Amylase production by Mucor pusillus and Humicola lanuginosa as related to mycelial growth

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Abstract

Mycelial dry weights of *Mucor pusillus* and *Humicola lanuginosa* reached maxima after two and eight days of incubation, respectively, in starch-yeast media. Maximum levels of extracellular amylase activities measured in the growth media were recorded after three to 11 days of incubation of *M. pusillus,* and after 9–25 days of incubation of *H. lanuginosa,* periods corresponding to observed reductions in mycelial dry weights. In both cases, abnormally high concentrations of reducing sugars were measured in the growth media prior to the attainment of maximum mycelial dry weight. It is suggested that a membrane-bound form of amylase might be principally responsible for providing reducing sugars necessary for growth, and extracellular amylase might be due principally to autolysis.

Introduction

Of the several previous studies which have demonstrated the presence of amylase (1, 3, 5, 9, 10), along with other enzymes (2, 5, 6, 8, 11, 12, 13, 14), in the extracellular growth media of various thermophilic fungi, at least two (5, 10), which report quantitative determinations of amylase activity, are based on a 1971 study by Barnett and Fergus (3). This study reported that extracellular amylase measured in the filtered growth medium of *Humicola lanuginosa* (Griff. & Maubl.) Bunce reached a maximum after about 22 days of incubation. This maximum level of amylase activity appeared remarkable because it was about seven times the maximum level noted in the incubation media of certain mesophilic fungi studied under similar growth conditions, and also because it was reported to occur about eight days after *H. lanuginosa's* maximum mycelial dry weight was recorded. Based on these results, as well as Fergus's previous qualitative survey of amylase production by a large variety of fungi (9), Barnett and Fergus (3) suggested that *H. lanuginosa* would be an excellent test organism for the study of extracellular amylase produced by thermophilic fungi, since it appeared to produce an abnormal abundance of this enzyme. However, later studies of growth (7) and extracellular amylase production (1) occuring in cultures of *Mucor miehei* Cooney & Emerson and *M. pusillus* Lindt suggested that these thermophilic fungi might produce amylase having greater activity than that of *H. lanuginosa* by a factor of as much as 3, an idea supported by the observation that both *M. miehei* and *M. pusillus* are prolific producers of lactase (13). Since the growth conditions in the original study of *H. lanuginosa* were different from those in that of *M. miehei* and *M. pusillus,* this study was undertaken to compare the growth and amylase production of *H. lanuginosa* with *M. pusillus* under the same conditions. The results reported herein are compared with the data and conclusions of the previous study (3).

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Materials and methods

The organisms *(H. lanuginosa* and *M. pusillus)* were obtained from Dr. J. Deploey (Department of Biology, The Pennsylvania State University, York Campus). They were cultured in a medium containing soluble starch 5 g., yeast extract (Difco) 2 g., KH_2PO_4 1 g., $MgSO_4.7H_20$ 0.5 g., distilled water 1000 ml. This medium was autoclaved (121 °C for 15 min.), allowed to cool, then aseptically added to sterile 250 ml Erlenmeyer flasks (50 ml added per flask). All chemicals used were of reagent grade.

The inoculum was obtained by culturing the fungi in petri dishes for 2 to 3 days at 45 °C containing the above-specified medium solidified with agar (20 g/liter). Each flask was inoculated with one agar disc bearing mycelium obtained from the agar culture by using a sterile cork borer (3 mm diam.). The flasks (containing medium and inoculum) were then placed in an incubator maintained at 45 °C ± 0.3 without shaking.

At the ends of specified time periods (from 1 to 35 days) three flasks were removed from the incubator at random. The mycelium in each flask was separated from the liquid medium by suction filtration through pre-weighed Whatman #1 filter paper (9 cm) contained in a Buchner funnel. The agar inoculum was removed from the mycelium and discarded. The filter paper with mycelium was washed with distilled water, dried to a constant weight at 80 °C and weighed to the nearest mg. The filtrates were separately assayed for amylase activity.

Amylase activity was measured using a modification of the Bernfeld method (4). For each assay, seven test tubes (designated, 0, X, 1, 2, 3, 4, 5) were prepared containing 1.0 ml of a 1% starch solution in M/150 NaCl and M/150 phosphate buffer pH 6.9. A 2.0 ml aliquot of a solution prepared from 1 g. 3, 5-dinitrosalicylic acid, 20 ml of 2N NaOH and 30 g Rochelle salt per 100 ml (DNSA reagent), was immediately added to the "0" (reagent blank) and "X" test tubes. Test tubes 1 through 5 were placed in a water bath maintained at 45 °C and a 0.2 ml aliquot of extracellular filtrate solution added to each. A 0.2 ml aliquot of filtrate solution was also added to tube "X" (not placed in water bath). After exactly 1, 2, 3, 4 and 5 minutes a 2.0 ml aliquot of DNSA reagent was added to the appropriate tube and it was removed from the water bath. All tubes were then heated in a boiling water bath for 5 mins. after which 20 ml of distilled water were added to each. The absorbance of each solution at 540 nm, measured using Bausch & Lomb Spectronic 20 spectrophotometer, was assumed to be proportional to the amount of reducing sugars present in that solution. The spectrophotometer was set to zero absorbance using solution "0" and the absorbance of solution "X" measured. This was assumed to indicate the amount of reducing sugars originally present in the 0.2 ml extracellular filtrate aliquot. The instrument was then re-set to zero absorbance with solution "X" and the absorbances of solutions 1 through 5 were recorded. These data were assumed to indicate the amounts of reducing sugars formed during the one to five minute assay-incubation period, and were the data upon which the amylase activity designation was based. Amylase activity was designated from the slope of the absorbance (reducing sugars present) versus time linear graph as calculated using the method of least squares. Correlation coeficients for all amylase assays used in this study were 0.95 or better. One unit of amylase activity is defined here as that amount of enzyme which catalyzes the formation from starch of 1 mg per minute of any reducing sugar which reacts with DNSA reagent at 45 °C. Maltose was used to construct a standard curve relating absorbance at 540 nm to amount of reducing sugar.

Results

Mucor pusillus proved to be the faster growing of the two fungi. An increase in mycelial dry weight from about 24 mg after 1 day of incubation to about 105 mg at the second day was recorded (Fig. 1). Mycelial dry weight remained at about this level until day 4, then declined sharply to about 80 mg at days 7–8. Thereafter the decline in mycelial dry weight was less steep, and a weight of about 65 mg was recorded after 35 days of incubation. The pattern of variation in mycelial dry weight of *H. lanuginosa* was similar, but slower than that for *M. pusillus* (Fig. 2). The maximum mycelial dry weight of this fungus (about 75 mg) was recorded after 8 days of incubation. This was followed by a rather steep decline in mycelial dry weight to about 40 mg at day 18, and a less steep decline thereafter. About 30 mg dry weight were still recorded after 30 days of incubation.

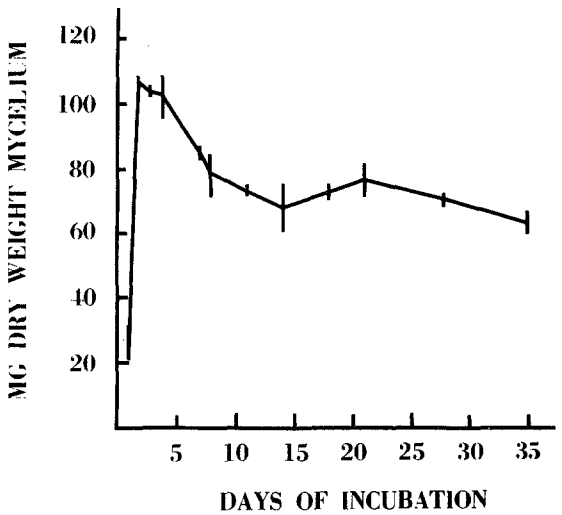

Fig. 1. Mycelium dry weight changes of *Mucor pusillus* at 45 °C. Incubation medium is defined in the text. Each value shows the range from three flasks selected at random.

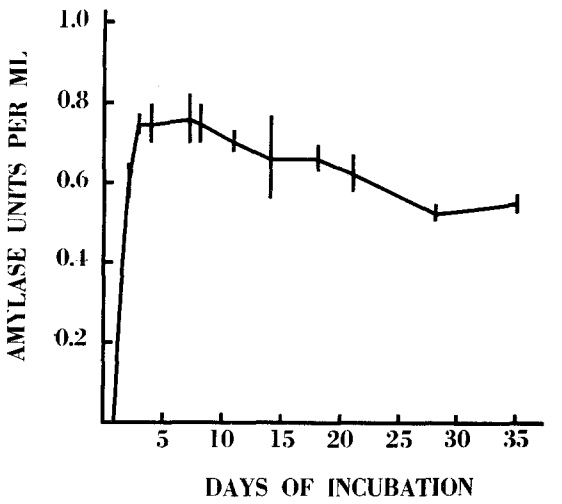

Fig. 3. Activity of extracellular amylase from *Mucor pusillus.* Filtrate aliquots were incubated at 45 °C for one to five mins in a 1% starch solution containing M/150 NaCl and M/150 phosphate buffer pH 6.9. Amylase units are defined in the text. Each value shows the range from three flasks selected at random.

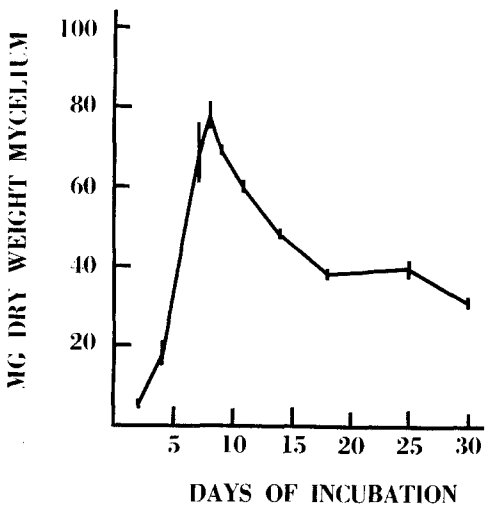

Fig. 2. Mycelium dry weight changes of *Humicola lanuginosa* at 45 °C. Incubation medium is defined in the text. Each value shows the range from three flasks selected at random.

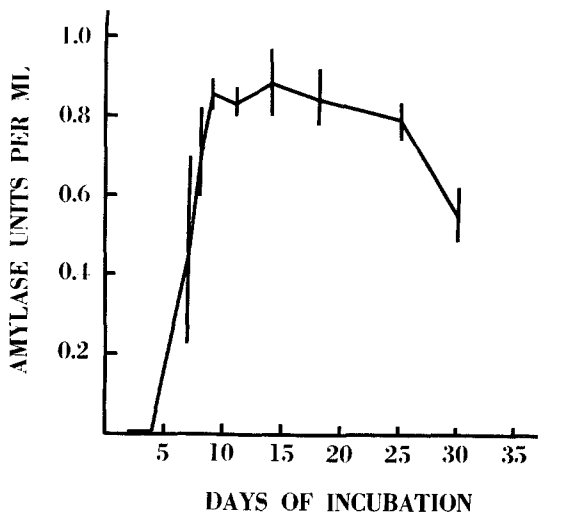

Fig. 4. Activity of extracellular amylase from *Humicola lanuginosa.* Filtrate aliquots were incubated at 45 °C for one to five mins in a 1% starch solution containing M/150 NaCl and M/150 phosphate buffer pH 6.9. Amylase units are defined in the text. Each value shows the range from three flasks selected at random.

In both cases, the pattern of amylase activity was related to that of mycelial growth. The units in the ordinates of Figures 3 & 4 were obtained by multiplying the measured amylase activity (using the 0.2 ml samples) by 5, thus standardizing the data in amylase units per ml of extracellular filtrate.

A maximum of about 0.75 units of amylase activity was measured in the extracellular filtrates from samples obtained from *M. pusillus* which had been incubated for 3 days (Fig. 3). This level of amylase activity remained fairly stable during the period of rapid decrease of mycelial dry weight, then amylase

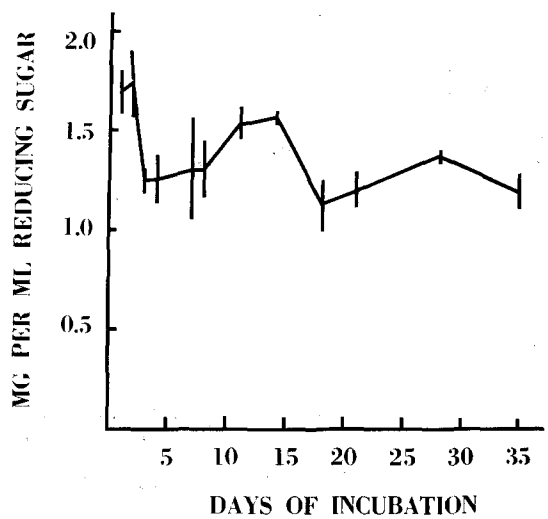

Fig. 5. Extracellular reducing sugar concentration changes from *Mucor pusillus.* Filtrate aliquots were added to DNSA reagent and analyzed for reducing sugar spectrophotometrically. Each value shows the range from three flasks selected at random.

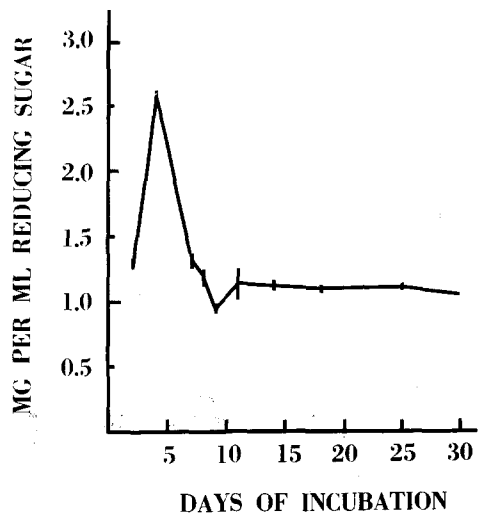

Fig. 6. Extracellular reducing sugar concentration changes from *Humicola lanuginosa.* Filtrate aliquots were added to DNSA reagent and analyzed for reducing sugar spectrophotometrically. Each value shows the range from three flasks selected at random.

activity slowly declined. Similarly, the amylase activity in filtrates from *H. lanuginosa* incubation samples (Fig. 4) reached a maximum of about 0.85 units after 9 days of incubation remaining near that level until day 25 before declining.

Relatively large amounts of reducing sugars present in the extracellular filtrate were noted for both fungi during the period of rapid initial growth (Figs. 5 & 6). This is shown most clearly during the growth of *H. lanuginosa* (Fig. 6). The relatively high level of over 1.25 mg of reducing sugar per ml of filtrate after 2 days of incubation is more than doubled by day 4 before declining to a steady 1.1–1.2 mg per ml through day 25. This high level of reducing sugar measured clearly prior to the time of *H. lanuginosa's* maximum mycelial dry weight and maximum amylase activity is more difficult to document for *M. pusillus* because of its much more rapid initial growth, but the probability of a similar pattern is evidenced by the relatively high amounts of reducing sugars measured after 1 and 2 days of incubation compared with subsequent measurements (Fig. 5).

Discussion

Mucor pusillus is shown here to be a much faster-growing organism than *H. lanuginosa.* This is consistent with previous observations of the growth patterns of these fungi. *Mucor pusillus* has been shown to achieve maximum mycelial dry weight after 3 days of incubation (7), although in a medium different from that used in this study. *H. lanuginosa* has been previously shown to reach maximum mycelial dry weight after about 14 days of incubation (3), using a medium identical with that used in this study. These data differ slightly from those reported herein, but the authors of the previous study do not report any measurements between 10 and 14 days, nor between 14 and 18 days. Such measurements, if they had been made, might have revealed a growth pattern closer to that reported here. The observations of Barnett and Fergus (3) with regard to the pH variation of the extracellular medium are entirely in agreement with the present study and are not otherwise noted here.

A greater difference is apparent in the patterns of extracellular amylase activity versus days of incubation between the previous and present studies. For *H. lanuginosa,* Barnett and Fergus (3) report an increase in "amylase units" from 3 units after 10 days of incubation to a maximum of over 7 units after 21 days of incubation. This pattern is in sharp contrast to maximum amylase activity measurements of less than one unit recorded after 6 days of incubation, or less, for three other fungi studied by them, (*Humicola grisea* Traaen, *H. brevis* (Gilman

& Abbott) Gilman and *H. grisea* var. *thermoidea* Cooney & Emerson) including one thermophilic fungus *(H. grisea* var. *thermoidea).* The present study shows *H. lanuginosa* to reach maximum extracellular amylase levels (in the range of 0.75 to 0.97 units) after 9 days of incubation, and to remain unchanged through the 25th day. This pattern is very similar to that noted for *M. pusillus,* except that the increase in amylase activity is faster. Maximum levels of extracellular amylase activity of *M. pusillus* were recorded in the range 0.70 to 0.82 units after three days of incubation, remaining unchanged through the 11th day. Thus, *M. pusillus* and *H. lanuginosa* appear to be about equally amylolytic. It is difficult to compare quantitatively the amylase activity of *H. lanuginosa* reported here with that reported by Barnett and Fergus (3) because their assay method of amylase was based on one-hour incubations using 1 ml aliquots of extracellular filtrates the initial reducing sugar concentrations of which were not reported. In this laboratory, the production of reducing sugar from the starch substrate using 1 ml of filtrate did not appear to be linear over a one-hour period. If the amylase activity data reported here are accurate, and if reducing sugars are necessary for growth, these reducing sugars might be provided by means of membrane-bound amylase which does not appear in the filtrate. Such membrane-bound amylase, possibly induced by the starch substrate included in the growth medium, could account for the relatively high levels of reducing sugars measured in the extracellular filtrates of *H. lanuginosa* in the present study during the period of most rapid growth (days 2–7). This idea is supported by the relatively low amylase levels measured in the extracellular filtrates during the same period, including a non-measurable reading after 4 days of incubation, and further suggests that extracellular amylase might be due in large part to autolysis. The highest levels of extracellular amylase have been measured in the period (days 9–18) corresponding to a rapid reduction in the measured mycelial dry weight. This general situation is more difficult to observe in *M. pusillus,* because it reaches its maximum mycelial dry weight after only 2 days of incubation, but the data are supportive. On the other hand, if the fungi are grown on agar in petri dishes, starch appears to be absent at an early stage of growth at the periphery of the colony, a region which is considered extracellular.

Studies of amylase production by other species of fungi are continuing.

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