reasonable to assume that the regular array of molecular chains will be disturbed at intervals by the occurrence of chain-ends. The disturbance in hydrogen bonding between chains in the vicinity of the chain-end may be insufficient to enable C_x, acting alone, to split off soluble sugars, but when both components are present, a single bond-rupture by the C_x enzyme might allow the hydrogen bonding to be further disturbed by the C_1 with consequent loosening of a short length of surface chain, which might then be susceptible to more extensive attack by the C_x enzyme.

Selby (1969) also found that the rates of C_x enzyme and cellobiase adsorption to cellulose were not affected by the presence of the C_1 component; nor was the adsorption of C_1 enzyme increased in the presence of the C_x enzyme. The function of the C_1 enzyme does not appear related to the adsorption of C_x enzyme to cellulose, or vice-versa.

Unlike cellobiase, temperature and pH effects on C_x enzymes of both cultures were governed by the type of CMC used in this assay. This was due to the degree of substitution which influenced the rate of enzymatic hydrolysis inversely. Although it has been shown by Reese et al. (1950) that the rate of hydrolysis is independent of the degree of polymerization, direct comparisons of C_x enzyme activities between different workers are only possible if the identical substrate is used.

Carboxymethylcellulose is a cellulose ether (Fig. 2) produced by reacting alkali-treated cellulose with sodium monochloroacetate under rigidly controlled conditions, and may be visualized as a chain similar to repeating cellobiose units. The degree of polymerization is indicated by the number of anhydroglucose units held together by beta linkages. Each anhydroglucose unit retains three hydroxyl groups capable of reaction and by substituting carboxymethyl groups for the hydrogens of these hydroxyl groups, CMC is obtained. The average number of carboxymethyl

groups substituted per anhydroglucose unit constitutes the degree of substitution (D. S.). Susceptibility of substituted cellulose derivatives to enzymatic hydrolysis increases as they become more water soluble and less rigidly ordered. Beyond the point of complete solubility, susceptibility decreases with increasing D. S. At a value greater than 1.0, complete loss of specificity necessary to enzymatic action results (Cowling and Brown, 1969).

Temperature and pH effects on C_x enzymes and cellobiases of both cultures were readily reproducible. The optimum temperature of an enzyme is reflected by the balance between the effect of temperature on the reaction velocity and its denaturative effect on the biological stability of the protein involved (Dixon and Webb, 1964). These effects of temperature combine, following the initial "Q_{10} effect," to produce a continuous fall in the concentration of active enzyme as measured by the reaction rate. The rate of thermal inactivation depends, typically, upon hydrogen ion activity, ionic strength, protein concentration and the amount of protection afforded, in many cases, by the attached substrate. Because any optimum temperature is based on the above factors, it may not be a definite characteristic of the enzyme per se, but merely a rough idea of its heat stability as an amino acid polymer (Haldane, 1965).

The variable optimum temperature for the C_x enzyme of C. achraspora and H. mediosetigera (700 LC_1) (Fig. 11 and 20) appeared to be lower than that for either cellobiase (Fig. 10 and 19). The apparent fall in the C_x enzyme optimum temperature from 50 C to 37 C, after the initial rise, probably was due to inactivation because of the increased period of incubation. The so-called optimum temperature, therefore, was a function of the time chosen for the rate measurement.

Optimum temperatures for fungal C_x enzymes have not been studied in great detail, but reports indicate a wide range depending on assay conditions (Gascoigne and Gascoigne, 1960). Again, most work has been done with terrestrial fungi; for example, several filtrates, obtained by growing Aspergillus fumigatus, Myrothecium verrucaria and Aspergillus luchuenis on native cotton, were tested for C_x enzyme activity at various temperatures by Reese et al. (1950). Their fungal filtrates were most active at 60 C after 60 min incubation while other studies showed maximum hydrolysis rates at 65 C when a 30 min incubation was used. A longer period of incubation resulted in significant inactivation of filtrate activity giving the appearance of a lower optimum temperature.

By comparison, optimum temperatures for bacterial cellulases vary between 36 and 40 C, but thermophilic organisms, such as Clostridium thermocellulaseum, may have an optimum cellulase activity as high as 57 C. Cellulases from animal sources appear to act best around 25 to 35 C, while little is known about the optimum temperature of plant cellulases (Gascoigne and Gascoigne, 1960). In the case of reports on so-called animal and plant cellulases, it must be borne in mind that the belief is strong in favor of microorganisms as the sole sources of cellulolytic proteins (Nord and Schubert, 1962).

The effect of hydrogen ion activity on the reaction rates of C_x enzymes and cellobiases of both marine fungi scarcely deviated from the generality that most enzymes are active over a distinct pH range and have an apparent optimum pH. This optimum may result from any one of three effects or their combination. Such effects are (1) reversal of the maximum velocity of the reactions catalyzed, (2) protein destabilization or (3) dissociation of the

enzyme from the substrate (Haldane, 1965). Since the substrates used in this study are normally viewed as nonpolar in nature, the critical importance of ionization may have been restricted to that of the catalytic protein structure.

Optimum pH values vary widely for fungal C_x enzymes over a range from 3.0 to 8.0, but usually are found to lie between 4.0 and 7.0. Frequently, for this reason, different results derived from the same organism may be partially explained by differing conditions of assay (Gascoigne and Gascoigne, 1960). The acidic optimum pH of cellobiase and C_x enzyme reported in this thesis correlated well with data on terrestrial fungal cellulases. For example, the effect of pH on C_x enzyme activity of CMC- and cotton-grown M. verrucaria, A. luchuenis and A. fumigatus was shown by activity curves which exhibited maxima at pH 5.1 for all organisms (Reese et al., 1950). The relative inactivity at pH 7.0 and 8.0 coincided with the known preference of these organisms for acidic growth conditions. It is obvious that pH optima for growth and cellulase activity were the same. How then can we account for our marine cultures which have cellulase pH optima in the 5 to 6 range while their growth pH optima are distinctly alkaline (Sguros and Simms, 1963)? One possible explanation could be that the pH of the micro-environment between the hyphae and the cellulosic substrate is acidic in comparison to the alkaline nature of the growth medium. Mycelial respiration results in the production of much carbon dioxide which can then react with water to form bicarbonate ion. This probably lowers the pH in the microcosm.

In pH and temperature stability studies, the rate of inactivation again must be assumed to depend largely on the hydrogen ion concentration,

ionic strength, protein concentration and/or the presence of other substances. Cellobiases from each fungus had similar stability patterns as did the C_x enzymes. However, the C_x enzyme of C. achraspora appeared to be much more stable than cellobiase from the same organism; i.e., 100 C for 15 min versus 50 C for 15 min, respectively. The same was true in comparing the C_x enzyme and cellobiase of H. mediosetigera (700 LC_1). Between species, on the other hand, the thermostability of H. mediosetigera (700 LC_1) C_x enzyme was found to be much lower than that for C. achraspora. This could indicate significant generically-based differences in these enzymes.

Since these studies were carried out with crude enzyme preparations, the heat stability of both the C_x enzyme and cellobiase may have been due to the protective action of unidentified impurities. The thermostability of a pure cellulase system is probably more of an exception than the rule (Gascoigne and Gascoigne, 1960).

Fungal extracts, in contrast to filtrates, had no C_x enzyme activity. This confirms the work of terrestrially-orientated mycologists who have shown that the enzymes actually concerned with cellulose attack are present normally in the growth medium. Because cellulose is insoluble and cannot permeate cell membranes, cellulases have been assumed to be extracellular. According to Norkrans (1966) this must be so since the activity of preparations from corresponding amounts of mycelia was always minimal. For most fungi tested, the highest cellulase activity was obtained from media containing young mycelia and hyphal tips.

In contrast to fungal cellulases, bacterial cellulases are found not only in culture filtrates, but in cell extracts as well. The physiological function of intracellular components may, however, be distinct from that

of extracellular components. Extensive studies of the cellulolytic enzyme system of Pseudomonas fluorescens have shown that two components are extracellular while one is cell-bound. The three purified components hydrolyzed various cellodextrans and celluloses,but none could split cellobiose (Suzuki, Yamane and Nisizawa, 1969).

Intracellular enzyme extracts from both species, in contrast to cell-free filtrates, displayed very high cellobiase activity indicating that cellobiose could enter the cells as the disaccharide precluding the necessity for cellobiase as an exoenzyme. In terrestrial fungal studies, Levinson et al. (1951) showed that cellobiose was utilized rapidly by fungal cells even though the filtrates had only trace amounts of cellobiase activity. Spontaneous autolysis occuring with culture age could have contributed cellobiase to the medium. Growth of filamentous fungi in liquid shake-cultures is characterized by three consecutive phases: (1) unapparent (2) rapid, linear and (3) partial autolysis with fluctuations in dry weight (Cochrane, 1958). In the last phase, the loss of weight varies from negligible to extreme with commensurate liberation of proteins and other nitrogenous compounds into the medium.

The large range of pH and temperature over which marine fungal C_x enzymes and cellobiases were active indicated a flexibility which could prove necessary in a marine microcosmic environment. Here, fluctuations in salinity, temperature and hydrogen ion concentration owing to physical, chemical and metabolic circumstances may require fungi to successfully adapt for survival.

The pH of sea water is usually in a state of change due to numerous factors, including its proximity to the land mass. As the carbon dioxide

content of the water increases, the bicarbonate ion concentration correspondingly increases, and the pH is lowered (Johnson and Sparrow, 1961). With a decrease in bicarbonate ion, for example, during intense photosynthetic activity, the pH may exceed 8.5. At night or during periods of low photosynthetic activity, the carbon dioxide evolved by respiration increases bicarbonate ion concentration resulting in a pH drop.

Temperature variations of the surface waters closely parallel variations in atmospheric temperature. However, while temperature decreases with depth, our marine cultures are littoral and would not be affected by this environmental parameter.

This work has been intended to establish fundamental parameters in the first detailed study of the cellulase-enzyme complex in marine fungi. Future work will include separation of the C_1, C_x and cellobiase proteinaceous components by Sephadex chromatographic filtration. Answers to questions concerning the action of these components separately and recombined will be sought and their responses to marine environmental factors noted.

SUMMARY

1. Cellulase components are present in both H. mediosetigera (700 LC_1) and C. achraspora (230), but only the latter can retain the ability to initiate growth on native cellulose after years of carriage on noncellulosic media. H. mediosetigera (700), continuously carried in cellulosic media, retained C_1 enzyme activity.

2. Cellulase components of these marine fungal species were inducible; only native cellulose can induce the C_1 enzyme.

3. Both species grow on CMC and Alphacel with the elaboration of C_x enzyme and lesser amounts of cellobiase into the medium while growth on cellobiose yields only cellobiase. Growth on glucose yields neither C_x enzyme nor cellobiase. The principle of sequential induction is rigidly followed.

4. Optimum pH values for both C_x enzyme and cellobiase of C. achraspora were 6.0 at 37 C while those for H. mediosetigera (700 LC_1) were 5.0 and 6.0 respectively, at 37 C.

5. In both cultures C_x enzymes had a variable optimum temperature at pH 6.0, depending on the length of the incubation period.

6. Both cellobiases had the same optimum temperature at pH 6.0.

7. Thermostability observations on C_x enzyme and cellobiase of C. achraspora showed almost complete deactivation at 100 C in 15 min for the former and at 50 C in 15 min for the latter.

8. Thermostability observations on C_x enzyme and cellobiase of H. mediosetigera (700 LC_1) showed almost complete deactivation at 45 C in 15 min for the former while the latter's activity became negligible at 50 C in 15 min.

9. Observations of pH stability showed a wide range for cellobiases and C_x enzymes of both marine fungal species.

10. Cellulase systems of these marine fungi are similar to many terrestrial species with respect to induction, temperature and pH characteristics.

11. Intracellular enzyme assays emphasize the extracellular nature of C_x enzyme, in contrast to that of cellobiase, in both fungi.

12. Data indicate that at least three enzymatic functions are involved in cellulose breakdown by these cultures.

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