Semi-continuous and Continuous Production of *Aspergillus niger* Spores in Submerged Liquid Culture

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Sporulation of *Aspergillus niger* was induced in continuous tower fermenters by restricting growth with nitrate limitation. Semi-continuous production of spores was achieved in a single-stage fermentation by alternating full-nutrient and nitrogen-deficient media to the culture. Continuous spore production was obtained in a two-stage fermentation system using the first stage for the growth of vegetative mycelium under optimum conditions and the second, larger, stage for sporulation induction in a constant nitrogen-deficient environment. Spores were produced from much simplified sporulation structures. Using a new sporulation index (Ω), which relates spore numbers produced to the quantity of substrate utilized, the average production efficiency of the two-stage continuous system was shown to be more than twice that of the semi-continuous production system. The tower fermenter was shown to be ideal for controlling organism morphology, thus providing conditions which allowed the development of continuous fungal sporulation.

INTRODUCTION

The induction of sporulation by nutrient limitation of *Aspergillus niger* and *Aspergillus ochraceus* in continuous submerged liquid culture in a tower fermenter has previously been described (Broderick & Greenshields, 1981). A shock decrease in the medium nitrate concentration or a gradual reduction of medium sucrose or starch concentration induced a transient sporulation response from both organisms, but spore production was not continuous. Nevertheless, the possibility of control of sporulation in continuous culture was indicated by these experiments and, although carbon limitation was an unreliable method, the nitrate limitation technique for induction of sporulation proved consistently repeatable. The technique was therefore used as a basis for the development of semi-continuous and continuous spore production systems to provide alternative and more efficient methods for mass production of conidia from commercially important fungal strains.

Recently, the ability of fungal spores to perform commercially valuable chemical transformation reactions has received considerable attention (Peterson, 1963; Charney & Herzog, 1967; Vezina *et al.*, 1968; Vezina & Singh, 1975) and fungal spores are now known to possess enzymes capable of the transformation of substrates apparently unrelated to their metabolism. Fungal spores are also used for biological pest control (Hall & Burges, 1979).

The aim of this work was to develop a fermentation system for the efficient continuous production of fungal spores based upon the organism retention capacity of the tower fermenter system. This factor allows the induction of low growth rates in continuous culture due to nutrient limitation without organism wash-out. An increased product yield results, particularly for spores, which require mycelial support structures.

METHODS

Organism. A culture of *Aspergillus niger* Van Tieghem 38 of the fermentation laboratory culture collection was selected. The suitability of this organism for work in the continuous tower fermenter system has previously been

Fig. 1. Diagrammatic representation of the two-stage fermentation system. V_1 , volume of stage 1 (l); V_2 , volume of stage 2 (l); x_{F1} , fermenter biomass concentration of stage 1, $g\ l^{-1}$; x_{F2} , fermenter biomass concentration of stage 2, $g\ l^{-1}$; x_{E1} , effluent biomass concentration of stage 1, $g\ l^{-1}$; x_{E2} , effluent biomass concentration of stage 2, $g\ l^{-1}$; f_1 , flow rate through stage 1, $l\ h^{-1}$; f_2 , flow rate through stage 2, $l\ h^{-1}$; f_3 , optional flow rate into stage 2, $l\ h^{-1}$; D_1 , dilution rate in stage 1; D_2 , dilution rate in stage 2.

confirmed and the physiological and morphological characteristics of *A. niger* in the continuous tower fermenter are similar to those of other commercially important filamentous fungi. A master culture was maintained on malt extract agar slopes (Broderick & Greenshields, 1981) and spore inocula for injection into the fermenter were prepared from spore mats on malt extract agar in 250 ml conical flasks by washing with 100 ml 0.1% (v/v) solution of Tween 80 wetting agent.

Culture conditions. Fermentations were conducted in a tower fermenter of either 10.0 or 4.2 l capacity as previously described (Cocker & Greenshields, 1975; Broderick & Greenshields, 1981) and the organism was grown on basal medium, constituents ($g\ l^{-1}$): sucrose, 5.0; $NaNO_3$, 1.3; $NaH_2PO_4 \cdot H_2O$, 0.5; KCl, 0.25; $MgSO_4 \cdot 5H_2O$, 0.1; $CaCl_2$, 0.05; yeast extract, 0.5.

Analytical methods. These were as described by Broderick & Greenshields (1981).

The semi-continuous production system. In order to increase spore production, optimal and nitrogen-lacking nutrient media were supplied alternately to a culture of *A. niger* growing at a dilution rate of $0.1\ h^{-1}$ or $0.2\ h^{-1}$ in the 4.2 l tower fermenter. This allowed the induction of alternate phases of vegetative growth and sporulation in single-stage continuous culture. During these experiments, the biomass yield (Y_{sub}), representing the amount of fungal biomass produced per unit substrate utilized was calculated by: $Y_{sub} = (x_E/dS)\ g\ mycelium\ (g\ sucrose\ utilized)^{-1}$, where x_E = effluent biomass concentration ($g\ l^{-1}$) and dS = substrate utilized ($g\ l^{-1}$). The number of spores produced per unit substrate utilized (Ω) was calculated by: $\Omega = n/dS\ spores\ g^{-1}$, where n = number of spores per litre. Ω_m was used to denote the maximum sporulation efficiency calculated during a fermentation, and did not necessarily coincide with maximum sporulation intensity.

The continuous production system. It was shown that a continuous sporulation response to nutrient limitation could not be maintained in a single-stage fermentation (Broderick & Greenshields, 1981) due to adaptation by the mould to the adverse nutritional environment; to overcome this, a two-stage system was developed. This consisted of a 4.2 l and 10.0 l tower fermenter in series with the outflow from the 4.2 l vessel (stage 1) fed directly to the base of the 10.0 l vessel (stage 2; Fig. 1). The first fermenter was used for continuous vegetative mycelium production under optimum steady-state conditions and the larger second fermenter, allowing increased residence time available for product formation, was used for the restriction of growth and induction of sporulation by provision of constant unfavourable environments. All fermentation parameters were calculated as described previously (Broderick & Greenshields, 1981) with the following exceptions (Fig. 1):

$$\text{Organism growth rate, } \mu, \text{ in stage 2} = [(D_2 \cdot x_{E2}) - f_1(x_{E1}/V_2)]/x_{F2}$$

$$\text{Productivity, } Y, \text{ of stage 2} = D_2(x_{E2} - x_{E1})$$

Replication. Values given represent the means and standard deviations of triplicate analyses from at least two experiments.

RESULTS

Semi-continuous spore production

To overcome the problem of adaptation by the organism to a growth-limiting medium causing sporulation to cease, the organism was supplied with a continually changing environment.

Fig. 2. Growth rate, μ (○) and sporulation efficiency, Ω (●) of *A. niger* in response to repeated cycles of nitrate limitation (medium nitrogen concentration 0.21–0.021 g l⁻¹) followed by full-medium recovery. A, nitrogen limitation initiated; B, restoration of optimal medium.

Alternating supplies of optimum medium and shock nitrate limitation to a culture of *A. niger* caused its growth phases to oscillate between optimum vegetative growth and peak spore production on a cycle length of approximately one week. The culture was allowed to reach steady-state growth at 30 °C, pH 3.0, aeration = 1 vol. vol.⁻¹ min.⁻¹ on full nutrient basal medium, with 0.21 g atomic nitrogen l⁻¹ (1.3 g NaNO₃ l⁻¹). The nitrate supply was then reduced to 0.021 g atomic N l⁻¹, while all other parameters were maintained constant, and the growth rate and sporulation responses monitored. Spores were produced for 3–5 d but when spore production decreased, full nutrient medium supply was restored until optimal steady-state growth was re-established. Limitation was then re-imposed and the cycle repeated. The effect of these nutrient cycles at a dilution rate of 0.1 h⁻¹ is shown in Fig. 2. Three cycles are illustrated, but the sequence appeared to be capable of indefinite repetition. Characteristically, in the nitrogen limited system, growth rate immediately decreased upon shock introduction of the low nitrogen medium and spores were immediately evident in every cycle except the first where there was a lag time of 18 h. All spores were produced from simplified reproductive structures consisting of single phialides on hyphal tips. Fermenter biomass concentration (x_F) increased during nitrogen-limited phases and decreased during the recovery period, but mycelial flocs remained loosely filamentous at all times (type 1, Cocker, 1980). Sucrose utilization was constant throughout the cycle and the specific sucrose utilization rate was therefore the mirror image of x_F . Effluent biomass concentration (x_E), productivity (Y) and biomass yield (Y_{sub}) all decreased during the limitation phases and returned to their original levels during the recovery phase. A sample set of results is summarized in Table 1.

When the cycling procedure was repeated at a dilution rate (D) of 0.2 h⁻¹, no sporulation was observed although conditions other than dilution rate were identical to those at $D = 0.1$ h⁻¹. *Aspergillus niger* had a higher original growth rate (0.16 h⁻¹) which was only reduced approximately 50% by nitrogen limitation, and it was concluded that the high growth rate prevented the onset of sporulation. A second inhibitory factor was that sucrose utilization decreased from 4.58 g l⁻¹ to 2.7 g l⁻¹ during the limitation phase at $D = 0.2$ h⁻¹, thus making less energy available for sporulation.

The continuous spore production system

To induce continuous sporulation this system had to overcome the transience of the sporing response to a change in environment exhibited by *A. niger* in previous experiments. This was achieved by maintaining two different but constant environments. The environment in the first stage was optimal for growth while that in the second stage was nitrogen-deficient to induce sporulation. A continuous supply of vegetative mycelium was therefore supplied to the second stage where it experienced a sudden change in environment that induced sporulation. Therefore, while the sporulation response was still transient, spores were produced continually.

Table 1. *Effect on growth and sporulation in A. niger of alternating supplies of full-nutrient medium and nitrate-limited medium*

D , 0.1 h⁻¹; T , 30 °C; pH 3.0; sucrose supply, 5.0 g l⁻¹. —, No spores observed; +, spore numbers below accuracy of counting method. Values are the means of triplicate analyses from at least two experiments $\pm$ S.D.

Day	Effluent biomass concn, x_E (g l ⁻¹)	Fermenter biomass concn, x_F (g l ⁻¹)	Productivity, Y (g l ⁻¹ h ⁻¹)	Growth rate, μ (h ⁻¹)	Yield, Y_{sub} (g g ⁻¹)	Sporulation efficiency [$10^{-9} \times \Omega$ (n g g ⁻¹)]
1-5	2.5 $\pm$ 0.71	3.85 $\pm$ 0.35	0.25 $\pm$ 0.07	0.066 $\pm$ 0.024	0.545 $\pm$ 0.163	—
Nitrogen limitation						
6	1.8 $\pm$ 0.17	5.4 $\pm$ 0.2	0.18 $\pm$ 0.017	0.051 $\pm$ 0.0035	0.039 $\pm$ 0.037	—
7	1.35 $\pm$ 0.06	6.0 $\pm$ 0.42	0.135 $\pm$ 0.006	0.033 $\pm$ 0.001	0.29 $\pm$ 0.02	14.7 $\pm$ 0.23
8	1.15 $\pm$ 0.05	7.2 $\pm$ 0.25	0.115 $\pm$ 0.005	0.026 $\pm$ 0.006	0.26 $\pm$ 0.01	1.93 $\pm$ 0.15
9	0.9 $\pm$ 0.13	7.0 $\pm$ 0.27	0.09 $\pm$ 0.013	0.013 $\pm$ 0.0022	0.2 $\pm$ 0.03	7.2 $\pm$ 0.55
Full medium restored						
10	2.4 $\pm$ 0.04	6.8*	0.24 $\pm$ 0.004	0.022 $\pm$ 0.0035	0.51 $\pm$ 0.01	—
11	3.5 $\pm$ 0.07	5.2 $\pm$ 0.47	0.35 $\pm$ 0.007	0.037 $\pm$ 0.0035	0.74 $\pm$ 0.01	—
12	1.95 $\pm$ 0.06	5.8 $\pm$ 0.11	0.195 $\pm$ 0.006	0.05 $\pm$ 0.003	0.43 $\pm$ 0.03	+

Nitrogen limitation

* S.D. not determined.

Initially the mycelium was fed directly to the second stage, which maintained an identical physicochemical environment but had no separate nutrient supply. The only nutrients therefore available to the mould in the second stage were those entering the vessel in the effluent from stage 1. On average, these were 0.3 g sucrose l⁻¹, 0.08 g atomic nitrogen l⁻¹ and traces of other nutrients. Although the mould had a residence time of 23.8 h in stage 2 compared with 10 h in stage 1, no sporulation was induced by alteration of temperature or pH, or addition of potassium ferrocyanide to the second stage. The conclusion from these tests was that sporulation was prevented by the relatively high nitrogen content, 0.08 g l⁻¹, and by the lack of an adequate carbohydrate source. It was known that sporulation of *A. niger* would be induced by the nitrate-limited technique used in single-stage experiments. The nitrate supply to stage 1 was therefore reduced to 0.1525 g atomic N l⁻¹, a level slightly above that of maximum utilization under optimum conditions at a dilution rate of 0.1 h⁻¹. Stage 1 was run at a dilution rate of 0.1 h⁻¹ so that growth was optimal, but only 0.025 g atomic N l⁻¹ entered stage 2 in the effluent from stage 1, which was a similar level to that of 0.02 g l⁻¹ used to induce sporulation in the single-stage work.

The other requirement for sporulation indicated by previous experiments was the supply of an adequate carbohydrate source. This was provided in the second stage by an additional sucrose-only supply of 5.0, 4.0, 3.0 or 2.0 g l⁻¹ fed continuously at a flow rate (f_3) of 0.58 l h⁻¹. Therefore, the dilution rate of stage 2 (D_2) = $(f_1 + f_3)/V_2$ = (0.42 + 0.58)/10 = 0.1 h⁻¹. Vegetative mycelium was, therefore, continuously supplied to stage 2 in which conditions were; D , 0.1 h⁻¹; T , 30 °C; pH 3.0; aeration, 1.0 vol. vol.⁻¹ min⁻¹; mean nutrient supply, 0.025 g atomic N l⁻¹, 5.0, 4.0, 3.0 or 2.0 g sucrose l⁻¹ and traces of other nutrients. The steady-state fermentation parameters are given in Table 2. With a sucrose supply of 5.0 g l⁻¹ to the second stage, the mean spore production efficiency (Ω) was 3.4×10^9 spores (g sucrose utilized)⁻¹, although production varied from this mean value by a factor of ten due to periodic wash-out of spores. Spore development was less than 10 h (the residence time in stage 2), and all spores were formed from simplified structures of single phialides on hyphal tips. The mycelium in stage 2 assumed the form of loose flocs with dense centres (types 3-4; Cocker, 1980) and this form persisted throughout the fermentation. The situation was stable and sporulation continued for several weeks although bacterial infection eventually caused the experiment to be terminated. The experiment was repeated with decreased sucrose concentrations in the medium supply to the second stage while all other parameters remained constant. Progressively fewer spores were produced in the

Table 2. Two-stage growth and sporulation of *A. niger*

Steady-state fermentation parameters of stages 1 and 2 with various sucrose concentrations supplied to stage 2: dilution rate, 0.1 h⁻¹; pH, 3.5; temperature, 30 °C. Stage 1, optimum growth; stage 2, nitrate-limited; —, no spores observed; +, spore numbers below accuracy of counting method. Values are the means of triplicate analyses from at least two experiments $\pm$ S.D.

Sucrose supply to stage 2 (g l ⁻¹)	Stage	Effluent biomass concn, x_E (g l ⁻¹)	Fermenter biomass concn, x_F (g l ⁻¹)	Growth rate, μ (h ⁻¹)	Sucrose utilization, dS (g l ⁻¹)	Nitrogen utilization, dN (g l ⁻¹)	Sporulation efficiency [10 ⁻⁹ $\times \Omega$ (n g ⁻¹)]
5.0	1	2.0 $\pm$ 0.25	3.6 $\pm$ 0.11	0.056 $\pm$ 0.008	4.7 $\pm$ 0.095	0.13 $\pm$ 0.004	—
	2	2.78 $\pm$ 0.58	4.6 $\pm$ 0.43	0.042 $\pm$ 0.01	4.73 $\pm$ 0.072	0.016 $\pm$ 0.006	3.4 $\pm$ 0.43
4.0	1	2.7 $\pm$ 0.25	6.4 $\pm$ 0.5	0.042 $\pm$ 0.006	4.75 $\pm$ 0.03	0.118 $\pm$ 0.005	—
	2	2.9 $\pm$ 0.058	4.47 $\pm$ 0.29	0.038 $\pm$ 0.007	2.75 $\pm$ 0.3	0.033 $\pm$ 0.009	1.06 $\pm$ 0.177
3.0	1	3.1 $\pm$ 0.03	5.32 $\pm$ 0.97	0.06 $\pm$ 0.016	4.5 $\pm$ 0.05	0.1	—
	2	3.17 $\pm$ 0.55	4.25 $\pm$ 0.37	0.044 $\pm$ 0.011	2.52 $\pm$ 0.13	0.049 $\pm$ 0.001	+
2.0	1	2.55 $\pm$ 0.21	6.15 $\pm$ 0.35	0.042 $\pm$ 0.0007	4.75 $\pm$ 0.007	0.118	—
	2	2.4 $\pm$ 0.14	3.25 $\pm$ 0.35	0.04 $\pm$ 0.0085	1.42 $\pm$ 0.61	0.018 $\pm$ 0.007	—

steady states achieved: 3.22×10^7 spores ml⁻¹ with 5.0 g sucrose l⁻¹, 1×10^6 spores ml⁻¹ with 4.0 g l⁻¹, a few spores with 3.0 g l⁻¹ and no sporulation was evident with a supply of 2.0 g l⁻¹. The results (Table 2) indicate a direct relationship between increasing intensity of sporulation and increasing availability of a carbon substrate in the nitrogen-limited system.

Comparison of the semi-continuous and continuous systems

A crude estimation of the production capacity and efficiency of both systems can be made. During peak spore production in the semi-continuous system, $\Omega_m = 1.93 \times 10^{10}$ spores (g sucrose utilized)⁻¹. This compared favourably with the Ω_m value of 1.4×10^{10} spores (g sucrose utilized)⁻¹ for the continuous system. This is essentially due to the fact that the continuous two-stage system requires two separate sucrose supplies which must both be taken into account when calculating a production efficiency. However, if the average spore production and nutrient consumption of the respective systems over a period of several days is compared, the efficiencies are reversed. If spore production during one cycle of the semi-continuous system (168 h), during which spores are produced for only approximately 60 h, is compared with 168 h output by the continuous system, the respective overall mean spore production efficiencies (Ω) are 2.83×10^9 and 6.83×10^9 spores (g sucrose utilized)⁻¹. Thus, when in production over a period of several days or weeks, the two-stage continuous system is shown to be more than twice as efficient as the semi-continuous cycled system despite utilizing two carbon supplies.

DISCUSSION

There is clearly a considerable difference between induction of sporulation in continuous culture and induction of continuous sporulation. Spore production was known to be a transient response to particular changes in the environment, but the organism eventually either adapted to, or was killed by the environmental change and sporulation ceased. The experiments described here therefore used either a continually changing environment or a continuously replaced organism to induce repeated sporulation responses to that both semi-continuous and continuous production of *A. niger* spores was possible. It should be stressed that the terms 'semi-continuous' and 'continuous' refer only to the type of spore production achieved and not to the fermentation systems themselves, which were both continuous culture systems. The importance of organism retention by the tower fermenter was illustrated in the single-stage semi-continuous production system, in that mycelial flocs maintained their morphology during nitrate limitation, which prevented organism wash-out and enabled spores to be produced. The semi-continuous system demonstrated that sucrose utilization remained approximately constant throughout the nutrient cycles, and that the energy derived from the sucrose was directed towards hyphal

growth during optimal medium supply and towards the high-energy-requiring sporulation process during nitrogen limitation. This energy was also required for the creation of storage compounds in spores and for other secondary metabolic functions associated with decreased growth rate. The failure of the cycled system to operate at a dilution rate of 0.2 h^{-1} suggested that there was an upper limit of growth rate of approximately 0.05 h^{-1} in this system which permitted vegetative growth but prevented the onset of sporulation. A similar effect was observed by Ng *et al.* (1973). The cycle length used (168 h) was the minimum possible to enable full recovery of the vegetative culture between each sporulation phase using $5.0 \text{ g sucrose l}^{-1}$, but further relationship between cycle time and sugar concentration was not investigated. Output from the system might be increased by shortening the length of sporulation phases and thus reducing the cycle time, as the majority of spores were produced in the first 48 h of nitrogen limitation. *Aspergillus niger* displayed a positive expression of commitment to sporulation in that spores continued to be produced for up to 18 h after full nutrient medium was restored to the culture. One can conclude that the portion of mycelium committed to sporulation before restoration of the full nutrient medium produced spores regardless of the nutrient environment, before switching again to vegetative growth.

The two-stage continuous spore production system demonstrated the dependence of the system both upon an adequate carbohydrate or energy source and upon an adequate growth rate during sporulation. Once established, spore production was continuous and overall output using $5.0 \text{ g sucrose l}^{-1}$ supplied to stage 2 was the maximum observed for a steady-state system. An increase in the volume of stage 2 might improve spore output of the system by allowing a greater residence time for spore development, and this would also increase the efficiency of the system. In a previous paper (Broderick & Greenshields, 1981) it was noted, in agreement with other workers (Righelato *et al.*, 1968; Ng *et al.*, 1973) that sporulation in carbon-limited continuous culture occurred with the carbon supply slightly above the maintenance requirement of the organism. The two-stage experiments demonstrated that sporulation intensity in nitrogen-limited culture increased as carbon supply was increased in excess of the maintenance requirement, although the upper limits of this relationship were not determined. It should be noted that the spores were produced from simplified sporulation structures of *A. niger*, yet Smith & Berry (1974) stated that the simplified form of spore production cannot compare quantitatively with that from sub-aerial morphologies. If such sub-aerial structures could be induced, the productivity of both systems might well be increased.

These experiments have demonstrated that continuous production of fungal spores is feasible on a laboratory scale, although it is difficult to compare the efficiency of these production systems with traditional surface culture techniques. The development of the Ω index to represent sporulation efficiency facilitates the comparison of yields from this work with those from surface culture techniques, and is therefore of greater commercial interest than the traditional β index, which is not energy related. The commercial use of spores has several advantages over other microbial systems, including greater specificity and efficiency of reaction, simpler product recovery, longer storage life and repeated usage of the enzyme units (Schleg & Knight, 1962; Vezina & Singh, 1975). Thus, if sporulation of other commercially important filamentous fungi can be induced in a similar manner to that of *A. niger*, this new continuous production system may improve the commercial feasibility of microbial reactions involving fungal spores.

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