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The physiology of erythromycin biosynthesis in cyclic fed batch culture

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Antibiotic production in *Saccharopolyspora erythraea* was significantly enhanced in cyclic fed batch culture (c.f.b.c) compared to batch culture, whereas chemostat culture resulted in reduced production. C.f.b.c. allowed the specific growth rate to be varied, with time, according to an asymptotically decreasing trajectory without the necessity for nutrient exhaustion. It was, therefore, possible to increase productivity by increasing the growth-limiting substrate concentration. It was necessary to apply the c.f.b.c regime to early-exponential-phase cultures in order to obtain a stable, nutrient-limited, c.f.b.c. The antibiotic production rate during any c.f.b.c. cycle was dependent on the relationship between the specific growth rate at the time and the growth rate at the start of the cycle.

Keywords: cyclic fed batch, erythromycin, antibiotic production

INTRODUCTION

Fed batch culture of micro-organisms in bioreactor culture has been used as a means of (1) controlling growth rate during exponential phase, (2) prolonging stationary phase and (3) supplying feeds of potentially deleterious nutrients and precursors to the culture at sub-inhibitory concentrations. Strategy 3 is well-established in antibiotic production processes (e.g. Queener & Swartz, 1979) and we have applied strategy 2 to antibiotic and monoclonal antibody production in, respectively, *Streptomyces* (Bushell, 1988) and hybridoma (Bushell *et al.*, 1994) cell cultures.

The control of growth rate (strategy 1) has been used to avoid oxygen limitation during exponential phase yeast production (Reed & Peppler, 1973). As the relationship between specific growth rate and oxygen uptake rate is stoichiometric (Bushell & Fryday, 1983), the oxygen requirements of the culture can be matched to the aeration capacity of the bioreactor. Cyclic fed batch culture (c.f.b.c), as implemented in the present study, is also a method for controlling growth rate, not at a constant rate, as in the yeast processes, but varying according to a defined profile.

The term cyclic fed batch culture has been used to describe a number of variants of the repeated fed batch culture technique. Gray & Vu-Trong (1987) used the term to describe regularly interrupted feeds of the growth-limiting substrate(s), resulting in enhanced tylosin yields in *Streptomyces fradiae* cultures. More recently, Lee & Low (1993) have used the term to describe algal cultures subjected to diurnally fluctuating lighting conditions. Theoretical treatments of cyclic feeding strategies have been based on the concept of uncoupled dilution and growth rates (e.g. Bosnak *et al.*, 1979; Bushell, 1989a), or of mycelial ageing factors or repressive substrates (e.g. Trilli *et al.*, 1976; Genon & Ruggeri, 1988).

In work reported here, the experimental design assumes that the down-regulation of culture growth rate acts as an effector for antibiotic production (see also Clark *et al.*, 1995) and also assumes the quasi-steady state concept of Pirt (1975) to describe the physiology of c.f.b.c.

Abbreviations: c.b.f.c., cyclic fed batch culture; c.f.c.c., cyclic fed chemostat culture.

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 antibiotic production medium for *S. erythraea* contained the following major nutrients (g l^{-1} in reverse-osmosis-purified water): glucose 6, $NaNO_3$ 11·21, KH_2PO_4 3·0, K_2HPO_4 7·0; and the following trace components (g l^{-1}): $MgSO_4.7H_2O$ 0·25, $FeSO_4.7H_2O$ 0·025, $CuCl_2$ 0·00053, $CoCl_2$ 0·00055, $CaCl_2.2H_2O$ 0·0138, $ZnCl_2$ 0·0104, $MnCl_2$ 0·0062, Na_2MoO_4 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 and *A. citreus* were routinely maintained on nutrient agar, and grown in 250 ml baffled Erlenmeyer flasks containing 50 ml medium at 30 °C on a rotary shaker at 250 r.p.m. A nutrient broth starter medium was inoculated with colonies picked off a plate. After 48 h agitation, 2 ml was removed and used (*S. erythraea*) to inoculate preculture flasks containing the defined medium. After 48 h further incubation, the precultures were used at approximately 5% (v/v) as an inoculum for bioreactors.

Bioreactor culture. The Braun Biostat MD bioreactor used had maximum and minimum working volumes of 10·5 l and 4·5 l, respectively. Agitation was provided by disc turbine impellers rotating at 1000 r.p.m., and sterile air was supplied through a sparger. The temperature was controlled at 30 °C. Dissolved oxygen concentration in the bioreactor was monitored with an Ingold polarographic dissolved oxygen electrode and maintained above 80% of air saturation by varying the airflow rate automatically. The pH was controlled at 7·0 using automatic additions of 0·1 M HCl and 0·1 M NaOH. Foaming was eliminated by including 0·01% (v/v) Breox FMT30 antifoam (Water Management and Gamlen) in the culture medium.

Medium feeds employed a Braun FE 211 high-precision piston-actuated dosing pump, controlled by microMFCS fermenter control software (Braun) running on a personal computer.

Determination of culture biomass concentration. Biomass samples (10 ml) were collected on pre-dried membrane filters (Whatman 0·45 μm). Filtrates were collected and frozen for later assays (erythromycin, glucose, nitrate and phosphate), and the filter was rinsed with distilled water (3 × 10 ml) before it was dried in a microwave oven (high power, 5 min). Dry weights were measured and biomass concentrations calculated after cooling and desiccation.

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.

Cyclic fed batch culture. Growth medium was pumped into the culture vessel at a constant rate, so that the culture volume increased with time from the initial value, V_{min}, (4·5 l) to the final value, V_{max} (9·9–10·5 l, depending on the desired dilution rate range). The time taken to complete this volume change constituted a single c.f.b.c. cycle. At the end of each cycle, the culture volume was reduced immediately from V_{max} to V_{min} using a fast peristaltic pump actuated by the microMCFS software. The dilution rate (flow rate/volume) therefore followed a constantly decreasing profile (Fig. 1) from D_{max} to D_{min}. The dilution rate at any point in the cycle is given by $D = F/[V_{min} + (F.t_c)]$, where D is the dilution rate (h^{-1}), F is the flow rate ($l\ h^{-1}$) and t_c is the elapsed cycle time (h).

Three c.f.b.c. dilution rate ranges, exploiting the full volume range of the bioreactor, were compared (Fig. 1).

Reproducibility and replication of experiments. All experimental data 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. Experiments were rejected where a χ-squared test indicated significant differences between replicates. Where error bars (representing SEM) are not shown, they were too small to be visible on the figures presented.

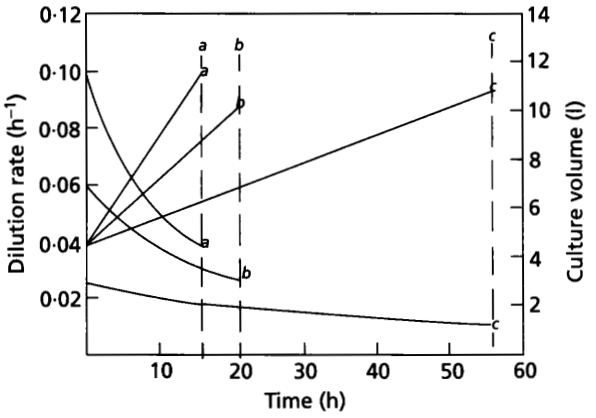

Fig. 1. Dilution rate profiles (curves), culture volume profiles (straight lines) and cycle times (dashed lines) for the three c.f.b.c. regimes studied: (a) 0·1–0·04 h^{-1}, (b) 0·06–0·027 h^{-1} and (c) 0·025–0·01 h^{-1}.

RESULTS AND DISCUSSION

Antibiotic production in c.f.b.c. compared to batch culture

At 6 g glucose l^{-1}, erythromycin titres in c.f.b.c. varied with dilution rate range (Table 1). Titres were higher than observed in batch culture at the low (Fig. 2) and medium dilution rate ranges. The erythromycin concentration at the low dilution rate range was 180% of the batch value (Fig. 2), and at the medium dilution rate range it was 113% of the batch value (Table 1). At the high dilution rate range a lower titre (88% of batch value) was obtained (Table 1). Overall specific productivity and gravimetric productivity both increased with increased dilution rate range. Specific productivity was, respectively, 80%, 115% and 192% of the batch culture value at the low, medium and high dilution rate ranges. However, the parameter with the most commercial significance is gravimetric productivity. Cyclic fed batch gravimetric productivity values were, respectively, 117%, 167% and 279% of the batch culture value. This represents 18·7 mg antibiotic h^{-1} in c.f.b.c. compared to 6·7 mg h^{-1} in batch culture. The c.f.b.c productivity was increased to 28·2 mg h^{-1} when the glucose concentration was increased to 15 g l^{-1} (Table 1).

Establishment of a quasi-steady state

Experimentation showed that the establishment of a successful c.f.b.c. regime was critically dependent on when the medium flow was begun during the initial batch

Table 1. Summary of erythromycin productivity data

Culture type	Maximum erythromycin concn (mg l^{-1})	Erythromycin overall specific productivity (mg g^{-1} h^{-1})*	Erythromycin gravimetric productivity in 10 l (mg h^{-1})†
Batch (glucose: 6 g l^{-1})	40	0·39	6·7
C.f.b.c.			
0·025–0·01 h^{-1} (glucose: 6 g l^{-1})	72	0·31	7·84
0·06–0·027 h^{-1} (glucose: 6 g l^{-1})	45	0·45	11·2
0·1–0·04 h^{-1} (glucose: 6 g l^{-1})	35	0·75	18·7
0·1–0·04 h^{-1} (glucose: 15 g l^{-1})	75	0·56	28·23
Chemostat (c.f.c.c.)			
0·04 h^{-1} (glucose: 6 g l^{-1})	10·36	0·16	4·14
0·07 h^{-1} (glucose: 6 g l^{-1})	9·99	0·25	6·99
0·10 h^{-1} (glucose: 6 g l^{-1})	8·28	0·31	8·25
0·12 h^{-1} (glucose: 6 g l^{-1})	6·15	0·27	7·38

*{[titre (g l^{-1}) at $V_{max} \times V_{max}$] − [titre (g l^{-1}) at $V_{min} \times V_{min}$]}/{biomass concentration × cycle time}.
†{[titre (g l^{-1}) at $V_{max} \times V_{max}$] − [titre (g l^{-1}) at $V_{min} \times V_{min}$]}/{cycle time}.

phase. If flow was initiated after early exponential phase, when growth-limiting substrate concentration had declined beyond 50% of its original value, progressive culture wash-out was observed during each cycle and a stable c.f.b.c was not obtained. Therefore, in the experiments reported here, flow was initiated immediately after the point at which an increase in biomass concentration was first observed.

After the third cycle, a quasi-steady state (Pirt, 1975) was observed, characterized by constant biomass and undetectable glucose concentrations. By analogy with the well-known expression

$$\mu = \frac{dx}{dt} \cdot \frac{1}{x}$$

where μ is the specific growth rate (h^{-1}) and $\frac{dx}{dt}$ the rate of biomass increase at biomass concentration x (g l^{-1}), if the culture volume is changing, the growth rate, μ, is given by:

$$\mu = \frac{x_t . V_t - x_0 . V_0}{t} \cdot \frac{1}{x_0 . V_0}$$

where x_t is the biomass concentration and V_t the volume after time t (infinitesimal), and x_0 and V_0 are the same parameters before time t. As the biomass concentration did not vary significantly during any of the c.f.b.c. cycles studied, it was assumed that $\mu \approx D$.

The product concentration increased with cycle number (Fig. 3). This can be partially accounted for by the carry-over effect of 4·5 l of culture from the previous cycle (containing antibiotic) being present at the start of each new cycle. Even allowing for this, however, the net titre continued to increase at 0·28 mg l^{-1} per cycle (approximately 7·7% per cycle). A possible explanation for this is that an increasing proportion of the culture adapted to c.f.b.c. with each cycle, resulting in an enhanced ability of the culture to respond to the constantly changing conditions (down-regulation of growth rate) by more rapid initiation of antibiotic production. If correct, this is encouraging as it indicates that selective pressures involved in prolonged exposure to c.f.b.c. may not result in deleterious effects on productivity.

Effect of dilution rate range on erythromycin productivity

Antibiotic titre and specific production rate varied with dilution rate range in c.f.b.c. (Fig. 4a, b). Both also varied throughout each cycle, the productivity tending to show a sustained rise during the later part of the cycle (Fig. 4b). There appeared to be no simple relationship between productivity and dilution rate *per se* during a cycle (Fig. 4c, d).

Despite the observation that maximum erythromycin concentration increased with c.f.b.c. cycle time, overall specific and gravimetric productivity decreased (Table 1), indicating the overriding economic importance of process rate compared to the significant yield increases obtainable in this system.

Erythromycin production at constant dilution rate

To assess the effects of the changing dilution rate regime in c.f.b.c., cultures were set up at constant dilution rate. In order to provide a culture system more comparable to c.f.b.c. than conventional chemostat culture, a variable-volume, variable-flow system was used in which the

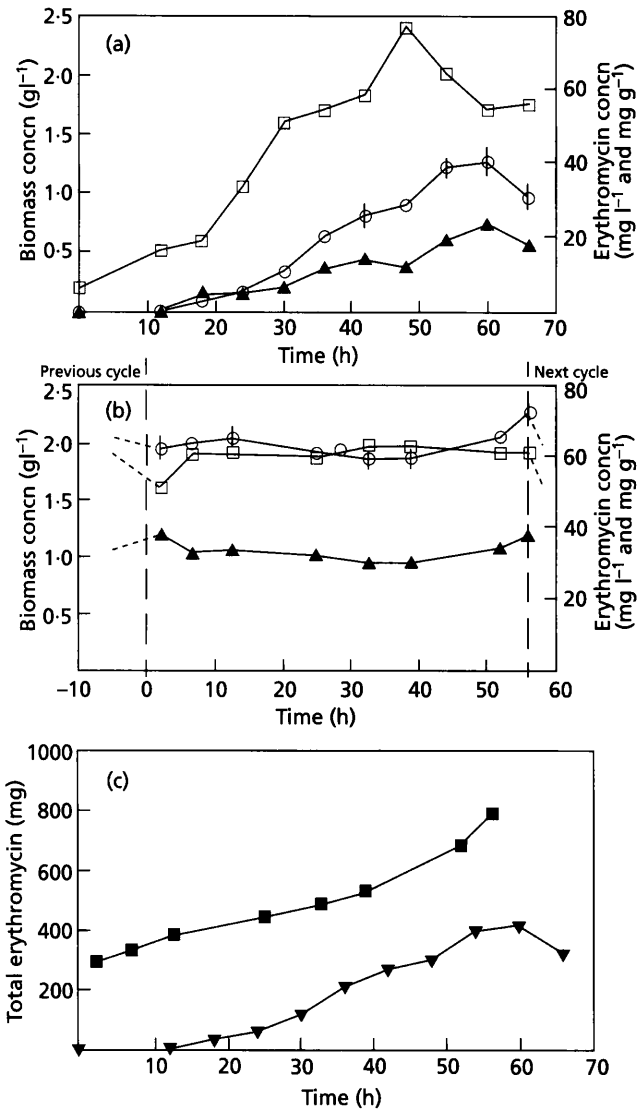

Fig. 2. Erythromycin concentration (□), biomass concentration (○) and specific erythromycin production [mg erythromycin produced (g biomass)$^{-1}$] (▲) in (a) carbon-limited batch culture and (b) a representative cycle of a c.f.b.c at 0·025–0·01 h^{-1}. (c) Total erythromycin produced in batch (▼) and c.f.b.c. (■).

culture vessel filled up at a variable rate so that the dilution rate was maintained at a constant value (Fig. 5). The additional advantage of this system over chemostat culture was that potential differential wash-out of hyphal fragments of different size (S. Martin, personal communication) was avoided. Chemostat steady-state conditions were obtained during which the specific growth rate was equal to the dilution rate, with biomass concentration remaining constant and culture growth-limiting substrate (glucose) concentrations maintained at undetectable levels.

Erythromycin titres obtained in this cyclic fed chemostat culture (c.f.c.c.) were less than those obtained in either batch culture or c.f.b.c. Specific productivity was also lower than in both of the other systems except for c.f.b.c at 0·025–0·01 h^{-1} (Table 1).

Titre increase due to increased growth-limiting substrate concentration

Increasing the growth-limiting substrate concentration to 15 g l^{-1} in c.f.b.c. resulted in corresponding increases in antibiotic titre and gravimetric productivity at a dilution rate range of 0·1–0·04 h^{-1} (Fig. 6, Table 1). This highlights one of the significant potentials of the technique in secondary metabolite production. Production of antibiotics in batch culture invariably requires the exhaustion of the growth-limiting substrate This leads to a process design conflict. Increasing growth-limiting substrate concentration is required to increase biomass, and hence antibiotic concentration. However, the time taken for assimilation of growth-limiting substrate levels to growth-rate-limiting concentrations will increase as the initial growth-limiting substrate concentration is raised, resulting in longer process times. In c.f.b.c., the growth rate decay profile during a cycle is the same, irrespective of the growth-limiting substrate concentration in the feed, once a quasi-steady state has been achieved. Thus, productivity and titre may be increased simultaneously. In this experiment, the specific yield [~ 19 mg (g biomass)$^{-1}$] was the same at 15 g glucose l^{-1} as that observed in cultures fed with 6 g glucose l^{-1}, indicating

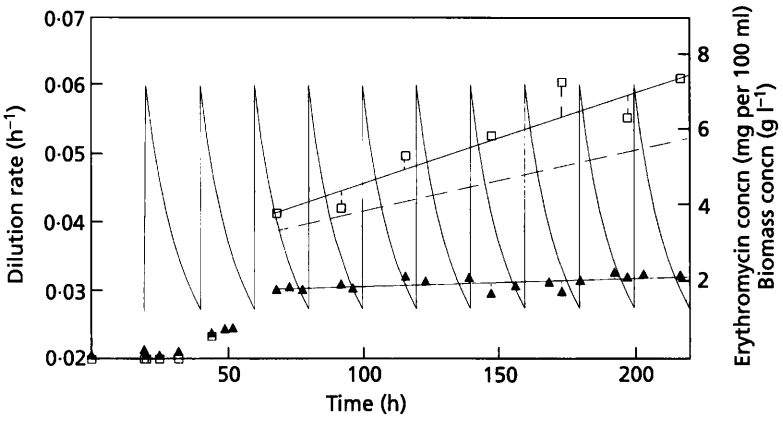

Fig. 3. Observed erythromycin concentration (□; linear regression, solid straight line), theoretical erythromycin concentration (dashed line), biomass concentration (▲) and dilution rate (curves) over successive cycles of a c.f.b.c. at 0·06–0·035 h^{-1}.

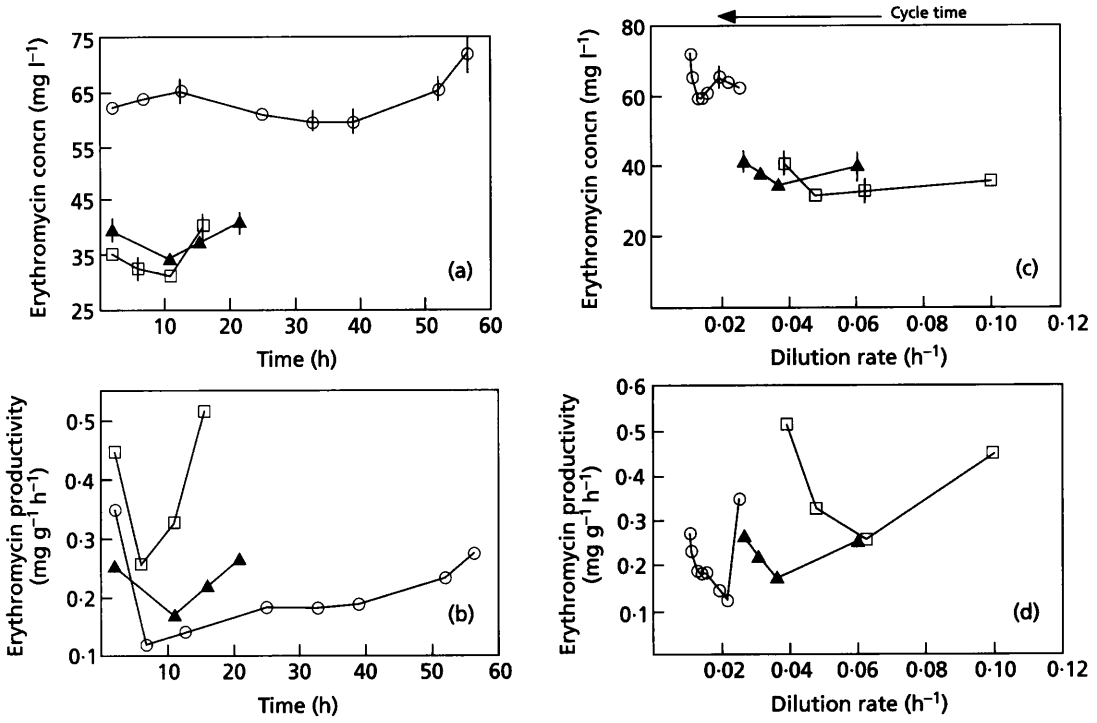

Fig. 4. (a, c) Erythromycin concentration and (b, d) specific erythromycin productivity rate [mg erythromycin produced (g biomass)$^{-1}$ h^{-1}] as a function of cycle time (a, b) and dilution rate (c, d) in c.f.b.c. at 0·025–0·01 h^{-1} (○); 0·06–0·027 h^{-1} (▲) and 0·1–0·04 h^{-1} (□).

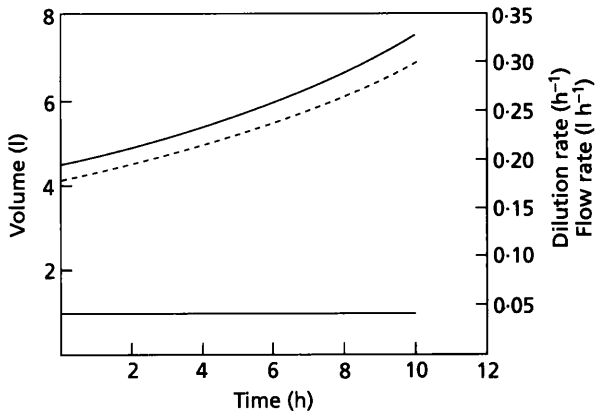

Fig. 5. Dilution rate (straight line), flow rate (dashed curve), and volume (solid curve) in a constant dilution rate continuously fed system (c.f.c.c.).

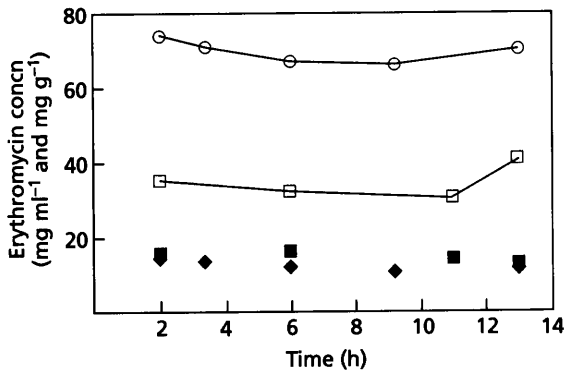

Fig. 6. Erythromycin concentration at 6 g glucose l^{-1} (□) and 15 g glucose l^{-1} (○), and specific erythromycin production rate at 6 g glucose l^{-1} (■) and 15 g glucose l^{-1} (◆) in c.f.b.c. at 0·1–0·04 h^{-1}.

that the titre increase observed was, indeed, due to the increase in biomass concentration.

Batch vs chemostat vs cyclic fed batch culture

Our detailed analysis of specific growth rate profiles in batch culture has elucidated the relationship between specific growth rate profile and initiation of antibiotic production in batch culture (McDermott *et al.*, 1993). That study demonstrated that antibiotic production in carbon-limited batch culture is initiated only when the specific growth rate is falling. If down-regulation of growth rate is the trigger for initiation of secondary metabolite production, then it is reasonable to expect high antibiotic production rates at the end of batch culture and throughout c.f.b.c. cycles. Furthermore, the steady-state

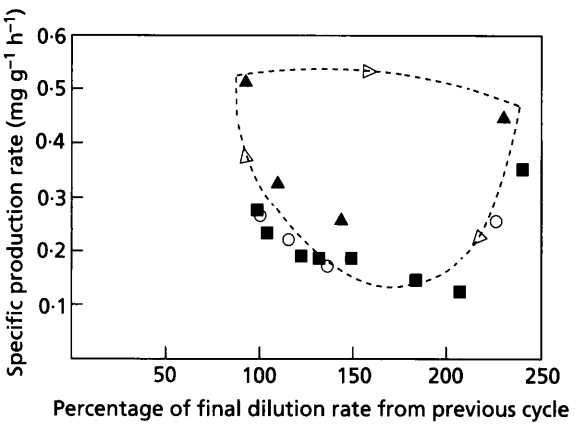

Fig. 7. Pooled data for specific erythromycin productivity from all of the c.f.b.c. experiments as a function of D_{max}/D (as a percentage), where D is the dilution rate when the productivity was measured and D_{max} is the maximum dilution rate for the cycle. The dotted line and arrowheads indicate the time trajectory (the locus and direction of the time axis). The dilution rate ranges were: 0·025–0·01 h^{-1} (■); 0·06–0·027 h^{-1} (○); and 0·1–0·04 h^{-1} (▲).

conditions of chemostat culture would be expected to support little antibiotic production. The observations reported in this paper are consistent with these ideas. The fact that any production occurs at all during chemostat culture is possibly a reflection of the heterogeneity amongst a population of hyphal fragments. Unlike unicells, each hyphal fragment will contain mycelium whose age is a function of its distance from the growing tip. The concept of a steady state in a culture consisting of a population of hyphal fragments of different size, branch number and growth rate may, therefore, be flawed.

The specific productivity values obtained during the course of a c.f.b.c. cycle appeared to be related to the growth rate up-regulation event taking place between the end of one cycle and the beginning of the next. Pooling the data suggested that the period of decreasing productivity trend observed at the beginning of each cycle (Fig. 4) was a consequence of the difference between the prevailing growth rate and the initial growth rate in each cycle (Fig. 7). An increase in productivity with time was observed only when the prevailing growth rate had dropped below 150% of D_{min}.

Our previous studies have described the induction of secondary metabolite production due to the effect of dissolved nutrient limitation (McDermott *et al.*, 1993) and oxygen depletion (Clark *et al.*, 1995) on culture growth rate in batch culture. In c.f.b.c. we have been able to down-regulate growth rate directly, without the necessity of nutrient exhaustion.

Production of antibiotic as a response to down-regulation of growth rate is compatible with the 'biowars' function for secondary metabolites (Bushell, 1989b). The arrival of a competitor organism in a micro-habitat resulting in a sufficient down-regulation in growth rate of the original inhabitant, due to competition for nutrients, would elicit production of antibiotic as a defensive measure. Production of secondary metabolites only when necessary would have an evolutionary advantage over the biosynthetic expense of constitutive production. Secondary metabolism at the end of closed batch culture may, therefore, be a consequence of the fact that rapid decay in growth rate is only observed when the growth-limiting substrate concentration approaches the value of the substrate affinity constant (K_s), which is 0·015 mg glucose ml^{-1} in *S. erythraea* (McDermott *et al.*, 1993). If this hypothesis is accepted, the growth rate down-regulation profile observed in c.f.b.c. is simulating the arrival of a competitor, resulting in enhanced production of the antibiotic.

REFERENCES

Bosnjak, M., Topolovec, V. & Johanides, V. (1979). Growth kinetics and antibiotic synthesis during the repeated fed-batch culture of Streptomycetes. *Biotechnol Bioeng Symp* **9**, 155–165.

Bushell, M. E. (1988). Growth, metabolism and fermentation technology. In *Biotechnology of the Actinomycetales*, pp. 185–215. Edited by M. Goodfellow, S. T. Williams & M. Mordarski. London: Academic Press.

Bushell, M. E. (1989a). The process physiology of secondary metabolite production. In *Microbial Products: New Approaches (Society for General Microbiology Symposium* no *44*), pp. 95–120. Edited by S. Baumberg, I. S. Hunter & P. M. Rhodes. Cambridge: Cambridge University Press.

Bushell, M. E. (1989b). Biowars in the bioreactor. *New Sci* **124**, 42–45.

Bushell, M. E. & Fryday, A. (1983). Application of materials balancing to the characterization of sequential metabolite formation in *Streptomyces cattleya* NRRL 8057. *J Gen Microbiol* **129**, 1733–1741.

Bushell, M. E., Bell, S. L., Scott, M. F., Spier, R. E., Wardell, J. N. & Sanders, P. G. (1994). Enhancement of monoclonal antibody yield by hybridoma fed-batch culture, resulting in extended maintenance of viable cell population. *Biotechnol Bioeng* **44**, 1099–1106.

Clark, G. J., Langley, D. & Bushell, M. E. (1995). Oxygen limitation can induce microbial secondary metabolite formation: investigations with miniature electrodes in shaker and bioreactor culture. *Microbiology* **141**, 663–669.

Genon, G. & Ruggeri, B. (1988). The stability conditions of the fed-batch reactor. Part 1: Monod type growth kinetics. *J Chem Technol Biotechnol* **42**, 261–277.

Gray, P. P. & Vu-Trong, K. (1987). Production of the macrolide antibiotic tylosin in cyclic fed batch culture. *Biotechnol Bioeng* **29**, 33–40.

Huck, T. A., Porter, N. & Bushell, M. E. (1991). Positive selection of antibiotic-producing soil isolates. *J Gen Microbiol* **137**, 2321–2329.

Lee, Y. K. & Low, C. S. (1993). Productivity of outdoor algal cultures in unstable weather conditions. *Biotechnol Bioeng* **41**, 1003–1006.

McDermott, J. F., Lethbridge, G. & Bushell, M. E. (1993). Estimation of the kinetic constants and elucidation of trends in growth and erythromycin production in batch and continuous cultures of *Saccharopolyspora erythraea* using curve-fitting techniques. *Enzyme Microb Technol* **15**, 657–663.

Pirt, S. J. (1975). *Principles of Microbe and Cell Cultivation*, pp. 211–213. Oxford: Blackwell Scientific Publications.

Queener, S. & Swartz, R. (1979). Penicillins; biosynthetic and semisynthetic. In *Economic Microbiology, 3. Secondary Products of Metabolism*, pp. 35–123. Edited by A. H. Rose. London. Academic Press.

Reed, G. & Peppler, H. J. (1973). *Yeast Technology*, pp. 664–668. Westport: Avi.

Trilli, A., Michelini, V., Mantovani, V. & Pirt, S. J. (1976). Estimation of productivities in repeated fed batch cephalosporin fermentation. *J Appl Chem Biotechnol* **27**, 219–224.

Tsuji, K. & Goetz, J. F. (1978). High performance liquid chromatographic determination of erythromycin. *J Chromatogr* **147**, 359–367.

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