Antibody Production by a Hybridoma Cell Line at High Cell Density Is Limited by Two Independent Mechanisms

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Abstract: Our previous attempt to model the stationary phase of production-scale hollow-fiber bioreactors using a scaled-down micro hollow-fiber bioreactor resulted in a predicted antibody production rate that was three- to fourfold lower than the actual value (Gramer and Poeschl, 2000). Medium limitations were suspected as the reason for the discrepancy. In this study, various increases in medium feed rate were implemented in the micro bioreactor by increasing the diameter of the silicone tubing that houses the hollow fibers. Because larger diameter tubing may induce oxygen limitations, we also explored the effect of medium recirculation to enhance oxygenation. Antibody production in the micro bioreactor increased both as a result of increased medium supply and due to medium recirculation. However, these parameters increased antibody production through two independent mechanisms. The increased medium supply resulted in a higher cell-specific antibody production rate, but not a higher viable cell density. Medium circulation resulted in the support of a higher viable cell density, but had little effect on the cell-specific secretion rate. The two mechanisms of enhanced antibody production were additive, demonstrating that simultaneous parameters can limit antibody production by this cell line in a hollow-fiber system. When the medium feed and circulation rates were increased to a volumetrically proportional scale, scale-up predictions from the micro bioreactor matched the actual data from the production-scale system to within 15%. These data demonstrate the usefulness of the micro bioreactor for characterizing cell growth and limiting mechanisms at high cell densities. *Biotechnol Bioeng* **79: 277–283, 2002.**

Keywords: scale-up; model system; optimization; medium limitation; oxygen limitation; hollow-fiber bioreactor

INTRODUCTION

A number of theoretical models have been proposed to describe performance of hollow-fiber bioreactors (reviewed in Brotherton and Chau, 1996). The models are based primarily on prediction of cell densities assuming oxygen as the limiting component; this assumption is justified due to the relatively low solubility of oxygen in cell culture medium. However, while these models are very useful for examining oxygen delivery, commercial hollow-fiber bioreactors are not necessarily running under oxygen-limited conditions. Factors affecting the performance of hollow-fiber bioreactors are not well understood. As a result, factors other than oxygenation are likely to limit the performance of a hollow-fiber system in some cases. For example, the proper distribution of growth factors on the cell side of the fiber is a key consideration (Gramer et al., 1999; Piret and Cooney, 1990).

Standard hollow-fiber bioreactors are not well suited for elucidation of limiting mechanisms. Direct cell enumeration in a standard hollow-fiber bioreactor is essentially impossible so that cell-specific parameters cannot be obtained. Another problem is that pumps are required for medium addition, removal, and circulation, which severely limits the number of conditions or replicates that can be examined at a time.

A micro hollow-fiber bioreactor was recently introduced to solve these problems (Gramer and Poeschl, 1998). Cells are placed inside of the fibers. The fibers are encased in a piece of silicone tubing, which is permeable to gasses and forms an outer compartment for use as a medium reservoir. After inoculation, the bioreactor is placed in an incubator and used essentially like a T-flask. The micro bioreactor was used to demonstrate that small molecular weight serum components, not oxygen, were limiting the growth of a murine hybridoma in a hollow-fiber bioreactor (Gramer and Poeschl, 1998). The micro bioreactor is very useful for such an investigation, and requires only a 3-day test with minimal effort and cost. Based on the micro bioreactor results, the large-scale solution was determined: include serum on both sides of the fiber for the duration of the growth phase, and then reduce the serum content on the non-cell side upon bioreactor confluency. Alternatively, the micro bioreactor can be used as a selection agent to quickly isolate subclones that do not require serum on the non-cell side for

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optimal growth (Gramer and Britton, 2000). The micro bioreactor has also been used to successfully formulate basal media such that serum- or conditioned medium-sensitive cell lines no longer require serum or conditioned medium on the non-cell side for optimal growth (unpublished results).

While the micro bioreactor is useful for quantitative prediction of initial cell growth in a production-scale hollow-fiber system, it was less useful for quantitative prediction of the steady-state antibody production rate (Gramer and Poeschl, 2000). The predicted antibody production rate was three- to fourfold lower on a volumetric basis in the micro bioreactor compared to the production-scale system. This discrepancy was attributed to the likelihood that the micro bioreactor medium reservoir provided fourfold less medium on a volumetric basis. One solution to increase medium availability in the micro bioreactor is to increase the diameter of the silicone tubing. However, the tubing diameter was limited in that study due to concerns regarding oxygen limitation.

This provides a dilemma; increasing the tubing diameter to accommodate medium limitations may result in the system now being oxygen limited. These suppositions are based on theoretical oxygen calculations using published procedures (Piret and Cooney, 1991). In this study, we explored the effect of increasing tubing diameter to alleviate potential medium limitations. We also explored the possibility of oxygen limitations by examining the effect of recirculating the medium.

MATERIALS AND METHODS

Cells and Media

A proprietary murine hybridoma secreting IgG was used for this study. This cell line is known as Clone 10 in a previous study (Gramer and Britton, 2000). The basal medium composition was a proprietary formulation developed specifically for murine hybridomas in hollow-fiber bioreactors. Serum-supplemented medium was prepared by adding 10% fetal bovine serum (FBS; Hyclone) to basal medium. Cells were routinely propagated in 10% FBS-supplemented medium in humidified incubators with 5% CO_2.

Assays

The viable and total cell concentrations were determined with a hemacytometer using trypan blue. Glucose and lactate concentrations were measured with a YSI-2700 Select Bioanalyzer (Yellow Springs Instruments). Antibody concentrations were determined by ELISA. Dissolved oxygen (DO) and pH were determined with an AVL-990 blood-gas analyzer (AVL Scientific).

Micro Bioreactors

Micro hollow-fiber bioreactors (US Patent No. 6,001,585) were constructed and used essentially as previously described (Gramer and Poeschl, 2000). Each bioreactor contained 30 cellulose acetate fibers (ID of 200 μm, OD of 230 μm, 10 kD molecular weight cut-off) encased in a 20-cm piece of silicone tubing. The intracapillary (IC) volume was 0.2 mL. The extracapillary (EC) volume, depending on the diameter of the silicone tubing, was 4.6 mL (ID 0.635 cm, OD 1.11 cm), 10 mL (ID 0.940 cm, OD 1.57 cm), or 20 mL (ID 1.27 cm, OD 1.91 cm). Cells were inoculated inside the fibers in 10% FBS medium at a nominal concentration of 50 million viable cells per mL. The EC contained basal medium, and was exchanged for fresh basal medium on a daily basis. Glucose, lactate, pH, and DO were measured in the spent medium on a daily basis. The IC medium was harvested after 3 days for cell counts and ELISA.

Circulation of EC Medium

The basal EC medium was circulated in some of the bioreactors. A 45-cm piece of #13 silicone tubing was hooked up to the EC ports on either end of a micro bioreactor. The circulation tubing added a negligible volume of medium (0.2 mL). The tubing was loaded into a peristaltic pump with a quick-release pump head, and the entire assembly was place in the incubator. Medium was circulated at 1 mL/min.

Determination of Cell-Specific Rates

Cell-specific rates are determined by plotting the change in concentration (antibody or glucose) vs. the integral of viable cells. In this study, the integral was estimated assuming a linear change in viable cell density between the day of inoculation and the day of harvest, 3 days later. Since the viable cell density changes little in 3 days, this method is useful for determining cell-specific rates without requiring a harvest every day (see Gramer and Poeschl, 1998 for five examples of every day harvest supporting this method).

Production-Scale Hollow-Fiber System

The Acusyst-MAXIMIZER hollow fiber bioreactor system (Biovest International) was used for this study. These results were previously published (Clone 10 cell line in Fig. 3 of Gramer and Britton, 2000).

Significance

Significance was determined using a two-tailed *t*-test. A $P < 0.05$ was considered significant.

RESULTS

Because of limited incubator space, only a few conditions could be tested at once. The micro bioreactor experiments were performed on three separate occasions, one each for the 4.6, 10, and 20-mL bioreactors. A control 4.6-mL stagnant bioreactor was inoculated in duplicate for each set of experiments. There were no statistically significant differences in any of the parameters reported in this article between the control bioreactors inoculated for each experiment, demonstrating that comparison between experiments is valid (data not shown).

Cell Density

There were no significant differences in the viable cell densities when the media feed rates were increased in stagnant or circulated bioreactors (Fig. 1). Circulation consistently increased the viable cell density from a range of 41–47 million/mL to 62–63 million/mL (significant difference), while the total cell density was about the same in each case (no significant difference). As a result, increased media had little effect on percent viability (not significant). Circulation consistently resulted in increased cell viability, which appeared more pronounced as the bioreactor diameter was increased; however, the difference in viability between stagnant and circulated conditions was only significant for the 20-mL bioreactor (Fig. 1).

Glucose Metabolism

The glucose concentration was a bit lower in the 4.6-mL bioreactors and about the same in the 10-mL and 20-mL bioreactors (Fig. 2). The glucose-uptake rate (GUR) increased little over the 3-d period for the 4.6-mL bioreactors, while an increased GUR was seen in the 10-mL and 20-mL bioreactors. Circulation always resulted in a lower glucose concentration and higher glucose uptake rate (GUR). This was not due to a significant change in the cell specific GUR (Fig. 4), but rather to the significant increase in viable cell density (Fig. 1). Increased media feed rates also resulted in an increased GUR (Fig 2). In contrast to circulation, the increase in GUR was due to do a significant increase in cell-specific GUR (from 0.2 to 0.5 pmol/cell/d as the bioreactor size increased from 4.6 to 20 mL) (Fig. 4), and not due to a significant change in the viable cell density (Fig. 1).

Lactate Metabolism

The lactate concentration decreased with increasing EC media feed rates and increased as a function of circulation due to the higher viable cell density (Fig. 2). The lactate concentration, and thus LPR, increased each day for all bioreactors, with larger increases associated with higher medium feed rates. The LPR/GUR ratio was higher in larger bioreactors with more available medium, and decreased each day. Clearly, the LPR/GUR ratio is a function of how spent the medium is with more spent medium resulting in a lower ratio (with spent medium being defined as the residual glucose concentration). Circulation resulted in a lower LPR/GUR. One interpretation is that more oxygen results in more efficient glucose usage. However, another interpretation is more likely; circulation resulted in a higher cell density

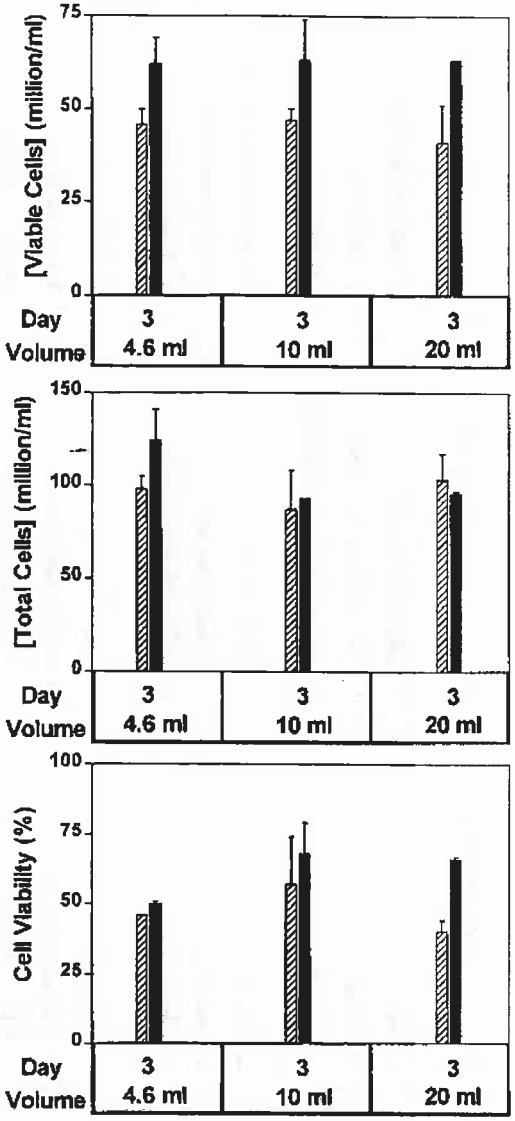

Figure 1. Effect of medium feed rate and circulation on cell growth in micro bioreactors. Cells were inoculated at a nominal concentration of 50 million/mL inside the fibers (0.2 mL volume) in 10% FBS. The basal medium outside the fibers was 4.6, 10, or 20 mL, depending on the diameter of the bioreactor. Half of the bioreactors were stagnant (hashed bars) while the basal medium was circulated at 1 mL/min for the other half (solid bars). The basal medium was changed on a daily basis, and the cells were harvested after 3 days. Data shown represent the average and standard deviation of duplication bioreactors. The media feed rate had little effect on cell growth, while circulation resulted in increased cell density and viability.

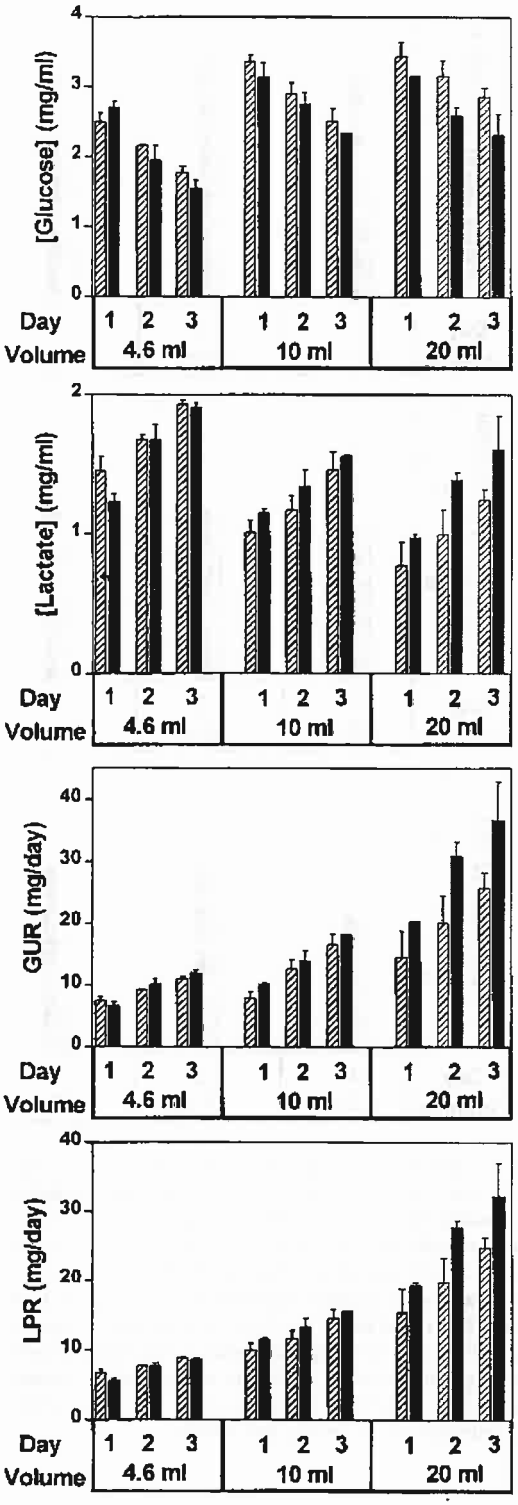

Figure 2. Glucose and lactate metabolism in micro bioreactors. See the caption to Figure 1 for details (hashed bars were stagnant; solid bars were circulated). Increased media feed and circulation both resulted in increased glucose uptake and lactate production.

and a more spent medium, which, in turn, resulted in a lower LPR/GUR. We have seen this phenomenon with other cell lines, and attribute it to the fact that a more spent medium results in a lower pH and higher lactate concentration (unpublished results). The reduced pH or elevated lactate concentration suppresses lactate production more so than glucose consumption, resulting in a decreased LPR/GUR ratio. Similar observations have been noted by others (Banik and Heath, 1996; Ozturk and Palsson, 1991).

Dissolved Oxygen (DO)

Oxygen delivery is expected to be higher for circulating bioreactors. The DO measured in the EC medium was clearly higher for the 4.6-mL bioreactors, but not for the rest (Fig. 3). There are a few possible explanations. One problem is that there is substantial error in this reading, so only very large differences are discernable. The other problem is that the reading is only an average of the EC medium. With the larger bioreactors, a larger percentage of the medium is closer to the surface of the silicone tubing. As a result, lower DO concentrations near the fiber surface are washed out by the bulk DO. In addition, the bioreactors with circulation had higher viable cell densities, so that the increased oxygen demand could offset some of the increase in DO that might be seen from circulation.

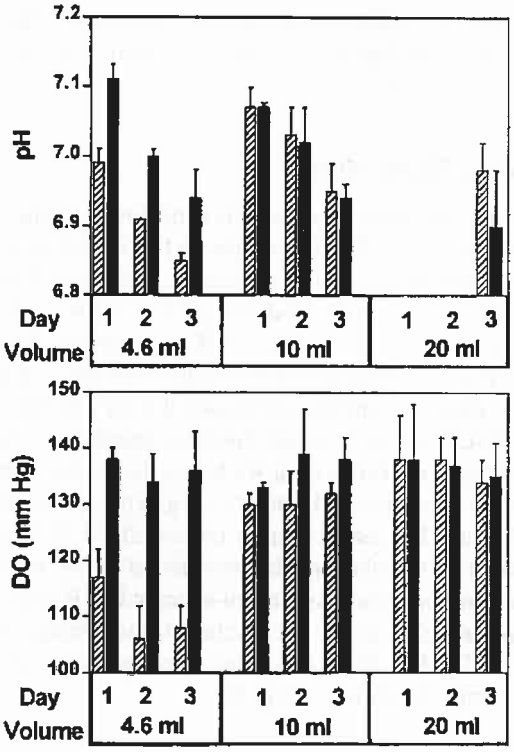

Figure 3. Effect of circulation on gas transfer in micro bioreactors. See caption to Figure 1 for details (hashed bars were stagnant, solid bars were circulated; pH readings were not available for the 20-mL bioreactor on days 1 and 2). The bulk medium pH and DO were clearly higher for 4.6-mL bioreactors, indicating higher gas transfer. Higher gas transfer was less apparent in the larger bioreactors, perhaps due to a washing out effect of the bulk medium.

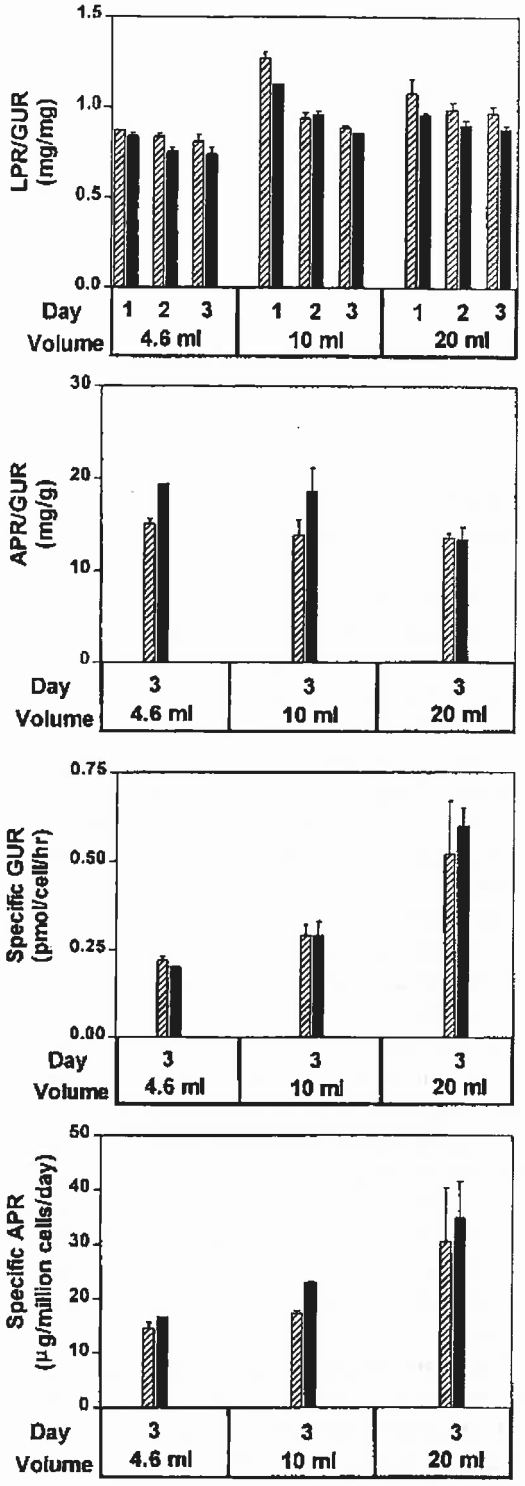

Figure 4. Metabolic rates and ratios in micro bioreactors. See caption to Figure 1 for details (hashed bars were stagnant; solid bars were circulated). The LPR/GUR ratio depends on residual glucose concentration and pH. The APR/GUR ratio is higher in circulated bioreactors when medium is limiting. The specific GUR and APR are strong functions of media feed rate, but not circulation.

pH

The pH is most dependent on the concentrations of lactate and CO_2. Higher metabolic activity results in more respired CO_2 and a higher production of lactate, which is neutralized by sodium bicarbonate to evolve additional CO_2. The excess CO_2 must be removed to provide pH buffering. Circulation is expected to remove CO_2 faster, resulting in faster equilibrium, and potentially a higher pH. For the 4.6-mL bioreactors, the lactate concentrations were similar when stagnant or circulated, and circulation clearly increased the pH of the bulk medium (Fig. 3). At higher feed rates, the pH was actually a bit lower in the circulated bioreactors. This is most likely explained by the fact that the lactate concentrations were higher in the circulated bioreactors due to higher cell-specific lactate production rates. Respiratory CO_2 is also expected to be higher in the circulating bioreactors due to the higher cell density.

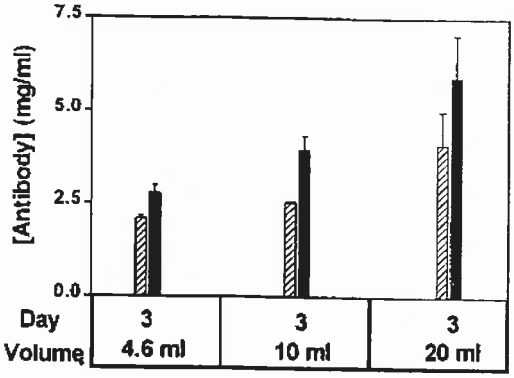

Figure 5. Antibody production by cells in the micro bioreactor. See caption to Figure 1 for details (hashed bars were stagnant; solid bars were circulated). Increased media feed resulted in higher antibody production through an increase in cell-specific APR, while circulation resulted in higher antibody production through an increased cell density.

Antibody

The antibody concentration increased both as a result of increased medium feed and from circulation (Fig. 5). There was a threefold difference in the lowest to the highest concentrations. Increased antibody production was achieved though two distinct mechanisms. Increased medium feed resulted in increased antibody production primarily by supporting a significantly higher cell-specific antibody production rate (APR). Circulation increased antibody production primarily by supporting a significantly higher viable cell density. These two mechanisms were additive, demonstrating that simultaneous mechanisms can limit antibody production in a hollow-fiber bioreactor. Circulation resulted in a significant increase in the APR/GUR ratio in the 4.6-mL and 10-mL bioreactors, but had no significant effect on this ratio in the 20-mL bioreactor (Fig. 4). The APR/GUR ratio may be a useful tool in determining whether the culture is medium limited. Overfeeding results in wasted medium while underfeeding results in lower overall production.

Table I. Scale-up predictions (glucose uptake rate, GUR; lactate production rate, LPR; antibody production rate, APR).

Media feed rate (mL/d)	Condition	GUR (mg/h)	LPR (mg/h)	APR (mg/d)
4.6	Stagnant	248	185	70
	Circulated	278	183	93
10	Stagnant	343	303	85
	Circulated	377	325	131
20	Stagnant	533	515	136
	Circulated	762	671	197
Production scale actuals		854	779	230

Scale-Up Predictions

Data from day 3 of the 0.2-mL micro bioreactors were used to predict the metabolic activity of a 100-mL production scale bioreactor, a 500-fold scale-up. Data from this cell line in a 100-mL bioreactor were previously published (Gramer and Britton, 2000). As shown in Table I, the original method using a 4.6-mL stagnant bioreactor underpredicted the metabolic activity of the production-scale bioreactor by about three- to fourfold. These data are consistent with the previous scale-up study using other cell lines and media formulations (Gramer and Poeschl, 2000). As the media feed is increased and circulation is implemented, the predicted metabolic activity approaches to within 10–15% of the actual metabolic activity in the 500-fold scaled-up system. The closest condition, 20 mL daily feed with 1 mL/min circulation, is a direct 500-fold scale-up in terms of media feed rate and circulation rate (the 100-mL bioreactor had a 500 mL/min circulation rate and a 10 L/d feed rate). These data demonstrate that both the increased medium feed and the circulation are necessary for reasonable quantitative prediction of scale-up performance.

DISCUSSION

The ultimate goal of this project is to define a fast, easy-to-implement scale-down model that is useful for quantitative prediction of production-scale hollow-fiber bioreactors. A commercial hollow-fiber bioreactor is typically inoculated at about 5 million cells per mL. The cells expand in the bioreactor over 1–2 weeks. Upon confluency, the system reaches a steady production phase. In the first attempt, cells were inoculated into micro bioreactors at 5 million/mL to simulate the growth phase and 50 million/mL to simulate the production phase, and the bioreactors were harvested over a 4–6 days (Gramer and Poeschl, 2000). Growth-phase correlations were quantitative. In addition, it was apparent that cell lines with faster initial growth and higher viability during the growth phase typically performed better in the production phase; this correlation is strictly qualitative, but has been useful for optimization (Gramer and Britton, 2000).

Quantitative production-phase correlations were problematic. The micro bioreactor appeared to be medium-limited at the higher inoculation density with 4.6 mL of medium feed per day. One approach to feeding more medium is to change medium manually more than once a day. Another approach is to continually pump fresh medium at an elevated rate. However, these approaches take away from the original goals of the project. Another approach is to increase the diameter of the silicone tubing, which in turn, increases the size of the medium reservoir. However, we were concerned with the probability that the bioreactors would then be oxygen limited.

Data presented here demonstrate that cells were in fact, medium-limited in the 4.6-mL bioreactor. Medium circulation somewhat enhanced the performance of the 4.6-mL bioreactor, but appeared to have a larger effect as the bioreactor size was increased to 10 mL and then 20 mL. Increased medium feed resulted in higher antibody concentrations by supporting a higher cell-specific production rate. Circulation resulted in increased antibody production by increasing the cell density. These two mechanisms were additive, demonstrating that simultaneous factors can be limiting. These findings should be considered when the goal is to design predictive mathematical algorithms for hollow fiber bioreactors.

The exact mechanism affected by circulation was not pursued in this study. The most likely interpretation is that enhanced performance is due to increased oxygenation. Because medium was limiting, reduction in medium component gradients could also result in better performance. However, this interpretation is unlikely since the mechanisms of enhanced performance were different for increased medium feed and circulation. An additional possibility is better CO_2 exchange, resulting in better pH control. However, we don't believe this is the case, because cell density appears to be independent of pH. More work is necessary to characterize this effect, for example, by placing stagnant micro bioreactors in an incubator with altered oxygen or CO_2 levels.

The availability of a simple, inexpensive, and quantitative prediction tool opens the door for efficient optimization of hollow-fiber bioreactor parameters. Optimization parameters include media feed rate, media formulation, cell line selection, and variation of operating conditions such as temperature. More experience is necessary to validate the general the usefulness of this approach.

References

Banik GG, Heath CA. 1196. High density hybridoma perfusion culture: Limitation vs inhibition. Appl Biochem Biotechnol 61:211–229.

Brotherton JD, Chau PC. 1996. Modeling of axial-flow hollow fiber cell culture bioreactors. Biotechnol Prog 12:575–590.

Gramer MJ, Poeschl DM. 1998. Screening tool for hollow fiber bioreactor process development. Biotechnol. Prog. 14:203–209.

Gramer MJ, Poeschl DM, Conroy MJ, Hammer BE. 1999. Effect of harvesting protocol on performance of a hollow fiber bioreactor. Biotechnol Bioeng 65:334–340.

Gramer MJ, Poeschl DM. 2000. Comparison of cell growth in T-flasks, in micro hollow fiber bioreactors, and in an industrial scale hollow fiber bioreactor system. Cytotechnology 34:111–119.

Gramer MJ, Britton TL. 2000. Selection and isolation of cells for optimal growth in hollow fiber bioreactors. Hybridoma 19:407–412.

Piret JM, Cooney CL. 1990. Mammalian cell and protein distributions in ultrafiltration hollow fiber bioreactors. Biotechnol Bioeng 36: 902–910.

Piret JM, Cooney CL. 1991. Model of oxygen transport limitations in hollow fiber bioreactors. Biotechnol Bioeng 37:80–92.

Ozturk SS, Palsson BO. 1991. Growth, metabolic, and antibody production kinetics of hybridoma cell culture 2: Effects of serum concentration, dissolved oxygen concentration, and medium pH in a batch reactor. Biotech Prog 7:481–494.