Effect of Feed and Bleed Rate on Hybridoma Cells in an Acoustic Perfusion Bioreactor: Part I. Cell Density, Viability, and Cell-Cycle Distribution

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Abstract: For the development of optimal perfusion processes the effect of the feed and bleed rate on cell growth in a perfusion bioreactor was studied. The viable-cell density, viability, growth, death, and lysis rate and cell-cycle distribution of a hybridoma cell line producing an IgG1 were studied over a range of specific feed and bleed rates. It was found that the feed and bleed rates applied in the different cultures could be divided into two regions based on the viable-cell density and cell-cycle distribution. The cultures in the first region, low feed rates (0.5 and 1.0 d⁻¹) combined with low bleed rates (0.05 and 0.10 d⁻¹), were nutrient-limited, as an increase in the feed rate resulted in an increase in the viable-cell density. The cultures in the second region, high feed and bleed rates, were nonnutrient-limited. In this region the viable-cell density decreased more or less linearly with an increase in the bleed rate and was independent of the feed rate. This suggests that the cells were limited by a cell-related factor. Comparison of Trypan-blue dye-exclusion measurements and lactate-dehydrogenase activity measurements revealed that cell lysis was not negligible in this bioreactor set-up. Therefore, lactate-dehydrogenase activity measurements were essential to measure the death rate accurately. The specific growth rate was nearly constant for all tested conditions. The viability increased with an increase of the bleed rate and was independent of the feed rate. Furthermore, the specific productivity of monoclonal antibody was constant under all tested conditions. For the optimal design of a perfusion process it should first be established whether viability is an important parameter. If not, a bleed rate as low as possible should be chosen. If low viabilities are to be avoided, the bleed rate chosen should be higher, with the value depending on the desired viability. Next, the feed rate should be set at such a rate that the cells are just in the nonnutrient-limited region. © 2004 Wiley Periodicals, Inc.

Keywords: acoustic perfusion; lactate dehydrogenase; cell-cycle distribution; growth limitation; hybridoma

INTRODUCTION

Worldwide there is a growing demand for monoclonal antibodies (Mab’s) for therapeutic and diagnostic purposes. To fulfil these demands reactor systems in which an increased volumetric productivity can be achieved, such as fed-batch and perfusion systems, are used more and more in industry. In perfusion systems, high viable-cell concentrations and, thereby, high volumetric productivities are obtained. However, high cell concentrations in perfusion systems are in general accompanied by low viabilities (de la Broise et al., 1991; Harigae et al., 1994; Hiller et al., 1993; Johnson et al., 1996; Mercille et al., 2000; Mercille and Massie, 1998; Smith et al., 1991; Trampler et al., 1994; Yabannavar et al., 1994). At high dead-cell concentrations a substantial amount of intracellular proteins, such as proteases and neuraminidases, are released. These proteins could have a negative effect on product quality (Goldman et al., 1997; Munzert et al., 1996), particularly when an extracellular protein is produced. Additionally, the released intracellular proteins as well as other cell debris could hamper the purification process (Maiorella et al., 1991) and could have a negative effect on the process by fouling of the probes used for process control. The key process parameters specific to a perfusion process, the feed and bleed rate and the feed composition, can be used to influence growth characteristics such as viable-cell density, viability, growth rate, and cell-cycle distribution. To develop a robust and optimal production process, more insight into the relationship between these process parameters and growth characteristics in perfusion processes is necessary.

Earlier investigations have shown that increasing the feed rate in general leads to an increase in the viable-cell density.
concentration at steady state, due to the enhanced supply of nutrients (Banik and Heath, 1995a, 1996; de la Broise et al., 1991; Fassnacht et al., 1999; Hiller et al., 1993; Mercille et al., 2000; Mercille and Massie, 1998; Robinson et al., 1992; Ryll et al., 2000; Yang et al., 2000). Besides removal of cell-free spent medium through the perfusion flow, also a small flow of cell-containing medium out of the reactor, the bleed, is necessary (Fig. 1). Since the growth of hybridoma cells cannot be arrested, they will always have a certain minimal growth rate (Smith et al., 1991). If no bleed is applied a steady-state viable-cell concentration will be reached where the death rate equals the mentioned minimal growth rate resulting in the infinite accumulation of dead cells in the bioreactor. Therefore, the removal of cells via a bleed is essential to reach steady state (Banik and Heath, 1995b; de la Broise et al., 1991; Hiller et al., 1993; Mercille et al., 1994, 2000; Trampler et al., 1994). When the bleed rate is increased the death rate generally decreases and the viability increases. Nevertheless, due to the bleed a larger amount of cells is removed resulting in a lower viable-cell density (Banik and Heath, 1996; Hiller et al., 1993; Robinson et al., 1992). Furthermore, the viable-cell density generally increases when the feed rate is increased. To find the optimal balance between a high viable-cell density and high viability, more information on the effect of the feed and bleed rate on these parameters is required.

Previous studies regarding the effect of feed and bleed rate on the process conditions were carried out with the use of either serum-containing (Banik and Heath, 1995a) or serum-free protein-containing media (Hiller et al., 1993). In this study a chemically defined protein-free medium was chosen, because during the last decade regulations for the production of pharmaceutical products have resulted in a shift towards media without components of an animal origin. Since serum contains many growth factors, it is expected that the influence of the feed and bleed rate on the growth characteristics will be different in protein-free media.

The objective of this study is to obtain insight into the effect of both feed and bleed rate on the viable-cell density, viability, growth, death and lysis rate, and cell-cycle distribution. In addition to this, glucose, lactate, amino acids, and ammonia concentrations were measured to obtain insight into the cell metabolism of hybridoma cells. The effect of feed and bleed rate on cell metabolism will be discussed in a second article. In this study an acoustic filter (BioSep) was used as a cell retention device. The experiments were carried out in a chemically defined protein-free medium at bleed rates that are within the range commonly used in industrial production processes. These relatively low bleed rates have not been extensively investigated. Furthermore, in this study each combination of feed and bleed rate was tested in a separate bioreactor run to exclude the effect of aging, selection, or adaptation of cells during the culture period that may occur when obtaining consecutive different steady states in a single run.

**THEORY**

In a perfusion bioreactor nutrients are provided by means of a feed. The medium is removed from the bioreactor via two flows, a cell-free perfusion flow and a small cell-containing flow, the bleed (Fig. 1).

The growth rate can be derived from the viable-cell balance, which is given by:

\[
\frac{dX_v}{dt} = \mu \cdot X_v - B \cdot X_v - P \cdot X_{vp} - \mu_d \cdot X_v \tag{1}
\]

where \( X_v \) is the viable-cell concentration (cells \( m^{-3} \)) in the bioreactor, \( X_{vp} \) is the viable-cell concentration (cells \( m^{-3} \)) in the perfusion flow, \( B \) is the specific bleed rate \( (m^3 \cdot m_{reactor}^{-3} \cdot d^{-1}) \), \( P \) is the specific perfusion rate \( (m^3 \cdot m_{reactor}^{-3} \cdot d^{-1}) \), \( \mu \) is the specific growth rate \( (d^{-1}) \), and \( \mu_d \) is the specific death rate \( (d^{-1}) \). For all cultures executed the separation efficiency of the acoustic filter was larger than 99% for both viable and dead cells. Therefore, the term \( P \cdot X_{vp} \) in the viable-cell balance (Eq. 1) is negligible. In steady state there is no accumulation of viable cells and the growth rate can be derived from Equation 1:

\[
\mu = B + \mu_d \tag{2}
\]
Thus, to calculate the growth rate the death rate should be determined. The specific death rate can in principle be derived from the dead-cell balance:

$$\frac{dX_d}{dt} = \mu_d \cdot X_v - B \cdot X_d - P \cdot X_{dp} - k_i \cdot X_d \quad (3)$$

where $X_d$ is the dead-cell concentration (cells·m$^{-3}$) in the bioreactor, $X_{dp}$ is the dead-cell concentration (cells·m$^{-3}$) in the perfusion flow, and $k_i$ is the first-order lysis-rate constant of dead cells (d$^{-1}$). As stated, for all cultures executed the separation efficiency of the acoustic filter for dead cells was larger than 99%. Therefore, the term $P \cdot X_{dp}$ in the dead-cell balance (Eq. 3) is negligible. In steady state the death rate can be calculated according to:

$$\mu_d = (B + k_i) \cdot \frac{X_d}{X_v} \quad (4)$$

From Eq. 4 it can be seen that this equation can only be used to determine the specific death rate when the lysis rate is negligible or measured. Cells are considered dead when the cell membrane is disrupted and is permeable to the Trypan-blue dye. Cells are considered lysed when they are completely disintegrated. In a perfusion system cells are retained, resulting in long residence times of both viable and dead cells. As a result, significant cell lysis may occur and the death rate may be underestimated. Therefore, the release of the intracellular enzyme lactate dehydrogenase (LDH) is used to calculate the specific death rate. It is assumed that the cells release all intracellular LDH when they die. The balance for the LDH activity in the medium is given by:

$$\frac{dLDH}{dt} = \mu_d \cdot X_v \cdot LDH_{int} - F \cdot LDH - k_{LDH} \cdot LDH \quad (5)$$

where $LDH_{int}$ is the intracellular LDH activity (U·cell$^{-1}$), $F$ is the specific feed rate (m$^3$·bioreactor$^{-3}$·d$^{-1}$), which is the sum of the specific perfusion rate and the specific bleed rate, $LDH$ is the LDH activity in the medium broth (U·m$^{-3}$), and $k_{LDH}$ is the first-order inactivation constant for LDH (d$^{-1}$). In steady state the death rate is derived from Eq. 5 and given by:

$$\mu_d = (F + k_{LDH}) \cdot \frac{LDH}{X_v \cdot LDH_{int}} \quad (6)$$

The intracellular LDH activity is measured at each steady state. In a stability study it was determined that the first-order inactivation constant for LDH at 37°C is negligible. The lysis rate of dead cells can now be attained from the dead-cell balance (Eq. 3) and is in steady state given by:

$$k_i = \mu_d \cdot \frac{X_v}{X_d} - B \quad (7)$$

A commonly used variable to describe the state of an animal-cell culture is the viability. In general, the viability is determined using the viable and dead-cell density determined via Trypan-blue dye-exclusion (TB):

$$Viab_{TB} = 100 \cdot \frac{X_v}{X_v + X_d} \quad (8)$$

Equation 8 can be rewritten using Eq. 4 resulting in the following equation for the Trypan-blue based viability:

$$Viab_{TB} = 100 \cdot \frac{B + k_i}{B + k_i + \mu_d} \quad (9)$$

Equation 9 shows that the Trypan-blue-based viability is not only dependent on the specific bleed and specific death rate, but also on the specific lysis rate of dead cells. When the lysis rate is not negligible the Trypan-blue based viability is clearly no longer a correct measure of cell death. Therefore, for comparison of the viability in the different steady states, viability based on LDH-activity measurements is a better measure. As stated, it is assumed that the cells release all intracellular LDH when they die. Therefore, all dead cells including lysed cells are included in the determination of the death rate and the LDH-based viability. Thus, Eq. 9 can be simplified into the following equation for the LDH-based viability:

$$Viab_{LDH} = 100 \cdot \frac{B}{B + \mu_d} \quad (10)$$

Another way to study the proliferation of cells is by looking at the distribution of cells over the cell cycle. The cell cycle can be divided into four phases, gap1 (G1), DNA synthesis (S), gap2 (G2), and mitosis (M). Besides the four phases of the cell cycle there is an additional phase, G0, the quiescent phase. At a certain point in the G1-phase, the restriction point, the cell decides to either commit to another cell cycle by entering the S-phase or to enter the G0-phase. Cells enter the G0-phase, for instance, when the circumstances are unfavorable for growth, because of nutrient limitation, growth factor deprivation, or inhibitory factors. Entering the G0-phase results in a loss of cell size, metabolic activity, and cellular activities related to initiation of chromosome replication (Park and Ryu, 1994). Cells can return to the cell cycle when conditions are favorable for growth again.

To determine the residence time of cells in the different cell-cycle phases, an age-structured population-balance model can be used. The age distribution of a cell population can be described by the age/time equation of M’Kendrick, which was applied to cell-cycle kinetics by von Foerster (1959) (Martens et al., 1995):

$$\frac{\partial n(a,t)}{\partial t} + \frac{\partial n(a,t)}{\partial a} = -(B + \mu_d) \cdot n(a,t) \quad with \ 0 \leq a \leq t_c \quad (11)$$

where $n$ population-density function (cells·m$^{-3}$·d$^{-1}$), $a$ is the cell age (d), and $t_c$ is the cycling time (d). The first term on the left represents the accumulation of cells of a certain age in time, which is zero at steady state. The second term on the left represents accumulation of cells at a certain age for a given cell age $a$. The rate of accumulation is determined by the probability of cell division $B$ and the death rate $\mu_d$.
due to movement of the cells through the cycle (aging). The terms on the right side of the equal sign represent loss of cells due to the bleed and cell death, which is assumed to be independent of cell age. It is for this moment that hybridoma cells do not go into quiescence (Al-Rubeai et al., 1991) and either grow or die. Under steady-state conditions the cell-age distribution does not change with time, and integration of Eq. 11 then gives:

\[ n(a) = n(0) \cdot e^{-(B + \mu_d)t} \quad \text{with} \quad 0 \leq a \leq t_c \]  

(12)

Knowing the growth rate (\( \mu \)), which equals \( B + \mu_d \) according to Eq. 2, the cycling time can be calculated according to:

\[ t_c = \frac{\ln 2}{\mu} \]  

(13)

The fraction of cells in a specific phase of the cell cycle is defined as the amount of cells in a certain phase divided by the total number of cells participating in the cell cycle, and can be determined via the following equation:

\[ f_x = \frac{\int_0^{t_1} f(x) \cdot e^{-\mu_d a} da}{\int_0^{t_c} f(x) \cdot e^{-\mu_d a} da} \quad \text{with} \quad 0 \leq a \leq t_c \]  

(14)

where \( f_x \) represents the fraction in a specific phase, \( t_1 \) represents the age a cell enters a specific phase (d), and \( t_x \) represents the residence time in the specific phase (d), with \( x \) being G1, S, and G2/M, respectively. The G2 and M-phase are taken together because it is difficult to discriminate between them experimentally. The equations for the different phases can next be rewritten to calculate the residence times in the different phases. Integration of Eq. 14 for the G1-phase gives the following equation for the residence time in this phase:

\[ t_{G1} = \frac{-\ln(1 - 0.5 \cdot f_{G1})}{\mu} \]  

(15)

Next the residence time in the S-phase can be calculated via:

\[ t_s = \frac{-\ln(e^{-\mu_d t_{G1}} - 0.5 \cdot f_s)}{\mu} - t_{G1} \]  

(16)

Finally, the residence time in the G2/M phase is given by:

\[ t_{G2/M} = t_c - t_{G1} - t_s \]  

(17)

MATERIALS AND METHODS

Cell Line and Growth Conditions

A mouse–mouse hybridoma producing an IgG1 was used. The cells were grown in a chemically defined protein-free medium (CD-CHO medium, Life Technologies, Paisley, UK), containing 30 mM glucose. It was supplemented with 40 ml\textsuperscript{-1} of a 200-mM glutamine solution (Life Technologies) and 10 ml\textsuperscript{-1} of a mixture of 10 mM hypoxanthine and 1.6 mM thymidine (Life Technologies).

Bioreactor Operation

The perfusion cultures were carried out in a 3L bioreactor (Applikon, the Netherlands) with a working volume of 1.7L. Cells were retained in the bioreactor using the BioSep (10L) acoustic cell-separation device (Applisens, the Netherlands). The reactor temperature was kept constant at 37°C. The pH was maintained at 7.0 by addition of CO\textsubscript{2} via the headspace and addition of 0.5 M NaOH. The dissolved oxygen level was controlled at 50% air saturation by adding a gas mixture of N\textsubscript{2} and O\textsubscript{2} to the headspace at a rate of 15 L\textsuperscript{-1}.h\textsuperscript{-1}. When necessary the oxygen transfer was increased by pumping part of the gas mixture in the headspace through the sparger.

Several bioreactor runs were carried out at different feed and bleed rates. The tested feed rates (F) were 0.5, 1.0, and 1.5 d\textsuperscript{-1}. At a feed rate of 0.5 and 1.0 d\textsuperscript{-1} bleed rates (B) of 0.05, 0.10, and 0.20 d\textsuperscript{-1} were tested. At a feed rate of 1.5 d\textsuperscript{-1} bleed rates of 0.10, 0.16, and 0.20 d\textsuperscript{-1} were tested. The feed rate is the sum of the bleed rate and the perfusion rate. Three sets of bioreactor runs were carried out. In each set three bioreactors operated at equal feed rates, but different bleed rates were carried out in parallel. To obtain the inoculum for the three bioreactors, cells from a frozen vial were used to inoculate a roller bottle. After six passages in roller bottles, enough cells were available to inoculate the three bioreactors. Cells were passaged in the exponential phase at a density of 2 \( \times \) 10\textsuperscript{6} cells\textsuperscript{-1} and diluted to a final density of 0.5-10\textsuperscript{6} cells\textsuperscript{-1}. An equal procedure was used for all bioreactor runs. After inoculation at a viable-cell concentration of \( \sim 0.5 \times 10\textsuperscript{6} \) cells\textsuperscript{-1} the cells were grown in batch mode for 3 days. Subsequently, the medium flow was increased daily with 0.25 d\textsuperscript{-1} until the desired feed rate was reached. The perfusion and bleed rates were controlled by peristaltic pumps. To maintain a constant culture volume the medium-supply pump was controlled by a level sensor in the bioreactor. The acoustic cell-retention device, the BioSep, was operated at a power input of 3 W, a run time of 300 sec, and a stop time of 4.5 sec. When necessary, the power input was increased to 4–5 W.

Analytical Methods

The viable- and dead-cell concentrations were determined with the Trypan-blue dye-exclusion method using a hemocytometer. Glucose and lactate concentrations were determined enzymatically using the automatic analyzer YSI 2700 (Yellow Springs Instruments, Youngstown, OH).

Lactate-dehydrogenase (LDH) activity was determined as total and extracellular enzyme activity. The intracellular enzyme activity is equal to the difference between the total and extracellular enzyme activity. To prepare extracellular
LDH-activity samples, the cell suspension was centrifuged for 10 min at 400 g and thereafter the supernatant was filtered through a 1.2-μm filter. To prepare total LDH-activity samples, an equal amount of a 0.2% Triton X-100 solution in medium was added to a cell suspension. The samples were stored at 4°C for a maximum of 2 weeks. No loss of LDH activity was observed during this time frame (results not shown) (Goergen et al., 1993). The LDH activity was determined using an analytical detection kit (Sigma, St. Louis, MO).

To prepare samples for cell-cycle analysis, DNA from dead cells was removed from the sample via a treatment with DNase (Roche, Nutley, NJ). Cells (1×10⁷ viable cells resuspended in 2 ml PBS) were incubated with 1 mg·ml⁻¹ DNase for 30 min at 37°C. After washing the cell pellet twice with 1 ml PBS, 4 ml ice-cold ethanol (70%) was added and the samples were stored at −20°C. Before staining, the ethanol was removed and the cells were resuspended in 1 ml PBS. To remove all RNA, 1 ml 0.5 mg·ml⁻¹ RNase (Sigma) was added. The samples were incubated for 30 min at 37°C. Thereafter, 1 ml 50 μg/ml propidium iodide was added. After 30 min incubation at room temperature in the dark the cell-cycle distribution was determined on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The data were acquired with Cell Quest software on a Macintosh Power G4 computer. Subsequently, the distribution of cells over the G1, S, and G2/M phase was estimated by the use of ModFit LT 3.0 software (Verity Software House, Topsham, ME).

RESULTS AND DISCUSSION

Perfusion Culture

A total of nine bioreactor runs were carried out at different feed (F) and bleed (B) rates as described in detail in Materials and Methods. Two representative characteristic growth patterns of the hybridoma cell line in a perfusion culture (F = 1.0 d⁻¹, B = 0.10 d⁻¹ and F = 1.0 d⁻¹, B = 0.20 d⁻¹) are shown in Figure 2. The courses of the viable-cell concentration, viability, glucose, and lactate concentration in both runs are illustrated. Steady state was defined when no significant variation of the viable-cell density, glucose, and lactate concentration was observed over 5 consecutive days. According to this definition steady state was achieved at day 59 for the culture shown in Figure 2A and at day 55 for the culture shown in Figure 2B. For the other seven cultures a similar cultivation time was necessary to achieve steady state (data not shown). For the tested conditions, different steady-state cultivation times were obtained as can be seen, for instance, for the viable-cell concentration in Figure 3A. The long cultivation time necessary to reach steady state was mainly due to oscillations in glucose, glutamine, and lactate concentrations, while the viable-cell concentration generally was constant from day 30 onwards. The fact that it takes so long for the glucose, glutamine, and lactate concentrations to become constant is probably due to the combination of this cell line with a medium containing relatively high nutrient concentrations. The results of the major culture parameters of these runs are individually reviewed and discussed below.

At a feed rate of 1.0 d⁻¹ and a bleed of 0.05 d⁻¹, a viable-cell density of 42×10⁶ cells·ml⁻¹ was reached. At such a high cell density significant foam formation occurred as a result of an increased gas flow through the sparger required to supply the culture with sufficient oxygen. As a consequence, it was difficult to achieve a clear steady state. A higher viable-cell concentration was expected at a feed rate of 1.5 d⁻¹ as compared to a feed rate of 1.0 d⁻¹ and, thereby, more difficulties were expected to achieve steady state at a feed rate of 1.5 d⁻¹. Therefore, it was decided to operate the bioreactor run with a feed rate of 1.5 d⁻¹ at a bleed rate of 0.16 d⁻¹ instead of 0.05 d⁻¹.

Viable-Cell Concentration

The steady-state viable-cell concentration as a function of the feed rate for the different bleed rates is shown in Figure 3A. At a bleed rate of 0.05 d⁻¹ the viable-cell density was found to be twice as high at a feed rate of 1.0 d⁻¹ as
compared to a feed rate of 0.5 d\(^{-1}\) and a bleed rate of 0.05 and 0.10 d\(^{-1}\) have a low specific glucose-consumption rate (<1 pmol-cell\(^{-1}\)-d\(^{-1}\)) and a low yield of lactate on glucose (~0.5 mol-mol\(^{-1}\)), which implies that there is a glucose limitation. Under the conditions of a higher feed and bleed rate the specific glucose-consumption rate is relatively high, 1-2 pmol-cell\(^{-1}\)-d\(^{-1}\), and is accompanied with a yield of lactate on glucose of ~1 mol-mol\(^{-1}\). The residual glucose and lactate concentration are shown in Table I. In the nutrient-limited region the glucose concentration is always smaller than 0.5 mM and lactate concentrations are smaller than 15 mM. In the nonnutrient-limited region the glucose concentration is higher than 1 mM and lactate concentrations are considerably higher, namely, >25 mM. Only the culture operated at a bleed rate of 0.16 d\(^{-1}\) has a glucose concentration smaller than 1 mM. However, based on the high lactate concentrations it clearly belongs to the nonnutrient-limited region. The observed behavior agrees nicely with the behavior expected by Vriezen et al. (1997) for waste metabolites. Based on the data of the viable-cell concentration and the specific glucose-consumption rate, a separation of the tested conditions over the two regions can be made (Fig. 5). The three cultures below the line, operated at low feed and bleed rates, have a low specific glucose-consumption rate, low yield of lactate on glucose, and
the viable-cell density is a function of the feed rate. The four cultures above the line, operated at high feed and bleed rates, have a higher specific glucose-consumption rate and a higher yield of lactate on glucose. In addition, the viable-cell density is independent of the feed rate and decreases with an increase in the bleed rate. Two cultures (operated at a feed rate of 0.5 d\(^{-1}\) with a bleed rate of 0.20 d\(^{-1}\) and at a feed rate of 1.0 d\(^{-1}\) with a bleed rate of 0.10 d\(^{-1}\)) are clearly an intermediate of the two regions. They have a low specific glucose-consumption rate and a low yield of lactate on glucose. However, the viable-cell density seems to be independent of the feed rate.

The observation of an increase of the viable-cell concentration with feed rate, as seen in the nutrient-limited region, was described thoroughly by others (Banik and Heath, 1995a; Hiller et al., 1993; Robinson et al., 1992). To our knowledge, the independence of the viable-cell density from the feed rate was only observed by Robinson et al. (1992). They studied growth patterns of a myeloma cell line in a spin-filter perfusion bioreactor at feed rates between 1.4 and 2.1 d\(^{-1}\) and a relatively high bleed rate of 0.7 d\(^{-1}\). At all tested feed rates, comparable viable-cell densities were obtained.

**Viability, Growth Rate, Death Rate**

To determine if significant cell lysis occurs, cell death was measured via the Trypan-blue dye-exclusion method as well as via lactate-dehydrogenase (LDH) activity measurements. The Trypan-blue-based viability (Eq. 8) and the LDH-based viability (Eq. 10) are both the ratio of viable cells to total cells. However, for the Trypan-blue-based viability the total cell concentration is formed by the sum of the viable and dead cell concentration, as measured using the Trypan-blue dye-exclusion method. Thus, this method does not include cells that have completely disintegrated, whereas for the LDH-based method the total cell concentration consists of viable, dead, and lysed cells. In Figure 6 the Trypan-blue-based viability (solid symbols) as well as the LDH-based viability (open symbols) are plotted as a function of the bleed rate. Under all conditions the LDH-based viability was lower compared to the Trypan-blue-based viability. This observation indicates that significant cell lysis occurs in our perfusion system. In Eq. 9, it is shown when significant lysis occurs the Trypan-blue-based viability overestimates viability because the disintegrated cells are not taken into account. The LDH-based viability takes into account both dead cells and cells that have disintegrated. Therefore, it is a more correct measure of viability. Under the tested conditions the LDH-based viability increases linearly with the bleed rate and is independent of the feed rate, as shown in Figure 6. The effect of feed and bleed rate on viability was studied for a broader range of bleed rates by Hiller et al. (1993) and Banik and Heath (1995a). In both cases a linear increase also of viability with the bleed rate is reported. From Eq. 10 it can be seen that at low bleed rates, where the specific death rate is relatively high, the viability will more or less linearly increase with the bleed rate. At higher bleed rates a smaller increase in viability will be seen. This is even more so because at higher bleed rates the specific death rate decreases. With respect to viability, no distinction between

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**Table 1.** Steady-state values and their standard deviations of the residual glucose and lactate concentration.

<table>
<thead>
<tr>
<th>Bleed rate (d(^{-1}))</th>
<th>0.05</th>
<th>0.09</th>
<th>0.10</th>
<th>0.16</th>
<th>0.52</th>
<th>0.97</th>
<th>1.47</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed rate (d(^{-1}))</td>
<td>0.50</td>
<td>0.98</td>
<td>0.49</td>
<td>0.91</td>
<td>1.49</td>
<td>1.41</td>
<td>0.52</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>(±0.07)</td>
<td>(±0.05)</td>
<td>(±0.11)</td>
<td>(±0.19)</td>
<td>(±0.64)</td>
<td>(±0.06)</td>
<td>(±0.22)</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>(±0.40)</td>
<td>(±1.24)</td>
<td>(±0.20)</td>
<td>(±1.46)</td>
<td>(±0.73)</td>
<td>(±1.30)</td>
<td>(±0.53)</td>
</tr>
</tbody>
</table>

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**Figure 5.** Division of the feed and bleed rates into a nutrient-limited region and a non-nutrient-limited region.

**Figure 6.** Steady-state viability based on Trypan-blue dye-exclusion (solid symbols) and based on LDH-activity measurements (open symbols) at a feed rate of 0.5 d\(^{-1}\) ( ), 1.0 d\(^{-1}\) ( ), and 1.5 d\(^{-1}\) ( ) as a function of the bleed rate.
At a bleed rate of 0.20 d\(^{-1}\) the Trypan-blue-based viability is independent of the feed rate (Fig. 6). Thus, under this condition the lysis rate is found to be independent of the feed rate. However, at a bleed of 0.05 and 0.10 d\(^{-1}\) the Trypan-blue-based viability increases with the feed rate, indicating an increase in lysis of cells. The lysis rate is calculated using Eq. 7. Under these conditions the viable-cell density increases with the feed rate (nutrient-limited region), which is accompanied with higher sparging rates to supply the culture with sufficient oxygen. The increased shear associated with these higher sparging rates forms a probable explanation for the observed increase in cell lysis.

Because significant cell lysis occurs, LDH-activity measurements should be used to determine the death rate, which in turn can be used to calculate the growth rate. In Table II it is shown that only small differences are observed in growth and death rate under the different test conditions. Only at a bleed rate of 0.20 d\(^{-1}\) was a slightly higher growth rate and a slightly lower death rate observed compared to the other bleed rates. Under all other conditions the growth and death rate are independent of the feed rate. Consequently, no distinction between the nutrient-limited and nonnutrient-limited region can be seen with respect to the growth and death rate. For perfusion systems the relationship between growth rate, death rate, and bleed rate has not been extensively studied. The fact that the growth rate is independent of the bleed rate at bleed rates below 0.20 d\(^{-1}\) indicates that a minimal growth rate is reached under the tested conditions. This pattern is observed also in continuous cultures for which the phenomenon of a minimal growth rate is described thoroughly. However, the minimal growth rate of 0.3 d\(^{-1}\) as obtained in our experiments is lower than the minimal growth rate of 0.4–0.5 d\(^{-1}\) that is generally observed in continuous systems (Frame and Hu, 1991; Hiller et al., 1991; Martens et al., 1993; Miller et al., 1988; Ray et al., 1989; Robinson and Memmert, 1991; Simpson et al., 1999).

To conclude, viability is mainly influenced by the bleed rate. In perfusion systems cell lysis may be substantial, leading to an overestimation of viability when only Trypan-blue dye-exclusion measurements are used. Therefore, cell lysis measurements are essential for studies on cell growth and death in perfusion systems. Furthermore, a minimal growth rate of 0.3 d\(^{-1}\) seems to exist in our perfusion system. The viability, growth rate, and death rate are not affected by the type of limitation, since the earlier observed separation of the cultures into the nutrient- and nonnutrient-limited region is not seen for these parameters.

### Cell-Cycle Distribution

The distribution of cells over the different phases of the cell cycle for the runs operated at a feed rate of 1.0 and 1.5 d\(^{-1}\) are shown in Figure 7. The cell-cycle distribution for the cultures in the nonnutrient-limited region (all three cultures operated at a feed rate of 1.5 d\(^{-1}\) and the culture operated at a feed rate of 1.0 d\(^{-1}\) and a bleed of 0.20 d\(^{-1}\)) is similar, which indicates that under these conditions the cell-cycle distribution is independent of the bleed and feed rate. For the two cultures in the nutrient-limited region, i.e., at a feed rate of 1.0 d\(^{-1}\) and a bleed of 0.05 and 0.10 d\(^{-1}\), a larger fraction of cells is present in the G1 phase and a smaller fraction in the S-phase (Fig. 7).

The residence times in the different phases of the cell cycle can be calculated using Eqs. 15–17 assuming that cells are not arrested in a specific phase of the cell cycle. For the nonnutrient-limited region this results in an average residence time in the G1 phase, S phase, and G2/M phase of 20–24 h, 20–28 h, and 6–8 h, respectively. In other perfusion systems, similar residence times were found at equal growth rates for both hybridoma and CHO cells (Leeelavatcharamas et al., 1999; Park and Ryu, 1994). In continuous cultures it was found that the lengths of the S and G2/M phase in mammalian cells are practically constant.

### Table II. Steady-state values and their standard deviations of the growth and death rate.

<table>
<thead>
<tr>
<th>Bleed rate (d(^{-1}))</th>
<th>0.05</th>
<th>0.10</th>
<th>0.16</th>
<th>0.20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed rate (d(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.24 (±0.06)</td>
<td>0.28 (±0.02)</td>
<td>0.29 (±0.04)</td>
<td>0.31 (±0.03)</td>
</tr>
<tr>
<td>0.98</td>
<td>0.31 (±0.01)</td>
<td>0.34 (±0.04)</td>
<td>0.35 (±0.01)</td>
<td>0.33 (±0.01)</td>
</tr>
<tr>
<td>0.49</td>
<td>0.31 (±0.01)</td>
<td>0.34 (±0.04)</td>
<td>0.35 (±0.01)</td>
<td>0.33 (±0.01)</td>
</tr>
<tr>
<td>0.91</td>
<td>0.31 (±0.01)</td>
<td>0.34 (±0.04)</td>
<td>0.35 (±0.01)</td>
<td>0.33 (±0.01)</td>
</tr>
<tr>
<td>1.49</td>
<td>0.31 (±0.01)</td>
<td>0.34 (±0.04)</td>
<td>0.35 (±0.01)</td>
<td>0.33 (±0.01)</td>
</tr>
<tr>
<td>1.41</td>
<td>0.31 (±0.01)</td>
<td>0.34 (±0.04)</td>
<td>0.35 (±0.01)</td>
<td>0.33 (±0.01)</td>
</tr>
<tr>
<td>0.52</td>
<td>0.31 (±0.01)</td>
<td>0.34 (±0.04)</td>
<td>0.35 (±0.01)</td>
<td>0.33 (±0.01)</td>
</tr>
<tr>
<td>0.97</td>
<td>0.31 (±0.01)</td>
<td>0.34 (±0.04)</td>
<td>0.35 (±0.01)</td>
<td>0.33 (±0.01)</td>
</tr>
<tr>
<td>1.47</td>
<td>0.31 (±0.01)</td>
<td>0.34 (±0.04)</td>
<td>0.35 (±0.01)</td>
<td>0.33 (±0.01)</td>
</tr>
</tbody>
</table>

*The death rate is determined using lactate dehydrogenase activity measurements (Eq. 6).*

Figure 7. Cell-cycle distribution at steady-state as a function of feed rate. Error bars indicate the standard deviation of five consecutive steady-state data points within a run.
with a typical duration of 6–10 h and 4–6 h, respectively (Martens et al., 1993; Prescott, 1976; Shields, 1978; Smith and Martin, 1974). There are two possible explanations for this phenomenon. First, all cells pass through the S and G2/M phase at a slower rate compared to continuous cultures or, in other words, both phases are prolonged. Second, a fraction of the cells move at a very slow or zero rate, while the rest of the cells move at a rate comparable to continuous cultures. In other words, a fraction of the cells are arrested in the S and G2/M phase. Since the measured cell-cycle distribution is based only on DNA content, it is impossible to discriminate between cycling and noncycling cells. Therefore, these measurements do not provide enough information to test both hypotheses.

Likewise, the exact cause for the increase in the G1 fraction for the nutrient-limited cultures is difficult to find. When nutrients are exhausted the cells are not able to pass the restriction point (Park and Ryu, 1994), and therefore cannot enter the S phase. This inability to pass the restriction point could possibly lead to either a transition into the G0 phase or a prolongation of the G1 phase. Cells in the G0 and G1 phase have an equal DNA content and, therefore, both appear in the G1 fraction.

**Nutrient Limitation Versus Nonnutrient Limitation**

Two regions can be distinguished within the tested feed and bleed rates. As shown in Figure 5, the cultures operated at a feed rate of 0.5 and 1.0 d−1, with a bleed rate of 0.05 or 0.10 d−1 appear to be nutrient-limited. In this region the viable-cell density increases with an increase in the feed rate and seems to be independent of the bleed rate. For the other conditions, at the higher feed and bleed rates the viable-cell density increases with an increase of the residence time of the cells in the bioreactor and is independent of the feed rate, suggesting a limitation of a cell-related factor. Robinson et al. (1992) observed this independence of the feed rate for the viable-cell concentration in a spin-filter perfusion bioreactor operated at a bleed rate of 0.7 d−1. This phenomenon was explained as a growth-rate limitation. Based on our results a growth-rate limitation seems unlikely, since under our tested conditions the highest bleed rate was 0.20 d−1 and the cells grew at their minimal rate. Although the exact nature of the limitation in our system is still unknown and needs further research, several explanations for hybridoma-growth limitation described in literature can be excluded, as discussed in more detail below (Banik and Heath, 1996; Europa et al., 2000; Follstad et al., 1999; Hawerkamp et al., 2001; Lee et al., 1995; Mahadevan et al., 1994; Zeng et al., 1998).

Cell growth could be negatively influenced by either intracellular or extracellular waste-product accumulation. High nutrient concentrations within the feed could lead to an increased production of inhibitory waste products such as lactate and ammonia. Especially at low feed rates this could be a problem, because waste-product removal is slower as compared to high feed rates (Banik and Heath, 1996; Mahadevan et al., 1994). If waste-product inhibition occurred, equal viable-cell concentrations would be expected under all nonnutrient-limited conditions. However, different cell concentrations were observed in steady state, depending on the bleed rate. Additionally, lactate and ammonia did not reach inhibitory levels (data not shown). Therefore, waste-product inhibition does not seem to be a cause for the observed effect.

Another explanation for the limitation could be the phenomenon of multiple steady states. Follstad et al. (1999) observed that in continuous cultures different viable-cell concentrations could be obtained at the same dilution rate due to differences in nutrient-consumption rates. The cells were forced into a more efficient nutrient consumption by adaptation to low nutrient levels during the preculture phase. Due to the adaptation, higher viable-cell concentrations were observed in the subsequent steady states (Europa et al., 2000; Follstad et al., 1999). For all precultures of the experiments described in this study the same procedure was followed. Furthermore, at a feed rate of 1.5 d−1 the viable-cell density increased linearly with a decrease in the bleed rate, while the glucose consumption rate was comparable for all three bleed rates tested (Fig. 4). Consequently, the phenomenon of multiple steady states is not the reason for the observed effect.

Several researchers have speculated about the existence of an extracellular autoinhibitor produced by hybridoma cells, which negatively influences cell growth (Hawerkamp et al., 2001; Lee et al., 1995; Zeng et al., 1998). The involvement of such an inhibitor or limiting factor results in a strong correlation between the ratio of the growth and death rate and the ratio of the viable-cell density and the feed rate as shown by Zeng et al. (1998). For the growth rates we tested, no correlation was observed between the ratio of the growth and death rate and the ratio of the viable-cell density and the feed rate. Therefore, we conclude that the effect is not caused by a single autoinhibitor.

Growth factors, as present in serum, are known to positively affect cell growth. The independence of the viable-cell density of the feed rate under serum-free conditions as found in this study was only observed by Robinson et al. (1992). This suggests that growth factors may be limiting under these conditions. If growth factors were limiting, an increase in feed rate would cause an even lower growth-factor concentration in the medium and, therefore, a decrease in viable-cell density. However, at a bleed rate of 0.20 d−1 the same viable-cell density was obtained at all feed rates (Fig. 3A). Therefore, a single growth-factor limitation is not responsible for the observed effect. However, cell growth is strongly regulated by both growth-promoting and inhibitory factors and a complex interplay of these factors causing the observed effect cannot be excluded.

In conclusion, the observed phenomenon that the viable-cell density increases with a decrease in bleed rate and is independent of the feed rate cannot be explained by the described mechanisms. The dependence on the bleed rate and independence of the feed rate indicates that at
least a cell-related factor is involved. More research is needed to find the mechanism behind the limitation. Understanding this mechanism could make it possible to increase the viable-cell density at higher bleed rates and thus at higher viabilities.

Design of a Perfusion System

Based on the viable-cell density, viability and specific productivity data an optimal feed and bleed rate can be found for a perfusion system. Because the total available production volume in the world is expected to be limiting in a few years (Garber, 2001), the optimal perfusion system is here defined as the system in which the highest volumetric productivity can be obtained. Of course, the effect of culture conditions on the specific productivity is also an important variable for designing an optimal perfusion process. For our system under all tested conditions the specific productivity of the monoclonal antibody was constant (data not shown). Therefore, the feed and bleed rate resulting in the highest viable-cell density is in principle the most optimal situation. Furthermore, the feed rate should be chosen at the border between the nutrient-limited and nonnutrient-limited region. A further increase in the feed rate would not result in a further increase in viable-cell density and would thus lead to a decrease in the product concentration and waste of expensive medium. However, another important point to consider is the effect of the viability of the culture on the product quality and the purification process. At the mentioned optimal condition the viability is relatively low compared to the other conditions. If a higher viability were desired, a higher bleed rate would be a better choice (Fig. 6). However, this would lead to a lower viable-cell density, resulting in a lower volumetric productivity. It is difficult to say what will be the optimal bleed rate in this case, since this will largely depend on the viability desired. However, one should realize that for bleed rates above the minimal growth rate the gain in viability per unit increase in bleed rate quickly declines. When a certain bleed rate is chosen, the feed rate should be set at such a rate that the cells are just in the nonnutrient-limited region. Under the test conditions the highest viable-cell density is obtained at the lowest bleed rate (0.05 d\(^{-1}\)) and a feed rate of 1.0 d\(^{-1}\). Under this condition a 10-fold increase in volumetric productivity is obtained as compared to the batch process.

CONCLUSIONS

Two regions can be distinguished within the tested feed and bleed rates. In the first region at low feed and bleed rates the viable-cell density increases with the feed rate. In this region the cells are nutrient-limited. In the second region at the higher feed and bleed rates the viable-cell density is independent of the feed rate and decreases with an increase in bleed rate. This dependence of the viable-cell density on the residence time suggests that a cell-related factor is involved. However, more research has to be carried out to find the explanation for this limitation.

In our perfusion process cell lysis is not negligible; therefore, LDH measurements are essential to accurately measure viability. Viability is more or less linear dependent on the bleed rate and independent of the feed rate. The growth and death rates were constant for all cultures, except the cultures operated at a bleed rate of 0.2 d\(^{-1}\). At this bleed rate a growth rate just above the minimal growth rate of 0.3 d\(^{-1}\) was observed and a death rate slightly lower than at the lower bleed rates.

For the optimal design of a perfusion process it should first be established whether viability is an important parameter with respect to product quality. If not, the bleed rate should be chosen as low as possible. If low viabilities should be avoided the bleed rate should be chosen higher, depending on the viability desired. To obtain the highest volumetric productivity the feed rate should be set at such a rate that the cells are just in the nonnutrient-limited region.

NOMENCLATURE

- \(a\) Cell age (d)
- \(B\) Specific bleed rate (m\(^3\)m\(^{-3}\)d\(^{-1}\))
- \(f_a\) Fraction of cells in a specific phase (-)
- \(F\) Specific feed rate (m\(^3\)m\(^{-3}\)d\(^{-1}\))
- \(k_l\) First-order lysis-rate constant of dead cells (d\(^{-1}\))
- \(k_{LDH}\) First-order inactivation constant of LDH (d\(^{-1}\))
- LDH LDH activity (U m\(^{-3}\))
- LDH\(_{act}\) LDH activity per viable cell (U cell\(^{-1}\))
- \(n\) Population density function (cells m\(^{-3}\))
- \(P\) Specific perfusion rate (m\(^3\)m\(^{-3}\)d\(^{-1}\))
- \(q_{glc}\) Specific glucose consumption rate (mol cell\(^{-1}\)d\(^{-1}\))
- \(t\) Time (d)
- \(X\) Cell density (cells m\(^{-3}\))
- \(Y_{lacglc}\) Yield of lactate on glucose (mol mol\(^{-1}\))
- \(\mu\) Specific growth rate (d\(^{-1}\))
- \(\mu_a\) Specific death rate (d\(^{-1}\))

Subscripts

- c Cycling cells
- d Dead cells
- dp Dead cells in perfusion flow
- G1 Gap1
- G2 Gap2
- LDH Lactate dehydrogenase
- M Mitosis
- S DNA synthesis
- v Viable cells
- vp Viable cells in perfusion flow

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References


