The Autolysis of *Aspergillus terreus* in a Physiologically Acid Medium

By R. LAHOZ, F. REYES, R. BELTRÁ AND C. GARCÍA-TAPIA

*Instituto 'Jaime Ferrán' de Microbiología, del Consejo Superior de
Investigaciones Científicas, Joaquín Costa 32, Madrid-6, Spain*

(Accepted for publication 9 May 1967)

SUMMARY

The behaviour of certain intracellular components contained in the mycelium of *Aspergillus terreus* during autolysis were studied. *Aspergillus terreus* grown in an acid medium underwent an 'acid autolysis' in which a decrease in mycelial dry weight amounting to 14·6% occurred. The content of fat present in the autolysing mycelium continuously decreased throughout the pre-autolytic and autolytic stages. The concentration of free glucose in the mycelium continuously increased at the beginning of autolysis, whereas xylose soon disappeared when autolysis began. At the end of the log phase and during the pre-autolytic stages the content of mannitol sharply decreased, at the beginning of autolysis its concentration decreased at a slower rate, being constant at the end of the autolytic period studied. Fourteen different amino acids were found in the autolysing mycelium of *A. terreus*. Eighty-six % of the maximum concentration of these free amino acids disappeared from mycelium before autolysis; 81 % of the remainder during autolysis.

INTRODUCTION

Studies on the chemistry of the autolytic phase of growth in filamentous fungi grown in physiologically alkaline and acid media have been reported only on three occasions for four different moulds: *Aspergillus niger* (Behr, 1930); *Cunninghamella elegans* (Schmidt, 1936); *Mucor adventitus* and *Absidia glauca* (Ritter, 1955). In these studies the behaviour of certain nitrogenous compounds during autolysis, in two types of medium (NO_3^- or NH_4^+ as N source) were described. However, very few papers have presented quantitative data on the variation of the amount of intracellular free sugars and free amino acids contained in autolysing mycelium in cultures on an acid medium.

Continuing our work on the chemistry of autolysis in fungi we reported in a previous paper (Lahoz, 1967) the behaviour of *Aspergillus terreus* grown in an alkaline medium, during autolysis. The present work describes the qualitative and quantitative chemical changes in lipids, free sugars, mannitol and free amino acids occurring in *A. terreus* grown in an acid medium during the autolytic phase of growth.

METHODS

Organism. *Aspergillus terreus* Thom (our collection no. 2426) was used as a physiological model in the present studies with 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 and cultivation of the organism. The organism was grown in static cultures in a modified Czapek-Dox medium, in which the source of nitrogen, NaNO_3 , was replaced by its equivalent amount of $(\text{NH}_4)_2\text{SO}_4$, thus leading to an 'acid autolysis'. The medium had the following composition: glucose (anhydrous) 50 g.; $(\text{NH}_4)_2\text{SO}_4$ 1.55 g.; KCl 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g.; KH_2PO_4 1.0 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g.; distilled water 1000 ml. Fifteen l. of this medium were prepared and distributed in 100 ml. portions in Jena glass conical flasks of 300 ml. capacity. The flasks were plugged with non-absorbant cotton wool and sterilized by steaming on three consecutive days, for 30 min. each day. When at room temperature, a heavy spore suspension of *Aspergillus terreus* was inoculated (about 2 ml.) into each flask. These flasks were then incubated at 24–25° in the dark.

Samples (5 flasks) were withdrawn at intervals from the incubator. The mycelia were separated from the culture fluids by filtration. These culture fluids from 5 flasks were pooled and diluted with distilled water to the original volume. The mycelia were washed free from culture fluid with distilled water, cut into small pieces and dried to constant weight at 60–70°.

Fractionation of mycelium. Weighed samples of powdered mycelium harvested at different times were exhaustively extracted with light petroleum (b.p. 50–70°). The solvent was removed from the extract by distillation and the crude lipids left in the flask dried in vacuum to constant weight; this constituted fraction 1. The defatted mycelium was carefully removed from the percolator, dried at 80°, weighed and extracted twice by cold (22°) distilled water (100 ml./g. dry defatted mycelium) with mechanical stirring for 1 hr. The cold-water extracts were combined and stored at –10° until needed (fraction 2). To test for completeness of extraction the residue was a third time extracted and the resulting extract concentrated under reduced pressure and a sample of it chromatographed to examine for sugars and amino acids.

One hundred ml. of fraction 2 were concentrated in vacuum at 70° on a water bath to a final volume of 10 ml. Portions (0.5 or 0.7 ml.) of this concentrate were used for chromatographic analysis of sugars and mannitol. The remainder of each concentrate was kept at –10°.

The determination of total nitrogen, fat, total free reducing substances, free sugars and mannitol were as described previously (Lahoz, Reyes & Beltrá, 1966).

Estimation of individual amino acids. One hundred ml. of fraction 2 were concentrated to dryness at 60–70° under reduced pressure and extracted with 50 ml. of the ethanol + water + conc. HCl (95 + 4.5 + 0.5, by vol.) mixture described by Baliga, Krishnamurthy, Rajagopalan & Giri (1955). This extract was concentrated and the amino acids separated and identified by paper chromatography using as solvent the butan-1-ol + acetic acid + 96% (v/v) ethanol + water (40 + 10 + 10 + 20, by vol.) mixture on Whatman no. 1 filter paper. The papers were developed by descending chromatography in three successive periods of 24 hr runs, each time air-dried and newly introduced into the tank for a total time of 72 hr. Elution of the spots and determination of the amount of each of the individual amino acids was done by the method of Giri, Radhakrishnan & Vaidyanathan (1953) as modified by Kay, Harris & Entenman (1956). Readings were done in a Beckman spectrophotometer, model B at 575 m μ . Standards were

prepared from leucine of analytical purity obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Results are expressed as g. amino acid/100 g. dry mycelium.

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

RESULTS

Autolysis of *Aspergillus terreus* in a medium containing NH_4^+ as N source took place at about pH 2 (Table 1). Dry weight of mycelium (Table 2) reached a maximum, 1.0824 g./flask, at the 18th day of incubation and was 0.9242 g./flask at the 150th day

Table 1. *The autolysis of Aspergillus terreus in cultures in an acid (pH 2) medium at 24–25° in the dark. pH values residual glucose, nitrogen and phosphorus of the culture fluid at various times of incubation*

Time of incubation (days)	Time of autolysis (days)	pH value	Residual glucose (g./100 ml.)	Nitrogen (mg./100 ml.)	Phosphorus (mg./100 ml.)
6	.	1.8	2.950	0.00	15.5
7	.	1.9	2.700	0.00	15.5
8	.	1.8	1.440	0.00	14.7
9	.	1.8	1.360	0.00	14.9
10	.	1.9	0.790	0.00	13.8
11	.	2.0	0.590	0.00	13.5
12	.	1.9	0.310	0.00	14.8
13	.	1.9	0.300	0.00	15.8
14	.	2.0	0.200	0.00	17.5
15	.	2.1	0.140	0.00	18.8
18	0	2.1	0.053	0.00	20.5
24	6	2.0	0.060	5.10	21.3
30	12	2.1	0.054	8.10	.
42	24	2.1	.	8.76	.
54	36	.	.	8.63	.
66	48	2.0	.	8.00	.
78	60	.	.	6.91	.
90	72	.	.	10.70	.
102	84	.	.	9.00	.
114	96	.	.	8.90	.
126	108	.	.	9.50	.
138	120	1.9	.	9.20	.
150	132	.	.	8.89	.
162	150	.	.	6.80	.

of autolysis. The loss in mycelial dry weight amounted to 14.6% during this autolytic period. The criterion of autolysis we have adopted throughout this work was the loss in mycelial weight. According to that, autolysis began on the 19th day of incubation. Carbon source (glucose) initially present at 5 g./100 ml. medium, reached an average constant value 0.055 g./100 ml. culture filtrate (Table 1), after the zero day of autolysis. A progressive increase in the content of total P in the culture filtrate during the pre-autolytic stages occurred (Table 1). Nitrogen was not detected in the culture filtrate between the 6th and the 18th days of incubation; at the 24th day 5.1 mg. N/100 ml. were present.

The percentage of nitrogen in autolysing mycelium of *Aspergillus terreus* (Table 2) grown with $(\text{NH}_4)_2\text{SO}_4$ as N source decreased slowly through the whole period of incubation, reaching a minimum constant value, 2.1%, at the 96th day of autolysis. The amount of total P present in mycelium during the incubation period here studied

decreased by about a half (54%) with respect to its initial maximum content, whereas the disappearance of P during the first 24 days of autolysis amounted to 75% (Table 2). The highest amount of fat present in mycelium of *A. terreus* was observed at the 9th day of incubation. From this stage on the content of fat continuously decreased during the whole cycle of autolysis, being 4.9 g./100 g. dry mycelium (Table 2), the final value observed at the end of the autolytic period (150 days).

Table 2. *The autolysis of Aspergillus terreus in cultures in an acid medium at 24–25° in the dark*

Amounts of nitrogen, phosphorus and fat in the mycelium at different times of incubation.

Time of incubation (days)	Time of autolysis (days)	Mycelium dry weight (g./flask)	N (%)	P (%)	Fat (g./100 g. dry mycelium)
6	.	0.6695	4.7	.	.
7	.	0.7988	3.4	0.96	19.0
8	.	0.8907	.	0.96	.
9	.	0.9264	2.8	0.92	21.6
10	.	0.9540	.	0.94	.
11	.	1.0090	.	0.87	20.5
12	.	1.0380	.	0.74	.
13	.	1.0652	.	0.68	.
14	.	1.0589	2.6	0.53	.
15	.	1.0770	.	.	15.8
18	0	1.0824	.	0.44	.
24	6	1.0764	.	0.29	13.6
30	12	1.0715	2.6	0.19	.
42	24	1.0661	.	0.11	13.5
54	36	1.0519	.	0.11	.
66	48	0.9796	.	.	10.0
78	60	0.9769	2.3	.	.
90	72	0.9625	.	.	9.4
102	84	0.9554	.	.	.
114	96	0.9677	2.1	.	8.8
126	108	0.9784	.	.	.
138	120	0.9628	2.1	.	.
150	132	0.9548	.	.	.
162	150	0.9242	2.1	.	4.9

Behaviour of the total free reducing substances, free sugars and mannitol. At the beginning of autolysis the amount of water-extractable reducing substances contained in the mycelium of *Aspergillus terreus* stayed nearly at a minimum (Fig. 1). At 36 days incubation the content of these materials were the highest observed, reaching to 1175 mg./100 g. dry mycelium. After that time the amount of total free reducing substances sharply decreased to values fairly constant in the range 300–400 mg./100 g. dry mycelium. The behaviour of free glucose showed a very similar pattern (Fig. 1) to that of total free reducing substances. Glucose accounted for an average of 70% of the total reducing power observed for the aqueous extracts. From the 40th day of incubation xylose (Fig. 1) tended to decrease. No appreciable amount of xylose was detected between 66 and 78 days of incubation.

During the phase of growth (9 days) mannitol was present in mycelium of *Aspergillus terreus* at (Fig. 2) 6.1 g./100 g. dry mycelium. As incubation proceeded this amount decreased sharply to the 18th day of incubation (zero days of autolysis) when 2.5 g.

mannitol/100 g. dry mycelium were present. At the beginning of autolysis its concentration decreased at a slower rate; between the 72 and 84 days the concentration of mannitol remained constant.

Fig. 1

Fig. 1. Changes in the concentration of total free reducing substances $\circ$; free glucose $\triangle$; xylose $\bullet$, in autolysing mycelium of *Aspergillus terreus* grown in a medium with NH_4^+ as N source.

Fig. 2

Fig. 2. Content of mannitol in autolysing mycelium of *Aspergillus terreus* grown in an acid medium.

Behaviour of the amino acids and its variation with age

The nature and amounts of free amino acids released from mycelium harvested at different times is shown in Table 3. Thirteen different amino acids were identified; one, present in relatively small amount, was not identified (unknown). The total content of these free amino acids increased between 7 and 9 days of incubation to a maximum. Thereafter the general picture was of decreasing concentration both at the end of the log phase and during the pre-autolytic stages. Asparagine, proline, tryptophan and phenylalanine disappeared (Table 3) in the pre-autolytic period. Spots due to these amino acids were not seen on the chromatograms at zero day of autolysis. The maximum concentration, (2.454 g./100 g. dry mycelium) at the 9th day, diminished by 86% during the 9 days before autolysis. From the beginning of the autolytic phase to the end of the period studied the total concentration of the free amino acids decreased again by 81%, with respect to the concentration present at zero day of autolysis. Apart from serine, which was easily detectable throughout the whole cycle, no other amino acid was observed at the later stages of autolysis. Leucine, serine, threonine and alanine were the most abundant constituents in the pool; the sum of these four components formed more than 50% of the total content of amino acids present at each date.

DISCUSSION

Cessation of growth of *Aspergillus terreus* in these experiments seemed to take place when the carbohydrate source (glucose) had been almost completely consumed. The amount of glucose present in the culture filtrate (determined by Somogyi's method)

Table 3. Intracellular free amino acids present in mycelium of *Aspergillus terreus* at various stages of growth

	Time of incubation (days)						Time of autolysis (days)					
	7	9	11	13	18	30	42	66	84	96	114	150
	Amounts of amino acids (g./100 g. dry mycelium)											
Amino acids												
Asparagine	—	*	*	—	—	—	—	—	—	—	—	—
Proline	*	*	*	—	—	—	—	—	—	—	—	—
Tryptophan	*	*	*	—	—	—	—	—	—	—	—	—
Phenylalanine	0.040	0.135	*	—	—	—	—	—	—	—	—	—
Unknown	0.034	0.030	0.018	0.012	0.010	*	—	—	—	—	—	—
Tyrosine	0.089	0.097	0.046	0.019	0.021	*	—	—	—	—	—	—
Aspartic acid	0.212	0.093	0.085	0.027	0.020	*	—	—	—	—	—	—
Norvaline	0.169	0.152	0.078	0.027	0.019	*	—	—	—	—	—	—
Lysine	0.072	0.302	0.220	0.064	0.016	*	*	*	—	—	—	—
Arginine	0.183	0.166	0.137	0.069	0.030	*	*	*	—	—	—	—
Leucine	0.189	0.344	0.147	0.065	0.046	*	*	*	—	—	—	—
Serine	0.281	0.352	0.261	0.102	0.072	*	*	*	*	*	*	*
Threonine	0.341	0.387	0.245	0.092	0.052	*	*	*	*	*	*	*
Alanine	0.382	0.396	0.212	0.075	0.052	*	*	*	*	*	*	*
Total amounts	1.995	2.454	1.454	0.552	0.388	0.288	0.162	0.060	—	—	—	—

* Too small amount to be individually estimated; — absent.

* Too small amount to be individually estimated; — absent.

stayed practically constant (Table 1) when autolysis set in. Crewther & Lennox (1953) found similar results for *A. oryzae* grown in a medium containing NH_4^+ as N source.

The decrease in mycelial dry weight by *Aspergillus terreus* (14.6%) in the present experiments is of the same order as that found by Ritter (1955) for *A. niger* (17.6%). A nearer value (14.4%) was reported by Ritter for a strain of *A. niger* which he called strain 'S'. The fungi in both cases were grown in an acid medium.

With respect to the behaviour of fat during autolysis it can be seen in Table 2 that a continuous decrease of mycelial lipids took place before and after the beginning of autolysis. Similar results were observed for *Aspergillus terreus* grown in a medium physiologically alkaline, in which there was a continuous diminution in the content of mycelial fat throughout the whole period of autolysis (Lahoz, 1967).

The increase in the concentration of free glucose in the mycelium of *Aspergillus terreus*, at a certain stage of autolysis, seems to be a common feature for both 'alkaline' (Lahoz, 1967) and 'acid' autolysis.

The disappearance of free amino acids from mycelium of *Aspergillus terreus* began at a stage in which autolysis, measured as decrease in mycelial weight, had not begun. Similarly the content of free amino acids in mycelium of *A. flavus* decreased during both the log and the autolytic phases (Pillai & Srinivasan, 1956), whereas for *Microsporum canis* the maximum concentration of the pool of amino acids was observed towards the end of the log phase, with a continuous decrease thereafter (Chattaway, Toothill & Barlow, 1962).

This research was financed in part by a grant made by the United States Department of Agriculture under P.L. 480. The work reported here formed part of a Ph.D. thesis submitted in the Faculty of Pharmacy of the University of Madrid (C. García-Tapia, 1966). One of us (C. G.-T.) is greatly indebted to the Comisaría de Protección Escolar del Ministerio de Educación y Ciencia for a generous grant.

REFERENCES

- BALIGA, B. R., KRISHNAMURTHY, K., RAJAGOPALAN, R. & GIRI, K. V. (1955). A simple method for desalting biological fluids for chromatography. *J. Indian Inst. Sci.* **37**, 18.
- BEHR, G. (1930). Über Autolyse bei *Aspergillus niger*. *Arch. Mikrobiol.* **1**, 418.
- CHATTAWAY, F. W., TOOTHILL, C. & BARLOW, A. J. E. (1962). The amino acid metabolism of *Microsporum canis*. *J. gen. Microbiol.* **28**, 271.
- CREWTER, W. G. & LENNOX, F. G. (1953). Enzymes of *Aspergillus oryzae*. III. The sequence of appearance and some properties of the enzymes liberated during growth. *Aust. J. biol. Sci.* **6**, 410.
- FISKE, C. H. & SUBBAROW, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375.
- GIRI, K. V., RADHAKRISHNAN, A. N. & VAIDYANATHAN, C. S. (1953). Circular paper chromatography. Part VI. The quantitative determination of amino acids. *J. Indian Inst. Sci.* **35**, 145.
- KAY, R. E., HARRIS, D. C. & ENTENMAN, C. (1956). Quantification of the ninhydrin color reaction as applied to paper chromatography. *Arch. Biochem. Biophys.* **63**, 14.
- LAHOZ, R. (1967). Quantitative changes in the content of non-nitrogenous compounds during autolysis of *Aspergillus terreus*. *J. gen. Microbiol.* **46**, 451.
- LAHOZ, R., REYES, F. & BELTRÁ, R. (1966). Some chemical changes in the mycelium of *Aspergillus flavus* during autolysis. *J. gen. Microbiol.* **45**, 41.
- PILLAI, N. C. & SRINIVASAN, K. S. (1956). The amino acid metabolism of *Aspergillus flavus*. *J. gen. Microbiol.* **14**, 248.
- RITTER, R. (1955). Physiologische Untersuchungen an Zygomyceten. *Arch. Mikrobiol.* **22**, 248.
- SCHMIDT, M. (1936). Makrochemische Untersuchungen über das Vorkommen von Chitin bei Mikroorganismen. *Arch. Mikrobiol.* **7**, 241.