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Effects of fermentation feeding strategies prior to induction of expression of a recombinant malaria antigen in *Escherichia coli*

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SUMMARY

A variety of feeding strategies have been described for attaining high cell densities in fed-batch fermentors. Although cell density is an important component in the productivity of recombinant fermentations, it must be achievable with high product expression levels. Experiments were conducted to study the influence of fermentation feeding strategies on the production of a recombinant malaria antigen in *Escherichia coli.* C-source feeding profiles were calculated to maintain specific growth rates at 0.1, 0.2, 0.35, and 0.5 l/h prior to induction in defined and complex media using an exponential growth model. Fed-batch fermentations employing these feeding profiles effectively controlled the specific growth rates prior to induction. Antigen yields per dry cell weight did not vary with specific growth rate. Antigen yields from fed-batch fermentations achieving high cell densities were similar to batch fermentations achieving low cell densities. These results show that C-feeding policies can limit growth without reducing expression levels in some systems, and suggest applications in managing oxygen demand and catabolic by-product formation during process scale-up.

INTRODUCTION

A variety of process strategies have been developed for the achievement of high cell densities in batch fermentation [1–5,7,10,12,14]. Common goals are the controlling of oxygen demand within the oxygen transfer capabilities of the fermentor, and the avoidance of the accumulation of partially oxidized catabolites such as acetate and ethanol. These goals can be met by feeding the carbon source to achieve C-limited growth, reducing temperature to decrease the growth rate, or increasing the oxygen transfer capability of the fermentor using oxygen as the sparging gas. Cell densities of 80 g/l and productivities of 6.2 g/l-h have been reported for the growth of *Escherichia coli* [7], and cell densities of 138 g/l and productivities of 5.8 g/l-h have been reported for yeast [12].

In situations involving microbial products other than biomass, fermentation results depend on the yield of product from biomass in addition to biomass concentration and growth rate. There are still

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relatively few published reports on the impact of the fermentation process on product yield, in spite of the importance of the variable on process performances. This contribution examines the effect of feeding strategies capable of achieving high cell densities on the yield of a recombinant protein in *E. coli*. This differs from most other reports by examining growth rate effects prior to induction of product expression rather than during product expression.

MATERIALS AND METHODS

Host/vector system

The clone used in these experiments was provided by Dr. James F. Young in the Molecular Genetics Department at Smith Kline & French Laboratories. The details concerning the preparation of the expression system are available in Ref. 13. The production vector pR32tet$_{32}$Kn is a derivative of pAS1, which has been developed for the efficient expression of a variety of heterologous proteins in *E. coli* [9]. It contains the λ phage promoter P_L, a kanamycin resistance gene, and the expression cassette. The AR58 host is a λ lysogen (F^-su^- galK2 $lacZ^-$ thr^- bio^- gal E::Tn10, tet^r λcI^{857} ΔHI Δ8 kil^- $chlA^-$ $uvrB^-$) derived from the standard NIH *E. coli* K12 strain N99. The host contains a defective λ lysogen, and produces a temperature-sensitive cI857 repressor. At 32°C, the repressor binds to the P_L promoter on the plasmid and blocks transcription of the expression cassette. At 42°C, the repressor is inactivated and the desired gene product is expressed.

Media preparation

Compositions of the complex and defined media employed are shown in Table 1. Solutions of kanamycin sulfate, biotin and threonine, N-feed and trace elements ($MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, and H_3BO_4) were sterilized separately using 0.2-μm membrane filters. The remaining components were steam-sterilized together in shaker flasks, fermentors, or feed aspirator bottles for 15–20 min at 121°C. The component solutions were combined after sterilization and cooling to give the compositions shown in Table 1.

To simplify this discussion, the yeast extract feed was assumed to be C-limiting, although the actual limiting nutrient(s) was not determined. The analysis to follow does not depend on the identification of the growth-limiting nutrient.

Fermentor inoculum

Cells were stored as 0.75 ml portions suspended in 10% glycerol and 20% dried skim milk at −70°C. Shake flasks (2.8-l) containing 1.0 l of sterile medium were inoculated with 100 μl of thawed cells. The shake flasks were incubated at 32°C and 200 rpm for 15 h, achieving an optical density (OD) of approximately 5. These cultures were used to inoculate the fermentors.

Fermentation

Fermentations were conducted in 15 l fermentors interfaced to an Emcon D3 direct digital control (DDC) system. Measurement of temperature, pressure, pH, dissolved oxygen (DO), sparging rate, agitation rate, feed reservoir weight, and off-gas composition (O_2, CO_2, N_2, H_2O, NH_3, C_2H_5OH) were acquired by the system at 10-s intervals. Digital proportional-integral-derivative (PID) feedback control loops were used to control temperature, pH, DO, agitation rate, sparging rate, and feeding rate. Calculated variables include culture volume, biomass concentrations (X), growth rate, specific growth rate (SGR), oxygen transfer rate (OTR), carbon dioxide transfer rate, respiratory quotient, O_2 mass transfer coefficient and feeding rate.

Fermentors were charged with sufficient medium to give volumes of 5, 7, or 10 l after sterilization and inoculation, depending on whether the fermentation was fed-batch using the defined medium, fed-batch using the complex medium, or batch, respectively. Shake flask cultures were transferred to 1.0-l aspirator bottles, and sufficient volume was pumped from the aspirator to the fermentor to give an OD of 0.5 after inoculation. In fed-batch fer-

Table 1

Media compositions

Media	Component	Shake flask	Initial fermentor charge	C-feed solution	N-feed solution	Unit
Complex	Yeast extract	24.0	24.0	300	0	g/l
	Bacto tryptone	12.0	120	0	0	g/l
	K_2HPO_4	15.3	153	0	0	g/l
	KH_2PO_4	1.7	17	0	0	g/l
	Kanamycin sulfate	50	0	0	0	mg/l
	PPG2000	0.05	2.0	0	0	ml/l
	NH_4OH	0	0	0	165	g/l
Defined	Glycerol	13	6	900	0	g/l
	KH_2PO_4	1.7	3.4	0	0	g/l
	K_2HPO_4	15.3	30.6	0	0	g/l
	$(NH_4)_2SO_4$	3.0	6.0	0	100	g/l
	$MgSO_4 \cdot 7H_2O$	0.3	0.6	3.0	0	g/l
	$CaCl_2 \cdot 2H_2O$	0.1	0.2	1.0	0	g/l
	$FeCl_3 \cdot 6H_2O$	0.027	54.0	270	0	mg/l
	$ZnSO_4 \cdot 7H_2O$	0.007	14.0	70	0	mg/l
	$MnCl_2 \cdot 4H_2O$	0.005	10.0	50	0	mg/l
	$Na_2MoO_4 \cdot 2H_2O$	0.0024	48.0	24	0	mg/l
	$CuSO_4 \cdot 5H_2O$	0.001	2.0	10	0	mg/l
	$CoCl_2 \cdot 6H_2O$	0.001	2.0	10	0	mg/l
	H_3BO_4	0.0003	0.6	3	0	mg/l
	Kanamycin sulfate	50	0	0	0	mg/l
	Biotin	0.1	0.2	1.0	0	mg/l
	Threonine	50	100	500	0	mg/l
	PPG2000 antifoam	0.05	2.0	0	0	ml/l
	NH_4OH	0	0	0	165	g/l

mentations, the sterile C-feed was pumped from a 4 l feed reservoir mounted on a load-cell to the fermentor using a peristaltic pump whose speed was controlled by the DDC computer system. Feeding rate set points were pre-programmed according to the calculations below. Pump speed was manipulated by differentiating the reservoir weight to obtain the measured feed rate and using a digital PID feedback loop to obtain agreement between the measured and setpoint feeding rates. In the case of the defined media fed-batch experiments, an initial charge of glycerol feed was made following inoculation to allow the culture to obtain a growth rate corresponding to the minimum flow rate obtainable from the pumping system (0.2 ml/min).

Other fermentation conditions were as follows. DO was maintained at or above 20% of saturation by varying the agitation rate between 300 and 1000 rpm and the sparging rate between 5 and 20 standard liters per minute (SLPM). Vessel pressure was controlled at 1.0 Bar (gauge). In some instances, a 40% oxygen in nitrogen gas mixture was used to sparge the fermentor when it was no longer possible to control DO above 20% using air. A 165 g/l solution of ammonium hydroxide was pumped into the fermentor to maintain the pH above 7. Temperature was controlled at 32°C during growth. Expression of the recombinant product was induced by increasing the temperature from 32°C to 42°C in 15 min. Samples were taken periodically for the determination of colony forming units (CFU), optical density (OD) at 650 nm, dry cell weights (DCW), glycerol concentration, and yield of antigen using the methods described below.

Table 2

Equations for a fed-batch fermentor

t is time, V is culture volume, F_C and F_N are feed rates of C-source and N-source solutions, respectively, X is the biomass concentration, μ is the specific growth rate, C and N are the concentrations of C-source and N-source in the culture, respectively, C_F and N_F are the concentrations of C-source and N-source in the feed solutions, respectively, $Y_{X/C}$ and $Y_{X/N}$ are the overall yields of biomass from C-source and N-source, respectively, Y_{growth} is the growth yield, and M is the maintenance coefficient.

Volume: $$\frac{dV}{dt} = F_C + F_N \quad (1)$$

Biomass: $$\frac{d}{dt}(V X) = V \mu X \quad (2)$$

C-source $$\frac{d}{dt}(V C) = F_C C_F - \frac{V \mu X}{Y_{X/C}} = 0 \quad (3)$$

N-source: $$\frac{d}{dt}(V N) = F_N N_F - \frac{V \mu X}{Y_{X/N}} = 0 \quad (4)$$

Solutions: $$V = \frac{V_0}{\mu}[X_0 A \exp(\mu t) + \mu - A X_0] \quad (5)$$

$$X = \frac{\mu X_0}{(\mu - A X_0)\exp(-\mu t) + A X_0} \quad (6)$$

$$F_C = \frac{V_0 \mu X_0 \exp(\mu t)}{Y_{X/C} C_F} \quad (7)$$

$$F_N = \frac{V_0 \mu X_0 \exp(\mu t)}{Y_{X/N} N_F} \quad (8)$$

where:

$$A = \frac{\mu}{C_F Y_{X/C}} + \frac{\mu}{N_F Y_{X/N}} \quad (9)$$

$$\frac{1}{Y} = \frac{M}{\mu} + \frac{1}{Y_{growth}} \quad (10)$$

Feeding control policy

Control policies have been classified as physiological or optimal depending on the formulation of the control problem [6]. A physiological approach was used in this work in which the control of the specific growth rate was the objective of the control policy. A simple mathematical model was used to calculate the feeding rate profile for the C-source required to limit growth to a specified growth rate during a fed-batch fermentation. The differential equations (Eqns. 1–4) and their solutions (Eqns. 5–10) for a fed-batch fermentor are shown in Table 2. The key assumptions are: volumes are additive, growth is exponential, a quasi-steady state exists for the concentrations of C-source and N-source, overall yields depend on the growth yields and maintenance coefficients as defined by Eqn. 10, and Y_{growth} and M are constant. The specific growth rate was controlled using an open-loop control policy by varying the feeding rate according to the calculated profile. A conventional PID feedback control loop was used to match the actual feed rate with the planned feed rate.

Preliminary experiments were used to define $Y_{X/C}$ and $Y_{X/N}$. Under the experimental conditions, maintenance requirements were small, allowing M to be set to zero. The N-source (NH_4OH) was added to control pH rather than according to the calculated feeding profile. It was necessary, however, to calculate a N-source feeding profile during the simulation to approximate the dilution of the culture by the N-source solution during fermentations employing the defined medium. Results of the simulation for the first 6 h used to calculate the C-source feeding profile for Run 151 are shown in Fig. 1. Note that a maximum biomass concentration is reached when the dilution of the culture by the feed is balanced by the increasing biomass so that no further increase in biomass concentration is possible. Since this theoretical limit is strongly dependent on C_F, N_F, $Y_{X/C}$, and $Y_{X/N}$, this simulation provides a convenient tool to plan fed-batch fermentations where high biomass concentration is an objective.

Analytical procedures

Biomass concentration was established by measuring optical density (OD) at 650 nm of samples diluted to give an OD of 0.005–0.6. The result was converted to a dry cell weight (DCW) concentration using a calibration curve relating OD measurements with independently measured dry cell weights.

Colony forming units (CFU) were used as a measure of culture viability and plasmid retention. Samples of whole broth were serially diluted (10^{-1}–10^{-7}) in sterile saline. A 100-μl portion was spread on Luria broth (LB) agar in a 100-mm petri dish. CFUs were counted on plates containing 30–300 colonies after incubation at 32°C for 24–48 h.

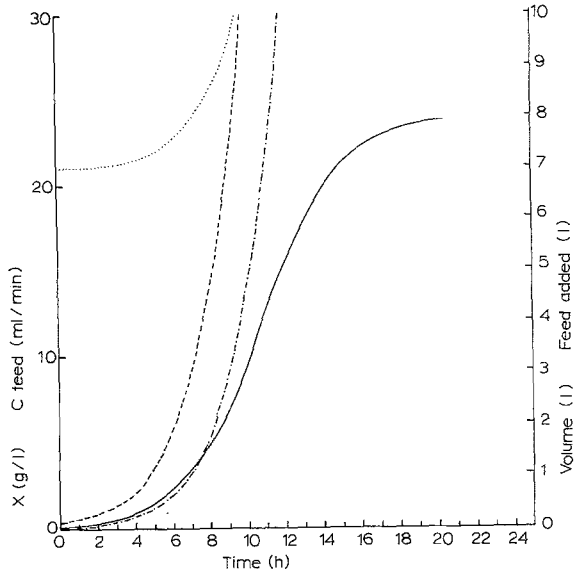

Fig. 1. Simulation of a fed-batch fermentation using a C-source feed containing 300 g/l YE and controlling specific growth rate at 0.5 l/h. An initial volume of 7 l, an initial biomass concentration of 0.12 g/l, and a yield of 0.08 g biomass/g YE were specified. ——, biomass concentration (g/l); ....., culture volume (l); – – –, C-source feeding rate (ml/min); ·–·–·, total feed added (l).

Glycerol concentrations in clarified broth samples were measured using an enzymatic assay kit (Boehringer Mannheim Cat. No. 148,270). The method phosphorylates the glycerol using ATP. ATP is regenerated by converting phosphoenolpyruvate to pyruvate. The pyruvate is reduced to form lactate, and the amount of NADH oxidized in this reaction is measured spectrophotometrically.

Antigen yields were measured using an automated particle concentration fluorescence immunoassay (PCFIA) system (Pandex Laboratories, Mundelein, IL). Culture samples of 50–100 μl were centrifuged and the supernatant removed to yield cell pellets. The cells were resuspended in 400 μl of Laemmli buffer (Tris 15.1 g/l, SDS 20 g/l, glycerol 20 g/l, 2-mercaptoethanol 50 g/l, bromphenol blue 20 g/l, pH 6.8). The cells were lysed by heating to 100°C for 5 min. After cooling to room temperature, the lysate was serially diluted in buffer and 50 μl of each lysate dilution was added to a microtiter well. A bead bearing a monoclonal antibody (MAB) against the antigen was immersed in the sample to bind the antigen. The bead was washed and was exposed to a second MAB against the antigen, bearing a fluorescence label, which bound to the antigen bound on the bead. The surface fluorescence of the bead was measured and the result was obtained using a standard curve prepared using pure antigen.

RESULTS

To determine the effect of C-limited growth at various specific growth rates on product expression levels, fermentations were run in which the C-source was fed according to the open-loop control strategy described above. A feed containing 30% (w/v) yeast extract was added according to feed rate profiles calculated to given specific growth of 0.50, 0.35 and 0.20 l/h prior to induction. After induction at an OD of 10, the feed rates were adjusted to give a specific growth rate of 0.5 l/h. In this manner, the effect of the specific growth rate during growth on expression levels could be isolated for study.

An experimental control using a batch fermentation containing 90 g/l of yeast extract was run, and the results are shown in Fig. 2. Exponential growth at a specific growth rate of 0.58 l/h was ob-

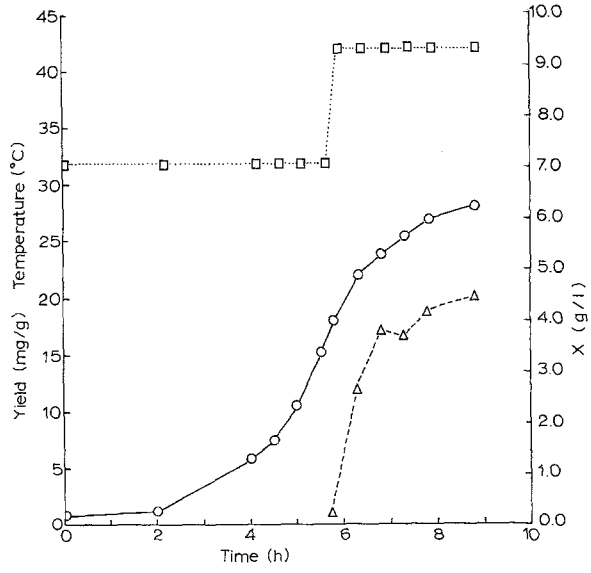

Fig. 2. Batch fermentation using the YE medium. ○, biomass concentration (g/l); □, temperature (°C); △, antigen yield (mg antigen/g biomass).

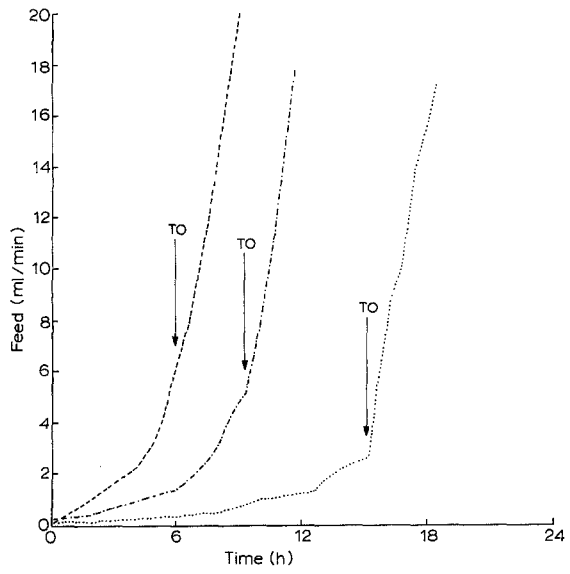

Fig. 3. YE (300 g/l) feeding rate profiles for fed-batch fermentations controlled at three different specific growth rates prior to induction. TO designates the time an OD of 5 was reached and expression was induced. Feeding rates appropriate to sustain a specific growth rate of 0.5 l/h were used after induction. ——, planned μ = 0.50 l/h (Run 151); ·—·—·, planned μ = 0.35 l/h (Run 152); ....., planned μ = 0.20 l/h (Run 153).

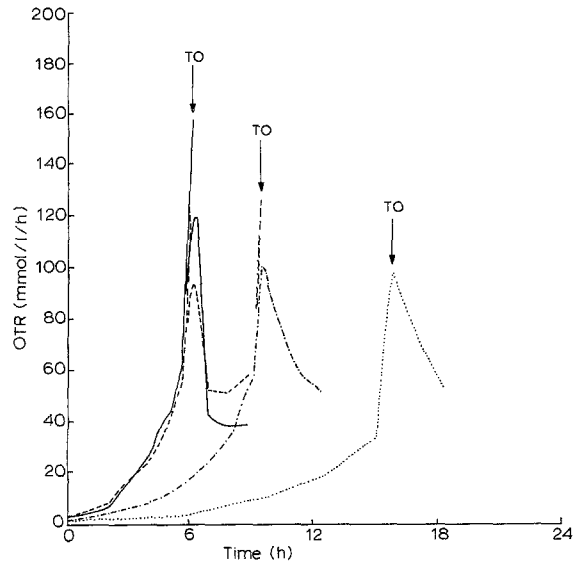

Fig. 4. Oxygen transfer rates for fed-batch fermentations controlled at three different specific growth rates prior to induction and a batch fermentation. TO designates the time that an OD of 5 was reached and expression was induced. Runs 150 and 151 were induced at the same elapsed time. ——, batch (Run 150); ——, planned μ = 0.50 l/h (Run 151); ·—·—·, planned μ = 0.35 l/h (Run 152); ....., planned μ = 0.20 l/h (Run 153).

tained up to induction. The culture was induced when it reached an OD of 10 at approximately 5.5 h by increasing the temperature from 32 to 42°C. Synthesis of the product followed shortly thereafter and the product was allowed to accumulate for 3 h at 42°C.

The feeding profiles used for the fed-batch experiments are shown in Fig. 3. All show an exponential-like curve up to the time corresponding to an OD of 10, when the cultures were induced. Thereafter, the curves are identical, corresponding to a specific growth rate of 0.5 l/h. The resulting oxygen transfer rates (OTR), which are representative of growth rate in these experiments, are shown for the four runs in Fig. 4. The exponential-like increase in OTR ends with a sudden increase, corresponding to the start of induction, followed by a gradual decrease during product accumulation. The linearity of the growth curves on semi-log coordinates shown in Fig. 5 demonstrate the effectiveness of feeding strategy at regulating the specific growth rate at a constant value. Good agreement between the planned and realized values of the specific growth rates was obtained. The similarity of the batch fermentation with the fed-batch fermentation with the highest specific growth rate is evident in Fig. 4 and 5. Yields of antigen per g of dry cell weight are shown in Fig. 6. The cellular rates of antigen synthesis in the fed-batch fermentors were the same within experimental error ($\pm$ 10%), and somewhat greater than those determined from the batch control. Maximum yields of 22 mg/g $\pm$ 10% were obtained in all four cases, indicating that the specific growth rate prior to induction has no significant effect on product expression levels.

Difficulties in obtaining C-limited growth and purification considerations were among the factors that prompted the development of fed-batch processes which employ defined rather than complex media. Another consideration is the inability to derive any practical benefit from the high specific growth rates obtainable with complex media at high cell densities owing to oxygen transfer limita-

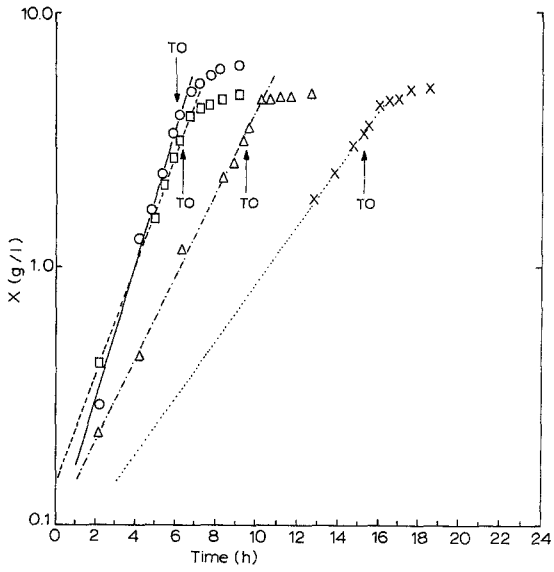

Fig. 5. Growth for fed-batch fermentations controlled at three different specific rates prior to induction, and a batch fermentation. TO designates the time that an OD of 5 was reached and expression was induced. ○, batch (Run 150); ——, measured μ = 0.58 l/h (Run 150); □, planned μ = 0.50 l/h (Run 151); – – –, actual μ = 0.48 l/h (Run 151); △, planned μ = 0.35 l/h (Run 152); ·–·–·, actual μ = 0.40 l/h (Run 152); × , planned μ = 0.20 l/h (Run 153); ....., actual μ = 0.23 l/h (Run 153).

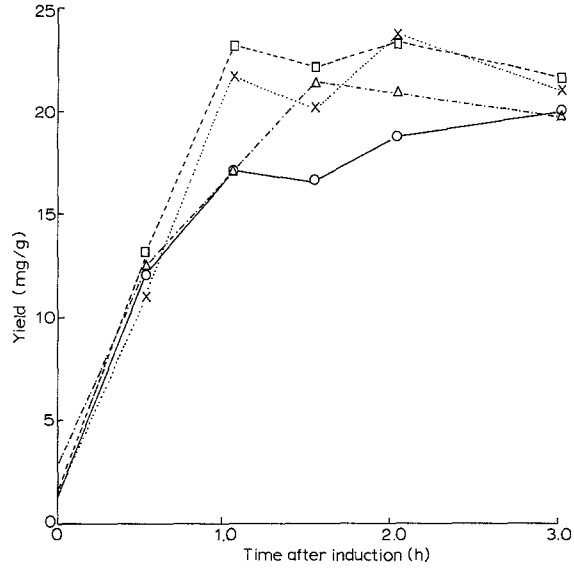

Fig. 6. Antigen yields for fed-batch fermentations controlled at three different specific growth rates prior to induction and a batch fermentation. ○, batch (Run 150); □, planned μ = 0.50 l/h (Run 151); △, planned μ = 0.35 l/h (Run 152); ×, planned μ = 0.20 l/h (Run 153).

tions. A maximum oxygen transfer rate of 600 mM/l-h is obtainable in our 15-l fermentors (temperature of 32°C, agitation of 1000 rpm, air sparging of 20 SLPM, pressure of 1.0 Bar, and DO of 20%), which supports a growth rate of 7 g/l-h in our experiments. A complex medium giving a specific growth rate of 0.8 l/h can only support a cell density of 9 g/l (OD 27) at 20% DO.

A defined medium was developed which used glycerol as the C-source. Glycerol has some advantages as a C-source feed. Glycerol is not a fermentative substrate, so that the accumulation of potentially harmful catabolites such as acetate or ethanol is not a significant problem. Glycerol is a liquid in pure form and leads to less dilution of the culture than is obtainable using concentrated aqueous solutions of carbohydrate, typically 40–50% w/w. Decreases in pH accompanying growth are smaller using glycerol rather than carbohydrates as a C-source. A disadvantage is a 3–4-fold cost premium relative to glucose on a weight basis.

Fermentations using the defined medium and a glycerol feed were evaluated for antigen expression at high cell densities. A typical run is shown in Fig. 7. Depletion of glycerol and growth near μ_{max} is shown during the initial batch phase up to hour 17. At that time the growth rate was sufficient to consume glycerol at a rate corresponding to the minimum flow capability of the pump, and feeding was initiated. Thereafter, the specific growth rate was reduced to 0.08 l/h and glycerol concentrations were approximately 3.0 g/l. During C-limited growth, one would expect glycerol concentrations in the mg/l range, suggesting an interference of the assay by substances in the culture supernatant.

Cell activity appeared to be constant during the growth phase based on the specific oxygen uptake rate (Q_{O_2}) data. During the initial batch phase with μ_{max} of 0.28 l/h, a constant Q_{O_2} of 15 mmol/g-h was obtained. After the shift to glycerol-limited growth with a specific growth rate of 0.08 l/h at hour 7, a constant Q_{O_2} of 7 mmol/g-h was obtained. The Q_{O_2} did not decrease during growth at 0.08 l/h as it would if dead cells were accumulating in the fermentor. On the other hand, viability based on

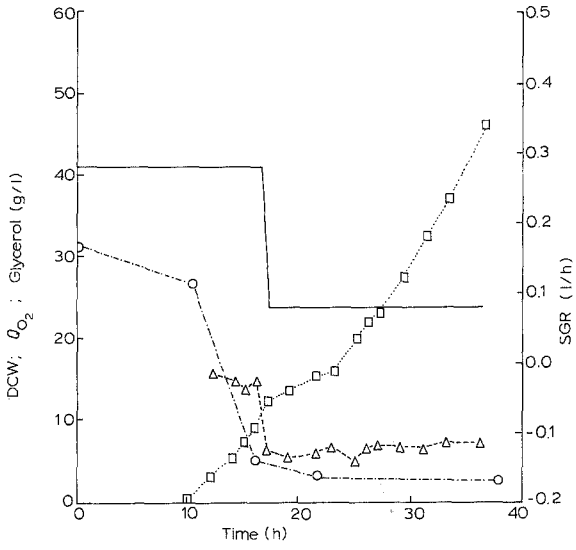

Fig. 7. Fed-batch fermentation Run 193 employing the defined medium. ——, specific growth rate (l/h); □, biomass concentration (DCW; g/l); △, specific oxygen uptake rate (mmol/g/h); ○, glycerol concentration (g/l).

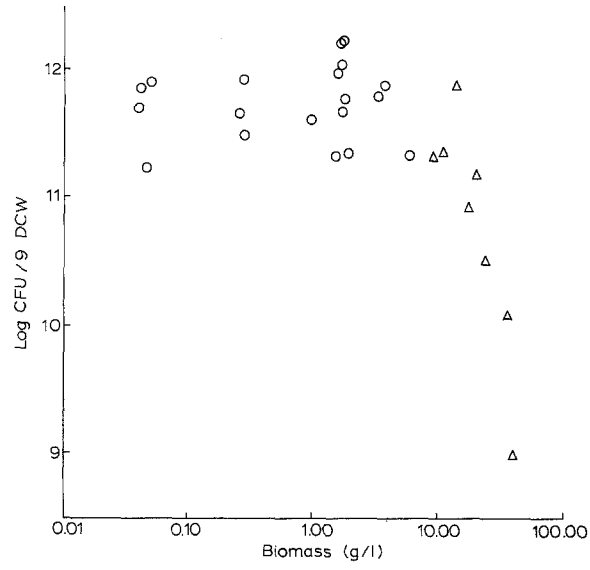

Fig. 8. Viability (CFU/g biomass) correlated with biomass concentration for batch (○) and fed-batch (△) fermentations.

CFU/g data appeared to decrease with growth by 100-fold. Fig. 8 shows the results of the fed-batch experiment together with results obtained from a variety of batch experiments. These results suggest that cells at OD > 20 may have an impaired ability to divide (i.e. decreased viability) without a loss in metabolic activity. Whether this trend is actual or an experimental artifact related to sample stability or plating efficiency has not been determined. This apparent loss in viability has prevented the use of replica plating as a means of measuring plasmid stability during growth in these high-cell-density fed-batch fermentations.

The culture was induced at an OD of 100 (44 g/l) and the glycerol limitation was removed by increasing the feed rate from approximately 1.5 ml/min to 4.6 ml/min. Antigen yields of 12 mg/g were obtained after 3 h of induction. As shown in Table 3, this is similar to the 15 mg/g obtained from a batch fermentation grown on the same medium and induced at an OD of 10. These results indicate that neither the imposed glycerol limitation to control the specific growth rate nor cell concentrations approaching 50 g/l resulted in a significant degradation of expression levels in the defined medium. However, there is a significant difference in antigen yields obtained using the complex medium (approximately 22 mg/g) and the defined medium (approximately 12 mg/g). Efforts to study this difference are just beginning. One possibility is that the rate of protein synthesis during induction in the defined medium may be limited by the de novo synthesis of amino acids that are available in the complex medium.

DISCUSSION

The open-loop C-feeding policy based on a simple macroscopic model of the fermentation was effective at controlling the specific growth rate to a specified value. This capability is a practical means of managing culture oxygen demand within the oxygen transfer capabilities of fermentors. Consequently, this approach can be useful during scale-up, where the decreased oxygen transfer capabilities of large fermentors can be offset by lowering the specified specific growth rate used to calculate the C-feeding profile. Implementation is practical in a production environment, since a variable flow

Table 3

Antigen yields in defined media

Medium	Fermentation	OD at induction	Yield (mg/g)
Defined	Batch	10	15
Defined	Fed-batch	100	12
Complex	Batch	10	22
Complex	Fed-batch	10	22

pump and a means of varying the flow rate set point according to the profile calculated off-line are the only specialized equipment.

The yield of recombinant product did not depend on the specific growth rate used before induction. In other chemostat studies with *E. coli* using the same regulated λ P_L promoter used in these studies, plasmid stability appeared to increase with specific growth rate when expression was repressed [11]. If this increase in plasmid stability leads to higher expression levels, the outcome would differ from our observations. Unfortunately, a direct connection between the results of these two studies can not be made, since we were unable to monitor plasmid stability by replica plating as discussed above, and the chemostat study did not report product yield as a function of specific growth rate.

Expression levels using the defined medium in low-cell-density batch fermentations and high-cell-density fed-batch fermentations were similar. Cell viability based on cellular respiration rates appeared to be constant during the fed-batch culture. On the other hand, cell viability based on CFU/g dry cell weight decreased by two orders of magnitude during the fed-batch culture. Work to reconcile these results is needed. Further studies are also needed to explain the different expression levels obtained using the complex and defined media.

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