PRODUCTION OF NUCLEIC ACID-RELATED SUBSTANCES BY *Aspergillus flavus* IN CHEMICALLY DEFINED MEDIA

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■ ABSTRACT: Chemically defined media suitable for *Aspergillus flavus* growth and UV light absorbing substances production were investigated and established. Growth and material production were found to be simultaneous in all growth phases. The most suitable carbon and energy sources, in the media, are: sucrose, D-mannose and D-glucose. With sucrose as carbon source the most adequate nitrogen source is gelatin for both, growth and substances accumulation. The results showed that the material production level depends on medium composition where phosphate concentration seems to be the most important factor increasing the UV light absorbing substances accumulation. Maxima growth and production was obtained in a medium containing, per liter, sucrose 50 g, gelatin 5 g, K_2HPO_4 0.52 g and $MgSO_4.7H_2O$ 0.2 g, after 258 hours of agitation. The results obtained indicated that the substances production is a process directly related to the microbial activity and not a process linked to the degradation of preformed nucleic acid in the decline phase.

■ KEYWORDS: *Aspergillus flavus*; nutrition; production of UV light absorbing substances.

Introduction

An intensive study was made on the fermentation production of nucleotides, nucleosides, nitrogenous bases and related substances by microorganisms.[2-4,7-18] Carvalho & Molinari[6] have studied the production of nucleic acid-related substances by *Streptomyces aureofaciens* in chemically defined media and have analyzed the composition of the complex mixture of such UV-absorbing substances.[5] Searching for microorganisms with high UV-absorbing substances secretion, similarly to the

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S. aureofaciens, the fungus *Aspergillus flavus* has shown such capacity in chemically defined media and this paper describes such fermentative accumulation by this microorganism.

Material and methods

Basal medium. The composition of the basic chemically defined medium is as follows: Sucrose – 50 g; $(NH_4)_2SO_4$ – 4 g; K_2HPO_4 – 1.31 g; $MgSO_4.7H_2O$ – 0.2 g; $ZnSO_4.7H_2O$ – 10 mg; $FeSO_4.7H_2O$ – 10 mg; $MnSO_4.H_2O$ – 10 mg; and $CoSO_4.7H_2O$ – 5 mg. Distilled water was added to give 1 l of medium. After autoclave sterilization the pH was asseptically adjusted to 6.0 – 7.0 when necessary.

Inoculum. To a slant sample of the sporulated culture (*Aspegillus flavus*, strain EPM 157 of our collection) in Agar-Sabouraud medium, 8 ml of sterile distilled water was added to produce a spore suspension by asseptical and gentle surface scrapping.

Enough Roux flasks containing each 200 ml of the basal medium solidified with 2% agar were inoculated with 2 ml of the above spore suspension and let grow to sporulation, at room temperature, for about 19 days. To each Roux sporulated flask 130 ml of sterile distilled water was added to collect the spore suspension as above. All the individual suspensions were pooled and asseptically homogenized in a sterile aluminum waring blendor and asseptically transferred, in 30 ml portions, to 50 ml vials, closed with butyl stoppers, quickly frozen to -30°C and kept frozen until use.

Sterilization. All the media were autoclave sterilized for effective 30 minutes heating at 121°C. The sucrose or any other carbohydrate used were separetely autoclaved and asseptically added to the sterilized sugar-free media just before inoculation. As described for the basal medium the final pH were adjusted to 6-7 when needed.

Fermentation. The microorganism was cultivated on submerged cultures on a rotary shaker at 250 rpm in revolving circles of 3 cm diameter and kept in a constant temperature room at 30°C, in 125 ml Erlenmeyer flasks containing 30 ml of fermentation medium inoculated with 1 ml of the spore suspension inoculum. The flasks were closed with 1 cm thick polyurethane foam covers. All experiments were made using duplicate pairs of flasks for each medium or condition studied. The fermentation time was fixed in 170 hours.

Analytical methods. The production of the nucleic acid-related substances was arbitrarily measured by the corrected average 260 nm optical density of the culture filtrate flasks pairs (A_{260}). The correction included the sterilized media blank UV absorption, the inoculum A_{260} contribution and the fermented volume corrected to the initial 30 ml.

One unit of the UV absorbing material was defined as the quantity per mililiter of the nucleic acid-related material needed to give absorption of 1.0 at 260 nm in 1 cm optical path cells, similar to that proposed by Bendich.[1]

The microorganism growth, in the chemically defined media, was measured by the average dry weight of the distilled water washed mycelium, dried at 100°C-105°C for 15 hours, corrected for the inoculum contribution and expressed as miligrams per mililiter referred to the initial 30 ml volume.

Results and discussion

Production and growth in chemically defined media. Following the initial finding on the ability of the studied strain to secrete the UV absorbing substances and to growth into the basal medium, the study of the influence of media composition on the filtrate A_{260} and growth was carried out.

Carbon source. The adequacy of several carbohydrates as carbon source for the *A. flavus* was studied by substituting the selected carbohydrates at the concentration of 50 g/l for the sucrose in the basal medium, as described in Methods and in another derived medium similar to that but eliminating the minor minerals (zinc, iron, manganese and cobalt). The first series will be called media A and the latter media B.

Table 1 contains the results obtained with six sugars in both media A and B. Table 1 indicates that both maximal A_{260} and maximal growth can be supported by sucrose, D-mannose and D-glucose. Lactose, appears not to be utilized by the fungus and that the minor minerals (Zn, Fe, Mn and Co) can be eliminated from the basal medium without production or growth reduction. From these data sucrose was selected as carbon source in media of the B serie.

Table 1 – The effect of the carbon source on *A. flavus* growth and on filtrate A_{260}

Carbon source	Media A		Media B	
	Filtrate A_{260}	Mycelium dry weight (m/gml)	Filtrate A_{260}	Mycelium dry weight (mg/ml)
Sucrose	1,7	2.3	2,6	3.0
D-Mannose	2,2	2.6	2,4	2.3
D-Glucose	1,5	2.1	2,0	2.4
D-Fructose	1,3	2.2	1,4	2.5
Maltose	1,0	1.3	1,3	1.5
Lactose	0,0	0.3	0,2	0.3

Nitrogen source. Table 2 shows the results obtained substituting several nitrogen compounds for the equivalent ammonium sulfate in the basal medium B, whose general composition is: sucrose 50 g/l; K_2HPO_4 1.3 g/l; $MgSO_4.7H_2O$ 0.2 g/l; nitrogen source variable.

Table 2 indicates that maximal A_{260} was achieved using gelatin as the nitrogen source in the basal medium B, containing sucrose.

Table 2 – The influence of the nitrogen source on filtrate A_{260} and growth

Nitrogen source		Final pH	Filtrate A_{260}	Mycelium dry weight (mg/ml)
Compound	Concentration			
Gelatin	5 g/l	3.0	10,9	3.9
Triethanolamine	60 mM	3.0	6,8	1.5
Potassium L-aspartate	60 mM	4.5	6,0	0.4
Glycine	60 mM	3.5	5,3	1.9
Potassium nitrate	60 mM	3.5	3,4	3.0
Ammonium sulfate	30 mM	4.0	2,6	3.0
None	–	7.0	0,8	0,5

Orthophosphate concentration. The dependence of UV substances secretion on the orthophosphate initial concentration was studied in the previous best medium, adjusted to constant potassium concentration (20 milieq. g/l) by the addition of potassium sulfate and varying the initial phosphate concentration up to 10.0 mM.

Table 3 contains the observed results, which indicates a strong dependence of the total UV substances secretion (A_{260}) and of the specific production (A_{260}/growth) on the initial phosphate concentration. A sharp peak, for A_{260}, is observed at 3.0 mM phosphate corresponding also to the maximal growth.

Tables 2 and 3 show that the filtrate pH associated with maximal A_{260} and also with adequate growth varies between 3.1 to 4.5.

Growth and A_{260} secretion time curves in the best defined medium developed. For both maximum UV absorbing substances secretion and maximum growth the following chemically defined medium was established as the most adequate: sucrose 50.0 g/l; gelatin 5.0 g/l; K_2HPO_4 0.52 g/l; and $MgSO_4.7H_2O$ 0.2 g/l.

The adequacy of this medium for growth and secretion of the UV absorbing substances is shown on Table 4.

Table 3 – The effect of initial phosphate concentration on *A. flavus* growth and on filtrate A_{260}

K_2HPO_4 (mM)	Filtrate pH	Filtrate A_{260}	Growth (mg/ml)	A_{260}/Growth
0.0*	3.1	9.0	3.1	2.9
0.5	3.1	10.1	5.5	1.8
1.5	3.1	35.6	7.2	4.9
3.0	3.2	90.0	8.6	10.5
4.5	3.3	31.1	7.8	4.0
6.0	3.3	9.4	5.9	1.6
7.5	3.4	10.9	3.1	3.5
9.0	3.5	6.4	2.7	2.4
10.0	3.5	6.8	2.7	2.5

(*) Contains the inoculum phosphate contribution.

Table 4 – *A. flavus* growth and UV absorbing secretion substances in the chemically defined medium

Growth time (hours)	Filtrate		Mycelium dry weight (mg/ml)
	pH	A_{260}	
0	7.0	–	–
19	7.0	0.0	0.1
28	6.5	0.6	0.3
41	5.0	0.9	0.9
52	4.3	3.6	2.0
65	3.7	12.3	4.1
77	3.6	20.8	3.6
89	3.6	29.8	3.8
100	3.5	35.1	4.4
113	3.5	45.3	5.7
161	3.5	73.3	7.9
185	3.7	115.3	9.1
209	4.0	186.8	11.2
258	4.1	231.8	11.7
332	5.7	17.3	11.5
400	5.9	16.3	10.3

The data indicates that both processes occur simultaneously with good parallelism, showing the maxima at 258 hours of growth. After this time the secreted UV substances quickly disappears and there are also a slighty pH rise and a mycelium reduction.

This observation suggests that S_{260} production is a process related to the microbial activity in all growth phases and not a process linked to the degradation of preformed nucleic acid in the decline phase.

Preliminary chromatografic analysis of the culture filtrate, made by classical methods, indicated, similar to a previous paper,[5] the presence of nucleic acid-related substances in the complex composition, as will be shown in a forthcoming paper.

CARVALHO, A. de et al. Produção de substâncias relacionadas a ácidos nucléicos por *Aspergillus flavus* em meios quimicamente definidos. *Ecl. Quím.*, v. 19, p. 49-55, 1994.

■ *RESUMO: Desenvolveram-se meios de cultivo, quimicamente definidos, adequados ao crescimento do* A. flavus *e à produção e acúmulo de substâncias que absorvem o ultravioleta. Entre as fontes de carbono e energia estudadas observou-se que as mais adequadas são a sacarose, a D-manose e a D-glicose. Usando-se sacarose como fonte de carbono, verificou-se que a melhor fonte de nitrogênio é a gelatina, tanto para o crescimento do microrganismo quanto para a produção do material. Constatou-se que a concentração inicial de fósforo é fundamental para a produção das substâncias em estudo e que, na composição dos meios nutrientes, a concentração do ortofosfato é o fator mais importante e que mais interfere no acúmulo do material nos caldos de cultura. A produção máxima das substâncias foi obtida em meio contendo, por litro, 50 g de sacarose, 5 g de gelatina, 0,52 g de ortofosfato dipotássico e 0,2 g de* $MgSO_4.7H_2O$. *Nesse meio o crescimento e a produção máximos são obtidos após 258 horas de agitação. Os resultados obtidos indicaram que a produção das substâncias é um processo diretamente relacionado à atividade microbiana, não se tratando de processo de degradação de ácidos nucléicos, pré-formados, na fase decrescente.*

■ *PALAVRAS-CHAVE:* Aspergillus flavus; *nutrição; produção de substâncias que absorvem luz ultravioleta.*

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