Quantitative Changes in the Content of Non-Nitrogenous Compounds during Autolysis of *Aspergillus terreus*

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SUMMARY

The behaviour of certain non-nitrogenous compounds in mycelium of *Aspergillus terreus* during autolysis in culture medium was studied. The mycelium of *A. terreus* lost during autolysis 45 % of its maximum dry weight. The amount of glucose, which was present from the beginning of autolysis throughout the whole further period of incubation, continuously increased to a maximum and then sharply descended to a constant value. Xylose decreased to half of its initial concentration during the first 30 days of autolysis and then disappeared. Mannitol descended in 95 % between the 18th and the 114th day of autolysis. Seventy-four % of the fat present at the beginning of autolysis was lost during the first 138 days of autolysis. The disappearance of fat and mannitol accounted for about 48 % of the dry wt. of mycelium lost during the whole period of autolysis.

INTRODUCTION

The earliest attempts to study the chemistry of autolysis in culture of filamentous fungi were confined almost entirely to the study of the behaviour of nitrogen-containing materials present in both mycelium and culture fluid (Behr, 1930; Schmidt, 1936; Woolley & Peterson, 1937; Bohonos, Woolley & Peterson, 1942; Ritter, 1955). Very few reports have appeared which deal with the behaviour of non-nitrogenous compounds in the mycelium during the autolysis of filamentous fungi in their culture media. Prill, Wenck & Peterson (1935) studied the effect of starvation on the formation and chemical nature of the fat produced by *Aspergillus fischeri*. Smithies (1953) examined autolysis of the mycelium of *Penicillium griseofulvum*, describing the total amount of carbohydrates liberated by enzymic action during autolysis. Some investigations have been made about changes in the amounts of organic acids and sugars, but in all cases these studies have been limited to detecting the presence or absence of these compounds. When these organic acids and sugars have been estimated, this has been done by a mere visual comparison (Tandon & Chandra, 1962). Emiliani & Ucha de Davie (1962) studied the liberation of glucose from the autolysing mycelium of *A. phoenicis* in 1 % acetic acid solutions. Arima, Uozumi & Takahashi (1965) and Uozumi, Takahashi & Arima (1965) described the autolysis of *A. oryzae* and the morphological changes of mycelium which occurred. Lahoz, Reyes & Beltrá (1966) studied some chemical changes occurring in mycelium of *A. flavus* during autolysis in culture. In the present work attention has been directed towards the qualitative and quantitative changes in the content of fat, free sugars and mannitol in the mycelium of *A. terreus* during autolysis in culture.

METHODS

Organism. *Aspergillus terreus* Thom (our collection no. 2426) was used throughout the present study of autolysis in a liquid medium.

Chemicals. Chemicals used in the preparation of the culture medium were of analytical purity, all obtained from Probus S.A. Barcelona, Spain. Other chemicals used were either from The British Drug Houses Ltd., Poole, Dorset, England, or from Sigma Chemical Co., St Louis, Mo., U.S.A.

Culture medium. The organism was grown in Czapek-Dox medium (g./l.): glucose (anhydrous), 50; NaNO_3 , 2.0; KCl, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; KH_2PO_4 , 1.0; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; distilled water, 1000 ml., pH 4.2. This medium was chosen as the standard medium in preference to other defined media because it contains its nitrogen as nitrate, which results in a greater 'alkaline autolysis' (Behr, 1930) accentuating the changes undergone by carbohydrates during autolysis.

Ten litres of this medium were prepared and distributed in 100 ml. amounts in Jena conical flasks (300 ml. capacity). The flasks were plugged with cottonwool and sterilized by steaming for 30 min. on three successive days. The flasks were inoculated with 1 or 2 ml. of a spore suspension obtained by gently rubbing the surface of each of 12 slopes of *Aspergillus terreus* grown on malt agar for 15 days at 24°. The inoculated flasks were incubated at 24–25° in the dark.

At regular intervals, generally 12 days, samples of five flasks were selected at random and withdrawn from the incubator. The pads of mycelium were separated from the culture liquid by filtration, pooled, washed on a Buchner funnel with distilled water, cut into small pieces and dried in an oven at 60–70° for 24 hr. Then they were dried in a desiccator (P_2O_5) to constant weight, and powdered in a small coffee mill (Turmix, model Moka-mix). The culture liquids from the five sample flasks were also pooled, and the pH value measured with Beckman pH meter, model G 2000.

An accurately weighed amount of powdered mycelium from each pooled, sample was continuously extracted in a Soxhlet apparatus with light petroleum (b.p. 50–70°) for 15–20 hr, the solvent removed by distillation (the last portions *in vacuo*) and the residue weighed. Results will be expressed as mg. component/flask. The defatted mycelium was re-extracted with diethyl ether in the same conditions as above and the resulting extract discarded.

In a typical experiment 1.0 g. air-dried, defatted mycelium was placed in a beaker and extracted twice with cold distilled water (2×100 ml.) for 1 hr with mechanical stirring. A third water extraction gave an extract which did not show any sugar spot when a portion of it after concentration was chromatographed. The first two aqueous extracts were pooled and protein removed by adding basic lead acetate (Eastham, 1949). Subsequently this treatment was found to be unnecessary, since samples of these extracts untreated with basic lead acetate gave good chromatographic separations as well as identical values for the content of total free reducing substances. A portion of this extract (100 ml.) was concentrated, under reduced pressure to final volume 10 ml. To 1 ml. of this concentrated extract, the reagents of Somogyi (1945) and Nelson (1944) were applied for the quantitative determination of total water soluble free reducing substances, by the use of a Klett–Summerson colorimeter. All readings were compared with standards treated in the same way. Results are expressed as mg. glucose/100 g. dry wt. mycelium. From the concentrates prepared as indicated

above individual sugars and mannitol were separated, identified and estimated as described previously (Lahoz *et al.* 1966).

Total nitrogen. The assays of total nitrogen were made by the micro-Kjeldahl method.

Total phosphorus. This was determined by a modification of the method of Fiske & SubbaRow (1925).

RESULTS

The criterion of degree of autolysis we have used through this work was the loss in weight of dry mycelium. Between 24 and 27 days of incubation of the cultures autolysis set in, as indicated by a loss in mycelial dry weight. The 25th day of incubation was therefore taken as the first day of autolysis; the word 'day' in the following text refers to 'day of autolysis'. By day 114 the mycelium lost 45 % of initial dry weight (Fig. 1). The pH value of the culture medium increased continuously during the first 18 days (Table 1) reaching about pH 8 at the 30 days, thereafter remaining practically constant. The nitrogen content of the mycelium steadily decreased throughout the whole period of autolysis; this decreased 54 % of the initial value (Table 1). The total loss of phosphorus was 17.8 mg./flask during the autolysis, representing about 90 % of the initial P in the mycelium at the beginning of autolysis.

Table 1. *The autolysis of Aspergillus terreus mycelium during incubation in Czapek-Dox medium at 25° in the dark*

Values for pH of culture filtrate, yield of mycelium, content of fat, nitrogen and phosphorus in mycelium. Autolysis began at 24–25 days of incubation.

Time of incubation (days)	Time of autolysis (days)	pH value	Yield of mycelium (g. dry wt./flask)	Content in mycelium of		
				Fat (mg./flask)	N (mg./flask)	P (mg./flask)
24	0	6.10	1.3261	295	23.0	—
27	3	6.85	1.3174	234	22.8	18.8
30	6	7.50	1.2368	229	20.5	16.0
42	18	7.75	1.0787	197	19.3	11.0
54	30	8.20	1.0050	208	19.0	7.0
66	42	8.10	0.9350	176	19.5	5.6
78	54	8.05	0.8410	150	17.6	4.2
90	66	7.70	0.8139	124	17.9	2.6
102	78	7.80	0.7863	128	17.1	2.4
114	90	7.80	0.7739	115	17.0	2.5
126	102	7.80	0.7498	117	16.4	2.2
138	114	7.80	0.7295	108	16.0	1.7
150	126	7.80	0.7464	—	—	1.2
162	138	7.80	0.7280	71	16.0	0.7
174	150	7.80	0.7012	82	10.5	0.9
186	162	7.80	0.7287	78	—	1.1

Behaviour of the mycelial fat. The content of fat at the beginning (Table 1) of autolysis, 295 mg./flask at the 0 day, decreased to a half by the 54th day, after 138 days amount of fat remained fairly constant. The total loss during autolysis being 74 % of the initial content.

Behaviour of the free sugars and of the free reducing substances in the mycelium. Apart from glucose and xylose the autolysing mycelium of *Aspergillus terreus* contained no other free sugar in significant amount.

Fig. 1

Fig. 2

Fig. 1. *Aspergillus terreus*. Change in the dry wt. of mycelium during autolysis in culture (25°, in dark).

Fig. 2. *Aspergillus terreus*. Changes of concentration of total free reducing substances, O; glucose, Δ; xylose, □, in mycelium during autolysis in culture (25°, in dark).

Fig. 3. *Aspergillus terreus*. Change in concentration of mannitol in mycelium during autolysis in culture (25°, in dark).

As shown in Fig. 2 there was an increase in the total amount of free reducing substances during the initial stages of autolysis. The initial values for reducing substances at the beginning of autolysis were 500 mg./100 g. dry wt. mycelium, increasing to 1600 mg./100 g. dry wt. mycelium by 18 days, 24 days later (42 days total) these reducing substances reached 1700 mg./100 g. dry wt. mycelium, followed by a sharp decrease (54–66 days) in their concentration to a final constant value. The curve for free glucose presented a very similar pattern (Fig. 2). We found an increase in the total amount of free glucose during autolysis. The initial values for the amount of glucose, 400 mg./100 g. dry wt. mycelium were nearly quadrupled by day 42, when there was a peak for total free reducing substances and glucose. Glucose decreased sharply from 42 to 66 days, then diminished at a slower rate to a constant value.

Between day 6 and day 126, glucose accounted for an average of 83 % of the total reducing power, at the end of the incubation time, day 174 to day 186, glucose accounted for the total reducing power of the aqueous extracts. Glucose was present from the beginning of autolysis through the whole period of incubation, xylose decreased half of its initial concentration (123 mg./100 g. dry wt. mycelium) during the first 30 days and then disappeared in the next 12 days.

Behaviour of mannitol

The concentration of mannitol in the mycelium of *Aspergillus terreus* during autolysis was at a minimum when autolysis began (Fig. 3). At day 18 it reached a peak (6.6 g./100 g. dry wt mycelium), decreasing then, rapidly at first, to a constant value after the day 114. Ninety-five % of the mannitol disappeared between days 18 and 114.

DISCUSSION

It is generally believed that carbohydrates present in fungal mycelia are continuously undergoing breakdown during autolysis in culture, but very few studies on carbohydrates in mycelia during autolysis of filamentous fungi are available for comparison with the present results. Our experiments indicate that carbohydrates in mycelium of *Aspergillus terreus* autolyzing in the culture fluid underwent enzymic hydrolysis in which glucose appeared, giving a concentration of glucose several times greater than in the initial stages of autolysis. Similar qualitative results were obtained by Tandon & Chandra (1962) who reported that the concentration of carbohydrates in the mycelium of *Colletotrichum gloeosporioides* decreased during autolysis.

In the autolysis of *Aspergillus phoenicis* in 1 % acetic acid solution glucose was one of the components more abundantly formed, amounting to 22 % of the dry weight of the original mycelium (Emiliani & Ucha de Davie, 1962). In our results free glucose reached much lower values (1.55 g./100 g. dry wt. mycelium) which represents only 7 % of the value for glucose released by *A. phoenicis*. However, in Emiliani & Ucha de Davie's experiments an induced autolysis took place, which in our opinion was partially due to the use of unnatural agents such as acetic acid to cause autolysis. The variety of methods used in autolysis studies partially explains the difficulties encountered in comparing different sets of results. Similar but less drastic conditions for autolysis, 'fresh mycelium' suspended in water, were used with mycelium of *Penicillium griseo-fulvum* but no reducing sugars were formed during autolysis (Smithies, 1953).

The formation of glucose in the mycelium of *Aspergillus terreus* during autolysis in

culture took place more actively in the initial stages, from day 3 or 6 to day 42 (Fig. 2), in which the highest values for glucose were formed. This recalls certain observations with yeast; Vosti & Joslyn (1954) stated that proteins and carbohydrates in yeast were involved in the early stages of autolysis.

Our experiments indicate that during autolysis fat continually disappeared from mycelium; similar results were obtained by Prill *et al.* (1935) who studied the effect of inanition on the chemical nature of the fat produced by *Aspergillus fischeri*. These workers found that the amount of fat continuously decreased, the loss being about 50% the initial value. In our experiments with *A. flavus* (Lahoz *et al.* 1966) the fat content remained constant.

A complete disappearance during autolysis of the free sugars in the mycelium of *Aspergillus terreus* seems not to take place; in the present work with an incubation period as long as 186 days, the glucose concentration at that time was greater than 200 mg./100 g. dry wt. mycelium (Fig. 2). With *A. flavus* grown in Raulin-Thom medium we have similarly found that the free sugars in the mycelium persisted up to 251 days of incubation (Lahoz *et al.* 1966).

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