Optimization and control of perfusion cultures using a viable cell probe and cell specific perfusion rates

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Abstract

Consistent perfusion culture production requires reliable cell retention and control of feed rates. An on-line cell probe based on capacitance was used to assay viable biomass concentrations. A constant cell specific perfusion rate controlled medium feed rates with a bioreactor cell concentration of $\sim 5 \times 10^6$ cells mL⁻¹. Perfusion feeding was automatically adjusted based on the cell concentration signal from the on-line biomass sensor. Cell specific perfusion rates were varied over a range of 0.05 to 0.4 nL cell⁻¹ day⁻¹. Pseudo-steady-state bioreactor indices (concentrations, cellular rates and yields) were correlated to cell specific perfusion rates investigated to maximize recombinant protein production from a Chinese hamster ovary cell line. The tissue-type plasminogen activator concentration was maximized (~ 40 mg L⁻¹) at 0.2 nL cell⁻¹ day⁻¹. The volumetric protein productivity (~ 60 mg L⁻¹ day⁻¹) was maximized above 0.3 nL cell⁻¹ day⁻¹. The use of cell specific perfusion rates provided a straightforward basis for controlling, modeling and optimizing perfusion cultures.

Introduction

With increasing biopharmaceutical industry demand for monoclonal antibodies and other complex recombinant proteins, it is increasingly important to maximize mammalian cell bioreactor productivity. Conversion of bioreactors from fed-batch to perfusion can increase volumetric productivities over ten-fold. However, perfusion culture production requires reliable cell retention and proper feed rate specification. A major objective of perfusion culture control is to balance the medium feed and cellular uptake rates to maintain pseudo-steady-state conditions.

Control of perfusion bioreactors can be especially challenging due to high and fluctuating cell concentrations (Vits and Hu, 1992) that can rapidly change environmental conditions. With infrequent daily sampling, the control system can have too little information on which to base an appropriate decision to manipulate the process. In Kurkela et al. (1993),

daily feed rate step changes by an operator were inaccurate and process deviations resulted. Recently, more advanced predictive modeling based on daily glucose analysis by Dowd et al. (1999) improved feed rate specification and bioreactor control by up to 7-fold compared to operator specification. Alternatively, the process information can be improved by increasing sampling frequency with automated flow injection analysis. Such control systems (Van der Pol et al., 1995; Konstantinov et al., 1996; Ozturk et al., 1997) resulted in <1 mM substrate concentrations variations.

Robust, automatic feed rate specification requires careful design of the three components of a control system: process information, controller logic and a decision to manipulate the process. A control system based on a constant cell specific perfusion often depends on manual daily samples and trypan blue exclusion hemocytometer cell counting (Heidemann et al., 2000).

Figure 1. Schematic of perfusion bioreactor with the viable cell monitor (VCM) and acoustic filter. The VCM signal was sent to the computer, which was running BioXpert. In the control algorithm, a cell specific perfusion rate was specified and the biomass density signal was averaged and converted to a perfusion flow rate. The acoustic filter was used to retain cells in the bioreactor, with an automated back-flush of clarified harvest.

Design of the controller logic is often overlooked, although it impacts the overall control system performance, especially in automatic systems. Konstantinov et al. (1996) described controller logic, manipulating the process at the same high frequency it was sampled, such that process noise caused oscillatory control. Van der Pol et al. (1995) sampled the process at similar high frequencies, but averaged information and manipulated the process at lower frequencies, improving the basis upon which to manipulate the process. Alternatively in Dowd et al. (1999), the control performance was designed *a priori* and, using predictive models so that from daily sampling, up to 8 manipulations of the process flow rate were performed per day.

Process information from on-line systems, such as mass spectrometers or probes can be used to calculate carbon dioxide evolution (Pelletier et al., 1994) and oxygen uptake rates (Kyung et al., 1994) to provide re-

latively noisy estimates of the cell density. Direct process information of the cell density has been obtained by a microscopic imaging analysis system developed by Maruhashi et al. (1994) to determine cell size and viability without sampling or staining. The dead cell concentrations, and corresponding viability, were correlated to the number of small, cell debris particles. Merten et al. (1985) described an infrared sensor for the determination of cell number, but the range of cell densities tested and the length of operation were limited. Laser turbidity probes have been used in batch cultures, with Zhou and Hu (1994) demonstrating a linear relationship up to 3×10^6 cells mL^{-1} and Konstantinov et al. (1992) calculating the specific growth rate (after signal noise filtering). Several light-based sensors were tested over a wide range of cell densities up to 25×10^6 cells mL^{-1} and up to 50 days in perfusion culture (Wu et al., 1995). Decreases in sensitivity at high cell densities with probes with longer detection

Figure 2. Model prediction of steady-states in perfusion cultures controlled with cell specific perfusion rates. In (A) cell specific glucose uptake rates for a batch culture were correlated to reactor glucose concentration. The relationship was used in (B) to predict changing glucose concentrations from a steady-state condition at 5.6 mM glucose ($0.18 \text{ nL cell}^{-1} \text{ day}^{-1}$ to 9.1 mM glucose ($0.28 \text{ nL cell}^{-1} \text{ day}^{-1}$). Confidence regions are the uptake-concentration correlation limits.

path lengths were observed. In general, probe performance was acceptable for high viability cultures. Once calibrated, a cell specific perfusion rate was specified and the feed rates were based on the cell probe output (Wu et al., 1995). However, the light-based probes did not distinguish between viable and nonviable cells. During process upsets, all the optical probe measures deviated from the cell densities measured by trypan blue exclusion.

With dielectric spectroscopy, the capacitance probe output is proportional to the membrane enclosed volume fraction (Harris et al., 1987). Since only intact

membranes should store charge, viable biomass is selectively measured. This method has been applied to microbial, animal and plant cells (Markx et al., 1991; Fehrenbach, 1992; Cerckel et al., 1993; Konstantinov et al., 1994) with reliable readings at minimum mammalian cell concentrations of $\sim 0.5 \times 10^6 \text{ cells mL}^{-1}$ (Degouys et al., 1993). A particular advantage of dielectric spectroscopy is that it can measure viable cell concentrations in packed bed and porous microcarrier systems (Guan et al., 1998; Guan and Kemp, 1998; Ducommun et al., 2001, 2002).

Figure 3. Viable cell concentrations and culture viabilities as a function of cell specific perfusion rate. In (A), the viable cell probe output and hemocytometer values are compared, while in (B), the culture viability obtained from a hemocytometer are plotted as a function of cell specific perfusion rate. The errors bars represent 95% confidence region around the average.

In this work, automatic perfusion feed rate control was based on estimation of the viable cell concentration using on-line dielectric spectroscopy. The cell density was captured and averaged for use in a control algorithm based on cell specific perfusion rates. Cell specific perfusion rates were maintained and the quality of the pseudo-steady-states analyzed. The dependencies of the substrate, metabolite and protein concentrations, along with uptake and production rates were analyzed as a function of cell specific perfusion rates. Cell specific rates that maximized the recombinant protein concentrations or volumetric productivity were defined.

Materials and methods

Cell culture

A DUKX-B11 derived CHO cell line (C5.23SFM1, Fann et al., 2000) was cultured in a serum- and methotrexate-free medium (SFM2, Cangene Corp., Winnipeg, MB). Thawed inoculum cultures were grown in T-flasks for 7 or 8 days (37 °C, 5% CO₂), before 2 subcultures in 250 mL spinners (Bellco, Vineland, NJ). Cell counts used trypan blue dye exclusion in a hemocytometer for both reactor and perfusion effluent. Approximately 10⁸ cells were used to inoculate a 0.8 L working volume (3 L overall) perfusion biore-

actor (Applikon, Foster City, CA) and run for 3 days in batch mode until perfusion feeding was initiated.

An acoustic filter (BioSep 10L, Applikon) retained cells in the perfusion reactor. The cell bleed rate was controlled by decreasing the acoustic filter duty (on : off ratio) to increase net cell bleed rates, between 45 on : 6 off and 15 on : 45 off (sec). At high on-off ratios, >99% cell retention was observed, vs. ~85% at the low on-off ratios. The acoustic filter was operated with a 25 mL medium back-flush every 15 min to avoid pumping cells through a circulation line (Merten, 1999). Temperature, pH and dissolved oxygen were maintained at 37 ± 0.2 °C, 7.2–7.3 and 60 ± 15 % of air saturation, respectively, by a digital controller (Applikon BioController 1040). An Aber Instruments Viable Cell Monitor (Applikon), along with probes for temperature, pH and dissolved oxygen were data logged (BioXpert). A conductance level sensor triggered an outflow pump to maintain a constant reactor working volume. In the first culture, perfusion rates were 0.3, 0.25 and 0.2 nL cell⁻¹ day⁻¹, while, in the second, 0.4, 0.3, 0.15, 0.1 and 0.05 nL cell⁻¹ day⁻¹ perfusion rates were investigated. The feed medium contained 30 mM glucose, except for the preliminary batch and perfusion cultures, performed with 25-mM glucose. In the initial perfusion culture, samples were taken from the reactor outflow using a refrigerated fraction collector. These samples were used for steady-state characterization.

Medium analysis

Manual samples were analyzed daily for glucose, lactate and ammonium concentrations using a Stat 10 Blood/Gas Analyzer (NOVA Biomedical, Waltham, MA). The enzymatic activity of t-PA was analyzed by a colorimetric assay (Fann et al., 2000). A conversion factor of 580000 U mg⁻¹ (WHO standard specific activity) was used to convert activity units to concentrations. The amino acid concentrations were determined by the Pico-Tag method (Cohen and Strydom, 1988; Hagen et al., 1993) followed by separation on a Waters C-18 reverse-phase column (Milford, MA) using a Shimadzu (Columbia, MD) HPLC system. Amino acid standards at 0.08, 0.2 and 0.8 mM were used, with an internal standard of 50 μM orleucine.

Specification of perfusion feed rates

The viable cell monitor (VCM) output was connected to the BioXpert software in a custom setup (Figure 1).

Every 30 min, the VCM output was sent to the BioXpert software, where a control algorithm automatically calculated the required flow rates, using a computer calibrated and controlled pump. In perfusion cultures, the flow rate was based on viable cell concentrations using:

$$F = X_v \cdot V \cdot p_{sp} \cdot 10^{-9}, \quad (1)$$

where F was the flow rate (L day⁻¹), X_v the VCM measured viable cell concentration (cells L⁻¹), V the reactor working volume (L) and p_{sp} the cell specific perfusion rate (nL cell⁻¹ day⁻¹). The volume of medium corresponding to the calculated flow rate was automatically added hourly, using the average of two VCM readings.

Statistical methods and analysis

In the analysis of pseudo-steady states, all data were linearly regressed versus time from 24 h after the change. The regressed line slope was compared to zero with a 95% confidence limit using the standard error for that slope estimate. It was assumed that if the slope was not significantly different from zero, and non-linear dynamics were not observed, then a steady-state condition had been achieved. All the data had an expected analysis measurement error. For calculated data (for example, cell specific glucose uptake rates are based on glucose and cell density measurements), an ANOVA analysis was performed to estimate the expected error for these calculations.

Results and discussion

Time to steady state

Perfusion cultures usually approach steady-state more rapidly than chemostat cultures without cell retention (Miller et al., 1988; Hiller et al., 1993), mainly because of much higher perfusion dilution rates due to greater cell concentrations. Figure 2B shows an example (6.3×10^6 cells mL⁻¹) of a step from 0.18 to 0.28 nL cell⁻¹ day⁻¹, where perfusion steady-state glucose concentrations were attained within 12–24 h of changing the cell specific perfusion rate. This was confirmed by mass balance based simulations using:

$$\frac{dC}{dt} = \frac{F}{V} \cdot (C_{in} - C) - \frac{q \cdot X_v \cdot 10^{-9}}{V}, \quad (2)$$

Table 1. Comparison of analysis errors to range of experimental data

	Analysis error	Range of experimental data
Glucose	<0.5 mM	7.0–20 mM
Lactate	<0.4 mM	9.0–22 mM
Ammonium	<0.2 mM	1.6–3.3 mM
t-PA	<1.7 mM L ⁻¹	22–45 mg L ⁻¹

where C and C_{in} are the reactor and inlet glucose concentrations (mM), q the cell specific glucose uptake rate (pmol cell⁻¹ day⁻¹). The flow rate, F , was specified in Equation (1). From batch culture data with medium that contained 25 mM glucose, the cell specific glucose uptake rate was correlated with the reactor glucose concentration (Figure 2A), and represented as:

$$q = 0.52 \cdot C - 0.91, \quad (3)$$

assuming no delay in the cellular response. Pelletier et al. (1994) has shown that batch kinetics can predict glucose concentrations in perfusion culture. The simulated and measured glucose concentrations changed quickly and attained 95% of the steady-state concentration within 12 h. The limits on the simulation correspond to the 95% confidence limits on the correlation (Equation (3)). Thus, in perfusion culture, where cell concentrations are controlled, pseudo-steady-state substrate and cell concentrations are possible within 12–24 h.

Analysis of steady states in perfusion cultures using viable cell monitor

In the perfusion cultures using the viable cell monitor, fixed cell specific perfusion rates were generally maintained for a minimum of 5 days each. All cell culture data (11 concentrations, cell specific rates and yields for the 8 perfusion cultures) were tested for statistical consistency with steady-state conditions. Overall, 73% of the 88 data sets tested for steady-state exhibited slopes not significantly different from zero. Of the remaining 24 data sets, 22 had standard deviations less than the error in analysis (Table 1). Therefore, analysis error was believed often responsible for apparent non-steady-state conditions. The 2 remaining data sets with significant slopes were t-PA concentrations and cell specific productivity at 0.05 nL cell⁻¹

day⁻¹, when culture viability was lowest at approximately 64% (Figure 3). Thus, overall good steady states were achieved.

Over the whole tested range of cell specific perfusion rates, the average ratio of t-PA production to glucose uptake was 1.9 g t-PA mol⁻¹ glucose. At lower than 0.15 nL cell⁻¹ day⁻¹ perfusion rates, the ratio of t-PA production to glucose uptake was more variable (± 0.9 g t-PA mol⁻¹ glucose), while above 0.2 nL cell⁻¹ day⁻¹, the ratio was more constant (± 0.04 g t-PA/mol glucose). The increase in t-PA variability at low cell specific perfusion rates was similar to previous work (Dowd et al., 2001), where, with the same cell clone and medium, a 3-fold higher t-PA concentration variability was observed at low glucose concentrations.

Cell concentration for a range of perfusion rates

Initial calibration of the viable cell probe was performed in a batch culture with greater than 90% viability. Above a cell specific perfusion rate of 0.2 nL cell⁻¹ day⁻¹, the average cell concentration was approximately 5×10^6 cells mL⁻¹ with a culture viability of approximately 90% (Figure 3). Hemocytometer counts in this range of perfusion rates were close to the VCM readings. However, below 0.2 nL cell⁻¹ day⁻¹, the viability of the culture declined, the VCM readings underestimated hemocytometer counts by approximately 35%, though this may be due to decreasing cell volumes under low viability conditions (Sonderhoff et al., 1992; Ducommun et al., 2002). These results contrast with Wu et al. (1995) who reported that several optical probe outputs overestimated viable cell concentrations (since nonviable cells scatter light).

In straightforward mass balance simulations with a stable protein, constant cell concentration and specific production rate, protein concentrations would increase with the reciprocal of perfusion rate. However, although lower perfusion rates allowed for higher protein concentrations, t-PA concentrations were limited due to increased extra cellular protein degradation and the inability to prevent cell death at low perfusion rates.

t-PA production in perfusion cultures with viable cell monitor

The cell specific t-PA productivity increased with increasing cell specific perfusion rate and approached a maximum around 0.2 nL cell⁻¹ day⁻¹ (Figure 4B). The highest t-PA titers were observed between 0.1 and

Figure 4. Steady-state recombinant protein production as a function of cell specific perfusion rates. In (A), the t-PA concentrations, in (B), the cell specific productivity and in (C), volumetric productivity.

0.3 nL cell⁻¹ day⁻¹ (Figure 4A). The downward trend in concentration at cell specific perfusion rates greater than 0.2 nL cell⁻¹ day⁻¹ was due to the increased flow rates flushing t-PA from the reactor, indicating that the maximal cell specific production rate had been obtained. The lower t-PA concentrations at low cell specific perfusion rates corresponded to lower culture viabilities of 60 to 80%. At lower culture viabilities, 3-fold higher extra-cellular t-PA degradation rates have been observed with this medium and cell clone (Dowd et al., 2000). Volumetric productivity was a function of both t-PA concentration and perfusion flow rate (Figure 4C). The low volumetric productivity observed at

low cell specific perfusion rates, reflected the lower culture viability and the low flow rate.

Reactor environment control using viable cell monitor

With increasing cell specific perfusion rates, nutrient and metabolite concentrations either changed linearly (increasing/decreasing) or exhibited a broad maximum. Glucose, glutamine and most other amino acids (data not shown) exhibited linear increasing concentrations (Figures 5 and 6). Lactate, ammonium and glycine (data not shown) exhibited linear decreasing concentrations with increasing cell specific perfusion

Figure 5. Steady-state glucose, lactate and ammonium concentrations and cell specific rates as a function of cell specific perfusion rates.

rate. Finally, serine, like t-PA, exhibited a broad maximum between 0.1 to 0.25 nL cell⁻¹ mL⁻¹. At low cell specific perfusion rates, along with a drop in viability, there may have been some limitation or inhibition in the culture conditions that reduced production and concentrations.

For cell specific rates, the relationships were generally of a saturation-type, with the changeable component being the inflection point for saturation. For glucose uptake and t-PA production, the inflection point was at approximately 0.2 nL cell⁻¹ day⁻¹. The majority of the measured components exhibited inflection points at approximately 0.3 nL cell⁻¹ day⁻¹. In contrast, maximal cell specific ammonium production

and glutamate uptake did not appear to be attained over the tested range of cell specific perfusion rates.

The yields of metabolites from substrates were generally constant over the range of cell specific perfusion rates (data not shown), which may be expected, as limiting conditions for substrates were not approached. The yield of lactate from glucose was typical in this respect (constant at 0.7 ± 0.2). The ratio of glucose to glutamine was also constant (6.9 ± 0.9), with no trends observed. The yields of ammonium and glutamate from glutamine increased slightly above 0.3 nL cell⁻¹ day⁻¹ cell specific perfusion rates.

Figure 6. Steady-state glutamine, glutamate and serine concentrations and cell specific rates as a function of cell specific perfusion rates.

Comparison of glucose and viable cell monitor based feed rate control

Specifying the cell specific perfusion rate to control the reactor environment resulted in greater glucose concentration variability of up to 1.4 mM. Dowd et al. (2001) used predictive control protocols based on daily samples to reduce glucose variation to ± 0.3 mM levels ($\pm$ S.D.). However controlling the reactor based on glucose concentrations requires some form of sampling and subsequent specifying pump flow rates as a result of assayed values.

Conclusions

Use of a viable cell probe and cell specific perfusion rates is a simple and relatively sample free control method for perfusion cultures. Steady states were rapidly achieved in the reactor after switching cell specific perfusion rates. The perfusion process feed rate was explored by manipulating the cell specific perfusion rate and observing the impact on bioreactor performance. The t-PA concentration peaked at approximately $0.2 \text{ nL cell}^{-1} \text{ day}^{-1}$ as protein was flushed from the reactor at higher cell specific perfusion rates. Cell specific glucose uptake and t-PA production rates reached a maximum above $0.2 \text{ nL cell}^{-1} \text{ day}^{-1}$. Volumetric t-PA productivity increased with increasing cell specific

perfusion rates up to 0.3 nL cell⁻¹ day⁻¹. Estimation of the viable cell concentration with a viable cell probe readily allowed cell specific perfusion rate selection for optimal process operation.

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