The Autolysis of *Aspergillus flavus* in an Alkaline Medium

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SUMMARY

The autolysis of *Aspergillus flavus* subjected to a combined action of a continuous flow of air and mechanical agitation has been studied. We have obtained a degree of autolysis amounting to 85 %. The total loss of nitrogen in autolysing mycelium of *A. flavus* reached 82 %, whereas the loss of phosphorus was 90 % from the beginning of autolysis. Total carbohydrates were reduced from 524 mg./sample, at 0 day, to 119 mg./sample by the 16th day of autolysis, which represents a loss of 77 %. Glucose was the only sugar found in significant amount in autolysing mycelium of *A. flavus*, being 96 % the total loss. The concentration of mannitol decreased to nearly a third at the end of the log phase. The disappearance of this substance during pre-autolysis and autolysis amounted to 97.7 % with respect to the maximum weight present at the 8th day of incubation. Fourteen different free amino acids were identified in the mycelium of *Aspergillus flavus* during the pre-autolytic and autolytic stages of growth. These amino acids increased their content between the 4th and the 6th days of incubation, to the highest concentration observed. Seventy-nine per cent of this maximum amount disappeared by the end of the log phase, whereas 78 % of the remainder disappeared from mycelium during autolysis (16 days). From the total amount of bound amino acids in mycelium of *A. flavus* it seemed that the make-up of the mycelial protein does not undergo great changes in the interval between pre-autolysis and the initiation of the autolytic phase of growth.

INTRODUCTION

During our investigations of the chemistry of autolysis in cultures of filamentous fungi (Lahoz, Reyes & Beltrá, 1966; Lahoz, 1967; Lahoz, Reyes, Beltrá & Garcia-Tapia, 1967) we have often suspected that the effect of a continuous flow of air on the process of autolysis might be to increase the amount of material released by the mycelium and consequently increase the degree of autolysis.

In exploratory experiments with *Aspergillus flavus* grown in a stirred alkaline culture medium and with a continuous supply of air (4 l./min.) we obtained a degree of autolysis amounting to 85 %, which is to our knowledge the highest value recorded for filamentous fungi. It was decided to examine autolysis under these conditions.

METHODS

Chemicals. The chemicals used in the preparation of the culture medium were of analytical purity and they were obtained from Probus S.A. (Barcelona, Spain) and from the British Drug Houses Ltd. (Poole, Dorset, England). Standards used in chromatographic work were from the Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Organism. *Aspergillus flavus* Link (our collection no. 2427) came from the Centraal-bureau voor Schimmelcultures, Baarn, Holland.

Culture medium and conditions of culture. The organism was grown in the Czapek-Dox medium of the following composition (g./l. distilled water): 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; pH 4.2. The fungus was grown in submerged culture with continuous aeration and mechanical stirring in a 14 l. fermentor (NBS Continuous Culture Apparatus, model CF 500, New Brunswick Scientific Co., Inc., New Brunswick, New Jersey, U.S.A.). Incubation was at 24–25°, aeration at 4.0 l./min. and the culture stirred at 150 rev./min. throughout. The empty fermentor was sterilized at 120° for 2 hr and afterwards the fermentor containing the basal medium was sterilized at 120° for 20 min. A concentrated solution of glucose autoclaved separately at 110° for 20 min., was added aseptically to the basal medium in the fermentor; the final volume of medium was 10 l.

Inoculum. A spore suspension (10 ml.) was obtained by gently rubbing the surface of a slope of *Aspergillus flavus* grown on malt agar incubated for 15 days at 24–25° in the dark. With this spore suspension two conical flasks of 300 ml. capacity each, containing 100 ml. of Czapek-Dox medium, were inoculated and incubated for 24 hr on a reciprocating shaker (60 × 10 cm. strokes/min.) at 25°. One of these flasks was used as inoculum for the fermentor.

The time of incubation was usually 30–40 days; the water lost by evaporation was daily replaced. At convenient intervals samples (250 ml.) of culture were taken, and mycelium separated from culture fluid by filtration. Culture filtrate was kept frozen until analysed. Samples of mycelium were washed on the filter with distilled water and dried at 60–70° to constant weight.

Analytical methods. Total carbohydrates were determined by the anthrone method of Morris (1948) with glucose as standard.

The intensity of the colour of the culture filtrate was measured with a Klett-Summers photometer with a blue filter.

Total N, P, crude fat, glucose, mannitol, amino acids and kojic acid were determined as previously described (Lahoz *et al.* 1966; Lahoz, 1967; Lahoz *et al.* 1967).

Fractionation of mycelium. Weighed samples of dry mycelium, harvested at different times, were exhaustively extracted with light petroleum (b.p. 50–70°) in a beaker (100 ml./g. dry mycelium) with mechanical stirring for 1 hr; the solvent was removed by distillation and the residue left in the flask constituted fraction I. This defatted mycelium was carefully removed from the beaker, dried at 80°, weighed and extracted twice by cold (22°) distilled water (100 ml./g. defatted mycelium) with mechanical stirring for 1 hr; the cold-water extracts obtained were combined (fraction II) and stored at –10°. To test for completeness of extraction the mycelial residue was extracted a third time and the resulting extract concentrated under reduced pressure and a sample of it chromatographed for sugars and amino acids. Free sugars and free amino acids contained in fraction II were separated by paper chromatography and

individually estimated as described previously (Lahoz *et al.* 1966; Lahoz, 1967; Lahoz *et al.* 1967). The water-extracted mycelium was dried at 80° to constant weight (fraction III). A portion (40 mg.) of fraction III was hydrolysed with 3 N-HCl (1 ml./10 mg. dry mycelium) in sealed ampoules at 100° for 12 hr. The hydrolysate was dried at 60–70°, the residue dissolved in the initial volume (4 ml.) of distilled water, again evaporated to dryness at 70°, again dissolved in 4 ml. distilled water and finally dried in a desiccator over NaOH + P₂O₅. This residue was dissolved in water (4 ml.), samples (0.10 ml.) chromatographed on Whatman no. 1 filter paper for carbohydrates (Lahoz *et al.* 1966).

A portion (40 mg.) of fraction III was heated with 6 N-HCl (1 ml./10 mg. dry mycelium) in sealed ampoules at 110° for 18 hr, the hydrolysate processed as for carbohydrates, and samples (0.07 ml.) of the final hydrolysate chromatographed for amino acids.

RESULTS

Aspergillus flavus incubated in medium containing nitrate as N source with aeration gave cultures showing pH values generally in the range pH 4.5–8.5, in some experiments rising to pH 8.6 and even 9.0. In a typical experiment (Table 1) the culture changed from pH 4.8 at day 11, when autolysis began, to pH 8.5 at the 9th day of

Table 1. *The autolysis of Aspergillus flavus during incubation in Czapek–Dox medium in submerged culture at 25° with aeration (4 l./min.) and mechanical stirring (150 rev./min.) in fermentors. Autolysis* began at 11–12 days of incubation*

Time of incubation (days)	Time of autolysis* (days)	pH	Residual glucose (g./l.)	Colour intensity (Klett values)	Kojic acid (mg./100 ml.)
4	—	5.0	33.0	n.t.†	0
6	—	4.5	23.5	n.t.	6
8	—	4.5	12.6	39	20
9	—	4.5	7.4	n.t.	60
11	0	4.8	0.7	65	125
12	1	5.3	0.6	80	162
13	2	6.5	0.6	175	195
14	3	7.1	0.5	260	158
16	5	7.6	0.3	355	61
18	7	8.0	0.2	395	21
20	9	8.5	0.1	390	0
24	13	8.5	0.1	490	0

* Autolysis was measured as decrease in mycelial dry weight.

† n.t. = not tested.

autolysis (day 20 of incubation), being constant thereafter. The criterion of autolysis adopted throughout this work was the loss in mycelial dry weight. According to that, autolysis begun on the 11th day of incubation. Residual glucose (Table 1) was present at about 0.5 g./l. at the beginning of autolysis. The colour of the culture fluid (Table 1) continuously increased in intensity and was equivalent to about 400 Klett scale units between 20 and 25 days. When kojic acid was produced its content in the culture fluid increased to a maximum value and thereafter decreased as the culture aged. At the end of the autolysis no kojic acid remained. In a typical experiment the maximum content of kojic acid in the culture fluid coincided with the beginning of autolysis.

In a representative experiment the highest amount of mycelial weight (Table 2), 1.81 g./250 ml. broth, was attained at day 11 of incubation, being of 0.28 g./250 ml. broth by the 16th day of autolysis—that is, an 85 % decrease of mycelial dry wt. The total-N content of the mycelium (Table 2) continuously decreased from the end of the

Table 2. *The autolysis of Aspergillus flavus mycelium during incubation in Czapek-Dox medium in submerged culture at 25° with aeration (4 l./min.) and mechanical agitation (150 rev./min.) in fermentors*

(Values for yield of mycelium, total N, P, carbohydrates and crude lipids from mycelium. The term 'sample' indicates the dry weight of mycelium contained in 250 ml. of broth.)

Time of incubation (days)	Time of autolysis (days)	Mycelium (g. dry wt./sample)	Total N (g./sample)	Total P (g./sample)	Total carbohydrates (g./sample)	Petroleum ether extract (g./sample)
6	—	1.214	0.050	n.t.*	0.327	0.174
8	—	1.623	0.057	n.t.	n.t.	0.290
11	0	1.807	0.046	0.010	0.524	0.386
12	1	1.599	n.t.	n.t.	n.t.	n.t.
13	2	1.550	n.t.	n.t.	n.t.	n.t.
16	5	1.214	0.031	0.007	0.461	0.162
18	7	0.625	0.017	0.003	n.t.	0.076
20	9	0.517	0.015	0.003	0.196	0.040
24	13	0.262	0.008	0.001	n.t.	0.018
27	16	0.278	0.008	n.t.	0.119	0.015

* n.t. = not tested.

Table 3. *Fraction II. variation in the content of water-extractable materials, total carbohydrates, mannitol and free glucose in mycelium of Aspergillus flavus during pre- and autolytic phase of growth*

Results are expressed as mg. substance(s)/sample of original dry mycelium. Other details are given in the text.

Time of incubation (days)	Time of autolysis (days)	Water extractable materials (mg./sample*)	Total carbohydrates in aqueous extracts (mg./sample*)	Mannitol (mg./sample*)	Glucose (mg./sample*)
6	—	476	50	70	42
8	—	514	57	90	43
11	0	361	43	37	25
16	5	221	24	21	13
18	7	104	11	9	5
20	9	75	7	7	4
24	13	30	4	2	2
27	16	—	3	2	1

* The term 'sample' indicates grams of dry mycelium contained in 250 ml. of brew.

log phase to the 24th day of incubation, being constant thereafter, the total loss of N during the period of autolysis amounting to 82 % with respect to the 11th day of incubation. Phosphorus lost during autolysis 90 % of its maximum initial content (Table 2). Total carbohydrates present in autolysing mycelium of *Aspergillus flavus*,

524 mg./sample, at the 11th day of incubation (Table 2) fell to 119 mg./sample by the 27th day of incubation, which represents a loss of 77% with respect to the content at the 11th day of incubation, whereas the content of lipids, measured as the total light petroleum extractable material, continually increased at the end of the log phase. During autolysis the loss of these materials was 96% with respect to the 11th day of incubation.

Behaviour of free glucose and mannitol. Free glucose was the only sugar present in significant amounts in the autolysing mycelium of *Aspergillus flavus* under these conditions. The decreasing concentrations of total carbohydrate, glucose and mannitol in fraction II are shown in Table 3. The behaviour of bound glucose in fraction III is shown in Table 4, where the amount of free glucose released on hydrolysis of fraction III is recorded.

Behaviour of free amino acids. Free amino acids increased in content between the 4th and the 6th days of incubation, being at the highest (Table 5) concentration on the 6th day of incubation. During pre-autolysis and autolysis the amount continuously decreased till the 27th day of incubation. Seventy-nine per cent of the maximum amount of the free amino acids contained in mycelium at the 6th day of incubation disappeared at the end of the log phase, whereas 78% of the remainder disappeared during autolysis. The bound amino acids released on hydrolysis of fraction III are shown in Table 6.

Table 4. *Total reducing substances and glucose released in the hydrolysis of the defatted, ex-water mycelium (fraction III) by 3 N-HCl during 12 hr at 100° in sealed ampoules (other details are given in the text)*

Time of autolysis (days)	Total reducing substances (mg./sample* of original dry mycelium)	Glucose (mg./sample* of original dry mycelium)
0	148	40
5	128	29
7	77	15
9	51	10
13	35	6
16	34	5

* The term 'sample' indicates the weight of dry mycelium contained in 250 ml. of brew.

DISCUSSION

These experiments seem to indicate that comparatively strong aerobic conditions during autolysis increase the extent to which the processes of breakdown of mycelium took place, which partially explains the high value for the degree of autolysis encountered in these conditions. Excepting actinomycetes, as a group of filamentous organisms, this is to our knowledge the highest loss of mycelial weight reported in the literature of filamentous fungi during autolysis.

In static cultures of *Aspergillus flavus* the maximum content of free amino acids (4.92 g./100 g. dry mycelium) occurred at the 5th day of incubation, with a subsequent decrease (Pillai & Srinivasan, 1956), the 15th day of incubation being the 0 day of autolysis. Similarly, in our experiments nearly 80% of the total free amino acids

Table 5. *Intracellular free amino acids present in the mycelium of Aspergillus flavus during pre-autolytic and autolytic phase of growth in a physiologically alkaline medium (Czapek-Dox) with aeration (4 l./min.) and mechanical agitation (150 rev./min.) at 25°*
Autolysis began at 11-12 days of incubation. Results are expressed as mg. amino acid/sample* (column A) and as g. amino acid/100 g. dry wt of initial mycelium (column B).

Time of incubation (days)		4		6		11		16		18		27	
Time of autolysis (days)		—		—		0		5		7		16	
Amino acids		A	B	A	B	A	B	A	B	A	B	A	B
Leucine		11.4	1.22	—	—	—	—	—	—	—	—	—	—
Phenylalanine		T	T	T	T	T	T	T	T	T	T	T	T
Valine		12.1	1.30	14.3	1.18	8.7	0.48	3.2	0.26	1.3	0.21	0.2	0.09
Tryptophan + tyrosine		3.4	0.37	24.0	1.98	6.1	0.34	4.6	0.38	1.8	0.30	—	—
Proline		T	T	T	T	T	T	T	T	T	T	T	T
Alanine		11.0	1.18	14.9	1.23	3.8	0.21	2.5	0.21	0.6	0.10	0.3	0.12
Glutamic acid		13.5	1.45	—	0.78	4.0	0.22	1.8	0.15	1.1	0.17	0.1	0.05
Serine + glycine + aspartic acid		11.0	1.18	13.4	1.10	4.7	0.26	2.5	0.21	0.9	0.14	0.2	0.06
Arginine		5.7	0.61	5.1	0.42	—	—	1.2	0.10	0.5	0.08	T	T
Histidine		6.2	0.67	8.3	0.68	—	—	—	—	0.4	0.07	T	T
Lysine		T	T	T	T	T	T	T	T	T	T	T	T
Total amount %			6.76		7.37		1.51		1.31		1.07		0.32

* The term 'sample' indicates g. dry mycelium contained in 250 ml. of brew.

T = Present in traces only.

— = No estimation was carried out.

Table 6. Bound amino acids in the mycelium of *Aspergillus flavus*, during the pre-autolytic and autolytic phase of growth, released in the acid hydrolysis of fraction III

Results are expressed as g. amino acid/100 g. fraction III. Other details are given in the text.

Time of incubation (days)	6	8	11	16	18	20	24	27
Time of autolysis (days)	—	—	0	5	7	9	13	16
Amino acid								
Leucine	1.14	1.98	1.48	2.00	0.92	0.80	0.88	0.86
Phenylalanine	T	T	T	T	T	T	T	T
Valine	1.28	2.16	1.52	1.84	1.20	0.75	1.08	0.96
Tyrosine	T	T	T	T	T	T	T	T
Proline	T	T	T	T	T	T	T	T
Alanine	0.80	1.20	1.48	1.32	0.72	0.74	0.66	0.76
Glutamic acid	1.16	1.18	1.40	1.44	1.04	1.10	0.94	0.84
Aspartic acid + glycine	1.30	1.40	2.04	2.00	1.00	1.16	1.14	0.92
Glucosamine	2.08	2.00	2.00	1.88	2.24	1.36	2.32	1.44
Arginine	0.52	0.88	0.68	0.74	0.36	0.46	0.46	0.28
Asparagine	T	T	T	T	T	T	T	T
Total amount	8.28	10.80	10.60	11.22	7.50	6.40	7.40	6.06

T = present in traces only.

present on the 6th day of incubation had (7.37 %) disappeared before the initiation of the autolytic phase of growth (1.51 % at the 0 day of autolysis). *Aspergillus terreus* behaved in a very similar fashion when grown in a physiologically acid medium as static cultures. In these cultures the maximum concentration (2.45 g./100 g. dry mycelium) of the free amino acids in mycelium at the 9th day of incubation diminished by 86 % during these 9 days prior to autolysis (Lahoz *et al.* 1967). The diminution of these compounds in the mycelium during the end of the log phase well before autolysis sets in (as measured by the loss of mycelial dry weight) seems to be a common pattern for these fungi irrespective of the medium and of the conditions of culture. Chattaway, Toothill & Barlow (1962) noted that the maximum concentration of the pool amino acid from growing mycelium of *Microsporum canis* was observed towards the end of the log phase of growth. In Pillai & Srinivasan's experiments, referred to above, with *Aspergillus flavus* grown as static cultures in Czapek–Dox medium the disappearance of free amino acids from mycelium during the first 15 days of autolysis amounted to 42 %, whereas in the present results this disappearance reached 78 %, thus indicating that in the presence of a continuous flow of air and mechanical stirring autolysis affected free amino acids to a greater extent than in stationary cultures.

The constancy in the content of glucosamine (values in the neighbourhood of 2 g./100 g. fraction III, almost throughout the whole period of incubation, Table 6) released upon acid hydrolysis of fraction III seemed to indicate that chitin is very little affected by autolysis in these conditions. This is in agreement with Emiliani & Ucha de Davie's (1962) observations working with *Aspergillus phoenicis*. These workers stated that chitin is not attacked, or very little attacked, during the induced autolysis of mycelium of *A. phoenicis* by 0.2 M-acetic acid.

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