Kinetic characterization of sporulation in *Streptomyces albidoflavus* SMF301 during submerged culture

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We report the first quantitative analysis of the relationship between environmental changes and sporulation of a streptomycete, *Streptomyces albidoflavus* SMF301, in submerged culture. A chemically defined medium was constructed for sporulation, over 10^9 spores ml^{-1} being formed in the submerged batch culture. Kinetic parameters calculated from batch and chemostat cultures showed that specific submerged spore formation rate (q_{spo}) was inversely related to the specific mycelial growth rate (μ). The optimum growth rate for submerged spore formation was 0.05 h^{-1} , when the maximum value of q_{spo} was 1.0×10^6 spores $\text{g}^{-1} \text{ h}^{-1}$. The turnover rate of biomass at maximum growth yield was 0.029 h^{-1} , when 5.6×10^6 spores were formed from 1 g mycelium. The present quantitative analysis of submerged spore formation using a controlled system opens the way for biochemical and molecular biological studies related to the morphological differentiation of *Streptomyces* spp.

Keywords: *Streptomyces albidoflavus*, submerged culture, kinetics of sporulation, differentiation

INTRODUCTION

Secondary metabolite production (physiological differentiation) in *Streptomyces* spp. accompanies the formation of spores (morphological differentiation), with both being induced by shift-down of some essential nutrients (Champness, 1988; Champness *et al.*, 1989; Chater, 1984; Demain, 1982). Although most streptomycete-derived antibiotics are produced in submerged liquid cultures, studies on the mechanisms involved in the morphological differentiation of *Streptomyces* spp. have mostly been carried out on solid medium (Chater, 1989, 1991; Chater *et al.*, 1988, 1989; Tan & Chater, 1993).

Some species of *Streptomyces* can sporulate in submerged culture, and this has been physiologically analysed in certain cases (Babcock & Kendrick, 1988; Kendrick & Ensign, 1983; Koepsel & Ensign, 1984; Huber *et al.*, 1987; Ochi, 1987; Daza *et al.*, 1989; Glazebrook *et al.*, 1990). However, the kinetics of sporulation of *Streptomyces* spp. in response to changes in culture conditions have not yet been thoroughly studied. Hence, adequate conclusions have not been made regarding the quantitative relationship between environmental changes and sporulation in these micro-organisms. In this context, we selected a strain of *Streptomyces* sp. which produced abundant spores

both in submerged culture and on solid media (Shin & Lee, 1986; Jeong *et al.*, 1988; Rho *et al.*, 1988, 1989). This strain was classified as *Streptomyces albidoflavus* by numerical analysis of the operating taxonomy unit characters (Williams *et al.*, 1983a, b) using TAXON, an identification probability matrix (Rho *et al.*, 1992). The aerial spores formed on solid medium and submerged spores formed in liquid culture were characterized in terms of their cellular composition and resistance to extreme conditions (Lee & Rho, 1993). In the work reported here, we evaluated the optimum culture conditions for submerged spore formation and analysed kinetic parameters which help elucidate the quantitative relationship between environmental changes and spore formation.

METHODS

Micro-organisms and media. The micro-organism used was *Streptomyces albidoflavus* SMF301 (Rho *et al.*, 1992; Lee & Rho, 1993). Rich medium consisted of (w/v): 1% glucose, 0.2% peptone, 0.1% yeast extract and 0.1% beef extract, with 1.8% agar for solid cultures. Chemically defined medium contained (w/v): 1–2% glucose, 0.15% NH_4Cl , 0.013% KH_2PO_4 , 0.009% Na_2HPO_4 , 0.06% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.0001% trace elements ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$). The initial pH of media was adjusted to 7.0

before steam sterilization; phosphate and magnesium salts were sterilized separately and added to the medium aseptically.

Strain maintenance and culture conditions. *S. albidoflavus* SMF301 was maintained by transferring to slopes of the rich agar medium each month and storing at 4 °C. Spores that developed on the rich agar medium were suspended in glycerol-rich broth medium and stored at -70 °C (Wellington & Williams, 1978). The frozen spore suspensions (about 10^9 spores ml^{-1}) were thawed at ambient temperature and used as inocula. For seed cultures, 1 ml spore suspension was inoculated into 50 ml chemically defined medium in 100 ml baffled flasks and incubated for 3 d at 28 °C on a rotary shaking incubator (150 r.p.m.). The seed cultures were inoculated into 2 l chemically defined medium in jar fermenters (model KFC-5, Korea Fermentor Co.) for batch cultures. Culture temperature was maintained at 28 °C, and pH was controlled at 7.0 by automatic addition of 1 M HCl and 1 M NaOH. In all the batch culture experiments (Figs 1–4), aeration was controlled at 1.0 vol. $\text{vol.}^{-1} \text{min}^{-1}$ (v.v.m.) and agitation speed was 500 r.p.m.; this maintained dissolved oxygen tension above 20% saturation. To commence chemostat operation, a standardized procedure was used in which the culture was grown batch-wise for 48 h, after which the chemostat operation commenced at the desired dilution rate. The medium was metered into the fermenter by a peristaltic pump. Culture temperature and pH, aeration and agitation speed were the same as for batch cultures. A steady-state condition was assumed to have been obtained when three samples over a period of two to three times the mean residence times showed no significant change in any of the measured parameters.

Analysis of growth, spore formation and chemical change.

Biomass, including mycelium and spores, in submerged culture was harvested by centrifugation at 15000 g for 10 min and washed twice with physiological saline solution and once with distilled water. The washed cells were dried at 100 °C for 12 h and the cell dry weight determined. The number of spores formed in submerged cultures was counted as follows. Culture broth (5 ml) was sonicated for 5 min at 100 W using a sonic desmembrator (model 300, Fisher). The sonicated cell suspension (0.5 ml) was mixed gently with 0.1 M HCl (4.5 ml) and the mixtures were allowed to stand for 5 min before diluting in physiological saline and spreading on rich agar medium. Colony counts were made after 4 d incubation at 28 °C to give initial spore numbers (Lee & Rho, 1993). The concentration of reducing sugars in the cell-free culture broth was determined using the dinitrosalicylic acid reagent of Miller (1959). The concentration of ammonium ions was analysed with a specific ion analyser (model EA940, Orion Research).

Analysis of kinetic parameters. The data from chemostat cultures were analysed in terms of various kinetic parameters, namely specific mycelial growth rate (μ), specific ammonium ion uptake rate (q_{amn}), specific glucose uptake rate (q_{glu}), and specific spore formation rate (q_{spo}). Kinetic parameters in chemostat cultures were calculated as follows: $\mu = D$, where D is the dilution rate (h^{-1}); $q_{\text{glu}} = D(s_0 - \bar{x})/\bar{x}$, where s_0 is the concentration of glucose in the medium, $\bar{x}$ is the steady-state concentration of glucose (g l^{-1}), and $\bar{x}$ is the steady-state concentration of biomass (g l^{-1}); $q_{\text{amn}} = D(n_0 - \bar{n})/\bar{x}$, where n_0 is the concentration of ammonium ions in the medium, and $\bar{n}$ is the steady-state concentration of ammonium ions (g l^{-1}); $q_{\text{spo}} = D(\bar{p})/\bar{x}$, where $\bar{p}$ is the steady-state number of spores; $Y_{\text{spo/x}} = \bar{p}/\bar{x}$; $Y_{\text{x/glu}} = \bar{x}/(s_0 - \bar{x})$. Maintenance energy coefficient (m_s) and biomass turnover rate (a) were calculated from the following equations: $q_{\text{glu}} = \mu/Y_{\text{x/s}}^{\text{max}} + m_s$, and $a = m_s Y_{\text{x/s}}^{\text{max}}$, as given by Pirt (1975).

Chemicals, reagents and reproducibility. Lysozyme, NTG

and amino acids were purchased from Sigma. All other chemicals were of reagent grade. Each experiment was repeated three times and the mean values are given.

RESULTS

Optimum culture conditions for mycelial growth and submerged spore formation

S. albidoflavus SMF301 grew well in the chemically defined medium containing glucose, fructose, maltose, starch or mannitol, but not in that containing sucrose, lactose or *myo*-inositol (Rho *et al.*, 1992). Since glucose was the best energy and carbon source, the effects of glucose concentration on mycelial growth and spore formation in submerged batch culture were evaluated. Mycelial growth and sporulation in submerged culture was not repressed by glucose at concentrations of up to 40 g l^{-1} when the culture pH was not controlled (Fig. 1a). However, repression was observed with higher concentrations of glucose (Fig. 1a). Repression at high concentrations of glucose was not evident when the culture pH was

Fig. 1. Effects of glucose concentration on the formation of biomass (■) and spores (□) in batch cultures of *S. albidoflavus* SMF301. (a) Initial culture pH was adjusted to 7.0 at the beginning of incubation, but was not controlled thereafter; (b) culture pH was maintained at 7.0 throughout the incubation period. The results shown were from 5 d cultures.

Fig. 2. Effects of culture pH on the formation of biomass (■) and spores (□) in batch cultures of *S. albidoflavus* SMF301. The results shown were from 5 d cultures.

Fig. 3. Effects of the concentration of (a) ammonium ions and (b) inorganic phosphate on the formation of biomass (■) and spores (□) in batch cultures of *S. albidoflavus* SMF301. The results shown were from 5 d cultures.

Fig. 4. Changes in the concentration of ammonium ions (○), glucose (●), biomass (■) and spores (□) in batch cultures of *S. albidoflavus* SMF301.

maintained at 7.0 (Fig. 1b). Since the culture pH became acid at higher glucose concentrations, the effects of culture pH on mycelial growth and spore formation were evaluated. The culture pH was maintained at a set point between 5 and 9 by the automatic addition of 1 M HCl or 1 M NaOH. It was found that the optimum pH for mycelial growth and spore formation was 7.0, but spore formation was more clearly repressed by alkaline culture pH than was mycelial growth (Fig. 2).

Although mycelial growth increased steadily with an increase in ammonium ion concentration, submerged spore formation was optimum at 1.5 g NH_4Cl l⁻¹ (Fig. 3a). Mycelial growth also increased with increasing inorganic phosphate concentration, but submerged spore formation was optimum with 2.5 mg inorganic phosphate l⁻¹ (Fig. 3b). The effects of other mineral salts on mycelial

Fig. 5. Steady-state values of ammonium ions (○), glucose (●), biomass (■) and submerged spores (□) in glucose- and NH_4Cl -limited chemostats of *S. albidoflavus* SMF301.

Fig. 6. Effect of specific growth rate on q_{amn} (○), q_{glu} (●), Y_{glu} (■) and $Y_{\text{spo/x}}$ (□) in glucose- and NH_4Cl -limited chemostats of *S. albidoflavus* SMF301.

growth and submerged spore formation were evaluated, but the effects were not significant. The optimum temperature for mycelial growth and submerged spore formation was 25–30 °C (data not shown).

Kinetic parameters for mycelial growth and submerged spore formation

The changes in concentration of glucose, ammonium ions, biomass and submerged spores in a batch culture using the chemically defined medium are shown in Fig. 4.

Fig. 7. Relationship between (a) μ and q_{spo} , (b) q_{glu} and q_{spo} , and (c) q_{amn} and q_{spo} of *S. albidoflavus* SMF301 in chemostat culture.

The number of submerged spores decreased with the initiation of mycelial growth, indicating germination of spores. Mycelial growth revealed a typical batch culture pattern, entering stationary phase when ammonium ions were completely utilized. However, the formation of spores was evident when the residual concentration of glucose was still relatively high.

Since the formation of submerged spores appeared to be closely related to the concentration of ammonium ions, experiments with ammonium-ion-limited chemostats were carried out in order to evaluate more precisely the relationship between the kinetic parameters. The steady-state values of glucose, ammonium ions, biomass and submerged spores are shown in Fig. 5. The steady-state concentrations of biomass decreased at lower dilution rates (below $0.1 h^{-1}$), but submerged spore formation was inversely related to dilution rate (specific growth rate). The optimum dilution rates were $0.1 h^{-1}$ for mycelial growth and $0.05 h^{-1}$ for submerged spore formation.

Kinetic parameters calculated from the steady-state data of the chemostat culture are shown in Fig. 6. Specific ammonium ion uptake rate (q_{amn}) and specific glucose uptake rate (q_{glu}) increased as the dilution rate increased. The maximum value of q_{glu} was $0.2 g g^{-1} h^{-1}$ at $0.1 h^{-1}$, whereas the maximum value of q_{amn} was $0.018 g g^{-1} h^{-1}$ at $0.15 h^{-1}$. The maintenance energy coefficient (m_s) was calculated to be $0.046 g g^{-1} h^{-1}$ by extrapolating the values of q_{glu} to zero growth rate. Maximum growth yield ($Y_{x/s}^{max}$) was calculated to be $0.636 g g^{-1}$, and the rate of biomass turnover (a) was estimated to be $0.029 h^{-1}$. By plotting q_{spo} against μ (Fig. 7a), q_{glu} (Fig. 7b) and q_{amn} (Fig. 7c), it was clear that q_{spo} was inversely related to all these parameters. The maximum value of q_{spo} ($1.0 \times 10^6 g^{-1} h^{-1}$) was obtained at $0.05 h^{-1}$, when 5.6×10^6 spores were formed from 1 g of mycelium.

DISCUSSION

Differing results have been obtained for the effect of glucose concentration on the formation of spores in submerged culture of a range of *Streptomyces* spp. Daza *et al.* (1989) found that sporulation of several species of *Streptomyces* in submerged culture was not inhibited by high concentrations of glucose. In contrast, Glazebrook *et al.* (1990) found that sporulation of *Streptomyces venezuelae* in submerged culture was repressed by high concentrations of glucose, and that this repression was not

merely a secondary result of acid accumulation. The same result was also obtained with *Streptomyces griseus* (Babcock & Kendrick, 1988). In addition, cellular differentiation of *Streptomyces coelicolor* A3(2) in surface culture was inhibited by high concentrations of glucose (Chater *et al.*, 1988).

In the present experiments, we found that sporulation of *S. albidoflavus* SMF301 in submerged culture was pH-dependent, with an optimum at 7.0, and that spore formation was not repressed by glucose up to $80 g l^{-1}$ when the culture pH was maintained at 7.0. Therefore, it was concluded that sporulation of *S. albidoflavus* SMF301 in submerged culture is more affected by culture pH than by high concentrations of glucose; however, the molecular mechanism remains to be elucidated.

It has been reported that sporulation of *S. griseus* in submerged culture is initiated by ammonium ion starvation (Kendrick & Ensign, 1983), and that an excess of nitrogen is inhibitory to submerged spore formation in *S. venezuelae* (Glazebrook *et al.*, 1990). We found that mycelial growth in submerged culture of *S. albidoflavus* SMF301 was enhanced by higher concentrations of ammonium ions or inorganic phosphate, whereas sporulation was repressed significantly by these conditions. Our data indicated that nitrogen or phosphate starvation influenced the shift from mycelial growth to submerged spore formation.

Prior to the present investigation, kinetic studies on the sporulation of *Streptomyces* spp. in submerged culture had not been reported, although the physiological analysis of sporulation of several species of *Streptomyces* in submerged culture has been carried out (Babcock & Kendrick, 1988; Kendrick & Ensign, 1983; Koepsel & Ensign, 1984; Huber *et al.*, 1987; Ochi, 1987; Daza *et al.*, 1989; Glazebrook *et al.*, 1990). From our batch and continuous culture kinetic analysis, it was very clear that the specific rate of formation of spores was inversely related to the specific growth rate (μ), and that q_{spo} was optimum (1.0×10^6 spores $g^{-1} h^{-1}$) when μ was $0.05 h^{-1}$. The rate of biomass turnover was $0.029 h^{-1}$, when 5.6×10^6 spores was formed from 1 g of mycelium.

This study is the first attempt to analyse quantitatively the relationship between mycelial growth and spore formation of *Streptomyces* spp. in submerged culture. The strain used produced abundant spores in submerged culture compared to other species of *Streptomyces*. Such quantitative analysis in controlled, submerged liquid

culture using a simple, chemically defined medium offers a system in which to elucidate the biochemical and molecular biological basis of morphological differentiation in *Streptomyces* spp.

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