Effect of hyphal micromorphology on bioreactor performance of antibiotic-producing *Saccharopolyspora erythraea* cultures

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When *Saccharopolyspora erythraea* biomass from submerged culture was filtered (100–120 µm sintered glass filter) the antibiotic yield of the retentate (mean hyphal particle diameter 103 µm) was significantly higher [1.740 mg (mg biomass)⁻¹] than that of the filtrate [1.286 mg (mg biomass)⁻¹; mean hyphal particle diameter 88 µm]. A hypothesis to explain this is that there is a critical hyphal particle diameter, below which the particle is incapable of producing antibiotic. This would be a consequence of the site of antibiotic production occurring at a fixed distance from the growing hyphal tip. A protocol is proposed for calculation of the hypothetical critical hyphal diameter (88 µm in this case). The proportion of retentate (more productive) fraction of biomass varied between 60% and 30% during the course of a batch culture. Bioreactor stirrer speed significantly affected mean hyphal particle diameter (70 µm at 1500 r.p.m.; 124 µm at 750 r.p.m.) and antibiotic productivity [0.867 mg (mg biomass)⁻¹ at 1500 r.p.m.; 0.913 mg (mg biomass)⁻¹ at 750 r.p.m.].

Keywords: hyphal micromorphology, bioreactor performance, secondary metabolism, antibiotic production, erythromycin

INTRODUCTION

We have reported that a significant hyphal breakage rate occurs in shake-flask liquid cultures of many *Streptomyces* species, a phenomenon that accounts for these species producing antibiotics on agar but not in liquid culture (Pickup *et al.*, 1993). We obtained evidence that resistance to hyphal breakage is dependent on the activity of a peptidoglycan-synthesizing enzyme, phospho-*N*-acetylmuramyl pentapeptide translocase, whose activity appears to determine hyphal tensile strength (Pickup & Bushell, 1995). Hyphal breakage appears to result in the formation of viable mycelial fragments, incapable of antibiotic production, whose growth rates are equal to those of the unbroken hyphal units from which they were derived (Pickup *et al.*, 1993).

Other laboratories have described correlations between product formation and morphology in cultures of *Streptomyces* species, reporting lack of production in cultures whose micromorphologies appeared fragmented (Shomura *et al.*, 1979; Roth *et al.*, 1982). Reports have also appeared relating antibiotic production to hyphal ultra-

structure (Williams *et al.*, 1974; Bewick & Williams, 1977; Bewick *et al.*, 1977; Liu *et al.*, 1984) but it has not been possible to relate these to fragmentation phenomena.

Our observations suggest that hyphal particles with a range of sizes coexist in submerged liquid culture of streptomycete species and that the antibiotic yield of these cultures is dependent on the ratio of the number of larger (antibiotic-producing) hyphal fragments to that of the smaller (non-producing) fragments.

METHODS

Strains and culture media. *Saccharopolyspora erythraea* NRRL 2338 was used throughout. Antibiotic concentrations were measured routinely by bioassay using a strain of *Arthrobacter citreus* (GL1) obtained from the Shell Laboratories culture collection, Sittingbourne, Kent, UK. The chemically defined (nitrogen-limited) antibiotic production medium for *S. erythraea* contained the following major nutrients (g l⁻¹ in reverse-osmosis-purified water): glucose, 15; NaNO₃, 1.19; KH₂PO₄, 3.0; K₂HPO₄, 7.0; and the following trace components (g l⁻¹): MgSO₄·7H₂O, 0.25; FeSO₄·7H₂O, 0.025; CuCl₂, 0.00053; CoCl₂, 0.00055; CaCl₂·2H₂O, 0.0138; ZnCl₂, 0.0104; MnCl₂, 0.0062; Na₂MoO₄, 0.0003. Glucose, salts and trace solution were autoclaved separately and then mixed. The pH was adjusted to 7.0 with 5 M KOH prior to autoclaving.

S. erythraea was maintained on tomato purée oatmeal agar (g l⁻¹:

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tomato purée, 10; oatmeal, 10; agar, 20) and *A. citreus* was maintained on nutrient agar. Both were grown in 250 ml baffled Erlenmeyer flasks containing 25 ml nutrient broth at 30 °C on a rotary shaker at 250 r.p.m. After 48 h agitation (*S. erythraea*), 2 ml was removed and used to inoculate preculture flasks containing 23 ml defined medium. After 48 h further incubation, the precultures were used at approximately 5% (v/v) as an inoculum for flask or bioreactors.

Bioreactor culture. An LH 2000 series bioreactor with a working volume of 7 l was used. Agitation was provided by disc turbine impellers, rotating at 500 r.p.m. (batch), 750 r.p.m. (chemostat low speed) or 1500 r.p.m. (chemostat high speed) and sterile air was supplied through a sparger at a flow rate of 0.8 v/v/m. The temperature was controlled at 30 °C. Bioreactor dissolved oxygen concentration was monitored using an Ingold polarographic dissolved oxygen electrode. The pH was monitored throughout but was not controlled. The cultures, which contained a phosphate buffering system (see above) increased in pH over the course of an experiment by no more than 0.5 of a pH unit. Foaming was eliminated by including 0.01% Breox FMT30 antifoam (Water Management and Gamlen) in the culture medium.

Chemostat cultures were performed using a peristaltic pump to introduce fresh medium into the vessel. A constant volume was maintained using a weir overflow pipe. A steady state was assumed when constant biomass concentration ($\pm 5\%$) was observed in four samples, taken at the rate of three per mean culture residence time.

Antibiotic assays. A bioassay employing *Arthrobacter citreus* was performed using procedures described previously (Huck *et al.*, 1991). Challenge strain seed cultures and assay plates were incubated at 30 °C, and the diameters of the zones of inhibition were recorded after 24 h. Examination of a number of samples using high-performance liquid chromatography (Tsuji & Goetz, 1978) confirmed that the bioassay values corresponded to erythromycin concentrations.

Residual glucose, nitrate and phosphate. A glucose-oxidase-based assay kit (Trinder system, Sigma) and a nitrate-reductase-based assay kit (Boehringer Mannheim) were employed. The colorimetric procedure described previously (McDermott *et al.*, 1993) was used for phosphate determination.

Culture filtration. A modification of the method described by Pickup & Bushell (1995) was used. Sintered glass filters (pore size 100–120 mm) were used instead of the muslin filters described by Pickup & Bushell (1995). The filters were mounted in autoclavable glass and plastic holders and asepsis was maintained throughout the filtration. Prior to filtration, 400 ml samples of culture were removed at regular intervals during batch fermentation and diluted with 100 mM MOPS buffer (pH 7.0) to give a final biomass concentration of 0.5 g l⁻¹.

Replacement culture experiments. Following filtration of a 10 h bioreactor sample, retentate, filtrate and unfiltered (centrifuged) mycelium were reinoculated into culture supernatant at 1 g l⁻¹. A control flask consisting of uninoculated supernatant was also incubated.

Reproducibility and statistical analysis. All experimental data presented in this paper were obtained from single cultures. Experiments were carried out in triplicate to ensure that the trends and relationships observed in the culture parameters measured were reproducible. Individual assays were replicated fourfold. Sampling shaker cultures involved sacrificing two cultures for each time point and assaying the contents in-

Fig. 1. Method used to estimate maximum diameter of hyphal fragments.

dividually. Experiments were rejected where a chi-squared test indicated significant differences between replicates. Biomass concentrations varied by no more than 5% between replicates. Error bars are not shown as they were mostly too small to be visible on the figures presented.

The Fisher–Berens test (Campbell, 1974) was used when it was necessary to determine whether two sets of data were significantly different. This requires the calculation of the *C* statistic where:

$$C = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\left(\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}\right)}}$$

and $\bar{x}$, σ and n are mean, sample deviation and sample size of the two populations. The calculated value of *C* is compared with those in an appropriate statistical table in order to test the null hypothesis that there is no difference between samples.

Hyphal fragment size measurement. A modification of the technique of Packer & Thomas (1990) was used to prepare slides for analysis. Culture samples were diluted 50-fold in 100 mM MOPS buffer (pH 7.0) and agitated to give a homogeneous suspension of mycelium. Samples of the suspension (40 µl) were spread over the surface of the slide and air dried. When dried, the slides were stained with methylene blue solution [0.3 g methylene blue, 30 ml 95% (v/v) methanol in 100 ml water] for 1 min. The slides were rinsed in water for 2 min and allowed to air dry for 2 h. Six slides were prepared for each sample. The slides were examined and photographed using a Leitz orthoplan microscope with phase-contrast optics and a magnification of 200. Sufficient fields were photographed to allow 250–300 separate fragments to be measured and counted. Photographs were examined and maximum particle diameter estimated using a stencil containing circles whose diameters ranged from 6 to 42 mm, with an interval of 2 mm. The scale was such that a length of 20 mm on the photograph corresponded to 100 µm. The maximum fragment diameter was defined as the smallest circle on the stencil into which a fragment could be completely fitted (Fig. 1).

RESULTS AND DISCUSSION

Antibiotic production during nitrogen-limited batch culture

Production kinetics similar to those obtained previously (McDermott *et al.*, 1993; Wilson & Bushell, 1995) were observed. Culture biomass and antibiotic concentrations increased in parallel (Fig. 2), an observation that contrasts with the growth-dissociated production observed in carbon-limited cultures (McDermott *et al.*, 1993; Lynch & Bushell, 1995).

Resolution of the culture into two fractions by filtration

Filtration using the sintered glass filter resulted in two populations of hyphal particles, with mean particle diameters of 88 μm (filtrate) and 102 μm (retentate) (Fig. 3). Frequency analysis indicated that the particle size distributions overlapped (Fig. 3), with the diameter at peak frequency being significantly smaller in the filtrate than in the retentate (Fig. 3, Table 1).

Fig. 2. Erythromycin concentration ($\circ$), biomass concentration ($\square$) and erythromycin yield [mg erythromycin produced (g biomass) $^{-1}$, since inoculation] ($\triangle$) in nitrogen-limited batch bioreactor culture.

Fig. 3. Distribution histograms for particle size in the filtrate ($\square$) and retentate ($\blacksquare$) for a 24 h nitrogen-limited bioreactor culture.

Table 1. Statistical analysis of the hyphal particle size distribution data obtained on filtering the culture through a sintered glass filter

$C = 7.354$, for the two sets of particle size data; therefore reject null hypothesis that there is no difference between particle size distribution in retentate and filtrate ($P = 0.01$).

Statistic	Filtrate	Retentate
Sample deviation (μm)	20.86	27.04
Sample mean (μm)	88.00	102.73
Variance (μm)	86.59	145.71
Sample size	300	284

Fig. 4. Erythromycin concentrations obtained when culture filtrate ($\triangle$), culture retentate ($\blacksquare$) and unfiltered centrifuged mycelium ($\circ$) were resuspended in culture supernatant and incubated. $\square$, Uninoculated control.

Antibiotic production in the filtrate and retentate fractions

During the replacement culture experiment, the erythromycin production potential of the two fractions was monitored at regular intervals for 14 h (Fig. 4). During the first 10 h the erythromycin titre in the retentate-derived culture was higher than that observed in the filtrate-derived culture. This relationship between particle size fraction and antibiotic production appeared to break down as the culture incubation time increased, presumably because elongation and fragmentation took place during the subsequent incubation, resulting in loss of homogeneity of hyphal morphology. If baffled flasks were used to incubate the two fractions, the relationship between filtration and antibiotic production broke down more rapidly (within 3 h), presumably due to an increased rate of fragmentation.

Specific erythromycin production rate

The replacement culture experiment was repeated over 12 h and the biomass concentrations of the two cultures estimated so that specific production rate of the retentate- and filtrate-derived cultures could be measured. The

retentate productivity was 32% higher than that of the filtrate (specific erythromycin production rate at 12 h 1.74 versus 1.29 mg g⁻¹ h⁻¹), providing clear evidence that the larger hyphal fragments had a higher specific production rate than that of the smaller fragments; this is consistent with the notion that there are more antibiotic producing sites per unit hyphal mass in the larger particles.

Calculation of the minimum hyphal particle size needed to support antibiotic production

If it is assumed that there is a threshold hyphal particle size, below which particles are incapable of producing antibiotic (hypothetical), then the culture biomass consists of a proportion of non-producing biomass (small fragments) and a proportion of producing biomass (large fragments). If it is also assumed that the yield ($\Delta P/\Delta X$) of the *producing* biomass is the same in retentate and filtrate, then the different production capabilities of the two fractions simply reflect the proportion of producing biomass in each fraction.

Let A = weight of producing biomass in 1 g filtrate, C = weight of producing biomass in 1 g retentate, B = weight of non-producing biomass in 1 g filtrate, and D = weight of non-producing biomass in 1 g retentate.

$$\text{Antibiotic yield in the filtrate} = \frac{A \cdot x}{A + B} \quad [\text{g erythromycin (g filtrate biomass)}^{-1}]$$

where x = product yield [g erythromycin (g producing biomass)⁻¹].

$$\text{Antibiotic yield in the retentate} = \frac{C \cdot x}{C + D} \quad [\text{g erythromycin (g retentate biomass)}^{-1}]$$

The units for the ratio of the specific production rates of the two fractions are:

$$\frac{\text{g erythromycin (g filtrate biomass)}^{-1} \text{ h}^{-1}}{\text{g erythromycin (g retentate biomass)}^{-1} \text{ h}^{-1}}$$

This simplifies to:

$$\frac{\text{g erythromycin (g filtrate biomass)}^{-1}}{\text{g erythromycin (g retentate biomass)}^{-1}}$$

which can be obtained from the ratios of the expressions for productivity, above:

$$= \frac{\left[\frac{Ax}{A+B} \right]}{\left[\frac{Cx}{C+D} \right]} \quad (1)$$

This ratio is equal to 1.29/1.74 = 0.739 (see specific production rates in preceding section).

At the minimum fragment diameter for production (assuming there is such a thing), $d_{\min}$, the ratio of filtrate biomass greater than $d_{\min}$ to retentate biomass greater than $d_{\min}$ is equal to:

$$\frac{A}{C} \quad (2)$$

Fig. 5. The y-axis shows the proportion of hyphal fragments in the total population having a fragment size greater than that shown on the x axis for filtrate (Δ) and retentate ($\square$).

Fig. 6. Ratio of filtrate to retentate data shown in Fig. 5 as a function of fragment diameter.

Since $[A + B] = [C + D] = 1$ g, expression (1) is equal to expression (2). Thus at the minimum diameter for production the ratio of filtrate:retentate biomass = ratio of filtrate:retentate productivity = 0.74.

Checking this value against the corresponding hyphal fragment diameter (Figs 5 and 6) yields a diameter of 88 μm. Thus, if there is a minimum productive particle size, it is in the order of 88 μm, and below this size, productivity is insignificant.

Variation in hyphal particle size during batch culture

A bioreactor was sampled at intervals and filtration performed using the sintered glass filter. The proportion of retentate (larger particles, higher antibiotic production rate) to filtrate (smaller particles, lower antibiotic production rate) fraction varied with time (Fig. 7). The proportion of the less productive filtrate fraction ranged between 60% and 30%, indicating that culture production potential is dependent on culture development. Dependence of branching rate on nutrient concentration (Bushell, 1988) may account for the variation in fragment size distribution with time if it is hypothesized that the hyphal branching rate influences susceptibility to breakage. The rise in proportion of the smaller particle size fraction between 60 h and 120 h may reflect the increased

Fig. 7. Percentage of total biomass existing as filtrate (less productive fraction) as a function of time during nitrogen-limited batch culture.

appearance of unbranched hyphae (as a result of nutrient depletion) with greater susceptibility to breakage. Similarly, the overall decrease in proportion of the smaller particle size fraction during the early phase of the culture is consistent with a gradual increase in branching frequency (as a result of adaptation to relative nutrient abundance), leading to greater resistance to fragmentation. Alternatively, a mechanism such as that proposed by Paul *et al.* (1994), that mycelial breakage rate in fungi is dependent on the hyphal vacuolation rate induced by nutrient limitation, may also be operating in filamentous prokaryotes.

The observation (Fig. 7) that the proportion of bioreactor culture biomass capable of producing antibiotic varies significantly with time (assuming our hypothesis is correct) clearly has implications for commercial antibiotic production in terms of process economics. Our findings may also help to explain batch-to-batch variation in antibiotic production cultures, as the breakage susceptibility of inoculum cultures will vary according to nutrient availability and, probably, hyphal branching rate. These parameters are unlikely to be monitored at the time of inoculum transfer and our results indicate that they could influence antibiotic yield and productivity significantly.

Influence of stirrer speed on particle size distribution and productivity

In order to test the effect of stirrer speed on hyphal particle size distribution and productivity, chemostat cultures were operated at 1500 r.p.m. and 750 r.p.m. at a constant dilution rate (0.08 h^{-1}). It has been reported that prolonged chemostat culture provides selective pressure for mutation to morphological variants in filamentous micro-organisms (Trinci, 1994). Experiments reported in the present paper were, therefore, replicated using fresh inocula to new cultures for each stirrer speed regime.

The particle size distribution was significantly affected by

Fig. 8. Hyphal particle size distributions obtained during nitrogen-limited bioreactor culture at 1500 r.p.m. (□) and 750 r.p.m. (■).

Table 2. Effect of stirrer speed on erythromycin productivity in a chemostat culture at $D = 0.08 \text{ h}^{-1}$ and statistical analysis of the hyphal particle size distribution data

$C = 30.315$, for the two sets of particle size data; therefore reject null hypothesis that there is no difference between particle size distribution at the two speeds ($P = 0.01$).

	Stirrer speed (r.p.m.)	
	750	1500
Biomass concn (mg ml^{-1})	2.04	1.92
Erythromycin concn (mg l^{-1})	23.32	20.80
Specific antibiotic production rate ($\text{mg g}^{-1} \text{ h}^{-1}$)	0.913	0.867
Sample size	371	361
Skew (μm)	0.03	0.20
Sample mean (μm)	124.01	70.13

stirrer speed (Fig. 8, Table 2), with a mean particle diameter of $70 \mu\text{m}$ recorded at 1500 r.p.m. and $124 \mu\text{m}$ recorded at 750 r.p.m. These observations are consistent with studies of growth and fragmentation in fungal bioreactor cultures which indicated that hyphal fragmentation rate is linearly related to the energy applied to the bioreactor, via the impeller (Shamlou *et al.*, 1994; Nielsen & Krabben, 1995). The specific antibiotic production rate responded accordingly (Table 2), with a higher erythromycin production rate ($0.913 \text{ mg g}^{-1} \text{ h}^{-1}$) at 750 r.p.m. than at 1500 r.p.m. ($0.867 \text{ mg g}^{-1} \text{ h}^{-1}$). Since the experiment was carried out in chemostat culture, it was possible to eliminate most sources of difference between the two cultures other than particle size distribution. Thus, culture specific growth rate, dissolved oxygen and pH were identical in the two cultures and steady-state biomass concentration differed by less than 5%.

These results are consistent with the hypothesis that there

Fig. 9. Postulated relationship between hyphal elongation and growth in a stirred bioreactor culture, indicating the significance of the location of the antibiotic secretion sites.

is a critical hyphal fragment diameter (88 μm), which must be exceeded if the fragment is to be able to produce antibiotic. This would be the case if the site of antibiotic secretion was located at a fixed distance behind the growing tip (Fig. 9). Particles below the critical size would grow but not produce antibiotic until they had increased in size sufficiently to include the hypothetical antibiotic secretion site. The concept of an antibiotic secretion site, at a fixed distance from the growing tip, has been hypothesized to explain the dynamics of antibiotic production in *Penicillium chrysogenum* cultures (Nestas & Wang, 1983).

Hyphal breakage can also result in a decrease in viability (e.g. Jadubansa *et al.*, 1994). Presumably, in a stirred bioreactor, the ability to produce antibiotic is influenced by a series of equilibria (Fig. 9). These will include the growth of hyphal particles from non-producing units (diameter below 88 μm) to larger antibiotic-producing particles, opposed by breakage into smaller viable, non-producing units and, even smaller, non-viable units (Fig. 9).

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