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Simple fed-batch technique for high cell density cultivation of *Escherichia coli*

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Abstract

A simple fed-batch process for high cell density cultivation of *Escherichia coli* TG1 was developed. A pre-determined feeding strategy was chosen to maintain carbon-limited growth using a defined medium. Feeding was carried out to increase the cell mass concentration exponentially in the bioreactor controlling biomass accumulation at growth rates which do not cause the formation of acetic acid ($\mu < \mu_{crit}$). Cell concentrations of 128 and 148 g per l dry cell weight (g l^{-1} DCW) were obtained using glucose or glycerol as carbon source, respectively.

Keywords: Escherichia coli; Fed-batch; Cultivation, high cell density

1. Introduction

Escherichia coli is still the most important host organism for recombinant protein production. To maximize the volumetric productivities of bacterial cultures it is important to grow *E. coli* to high cell concentrations. Preventing the accumulation of toxic levels of acetic acid is the main task to achieve high cell concentrations in the bioreactor. Growth-inhibiting acidic by-products of incomplete substrate oxidation such as acetic acid are produced in response to oxygen limitation or excess carbon. Different strategies to grow *E. coli* to high cell densities in fed-batch cultures have been reviewed recently (Riesenberg, 1991; Yee and Blanch, 1992). The highest cell concentrations of non-recombinant *E. coli* reported in the literature were obtained by feeding solid glucose via a special apparatus into a pressurized bioreactor (134 g l^{-1} DCW; Matsui et al., 1989) or by removing inhibitory substances by dialysis (174 g l^{-1} DCW; Märkl et al., 1993). Using feed-back control strategies for glucose feeding to maintain the dissolved oxygen in a certain range (Cutayar and Poillon, 1989; Mori et al., 1979) and to keep the specific growth rate approximately constant (Riesenberg et al., 1991) cell densities of 110–125 g l^{-1} DCW have been obtained.

All these strategies require considerable experimental effort. For instance, in the high cell density cultivation proposed by Riesenberg et al.

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(1991) control of growth was realized by a pO_2 control loop (by variation of glucose feeding) and a μ control loop (by variation of impeller speed), while the actual μ was calculated from the off-gas composition. Therefore, we report here on a simple fed-batch technique using liquid feed medium and a pre-determined feeding rate to maintain carbon-limited growth during the fed-batch process at growth rates which do not cause the formation of acetic acid ($\mu < \mu_{crit}$). Thus, no sophisticated feed-back control to prevent the accumulation of toxic levels of acetic acid or removal of acetic acid by dialysis is necessary to grow *E. coli* to cell densities of up to 130 and 150 g l^{-1} DCW using glucose and glycerol, respectively, as the carbon and energy source.

2. Materials and methods

2.1. Bacterial strain

The organism used in this study was *Escherichia coli* TG1 (DSM 6056) (Riesenberg et al., 1991; Carter et al., 1985).

2.2. Medium preparation

The medium was prepared essentially as described by Riesenberg et al. (1991). The composition of the batch and feed medium is given in Table 1. For preparation of 2.5 l batch medium, $(NH_4)_2HPO_4$, KH_2PO_4, citric acid, EDTA and trace elements were dissolved in 2.3 l distilled water in the bioreactor, pH was adjusted to 6.3 with 5 N NaOH and the solution was sterilized for 30 min at 121°C. Stock solutions of $MgSO_4$ and glucose or glycerol were each sterilized separately for 30 min at 121°C. Thiamine was sterilized separately by filtration. After cooling, all solutions including antifoam reagent SP1 (Th. Goldschmidt AG, Essen, Germany) were combined and the pH was adjusted to 6.7 with aqueous NH_3 (25% w/w) prior to inoculation. All components for the feed medium were sterilized separately and mixed afterwards. The following stock solutions were prepared: glucose (826.0 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (1.0 g ml^{-1}), EDTA (8.4 mg ml^{-1}) and trace elements per ml: $CoCl_2 \cdot 6H_2O$ (2.5 mg), $MnCl_2 \cdot 4H_2O$ (15.0 mg), $CuCl_2 \cdot 2H_2O$ (1.5 mg), H_3BO_3 (3.0 mg), $Na_2MoO_4 \cdot 2H_2O$ (2.5 mg), $Zn(CH_3COO)_2 \cdot 2H_2O$ (13.0 mg) and Fe(III)citrate (12.5 mg). Glucose, glycerol and $MgSO_4$ were sterilized for 30 min at 121°C. EDTA and trace elements were sterilized by filtration. Stock solutions of thiamine (10 mg ml^{-1}) were prepared directly prior to use (sterilized by filtration). Antifoam reagent SP1 was dissolved in ethanol (50% v/v).

Table 1
Medium composition

Components	Batch medium (per l)	Feeding solution (per l)
Glucose (glycerol) [a]	25.0 (30.0) g	795.0 (1021.0) g
KH_2PO_4	13.3 g	–
$(NH_4)_2HPO_4$	4.0 g	–
$MgSO_4 \cdot 7H_2O$	1.2 g	20.0 g
Citric acid	1.7 g	–
EDTA	8.4 mg	13.0 mg
$CoCl_2 \cdot 6H_2O$	2.5 mg	4.0 mg
$MnCl_2 \cdot 4H_2O$	15.0 mg	23.5 mg
$CuCl_2 \cdot 2H_2O$	1.5 mg	2.5 mg
H_3BO_3	3.0 mg	5.0 mg
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg	4.0 mg
$Zn(CH_3COO)_2 \cdot 2H_2O$	13.0 mg	16.0 mg
Fe(III)citrate	100.0 mg	40.0 mg
Thiamine · HCl	4.5 mg	–
Antifoam SP1	0.1 ml	–

[a] Initial concentrations of glucose or glycerol for precultures were 15.0 g l^{-1}

2.3. Culture conditions

Precultures

The first preculture (10 ml batch medium in 100-ml shake flask) was inoculated with a single colony of *E. coli* TG1 from M9 minimal medium agar plates and incubated on a rotary shaker at 30°C for 10–14 h. The second precultures (four precultures, each 100 ml batch medium in 1000-ml shake flasks) were inoculated with the first preculture (1% v/v) and incubated on a rotary shaker at 30°C for 10–12 h. Cells were harvested by centrifugation, resuspended in 60 ml batch medium (8-times concentrated) and used for inoculation of the main batch culture.

Batch culture and controls

Main cultivations were carried out at 28°C in a 5-l bioreactor (Type Biostat MD; B. Braun Diessel Biotech GmbH, Melsungen, Germany) equipped with an additional cooling device. The initial batch culture conditions were as follows: initial culture volume = 2.5 l, air flow = 2.5 l min^{-1}, oxygen flow = 0.1 l min^{-1}, stirrer speed = 500 min^{-1}. Thermal mass flow meters (Brooks Instruments B.V., Veenendaal, Netherlands) were used for mixing air and oxygen. The dissolved oxygen was maintained at 20% of air saturation by increasing the stirrer speed. pH was controlled at 6.7 by addition of aqueous NH_3 (25% w/w). Control of pH and dissolved oxygen was carried out by the digital control unit (DCU) of the bioreactor. The concentrations of oxygen and carbon dioxide in the exhaust gas were determined by paramagnetic and infrared gas analysis systems, respectively (Maihak, Hamburg, Germany).

Fed-batch culture and controls

After consumption of the initial glucose or glycerol and metabolic by-products (e.g., acetic acid) indicated each by an increase of the dissolved oxygen concentration, the fed-batch phase was started. Feeding was carried out to allow the volumetric cell mass concentration to increase exponentially. In the C-limited fed-batch culture without significant product formation the C substrate is solely used for growth and maintenance. Hence, for a desired specific growth rate μ and a given biomass concentration the actual feed rate of C substrate is given by

$$m_S(t) = F(t)S_F(t) = \left(\frac{\mu(t)}{y_{X/S}} + m\right)V(t)X(t) \quad (1)$$

where m_S is the mass flow of substrate (g h^{-1}), F the volumetric feeding rate (l h^{-1}), S_F the concentration of the substrate in the feeding solution (g l^{-1}), μ the specific growth rate (h^{-1}), $y_{X/S}$ the biomass/substrate yield coefficient (g g^{-1}), m the specific maintenance coefficient (g g^{-1} h^{-1}), X the biomass concentration (g l^{-1}) and V the cultivation volume (l). In a fed-batch system which is essentially variable in volume the following growth equation applies

$$\frac{d(XV)}{dt} = \mu XV \quad (2)$$

Assuming μ as time invariant one obtains an integration when starting the feeding at time t_F

$$X(t)V(t) = X_{t_F}V_{t_F}e^{\mu(t-t_F)} \quad (3)$$

Thus, by introducing Eq. 3 into Eq. 1 the substrate mass feeding rate for a constant specific growth rate (μ_{set}) follows as

$$m_S(t) = \left(\frac{\mu_{set}}{y_{X/S}} + m\right)V_{t_F}X_{t_F}e^{\mu_{set}(t-t_F)} \quad (4)$$

where the yield coefficient $y_{X/S}$ was set to 0.5 and 0.45 for glucose and glycerol, respectively, and $m = 0.025$ g g h^{-1} was used for both substrates. Eq. 4 applies to large-scale reactors where the volume increase is predominantly governed by the feeding rate, and volume changes due to sampling and pH control are negligible. However, in lab-scale fed-batch reactors the cultivation volume does not only depend on the feeding rate but also on sample volume, sampling frequency and ammonia addition. Such volume changes may be significant and are not negligible in small scale when balancing the overall process. Therefore, we found it more convenient to calculate the time-dependent feeding rate $m'_S(t)$ with the actual cultivation volume $V(t)$ instead of V_{t_F}. Thus

$$m'_S(t) = m_S(t)V(t)/V_{t_F} \quad (5)$$

However, when using Eq. 5 for the feeding rate the specific growth rate μ is no more constant. It can be shown that

$$\mu(t) = \mu_{set} + \frac{1}{t - t_F} \ln \frac{V(t)}{V_F} \quad (6)$$

The deviation of $\mu(t)$ from μ_{set} increases with increasing fed-batch time but is usually less than 20% towards the end of the cultivation. The changes in μ as a result of the feeding profile given by Eq. 6 are not important as long as it is guaranteed that $\mu < \mu_{crit}$, i.e., below the critical specific growth rate above which by-products like acetate are formed which inhibit growth. For the *E. coli* strain applied in this study μ_{crit} is about 0.17 h^{-1} but may be less at high cell densities and/or if other limitations occur.

The feeding solution and the aqueous NH_3 (25% w/w) were each placed on a balance in order to allow the determination of the time-dependent volume of the culture broth (densities of glucose or glycerol feeding solutions and aqueous NH_3 were 1.3 g cm^{-3}, 1.23 g cm^{-3} and 0.91 g cm^{-3}, respectively). The bioreactor was not placed on a balance in order to minimize disturbance caused by stirring and sampling. Changes in culture volume caused by sampling were corrected manually by subtracting sample volume from the volume of the culture medium. The dissolved oxygen was maintained at 20% of air saturation by increasing the stirrer speed and/or blending air with pure oxygen. Foam was suppressed, when necessary, by the addition of antifoam reagent SP1. DCU, mass flow meters, balances, feeding pumps and exhaust gas analysis systems were interfaced to a VME-bus microcomputer using UBICON (Universal Bio-Process Control System) software (Bellgardt et al., 1992). In addition to control functions carried out by the DCU (temperature, pH, dissolved oxygen concentration by

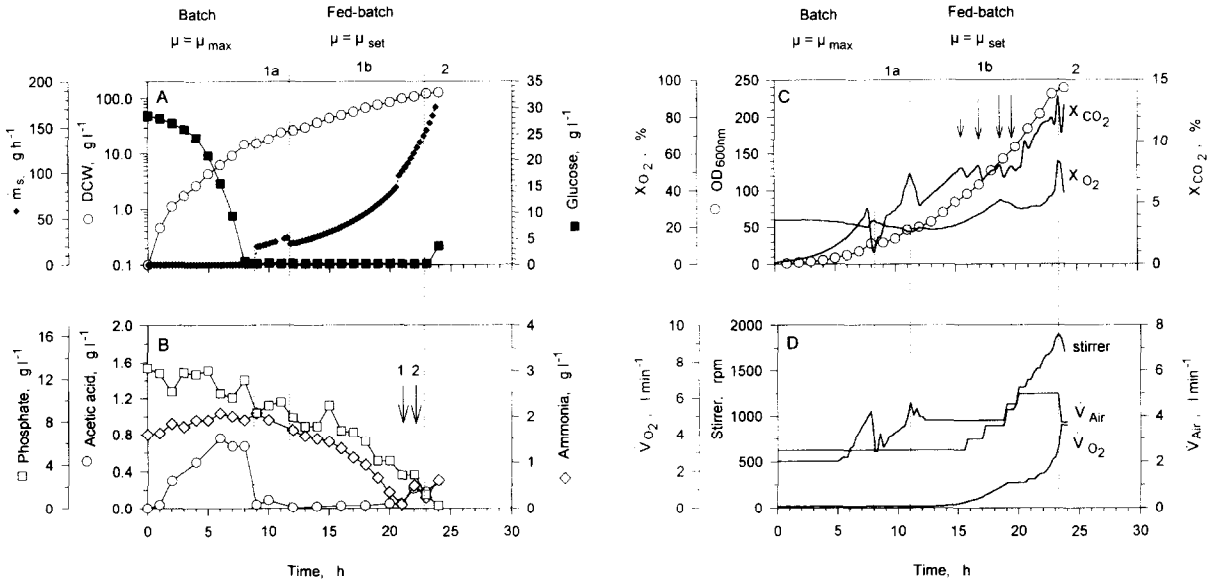

Fig. 1. High cell density cultivation of *E. coli* TG1 using glucose as carbon source. After unlimited growth during batch mode ($\mu_{max} = 0.45$ h^{-1}), fed-batch mode was started with a desired growth rate of $\mu_{set} = 0.17$ h^{-1} (phase 1a), after 3 h of fed-batch cultivation the desired growth rate was reduced to $\mu_{set} = 0.14$ h^{-1} (phase 1b). (A) Time-course of DCW (g l^{-1}), mass flow of glucose into the bioreactor (g h^{-1}) and glucose concentration (g l^{-1}) in the cell-free culture medium. (B) Time-course of acetic acid; phosphate (PO_4^{3-}) and ammonium (NH_4^+) concentrations (g l^{-1}) in the cell-free culture medium. Arrows indicate the addition of 7.2 g PO_4^{3-}, 2.7 g NH_4^+ and 20 mg thiamine (1) and 3.0 g NH_4^+ (2) to the culture medium. (C) Time-course of optical density (OD_{600}) and concentrations of oxygen and carbon dioxide in the exhaust gas (%). Arrows indicate the time when the air flow rate was increased. (D) Time-course of stirrer speed (rpm) and air and oxygen flow rates (l min^{-1}).

changing stirrer speed), UBICON was used to control the mass flow meters (control of dissolved oxygen concentration by blending air with pure oxygen), to calculate the time-dependent volume of the culture medium, to control the substrate feeding pump (concentrations of glucose or glycerol feeding solutions were 0.61 g g^{-1} and 0.83 g g^{-1}, respectively) and for data acquisition (exhaust gas analysis) and processing (e.g., calculation of oxygen uptake rates).

2.4. Analytical methods (off-line)

Cell growth was followed by measurement of the optical density at a wavelength of 600 nm (Novaspec II, Pharmacia LKB, Freiburg, Germany; 1 OD_{600} corresponds to 0.52 g l^{-1} DCW). In addition, dry cell weights (DCW) were determined from 1-ml aliquots of culture medium collected in balanced 1.5-ml centrifugation tubes. Cell pellets were collected by centrifugation for 3 min at 3300 × g, resuspended in distilled water, centrifuged again and dried at 40°C under vacuum until constancy of weight. Test kits from Boehringer-Mannheim GmbH (Germany) were used to analyze the concentrations of glycerol and acetic acid in the medium. A glucose analyzer (Yellow Springs, OH, USA) and an ammonium electrode (Type Orion 95-12; Colora, Lorch, Germany) were employed to analyze the concentrations of glucose and ammonium, respectively. The concentration of phosphate was analyzed according to a modified procedure described in the German Standard Methods (1983).

3. Results and discussion

The results of the high cell density cultivation using glucose as carbon source are shown in Fig. 1. After unlimited growth during the batch mode ($\mu = \mu_{max}$), glucose-limited growth at reduced

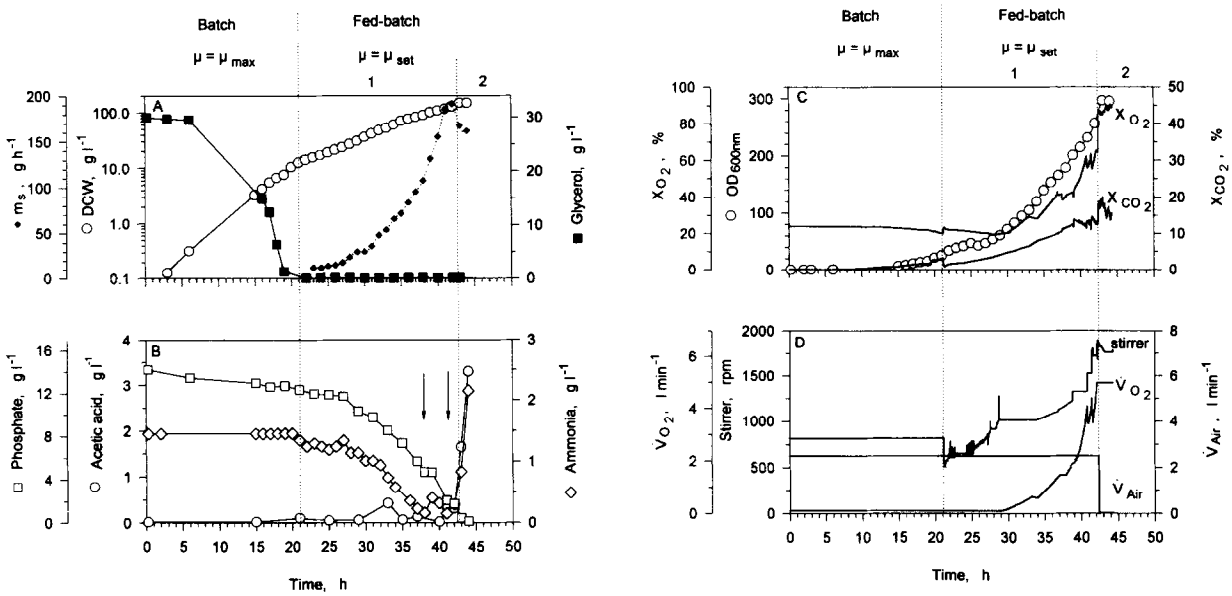

Fig. 2. High cell density cultivation of *E. coli* TG1 using glycerol as carbon source. After unlimited growth during batch mode ($\mu_{max} = 0.29\ h^{-1}$), fed-batch mode was started with a desired growth rate of $\mu_{set} = 0.12\ h^{-1}$ (phase 1). (A) Time-course of DCW (g l^{-1}), mass flow of glycerol into the bioreactor (g h^{-1}) and glycerol concentration (g l^{-1}) in the cell-free culture medium. (B) Time-course of acetic acid; phosphate (PO_4^{3-}) and ammonium (NH_4^+) concentrations (g l^{-1}) in the cell-free culture medium. Each arrow indicates the addition of 4.4 g PO_4^{3-} and 1.6 g NH_4^+ to the culture medium. (C) Time-course of optical density (OD_{600}) and concentrations of oxygen and carbon dioxide in the exhaust gas (%). (D) Time-course of stirrer speed (rpm) and air and oxygen flow rates (l min^{-1}).

growth rates (μ_{set} = const. < μ_{crit}) was realized by increasing the substrate feed during the fed-batch mode as described in Materials and methods (Fig. 1A). Feeding was started after acetic acid – produced as by-product during batch mode – was consumed (Fig. 1B). Exponential increase in the volumetric cell mass concentration was observed for 14 h or 2.6 doubling times of the biomass concentration during the fed-batch phase 1 (Fig. 1A). Cell growth stopped (phase 2) after depletion of phosphate. Phosphorylation of glucose as a prerequisite of glucose uptake became improbable and, consequently, glucose accumulated in the culture medium (Fig. 1A). The concentration of acetic acid started to increase in fed-batch phase 2, however, it did not exceed 0.3 g l^{-1} in the cell-free culture medium (Fig. 1B). The increase of the optical density of the culture and the concentrations of oxygen and carbon dioxide in the exhaust gas are shown in Fig. 1C. Oxygen and air flow rates and the stirrer speed are depicted in Fig. 1D.

The results of the high cell density cultivation using glycerol as carbon source are shown in Fig. 2. After unlimited growth during the batch mode ($\mu = \mu_{max}$), the fed-batch phase 1 with glycerol-limited growth at a reduced growth rate (μ_{set} = const. < μ_{crit}) followed (Fig. 2A). Feeding was started after glycerol was consumed. Acetic acid was not produced during batch mode due to the lower maximum growth rate supported by glycerol as carbon source (Fig. 2B). Exponential increase in the volumetric cell mass concentration was observed for 18 h or 3.1 doubling times of the biomass concentration (phase 1). At the end of the fed-batch process, the actual specific growth rate decreased and the concentration of acetic acid started to increase (phase 2). Acetic acid accumulated up to 3.3 g l^{-1} in the cell-free culture medium (Fig. 2B). As a result of using aqueous NH_3 for pH control, the concentration of ammonium also increased during phase 2 of the fed-batch process (Fig. 2B). Cell growth stopped completely after 22 h of fed-batch cultivation. Again, phosphate was identified as the limiting substrate (Fig. 2B). The time-course of the optical density and the concentrations of oxygen and carbon dioxide in the exhaust gas are shown in Fig. 2C. Oxygen and air flow rates and the stirrer speed are presented in Fig. 2D. A summary of the results of high cell density cultivations using glucose or glycerol as carbon source is shown in Table 2.

Using the described culture conditions the critical desired growth rate which did not cause the accumulation of acetic acid was approx. 0.17 h^{-1} with either glucose or glycerol as carbon source. However, a desired growth rate of 0.12–0.14 h^{-1} was chosen during the fed-batch process in order to keep the desired growth rate definitely below the critical value and to consider the increasing tendency to excrete acetic acid at high cell mass concentrations. Increased acetate formation at high cell mass concentrations may be caused by other by-products which accumulate to inhibitory concentrations and may enhance acetate excretion and growth cessation at elevated cell concentrations (e.g., the color of the cell-free culture broth is turning brown with increasing biomass concentration).

Table 2
Fed-batch cultivation

Carbon source	Glucose	Glycerol
Desired growth rate μ_{set} (h^{-1})	0.14	0.12
Final biomass X (g l^{-1})	128	148
Yield coefficient (carbon) $Y_{X/S}$ (g g^{-1})	0.506	0.43
CO_2 formation rate q_{CO2} (g g^{-1} h^{-1})	0.205 ± 0.01	0.18 ± 0.1
O_2 uptake rate q_{O2} (g g^{-1} h^{-1})	0.131 ± 0.02	0.163 ± 0.1
Respiratory quotient RQ	1.04 ± 0.02	0.77 ± 0.1

Applying this simple feeding strategy, final biomass concentrations obtained using glucose and glycerol as carbon source correspond to 58% and 67%, respectively, of the maximum theoretical cell mass concentration with respect to the viscosity of the culture medium (the viscosity of the culture medium for *E. coli* increases steeply above 200 g l^{-1} DCW and the culture medium looses fluidity at around 220 g l^{-1} DCW (Mori et al., 1979)). The higher biomass concentration obtained with glycerol as carbon source is mainly caused by the higher concentration of glycerol in the feeding solution. The concentration of glucose in the feeding solution cannot be increased further because of the limited solubility of glucose. However, using this fed-batch technique and supplementary feeding of phosphate it should be possible to increase the final cell mass concentration in the bioreactor even further. This fed-batch technique is insensitive to short-term disturbances in the dissolved oxygen concentration caused by the addition of antifoam reagent making this pre-determined feeding strategy superior to feed-back control strategies for carbon source feeding to maintain the dissolved oxygen in a certain range. It is sufficient to meet the oxygen requirements of the microorganisms (Table 2) and to maintain the dissolved oxygen concentration around 20% of air saturation by increasing the stirrer speed and/or blending air with pure oxygen. Off-gas analysis systems are not required to grow *E. coli* to extreme high cell densities.

In addition to biomass production, this fed-batch technique can be applied for production of recombinant proteins. Modifying the equation used for calculating the substrate mass flow rate as described in Materials and methods, this fed-batch protocol can be used to maintain a constant specific growth rate during the fed-batch process allowing the investigation of growth rate effects on host-vector interactions in *E. coli* (Hellmuth et al., 1994).

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