**The use of fed batch cultivation for achieving high cell densities for the pilot scale production of a recombinant protein (Phenylalanine dehydrogenase) in *Escherichia coli*.**

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## Introduction

High cell density techniques have been developed to improve productivity and also to provide advantages such as reduced culture volume, enhanced downstream processing, reduced wastewater, lower production costs and reduced investment equipment (Lee, 1996). The most frequently employed technique used to obtain high cell density cultures is fed-batch fermentation.

The feed rate of substrate during fed-batch fermentation affects many physiological variables such as specific growth rate, cell mass accumulation, substrate feed per cell, substrate feed to maintenance, carbon starvation stress, by-product formation and population segregation. These variables are known, or can be theoretically assumed, to influence protein production. Feeding strategies such as DO-stat (Cutayar and Poillon, 1989; Castan and Enfors, 2000), pH-stat (Robbins and Taylor, 1989) constant feeding (Wong *et al*., 1998), exponential feeding (Korz *et. al*., 1995) and linearly changing feeding rate (Wong *et al*., 1998), have been employed in fed-batch cultures of various recombinant *E. coli* strains.

Industrial production of recombinant protein often begins with an exponential feed phase of a limiting substrate, most commonly the carbon/energy source. Exponential feeding involves increasing the feed rate of the growth rate limiting substrate in proportion to cell growth. This allows the cells to grow at a constant specific growth rate which is below the critical growth rate for harmful by-product formation. During fed-batch operation, it is critical to control the specific growth rate as the formation of inhibitory by-products such as acetic acid; cell productivity and plasmid stability are related.

## Results and Discussion

In this work, simulations of fed-batch cultivation of *E. coli* were run using Matlab (Version 6.5) algorithms based on Monod kinetics with overflow metabolism and incorporating acetate production, acetate inhibition, substrate accumulation, substrate inhibition and cell maintenance (Xu *et. al.,* 1999) (See Table 1, Appendix 1 for equations used in the simulations).

The feed of substrate into the system, F (L/h), was increased exponentially as described by the following equation:

$$F = F_0 * e^{\mu t}$$

where $F_0$ is the initial substrate feed rate into the system (L/h), $\mu$ is the desired specific growth rate of cells (1/h) and t is the time (h). The exponential feeding period was followed by a constant feeding period. This constant feeding phase is to allow for the declining biomass yield and the increasing oxygen demand per energy substrate at increasing cell density.

The specific growth rate is set so as to maintain the growth rate below the critical growth rate, $\mu_{crit}$, above which inhibitory by-products, such as acetic acid, are formed. The threshold value for acetate production for the *E. coli* strain which was used in subsequent laboratory trials was previously found to be 0.35 $h^{-1}$. Thus, the specific growth rate was set at 0.3 $h^{-1}$ for the simulations.

Growth inhibiting concentrations of acetate for different strains of *E. coli* vary between 5 and 10 g/L (Gschaedler *et al*., 1994; Kleman *et al*., 1994). Preliminary laboratory trials indicated that cell growth of the *E. coli* strain used in this work ceased when acetic acid levels in the system exceeded 5 g/L. Therefore, this criterion was included in the simulations.

The initial feed rate and maximum feed rate were varied in order to determine the effect on the specific growth rate, the final biomass concentration, the acetate concentration and the substrate concentration in the cell free medium. The kinetic data used in the simulations is shown in appendix 1 (table 2).

Figure 1 shows a typical simulation obtained using the exponential feed profile with an initial feed rate of 0.03 L/h and a maximum of 0.8 L/h. Initially the biomass concentration increases exponentially as the substrate is fed into the system at an exponential rate. However, the acetate level in the system increases steadily from the start of the feeding phase and reaches the threshold value of 5 g/L after less than 10 hours of feeding. Thereafter, the biomass concentration does not increase and therefore the substrate begins to build up in the culture which results in further acetate production by overflow metabolism.

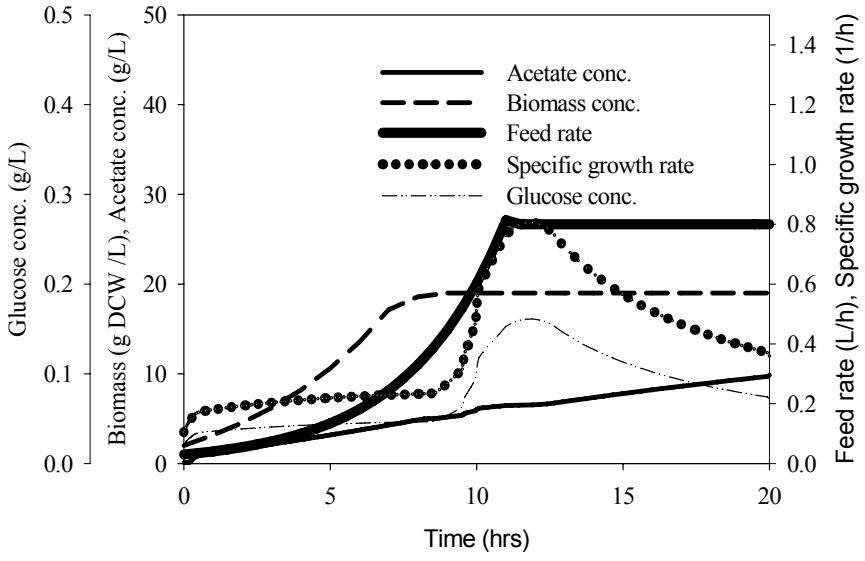


**Figure 1.** Simulation of fed-batch cultivation of *E. coli* with initial feed rate of 0.03 L/h and a maximum of 0,8 L/h.

The simulation was rerun varying the initial feed rate, the maximum feed rate and the specific growth rate with the aim of reducing the rate of acetate production and subsequently increasing the final biomass concentration. In order to maintain the acetate concentration below the threshold value which is harmful to cells it is necessary to reduce the maximum feed rate to 0.5 L/h and to grow the cells at a much lower specific growth rate of 0.08 $h^{-1}$. At this growth rate, it takes almost 20 hours to reach the same biomass concentration as in the original simulation (see figure 2). From a commercial and economically point of view, it is not practical to run fermentations for extended periods of time if it can be avoided.

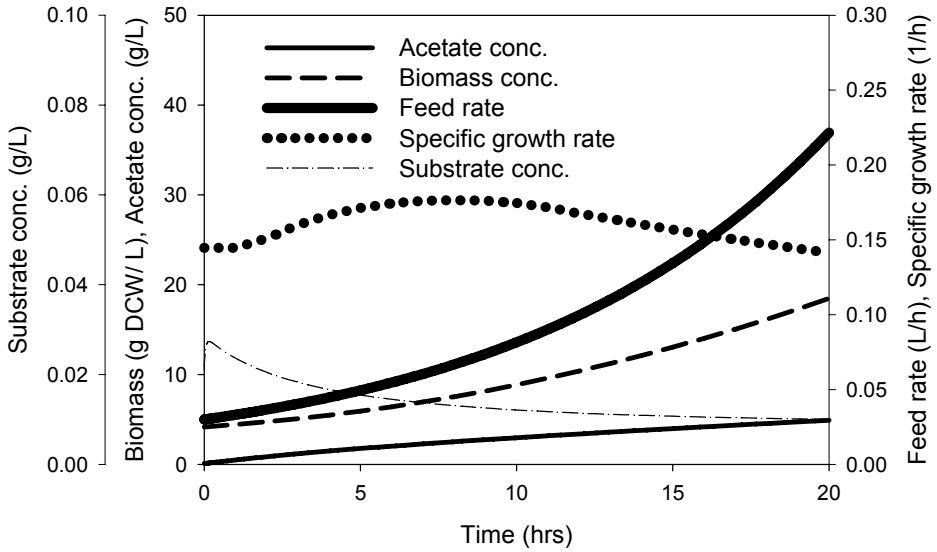


**Figure 2**. Simulation of fed-batch cultivation of *E. coli* with an initial feed rate of 0.03 L/h and a maximum of 0.5 L/h. The feed rate was reduced in order to reduce the specific growth rate with the aim of maintaining the acetate production levels below the level which is inhibitory to cell growth.

It is known that glucose represses cellular use of exogenous acetate, which is consumed only after glucose has been exhausted in the culture (Holms, 1986). Robbins and Taylor, 1989, also noted the diauxic pattern of growth of *E. coli*, first metabolising glucose and then acetate. A decline in the concentration of acetate upon glucose depletion was observed as it was metabolised through the tricarboxylic acid cycle and the glyoxalate bypass. Therefore, *E. coli* cells will reutilise acetate when substrate levels in the system fall to zero. Thus, if the substrate feeding into the system was stopped for short periods of time during the cultivation, acetate re-utilisation would be initiated preventing inhibitory levels of acetate building up in the system. Thus, a high cell density culture could be obtained in a shorter period of time.

By adjusting the feed rate in a trial and error manner and running the simulation it was possible to predict a feed profile that would increase the biomass concentration, maintain glucose levels at growth rate limiting concentrations and result in minimal acetate accumulation.

Figure 3 shows the feed profile designed using the simulations. Two starvation periods are included in the profile in order to initiate acetate re-utilisation. The first period is after 10 hours of feeding as simulations using the exponential feed profile indicated the

accumulation of acetate approached threshold values after this time. During laboratory trials, induction of the recombinant protein, PheDH, occurs after approximately 15 hours of feeding. It has been postulated that acetic acid is formed due to a temporary block of the citric acid cycle caused by a temporary imbalance at induction (Sanden *et. al*., 2003). Therefore, the second starvation period is after induction.

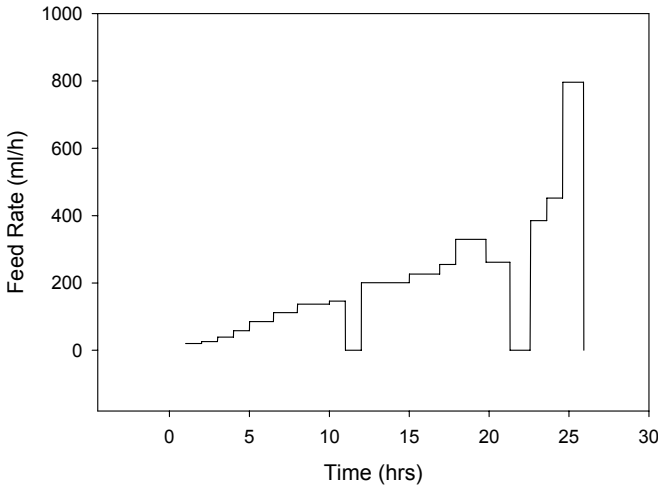


**Figure 3.** Feed profile designed using the simulations with the aim of increasing the biomass concentration, maintaining glucose levels at growth rate limiting concentrations and causing minimal acetate accumulation.

The concentrations of biomass, acetate and glucose and the growth rate of the cells obtained using this feed profile in the simulation are shown in figure 4. The biomass concentration is more than twice as high as the concentration predicted using the exponential feed profile in the simulation. The specific growth rate is maintained below the critical growth rate for by-product formation throughout the fermentation. At the feed starvation points, the glucose in the system falls to 0 g/L and acetate utilisation is initiated. Thus these glucose starvation periods help maintain the acetate level in the system below the threshold value which is inhibitory to cell growth.

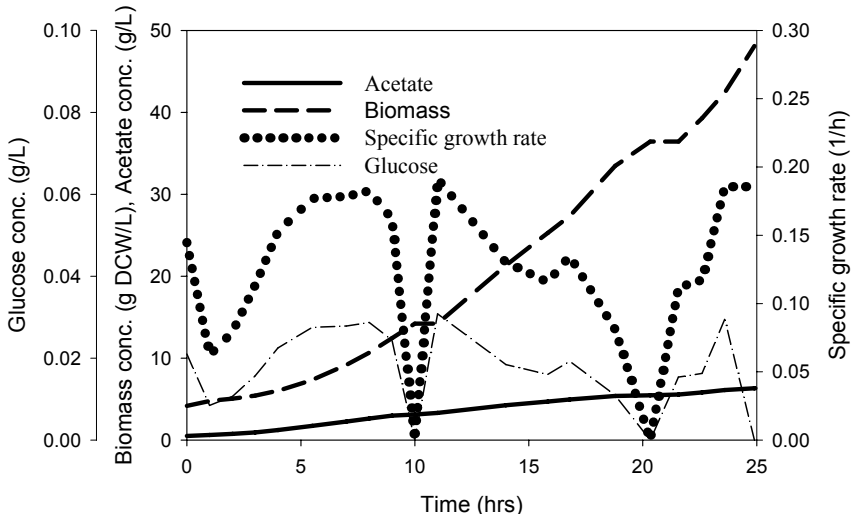


**Figure 4**. Concentration of biomass, acetate and glucose and the growth rate of the cells obtained using the feed profile shown in figure 3 in the simulation.

Pilot scale laboratory trials for the production of the recombinant protein, PheDH, in *E. coli* were carried out using the designed feed profile. Fermentations commenced with an initial batch phase. Feeding was started when glucose was exhausted in the cell free culture medium after 13 hours of batch cultivation. Glucose limited growth at reduced growth rates was realised by implementing the substrate feed during the fed-batch mode as described in figure 3. Feeding continued for 25 hours. The feed profile and acetate and glucose concentrations in the cell free medium at intervals throughout the cultivation are shown in figure 5. Acetate levels in the system remained below growth inhibitory levels until the point of induction (15 hours). After induction, acetate levels increased to 0.5 g/l but fell to 0.05 g/l 3 hours later when the system was starved of substrate for one hour. Acetate levels rose thereafter as the feed rate was increased, reaching a maximum concentration of 10 g/l. This trend continued until the feed was terminated. (See figure 5).

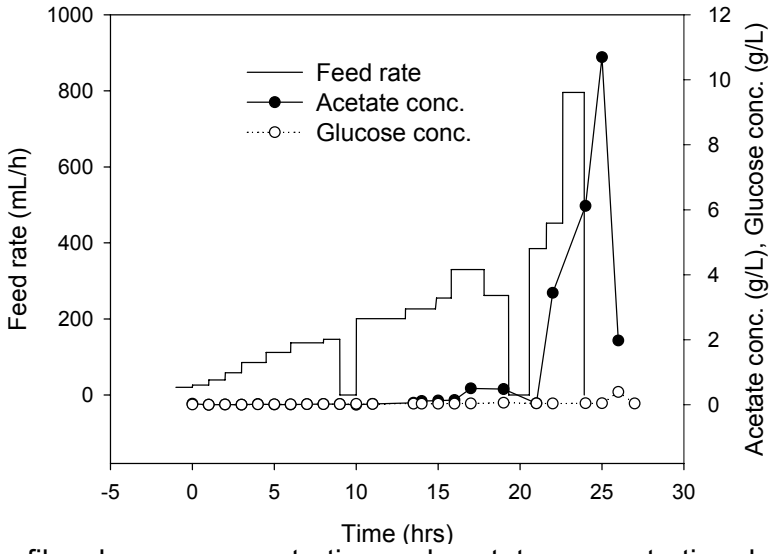


**Figure 5.** Feed profile, glucose concentration and acetate concentration during the fed-batch phase of the pilot scale cultivation of *E. coli* for the production of the recombinant protein PheDH.

During the fed-batch phase, the mass flow of glucose into the system was limited so that the specific growth rate did not rise above the threshold value for acetate production (figure 6). At the end of the feeding phase (25 hours), 53g/l dry cell weight (DCW) of biomass with a high intracellular product concentration of recombinant protein (1.5% of DCW) had formed. The specific activity of the unpurified protein was 1.18 U/mg DCW.

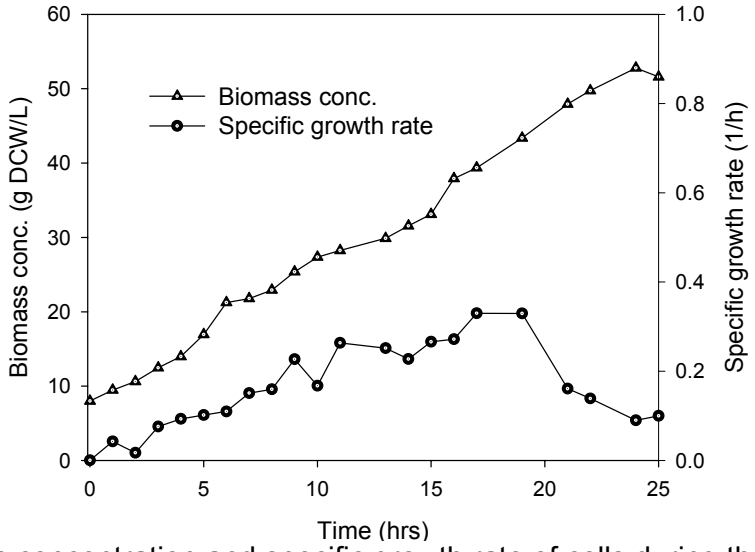


**Figure 6.** Biomass concentration and specific growth rate of cells during the fed-batch phase of the pilot scale cultivation of *E. coli* for the production of the recombinant protein PheDH.

## Conclusions

The aim of this work was to use computer simulations to design a novel feed profile which could be used during laboratory cultivations to produce a high cell density culture of *E. coli* with high over-expression and activity of the recombinant protein. The feed profile was designed according to the criteria in order to obtain high biomass concentrations and over-expression of recombinant protein, the specific growth rate must be controlled and the acetate accumulation in the system kept to a minimum. The two starvation points in the feed profile were introduced in order to induce acetate re-utilisation and thus avoid high levels of acetate accumulation and were successful in doing so.

**Appendix 1**

**Table 1**. Model based on Monod kinetics with overflow metabolism and incorporates acetate production, acetate inhibition, substrate accumulation, substrate inhibition and maintenance levels (as described by Xu *et. al.,* 1999).

| | |
|---|---|
| $qS = \left( \frac{qS_{max}}{1 + \frac{A_c}{K_{is}}} \right) \left( \frac{S}{S + K_S} \right)$ | Rate of substrate uptake in the system (g/g h) |
| $qS_{ox,an} = (qS_{ox} - q_m) Y_{X/S,ox} \frac{C_X}{C_S}$ | Substrate flux to oxidative anabolism. (g/g h) |
| $qO_S = qS_{ox,en} Y_{O/S}$ | Oxygen used for glucose oxidation. (g/g h) |
| $qS_{of} = qS - qS_{ox}$ | Rate of glucose channelled to overflow metabolism. (g/g h) |
| $qS_{of,an} = qS_{of} Y_{X/S,of} \frac{C_X}{C_S}$ | Component of flux used for anabolism. (g/g h) |
| $qS_{of,en} = qS_{of} - qS_{of,an}$ | Component of flux used for energy production via acetate formation. (g/g h) |
| $qA_p = qS_{of,en} Y_{A/S}$ | Rate of acetate production. (g/g h) |
| $if\ (qS_{ox}) \le q_m$<br>$qA_c = qA_{c,\max} \frac{A}{A + K_A}$ | Rate of acetate consumption. (Acetate consumption occurs when the substrate levels in the system fall below those required for maintenance) (g/g h) |
| $\mu = (qS_{ox} - q_m) Y_{x/s} + qS_{of} Y_{x/s,of} + qA_c Y_{xa\_c}$ | Specific growth rate. (1/h) |
| $\frac{dX}{dt} = \mu X - \left( \frac{F(t)}{V} \right) X$ | Mass balance equation for biomass (g/L) |
| $\frac{dV}{dt} = F(t) - F_{sample}$ | Mass balance equation for culture volume (g/L) |
| $\frac{dS}{dt} = \frac{F(t)}{V} (S_F - S) - qS.X$ | Mass balance equation for substrate (g/L) |
| $\frac{dA}{dt} = (qA_p - qA_c) X - \left( \frac{F(t)}{V} \right) A$ | Mass balance equation for acetate (g/L) |

**Table 2.** Kinetic data used in simulations

| Parameter | Unit | Value | Origin and Source |
|---|---|---|---|
| $C_S$ | mol C/g | 0.0333 | chemical formula |
| $C_X$ | mol C/g | 0.04 | chemical formula |
| $K_A$ | g/L | 0.05 | data fitting |
| $K_{is}$ | g/L | 5.0 | data fitting |
| $K_s$ | g/L | 0.05 | data fitting |
| $qA_{c,max}$ | g/g h | 0.06 | experimental data |
| $q_m$ | g/g h | 0.04 | Andersson *et. al*., 1994; Paalme *et. al*., 1997 |
| $qO_{max}$ | g/g h | 0.44 | experimental data |
| $qS_{max}$ | g/g h | 1.3 | experimental data |
| $S_{feed}$ | g/l | 332 | experimental procedure |
| $Y_{A/S}$ | g/g | 0.667 | stoichiometric constant (Xu *et. al*., 1999) |
| $Y_{ax\_c}$ | g/g | 0.4 | data fitting |
| $Y_{O/S}$ | g/g | 1 | data fitting |
| $Y_{x/s\ of}$ | g/g | 0.15 | Chen *et. al*., 1997 ; Varma & Palsson, 1994 |
| $Y_{x/s\ ox}$ | g/g | 0.42 | data fitting |

All simulations were run on Matlab version 6.5. The kinetic analyses were made by curve-fitting techniques. The experimental data was collected during preliminary laboratory trials.

**Symbols and Nomenclature**

| | |
|---|---|
| A | acetate concentration (g/L) |
| C | carbon concentration in moles per gram of substrate (mol C/g) |
| F | Feed rate (L/h) |
| $K_s$ | saturation constant (g/L) |
| $K_i$ | inhibition constant (g/L) |
| qA | specific acetate uptake rate (g /g h) |
| qO | specific oxygen uptake rate (mmol /g h) |
| qS | specific glucose uptake rate (g /g h) |
| S | glucose concentration (g/L) |
| t | time (h) |
| V | culture volume (L) |
| X | biomass (g/L) |
| $\mu$ | specific growth rate (1/h) |

**Subscripts**

| | |
|---|---|
| A | acetate |
| an | anabolic |
| c | consumption |
| en | energetic |
| F | feed |
| i | inhibition |

| | |
|---|---|
| m | maintenance |
| max | maximum |
| O | oxygen |
| of | overflow |
| ox | oxidative |
| p | production |
| S | glucose |
| Sample | sample taking |
| X | biomass |
| o | initial |

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