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Sporulation of *Aspergillus niger* and *Aspergillus ochraceus* in Continuous Submerged Liquid Culture

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Sporulation of *Aspergillus niger* and *Aspergillus ochraceus* was induced in a continuous tower fermenter by restricting growth by nutrient limitation. Shock carbon limitation produced no sporulation, but the gradual decrease of sucrose or starch supply to *A. niger* produced slight sporulation. Gradual nitrate limitation produced no sporulation, while a shock decrease in nitrate concentration caused heavy sporulation of both organisms. The previously unobserved morphology of the sporulating structures produced was much simplified under nitrate limitation, but similar to sub-aerial morphology under carbon limitation. Maintenance energy values for sucrose and starch were calculated for *A. niger* and for starch for *A. ochraceus*. The continuous tower fermenter system was found to be ideal for controlling organism morphology and thus sporulation.

INTRODUCTION

Suppression of submerged conidiation of filamentous fungi has been described as a feature characteristic of shake cultures (Cochrane, 1958). Several studies since have examined the induction of submerged sporulation by manipulation of cultural conditions (Smith *et al.*, 1977). Conidiation is considered to be a response to environmental conditions which severely restrict vegetative growth. Vezina *et al.* (1965) achieved conidiation of several filamentous fungi, including *Aspergillus ochraceus*, in submerged culture, and emphasized the importance of equilibrium between medium composition and physicochemical conditions. Sporulation may be induced by exhaustion of the carbohydrate source while nitrogen is in excess [Galbraith & Smith (1969*a*), for *A. niger*], or by exhaustion of available nitrogen in the presence of assimilable carbohydrate [Morton *et al.* (1960) and Morton (1961), for *Penicillium griseofulvum*; Hadley & Harrold (1958), for *P. notatum*; Carter & Bull (1969), for *Aspergillus nidulans*; Weiss & Turian (1966), for *Neurospora crassa*]. Galbraith & Smith (1969*a*) concluded that conidiation of *A. niger* depended upon the type and concentration of the nitrogen source. Batch cultivation consists of transient environments, while chemostat culture allows steady-state selective enhancement of specific morphological life cycle stages. Ng *et al.* (1973) described the conidiation of *A. niger* in continuous culture under citrate and nitrogen limitation, which showed that growth and sporulation are not necessarily mutually exclusive. Righelato *et al.* (1968) and Hsu & Ordal (1969) have also studied sporulation of fungi in chemostat culture; their results indicate that both the nature and concentration balance of the medium are important. The aim of this study was to develop a commercial fermentation system for the continuous production of conidia of *A. niger* and *A. ochraceus* using the continuous tower fermenter, and to study the sporulation morphologies induced.

METHODS

Organisms. Master cultures of *Aspergillus niger* Van Tieghem (no. 38 of the fermentation laboratory culture collection; ex Dr Drysdale, Department of Genetics, Birmingham University) and *Aspergillus ochraceus* Wilhelm

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(CMI 16247iv) were maintained on malt-extract agar slopes at 4 °C and subcultured every 6 months. The suitability of *A. niger* for work in the continuous tower fermenter system has previously been confirmed, and *A. ochraceus* was used because of its similarity to *A. niger* and because the importance of the spores in pharmaceutical reactions has been reported (Dulaney *et al.*, 1955; Schleg & Knight, 1962; Sehgal *et al.*, 1968; Haines & Collingworth, 1953).

Fermentation equipment. Fermentations were conducted at 30 °C in one of two glass tubular fermenter vessels, one of 10 l working volume, the other of 4·2 l. The design, construction and applications of the tower fermenter are described by Greenshields & Smith (1971, 1974), Smith & Greenshields (1974) and Cocker & Greenshields (1975). The basic 10 l design consists of three 40 cm lengths of standard 10 cm internal diameter Pyrex glass pipework, joined by polytetrafluoroethylene (PTFE) gaskets and clamped water tight. Spent medium and gases pass out of the top of the vessel via an 8 mm internal diameter glass swan-neck, and sterile compressed air (supplied to each fermenter at 1 vol. vol.$^{-1}$ min^{-1}) enters at the base and is dispersed before passing upwards by a PTFE air distributor plate. The 4·2 l fermenter has an identical aspect ratio to the 10 l fermenter (90·0 × 7·5 cm) but the main body of the fermenter is in one piece. Both vessels have facilities for continuous monitoring and control of pH, temperature and dissolved oxygen.

Preparation of inocula. A malt-extract agar mat was prepared in the base of a 250 ml conical flask, inoculated with the appropriate mould from the sub-master culture and incubated at 30–35 °C for 3 weeks. After this time, a full spore mat had formed, and the spores were removed by addition of 100 ml of a 0·1% (v/v) sterile solution of Tween 80 wetting agent, followed by vigorous agitation. The resulting spore suspension was aseptically injected into the fermenter.

Media. The basal medium constituents were (g l^{-1}): sucrose, 5·0; $(NH_4)_2SO_4$, 1·0; $NaH_2PO_4.H_2O$, 0·5; KCl, 0·25; $MgSO_4.7H_2O$, 0·1; $CaCl_2$, 0·05; yeast extract, 0·5. Sucrose was replaced in some experiments by glucose or starch equivalent. $(NH_4)_2SO_4$ was sometimes replaced by $NaNO_3$ (1·3 g l^{-1}). Media were sterilized by autoclaving in 20 l batches at 121 °C for 20 min.

Methods of nutrient limitation. Limiting levels selected were based upon the principle of decreasing nutrient supply until equal to or below the maintenance requirements of the organism. The criteria for nitrogen limitation were determined in two ways: firstly, by lowering the nitrogen content of the medium to below the level of utilization at maximum growth rate under optimum conditions at a steady state, while maintaining all other parameters optimal; secondly, by decreasing the nitrogen level to that used by other workers to induce sporulation in batch culture. Galbraith & Smith (1969*a*) stated that conidium formation is inhibited by the presence of nitrate at concentrations at or above 60 mg N l^{-1} and conidiophores are inhibited by concentrations at or above 120 mg N l^{-1}. In our experiments the nitrogen content of the medium was therefore decreased to 20 mg N l^{-1}, and at times to zero [Anderson & Smith (1971*a*) used a nitrogen-free medium to induce conidiophore elongation]. To provide gradual nitrogen limitation, stepwise reductions in the nitrogen content of the medium were made between the establishment of each steady state. Shock treatment was introduced by removing the medium supplying the optimum nitrogen supply (0·21 g N l^{-1}) and replacing this immediately with one containing approximately 0·02 g N l^{-1}, without altering any other environmental parameter. The effect upon growth of the organism was then monitored. The principles of carbon limitation (gradual and shock) were identical to those of nitrogen limitation, except that a concomitant decrease in dilution rate with carbon level was examined for both shock and gradual limitation. Details of each limitation are given in Results.

Dilution rate. The majority of experiments were conducted at dilution rates of 0·2 h^{-1} or 0·1 h^{-1}, although lower rates were used during carbon-limited fermentations.

Analytical methods. Total sugar concentration of medium and effluent was determined by the method of Dubois *et al.* (1956), with reference to a standard curve constructed for each kind of carbohydrate supply (expressed as g sugar l^{-1}). Nitrate and nitrite content of medium and effluent (expressed as g N l^{-1}) were determined by the method of Chapman *et al.* (1967). Ammonium nitrogen (expressed as g N l^{-1}) was determined by the Kjeldahl distillation method of Markham (1942).

Fermenter sampling. Samples of 100 ml were collected from (*a*) the effluent stream and (*b*) the tower, and filtered through fine muslin; the mycelial dry weight (g l^{-1}) was determined by drying the residual biomass at 105 °C for 24 h. These measurements gave (*a*) the effluent biomass concentration (x_E), and (*b*) the fermenter biomass concentration (x_F).

Microscopy. The production of sporulation structures was assessed by direct observation of fresh mycelial samples. Spore numbers were recorded by counting free conidia in an effluent sample after vigorous agitation, using an Improved Neubauer counting chamber.

Fermentation parameters. Dilution rate (D) was calculated by f/V, where f is the flow rate of medium supply (l h^{-1}) and V is the volume of the fermenter (l). The specific growth rate (μ) during a steady state was then calculated from the equation $\mu = Dx_E/x_F$. The factor Dx_E represents the productivity (Y) of the system (g l^{-1} h^{-1}). Growth rate in transient states was calculated by the equation

$$\mu = (1/x_{F\,total}) \times (\text{New biomass produced}/\Delta t)$$

where $$x_{\text{Ftotal}} = V(x_{\text{F1}} + x_{\text{F2}})/2$$

$$\text{New biomass produced} = f\bar{x}_{\text{E}}\Delta t + V(x_{\text{F2}} - x_{\text{F1}})$$

Δt is the time elapsed between time 0 and time t, x_{F1} and x_{F2} are the fermenter biomass concentrations at time 0 and time t, respectively, and $\bar{x}_{\text{E}}$ is the mean effluent biomass concentration between time 0 and time t. Where dilution rate varied during an experiment, the substrate supply rates (K_{s} for sugar, K_{N} for nitrogen; g h^{-1}) were calculated by $K_{\text{s}} = s_{\text{m}}f$ and $K_{\text{N}} = N_{\text{m}}f$, where s_{m} and N_{m} are the concentrations of sugar and nitrogen in the medium, respectively. The maintenance coefficient (m; g g^{-1} h^{-1}), representing the quantity of substrate consumed by cells for functions other than growth, was calculated by the method of Schulze & Lipe (1964), using regression analysis. The specific substrate utilization rate for sugar (q_{s}; g g^{-1} h^{-1}), was calculated by the equation $q_{\text{s}} = \Delta sD/x_{\text{F}}$, where Δs is sugar utilization (g l^{-1}). To express the intensity of sporulation, a sporulation index (β) was used (Hadley & Harrold, 1958) which represents the number of spores per g dry wt mycelium.

Replication. Where no time scale appears on the graphs, a result represents the mean of steady-state values recorded over at least 3 d. Mean nutrient levels were calculated from at least three analytical replicates, and mean growth rate and productivity values were obtained from triplicate x_{E} and x_{F} sample analyses. Where a time scale monitors a transient state, a point represents the mean value of the means of two or three replicates of the experiment.

RESULTS

Several authors have expressed the opinion that ammonium ions are either inhibitory or detrimental to sporulation (Galbraith & Smith, 1969*a*). The $(NH_4)_2SO_4$ of the basal medium was therefore replaced by $NaNO_3$. This had no adverse effect upon the optimum growth rates of either organism at a dilution rate of 0·1 h^{-1} in continuous culture. The mean growth rates of *A. niger* and *A. ochraceus* in $(NH_4)_2SO_4$ medium were 0·062 h^{-1} and 0·051 h^{-1}, respectively, while in $NaNO_3$ medium the respective growth rates were 0·06 h^{-1} and 0·05 h^{-1}. Thus the growth rate of *A. ochraceus* is lower than that of *A. niger* in this system.

Carbon limitation

Shock limitation. Several fermentations were performed using sucrose as the carbon source at $D = 0{\cdot}2$ h^{-1}; after achieving steady state (on 5 g l^{-1} sucrose plus normal basal medium) the sucrose concentration of the medium (s_{m}) was lowered to 0·25 g l^{-1}, i.e. K_{s} decreased from 4·2 to 0·21 g h^{-1} (Fig. 1). In this case limitation was imposed on day 8, but the limitation was too severe at this dilution rate, and washout occurred within 3 d, with no sign of sporulation. The peaks of productivity (Y) and growth rate (μ) the day after limitation was imposed are false values due to the high x_{E} values resulting from washout.

These experiments were repeated imposing a similar shock carbon limitation (K_{s} reduced from 4·2 to 0·19 g h^{-1}) but with higher s_{m} values and lower D values. Again, no sporulation resulted. Washout was more gradual but at longest the fermentation only lasted 3 d after initiation of sucrose limitation.

Gradual limitation. Dilution rate and sucrose concentration were gradually reduced to *A. niger* growing in otherwise optimal conditions (Fig. 2). Sporulation was achieved during this type of fermentation. In one example, sporing structures became visible in the tower contents on day 16 (K_{s} 0·4 g h^{-1}), although spores were too few to count. All the structures produced were complex sub-aerial forms with long conidiophores and large vesicles supporting many phialides, each producing spores. No spore chains were seen. Sporulation continued until the fermentation was terminated due to yeast infection. The gross morphology of the mycelium during sporulation corresponded to the type I–II form as proposed by Cocker (1980), accounting for the low x_{F} values encountered. A maintenance coefficient of 0·018 g g^{-1} h^{-1} was calculated. This experiment was repeated using *A. ochraceus* but the organisms washed out of the fermenter when K_{s} was lower than 1·0 g h^{-1}.

Starch is a carbohydrate source which is biochemically less available to the aspergilli, due to the need for hydrolysis before utilization, and it was anticipated that starch limitation would prove more severe than sucrose limitation and thus lead to increased sporulation. The

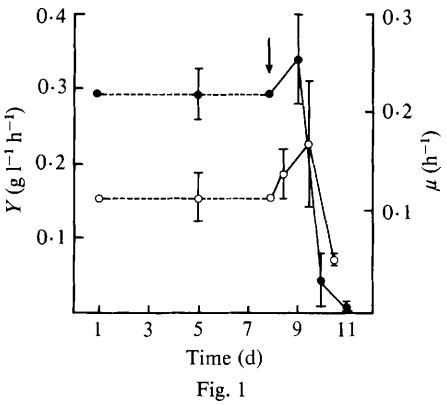

Fig. 1

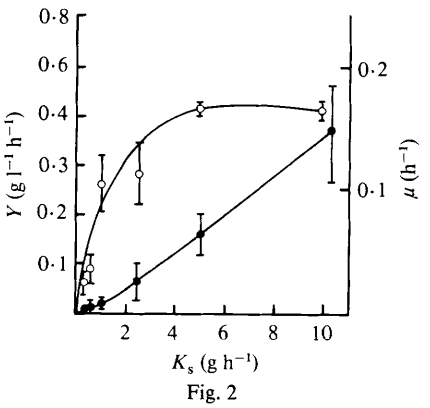

Fig. 2

Fig. 1. Effect of shock sucrose limitation [concentration in the medium suddenly decreased (arrow) from 5·0 to 0·25 g l^{-1}] on the growth rate, μ (○) and productivity, Y (●) of *A. niger*. D, 0·2 h^{-1}; temp., 30 °C; medium N concentration, 0·21 g l^{-1}. The bars represent the standard error of three replicates.

Fig. 2. Combined effect of gradually decreasing the medium sucrose concentration from 5·0 to 1·0 g l^{-1} and decreasing the dilution rate from 0·2 to 0·04 h^{-1} on the growth rate, μ (○) and productivity, Y (●) of *A. niger*. (Substrate supply rate, K_s, decreased from 10·0 to 0·4 g h^{-1}.) Temp., 30 °C; medium N concentration, 0·21 g l^{-1}. The bars represent the standard error of three replicates.

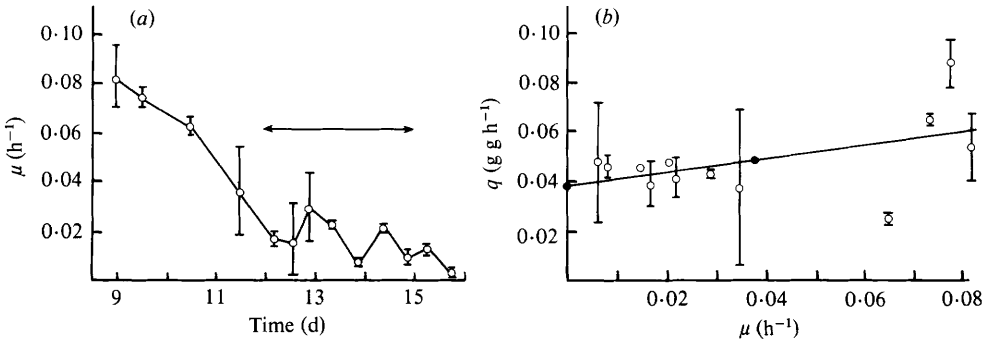

Fig. 3 (*a*) Gradual decrease in growth rate (μ) of *A. niger* after the imposition of a limiting starch supply of 0·6 g glucose equivalent l^{-1}. D, 0·1 h^{-1}; temp., 30 °C; medium N concentration, 0·21 g l^{-1}. The double-headed arrow indicates the period of sporulation. The bars represent the standard errors of three replicates.

(*b*) Estimation of the maintenance coefficient (m) of 0·0383 g g^{-1} h^{-1} for starch for *A. niger*, from the data of the experiments shown in (*a*). Specific substrate utilization rate (q) is plotted against growth rate (μ). ○, Observed values; ●, regression plot. The bars represent the standard error of three replicates.

effect of the lowest starch supply to *A. niger* at $D = 0{\cdot}1$ h^{-1} is shown in Fig. 3(*a*). Sporulation was first noticed on day 12 (48 h after the onset of the lowest carbon feed state), when conidiophore initials were produced from foot cells. Vesicles were visible 8 h later, and 24 h later phialides and spores were present. Sporulation was not synchronous, all structures being present together, but a sporulation development time of approximately 18–24 h from vegetative mycelium to spore production was indicated. The morphology of the sporing structures was simplified, only three or four phialides being produced on each vesicle.

Sporulation was evident for 72 h, but insufficient free spores were produced for accurate counts. At the time of sporulation the floc morphology was type I–II (Cocker, 1980). After 72 h, sporulation ceased and the culture died. This starch limitation experiment was repeated

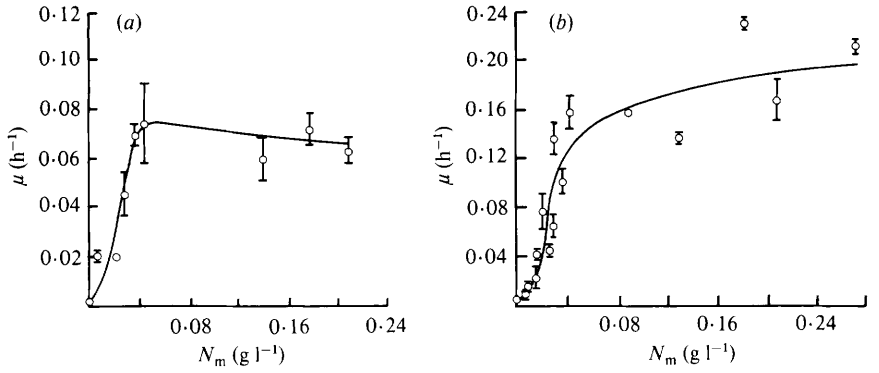

Fig. 4. Effect of gradual reduction of medium N concentration (N_m) on the growth rate (μ) of (*a*) *A. ochraceus* and (*b*) *A. niger*. *D*, 0·1 h^{-1} in (*a*) and 0·2 h^{-1} in (*b*); temp., 30 °C; medium sucrose concentration, 5·0 g l^{-1}. The bars represent the standard errors of three replicates.

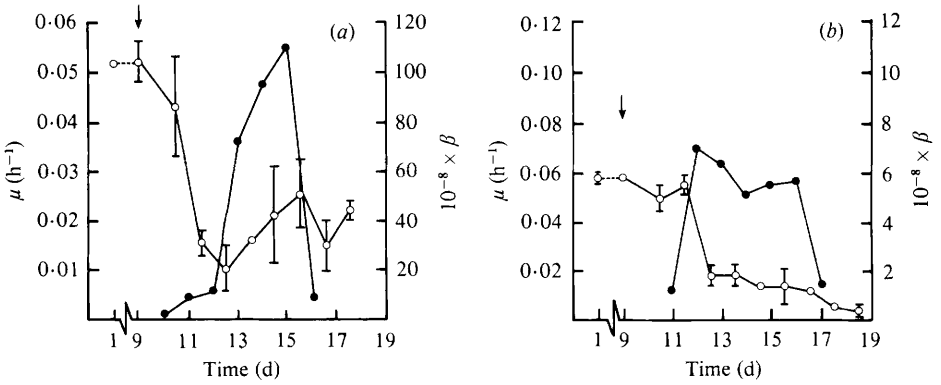

Fig. 5. Effect of shock nitrate limitation on the growth rate, μ (○) and sporulation index, β (●) of (*a*) *A. ochraceus* [medium N concentration suddenly reduced (arrow) from 0·214 to 0·0214 g l^{-1}] and (*b*) *A. niger* [medium N concentration suddenly reduced (arrow) from 0·21 to 0·021 g l^{-1}]. β represents the number of spores per g dry wt mycelium. *D*, 0·1 h^{-1}; temp., 30 °C; medium sucrose concentration, 5·0 g l^{-1}. The bars represent the standard errors of three replicates.

several times using *A. ochraceus*, but the organism died when K_s was decreased to 0·4 g h^{-1}, and no sporulation resulted.

Maintenance coefficients for starch of 0·0383 g g^{-1} h^{-1} for *A. niger* (Fig. 3*b*) and 0·123 g g^{-1} h^{-1} for *A. ochraceus* were calculated.

Nitrogen limitation

Gradual limitation. Maintaining a carbon supply of 5·0 g sucrose l^{-1}, at $D = 0{\cdot}2\ h^{-1}$, the nitrogen content of the medium was reduced stepwise, allowing the fermentation to approximate to a steady state between each stage (during nutrient limitation, steady-state operation is rarely encountered). The results for *A. niger* and *A. ochraceus* are presented in Fig. 4 (*a, b*). In both cases there was no sporulation, although growth was very slow, the mould adapting to the change in environment. One particular *A. niger* fermentation was run for 1536 h with a nitrogen concentration of 0·02 g l^{-1} or less with no sign of sporulation. It is suggested that the organism obtains sufficient nitrogen supply for maintenance and growth from the autolysis of dead cells at the low dilution rate. Pannell (1976) also observed this effect.

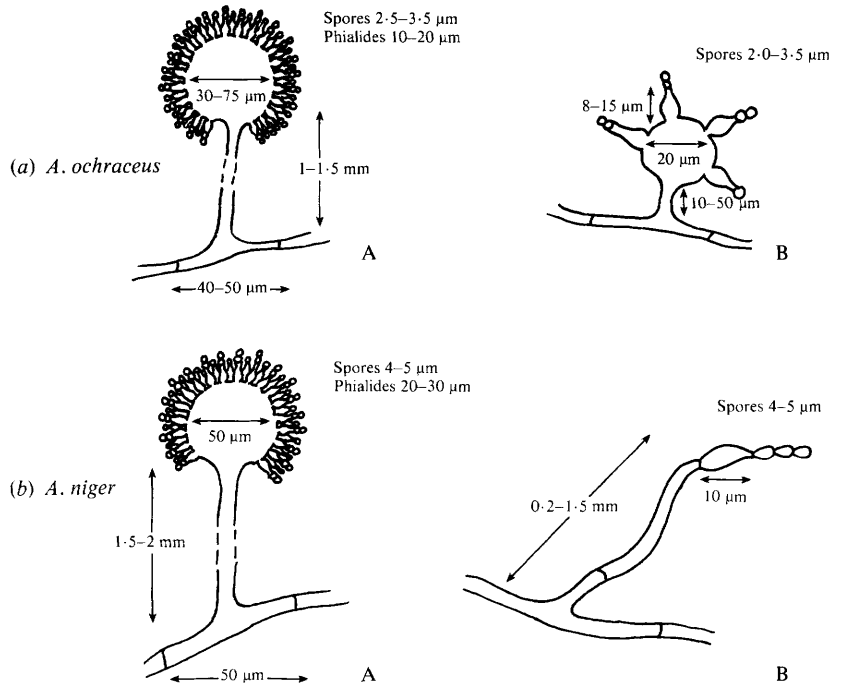

Fig. 6. Semi-diagrammatic representation of submerged sporulation structures of (*a*) *A. ochraceus* and (*b*) *A. niger*. A, sub-aerial form; B, simplified form in continuous culture after imposition of shock nitrogen limitation (see Fig. 5).

Shock limitation. After several days at optimum conditions and at a steady state, the nitrogen supply for *A. ochraceus* was suddenly reduced from 0·214 to 0·0214 g l^{-1} (Fig. 5*a*). Sporulation began approximately 10 h after the initiation of nitrogen limitation, when conidiophore initials were seen. After 16 h vesicles were present, and sporulation was complete after 18 h, upon the production of phialides and spores. Again, sporulation was not synchronous and continued for 7 d, after which time the fermentation was stopped due to infection. Although sub-aerial sporing structures were seen, the majority of structures were unusual, having a much reduced conidiophore bearing an apparently normal vesicle which held few (sometimes only two) phialides (Fig. 6*a*). The spore size was normal (2·0–3·5 μm diam.); no spore chains were seen. The sporulation index values from one experiment (Fig. 5*a*) show that spore production reached a peak after 6 d of nitrogen limitation, and then declined rapidly until, after 9 d, there were no spores visible, when the fermentation was stopped. An important observation during this fermentation was that of 'microcycle' conidiation. The incidence of the structures associated with this phenomenon was relatively low, but their presence indicates that the environment of the tower was capable of supporting the germination of spores produced in that system, and was also such that almost immediate re-sporulation of the germ-tube was induced. The nutrient interactions here are complex and an immediate explanation of this phenomenon was not apparent.

The experiment of shock nitrate limitation was repeated using *A. niger*, and an optimum steady state was achieved on a full medium supply at $D = 0{\cdot}1\ h^{-1}$. The nitrate supply was then decreased from 0·21 to 0·021 g N l^{-1} (Fig. 5*b*). As with the carbon limitation experiments, μ decreased very shortly after nutrient limitation, due to washout of the organism (at a particular dilution rate and nutrient concentration, the fermenter is only capable of supporting a limited fermenter population). Sporulation occurred approximately 12 h after the onset of limitation and continued for 8 d, when sporulation ceased and the

fermentation was terminated. Spore production was not as heavy as with *A. ochraceus* (sporulation index, Fig. 5*b*) but was found to be equally easily repeated. There were a variety of sporing structures produced, ranging from complex (sub-aerial type) to much simplified structures (those produced in the shortest time of 12 h) of single large phialides borne on hyphal tips (Fig. 6*b*). Sporulation was not synchronous and a complete range of structures were produced together. One should note that μ never reached zero during sporulation and carbon utilization did not drop appreciably at any time during nutrient limitation. The fermentation parameters exhibited damped oscillations after the shock stimulus, during which time sporulation occurred, and later gradually levelled out to an almost steady state, by which time sporulation had ceased.

DISCUSSION

It was possible with the continuous tower fermenter system to estimate the maintenance level of the carbohydrate supply to the fungi by extrapolating graphs of growth rate (μ) against specific substrate utilization rate (q) to the point of zero growth. The values of 0·018 g sucrose g^{-1} h^{-1} and 0·0383 g starch g^{-1} h^{-1} for *A. niger* calculated from our results are similar to published values for the carbohydrate maintenance requirement of other filamentous fungi (Table 1), but the value of 0·123 g starch g^{-1} h^{-1} for *A. ochraceus* is considerably higher than any previously calculated value. This may be due to the slow growth rate of this organism. It was not possible to calculate a maintenance coefficient value for nitrogen, as growth rate and productivity were still positive when utilization had apparently reached zero with no nitrogen supply to the culture. This agrees with the fact that the organism can utilize a carbohydrate source for respiration and growth in the absence of a nitrogen supply, while the reverse is not possible. Thus, growth ceases when sufficient carbohydrate is removed from the supply, but continues when all nitrogen is removed from the medium. At this point the organism is assumed to obtain sufficient nitrogen from autolysis of dead cells. This was most evident in the *A. niger* cultures, which were able to survive for long periods on their own lysis nitrogen products with medium that contained no nitrogen source but had all other nutrients in excess. This condition could not last indefinitely, because only sufficient nitrogen was available for the maintenance of existing cells and not for the production of new cells, i.e. there was no increase in mycelial mass. The existing cells therefore gradually die and are not replaced, although this is a very slow process.

Our results provide evidence that sporulation occurs at substrate levels slightly above the maintenance ration (sucrose 0·102 g g^{-1} h^{-1} to *A. niger*, starch 0·093 g g^{-1} h^{-1} to *A. ochraceus* and 0·04 g g^{-1} h^{-1} to *A. niger*), a phenomenon which was also noted by Righelato *et al.* (1968) and Ng *et al.* (1972).

We agree with Smith *et al.* (1977) that it is 'better to consider that vegetative growth and sporulation are cellular processes competing for limiting metabolic intermediates rather than as mutually exclusive phenomena', because growth continued throughout each sporulative phase. This observation may be distorted because fermenter productivity, and hence growth rate, is influenced not only by the physical and nutritional parameters but also by the organism morphology induced by these restraints. As a consequence of nutrient limitation, the gross mycelial floc morphology alters to a form of type I–II (Cocker, 1980), more easily removed from the fermenter; this results in a gradual washout which lowers the fermenter biomass concentration and falsely increases fermenter output. Growth, as calculated by the increase in mycelial dry weight, is probably due to the formation of sporulation structures and not to hyphal biomass.

The most intriguing aspect of this work was the unusual morphology of the sporulating structures observed. There are conflicting reports on the submerged reproductive structures of filamentous fungi. Carter & Bull (1969) describe submerged structures of *Aspergillus nidulans* as identical to sub-aerial structures. Galbraith & Smith (1969*a*) also state that the

Table 1. *Calculated maintenance coefficients of carbohydrate supply for filamentous fungi in continuous culture*

Organism	Substrate	Maintenance coefficient, m (g g^{-1} l^{-1})	Reference
Penicillium chrysogenum	Glucose	0·022	Righelato *et al.* (1968)
Aspergillus nidulans	Glucose	0·018	Carter *et al.* (1971)
Aspergillus nidulans	Glucose	0·029	Bainbridge *et al.* (1971)
Aspergillus niger	Citrate	0·045	Ng *et al.* (1973)
Aspergillus niger	Sucrose	0·015	Pannell (1976)
Aspergillus niger	Starch	0·04	Spensley (1977)

submerged sporulation structures observed were identical to those of static cultures, although lacking conidial chains. Similarity between submerged and sub-aerial structures has also been reported by Righelato *et al.* (1968), Vezina *et al.* (1965) and Hadley & Harrold (1958). Evidence for the reduction of complexity of submerged sporing structures is equally well documented. Anderson & Smith (1971*b*) observed direct production of phialides on tips of germ-tubes of *A. niger* and Ng *et al.* (1973) reported considerable reduction of complexity of the conidial apparatus of *A. niger* in chemostat culture. Various 'microcycle' sporulation structures have also been described for *A. niger* (Anderson & Smith, 1971*b*) and *Penicillium urticae* (Sekiguchi *et al.*, 1975). Our experiments produced much simplified reproductive structures of both *A. niger* and *A. ochraceus*. Structures produced in continuous culture under carbon limitation were all similar to the sub-aerial form, and any simplification (e.g. *A. niger* on starch) was reflected in the decreased number of phialides and spores. The structures produced by *A. niger* and *A. ochraceus* during nitrogen limitation were predominantly much simplified. However, in the nitrogen-limited cultures, a range of reproductive structures, from complex to very simple, were frequently observed along the same hypha. Explanation of the simplification of sporulation morphology is thus complicated; we consider that the exact switch-over point to a sporulative metabolism has not yet been defined. Ng *et al.* (1973) suggested that the simplification of morphology is due to a partial switching on of the conidiation mechanism, with the morphological and biochemical events of development that preclude spore formation being by-passed. They also suggested that the residence time of the mycelium in the fermenter may determine the morphology of sporulation, but this seems unlikely on the evidence of our work. Dilution rate, and hence residence time, determined the incidence of sporulation (under nitrogen-limited conditions, a dilution rate of 0·4 h^{-1} inhibited sporulation, while spores were formed under identical conditions at dilution rates of 0·2 h^{-1} and 0·1 h^{-1}), but exerted no control over the morphology of sporulation. Sporing structures were produced primarily at the edges of a mycelial floc, with the centre of the floc remaining vegetative. This indicated that those hyphae at the edge of the floc which have access to the limited nutrients available use these nutrients to produce spores. The observation of adjacent complex and simplified structures suggests that the morphology produced depends upon the availability of nutrients and oxygen along the hypha within the peripheral zone of the floc. Hence those structures near the hyphal tip, which has greater access to nutrients, would be complex, while further back along the hypha, structures would become increasingly simplified. However, there is as yet no evidence of this.

The induction of the reproductive phase of the organisms is considered analogous to the induction of a non growth-associated secondary metabolite, and as such the production of the desired product (i.e. spores) continues only for a finite period of time. Dawes & Thornley (1970) reported that the steady-state conditions of spore formation by *Bacillus subtilis* were not maintained indefinitely, due to the spontaneous appearance of asporogenous mutants, which replaced the sporing organism. This was not the case with either of the fungi we tested, because samples taken after the sporulation phase, and plated out at 30 °C produced perfect spore mats in 10–12 d. It is a feature of submerged continuous culture of filamentous fungi

that the capacity for sporulation is gradually lost, but Pannell (1976) considered this to be physiological rather than genetic, although the precise cause is not known. Such loss of sporulative capacity was only noted in our work with any fermentation conducted for 6 months or longer. Pannell (1976) found that sporulative ability was restored in subcultures of low-sporing strains. This was also the case in our experiments, but new fermentations were always started from a new master-culture. The loss of sporulative capacity is considered to be a response to the physicochemical stress and continual growth restraint imposed upon the fungus in this system.

During sporulation, overall growth rate decreases and therefore x_F decreases. As a result, the substrate supply per g dry wt mycelium increases, thus slowly removing the stimulus of nutrient limitation and ending sporulation. A cycling of nutrient levels in this way may explain the 'microcycle' conidiation observed.

It is proposed to establish a semi-continuous system by cyclic medium supply using one medium to induce conidiation, and a second to increase growth (and x_F) after sporulation has ceased, before imposing new growth restraint. The effects of physical parameters such as pH, temperature, dilution rate, and oxygen and carbon dioxide supply, which are easily controlled in the continuous tower fermenter, will also be investigated. It is already known that pH exerts a major effect upon mould morphology (Galbraith & Smith, 1969*b*; Stockbridge, 1979).

The effect of nutrient limitation upon sporulation is a complex phenomenon and is closely linked with the influence of the physicochemical environment of the continuous tower fermenter. We consider that continuous sporulation is the result of the summation of two or more elements of the physicochemical environment. However, the work described here indicates that control over sporulation in continuous submerged culture is possible using the continuous tower fermenter system, and leads to previously unobserved morphological forms.

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