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Comparison of cell growth in T-flasks, in micro hollow fiber bioreactors, and in an industrial scale hollow fiber bioreactor system

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Abstract

In this article, cell growth in a novel micro hollow fiber bioreactor was compared to that in a T-flask and the AcuSyst-Maximizer®, a large scale industrial hollow fiber bioreactor system. In T-flasks, there was relatively little difference in the growth rates of one murine hybridoma cultured in three different media and for three other murine hybridomas cultured in one medium. However, substantial differences were seen in the growth rates of cells in the micro bioreactor under these same conditions. These difference correlated well with the corresponding rates of initial cell expansion in the Maximizer. Quantitative prediction of the steady-state antibody production rate in the Maximizer was more problematic. However, conditions which lead to faster initial cell growth and higher viable cell densities in the micro bioreactor correlated with better performance of a cell line in the Maximizer. These results demonstrate that the micro bioreactor is more useful than a T-flask for determining optimal conditions for cell growth in a large scale hollow fiber bioreactor system.

Introduction

There are a number of advantages to using hollow fiber bioreactors for the production of biologicals such as proteins (Liu et al., 1991; Ala-Uotila et al., 1994; Schlapfer et al., 1995), cells (Knazek et al., 1990; Stronek, 1999), and viruses (Ratner et al., 1978). These advantages are a direct result of cell retention and the high density cell growth that hollows fiber systems provide. The use of a semi-permeable membrane to retain high molecular weight proteins on the cell side allows for more efficient use of expensive medium components while producing a highly concentrated product. As a result, cost reductions are obtained through continuous production, lower overhead, lower labor, reduced medium costs, and lower purification costs.

However, hollow fiber systems introduce some unique optimization issues not encountered in low density suspension cultures such as T-flasks and stirred tanks. Two separate media are used in a hollow fiber system, one for the cell side of the membrane and one for the non-cell side of the membrane. Media components and autocrine factors will partition across the membrane based on permeability, cellular consumption (or production), and diffusivity. As a result, the traditional research tools (such as T-flasks) for medium development or cell growth assessment are not useful for optimizing conditions for a hollow fiber system (Schlapfer et al., 1995). In addition, traditional hollow fiber systems are too cumbersome or expensive for routine screening purposes.

To address these problems, a micro hollow fiber bioreactor which does not require a pump was recenly developed (Gramer and Poeschl, 1998). This was accomplished by potting fibers in luer T fittings within a piece of silicone tubing. The cells are inoculated inside the fibers in the intracapillary (IC) space which has a volume of about 0.2 ml. The extracapillary (EC) space between the fibers and the silicone tubing is large enough to serve as the medium reservoir (about 4.6 ml), but still small enough to allow adequate diffusion of gasses through the silicone tubing to the fibers. Medium is added to and removed from the bioreactor with syringes. The protocol for use is to flush, inoculate, and place the bioreactor in an incubator for a

few days, after which the bioreactor is harvested for analysis.

In a previous study, the micro hollow fiber bioreactor was used to demonstrate that small molecular weight serum components (under 10 kD) are important during the initial growth phase of a murine hybridoma cell line (Gramer and Poeschl, 1998). This result correlated with a traditional hollow fiber system that was 425 times larger based on cell culture volume.

The purpose of this study to generate a more extensive comparison of cell growth in the micro bioreactor to that in a large scale industrial hollow fiber bioreactor system. One cell line was grown in three different media, and three additional cell lines were grown in one of the three media. Cells were placed in the micro bioreactor at the normal inoculation density of 5×10^6 ml^{-1} to simulate the growth phase and at an elevated density of 5×10^7 ml^{-1} to simulate the stationary production phase of a large-scale hollow bioreactor fiber system. The results from the micro and large scale hollow fiber bioreactors are compared to results from cells grown in a T-flask which is a more standard method to compare cell lines.

Materials and methods

Media

Basal media are proprietary formulations; they are designated Medium 1, Medium 2, and Medium 3. Each basal medium had 4 to 4.5 mg ml^{-1} glucose, 4 mM glutamine, 3.4 g l^{-1} sodium bicarbonate and antibiotics. Serum-supplemented media were prepared by adding 10% fetal bovine serum (FBS; Hyclone), to basal media.

Cells

The four cell lines used in this study were all IgG producing murine hybridomas chosen randomly from cell banks at Cellex Biosciences. The cell lines (fusion partners) were MH70 (NS-1), MH478 (P3 × 63Ag8.U1), MH564 (NS-1), and MH617 (NS-1). Cells were routinely propagated above 95% viability in 10% FBS-supplemented medium at 37 °C in a humidified incubator with 5% CO_2. Cells were passaged every 1 to 3 days. MH70 cells were cultured in Medium 1, Medium 2, or Medium 3, while the other cell lines were all cultured in Medium 3.

Assays

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

Hollow fiber micro bioreactor construction and preparation

Th micro bioreactors were constructed essentially as previously described (Gramer and Poeschl, 1998). 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 (ID of 0.635 cm, OD of 1.11 cm). The EC volume was 4.6 ml and the IC volume was 0.2 ml. The bioreactors were flushed with basal medium before use as described (Gramer and Poeschl, 1998). The IC was then primed with 10% FBS-supplemented medium, and the EC was filled with basal medium.

Hollow fiber micro bioreactor normal inoculation density growth curves

Cells at about 5×10^5 ml^{-1} were pelleted by centrifugation at 200 × g and resuspended in fresh 10% FBS-supplemented medium at 5×10^6 ml^{-1}. Twenty-one bioreactors were inoculated by passing 0.5 ml of the cell suspension through the fiber IC space, and the bioreactors were placed in a 5% CO_2 37 °C incubator. Three bioreactors were harvested each day; cells were quantitatively recovered by injecting air in one IC port with a 1-ml syringe while collecting the effluent from the opposite port with another 1-ml syringe. The EC medium was then removed with a syringe for determination of pH, dissolved oxygen, and glucose. Cells were counted in the IC medium, and the remainder of the IC sample was clarified by centrifugation and frozen for later ELISA analysis. The EC medium was exchanged with 4.6 ml of fresh, pre-warmed basal medium on the third day for each bioreactor, and every day thereafter if necessary based on glucose consumption (if the glucose was or would likely drop below 2 mg ml^{-1} before the next medium change).

Hallow fiber micro bioreactor high inoculation density growth curves

Cells at about 5×10^5 ml^{-1} were pelleted by centrifugation at 200 × g and resuspended in fresh 10% FBS-supplemented medium at about 5×10^7 ml^{-1}. Twelve bioreactors were inoculated with three harvested per day as described for normal density inoculation. The EC medium was exchanged with 4.6 ml of fresh, prewarmed basal medium every day as needed based on glucose consumption (if the glucose was or would likely drop below 2 mg ml^{-1} before the next medium change).

T-flask growth curve

Cells at about 5×10^5 ml^{-1} were pelleted at 200 × g and resuspended in the same fresh medium (basal medium plus 10% FBS) at about 5×10^4 ml^{-1}. Thirty T-25 flasks were filled with 5 ml of the cell suspension. Three flasks were harvested each day for cell counts until the viability dropped below 25%. Samples from each flask were centrifuged, and the cell-free supernatant was frozen at –20 °C for later determination of glucose and antibody concentrations.

Large-scale hollow fiber bioractor

The AcuSyst-Maximizer (Cellex Biosciences), an automated hollow fiber system was used for this study (Hirschel and Gruenberg, 1988). The bioreactor fibers were identical to those used in the micro hollow fiber bioreactor. The total bioreactor surface area based on fiber ID was 1.1 m^2 and the EC volume was about 100 ml. The cultureware was flushed with 10 l of basal medium over a 4-h period, and process control was initiated. Temperature was controlled at 37 °C and pH was controlled at 7.2 by an air/CO_2 gas blend. Just before inoculation, serum was injected into the EC space to bring the serum concentration to 10%.

Cells were expanded in a 1-l spinner flask in basal medium supplemented with 10% FBS. A total of 5×10^8 cells at about 5×10^5 ml^{-1} were pelleted by centrifugation and resuspended in 50 ml of fresh 10% FBS-supplemented medium. Cells were inoculated into the EC compartment with the excess medium forced across the fibers into the IC chamber. Basal medium was circulated in the IC at an initial rate 250 ml min^{-1}; the rate of IC circulation was increased up to the instrument maximum of 500 ml min^{-1} based on oxygen demand as determined by off-line dissolved oxygen readings. Fresh basal medium was continually added to and removed from the IC medium reservoir, starting at an initial of 25 ml h^{-1}. Off-line glucose readings were taken daily, and the IC medium addition rate was adjusted if necessary to keep the glucose above 2 mg ml^{-1} up to a maximum of 400 ml h^{-1}. After the maximum pump rate was reached, the glucose concentration dropped freely below 2 mg ml^{-1}. EC cycling (Gramer et al., 1999) was initiated on day 3 (70 ml transfer volume in 15 min). On day 4, 10% FBS-supplemented medium was added to the EC circuit at a rate of 1 ml h^{-1}; this rate was increased at about 1:100 of the rate of IC medium addition up to 4 ml h^{-1}. The rate of medium harvest from the EC compartment matched the rate of EC medium addition. Cells were removed from the EC chamber 2–3 times per week, and the medium harvested from this procedure (about 50 ml) was pooled with the regular harvest.

Determination of metabolic rates

Cell doubling times were determined from the slope of the natural log of the viable cell density vs. time; only the initial points in the linear region were used. Cell-specific metabolic activities were determined from the slope of the integrated viable cell density vs. metabolite consumption (or production); for cells in T-flasks, the correlation was linear only in the exponential growth phase, while the correlation was linear throughout the growth curve for cells in micro bioreactors. The glucose uptake rate was estimated as the basal medium addition rate times the change in glucose concentration from fresh media to waste media.

Results

For comparing growth in the different systems, one cell line (MH70) was cultured in three different media designated Medium 1, Medium 2, and Medium 3. The other three cell lines (MH478, MH654, and MH617) were cultured only in Medium 3.

T-flask growth curves

The T-flask growth curves were typical for murine hybridomas (Figure 1). MH564 cells showed a slight lag phase (1 day), whereas no lag phase was apparent for the other cell lines. Antibody production was stongly growth associated for the MH478 cell line and for MH70 cells in Media 1 and Media 3, whereas MH70

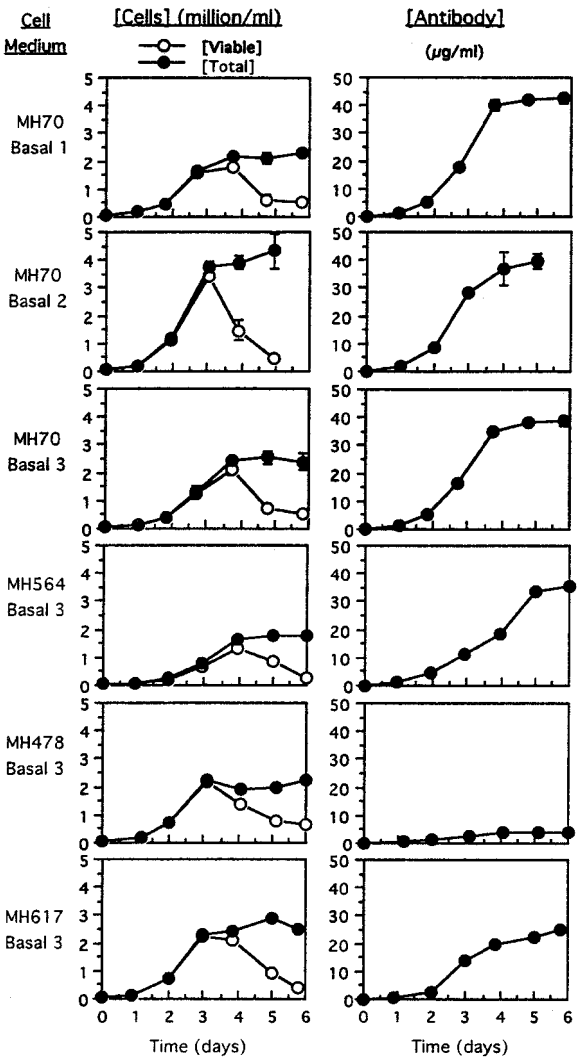

Figure 1. Growth and antibody concentration profiles for cells grown in T-flasks. Cells were inoculated at 5×10^4 ml^{-1}. Data shown are the average and standard deviation of triplicate determinations. Some of the error bars are obstructed by the size of the data points shown.

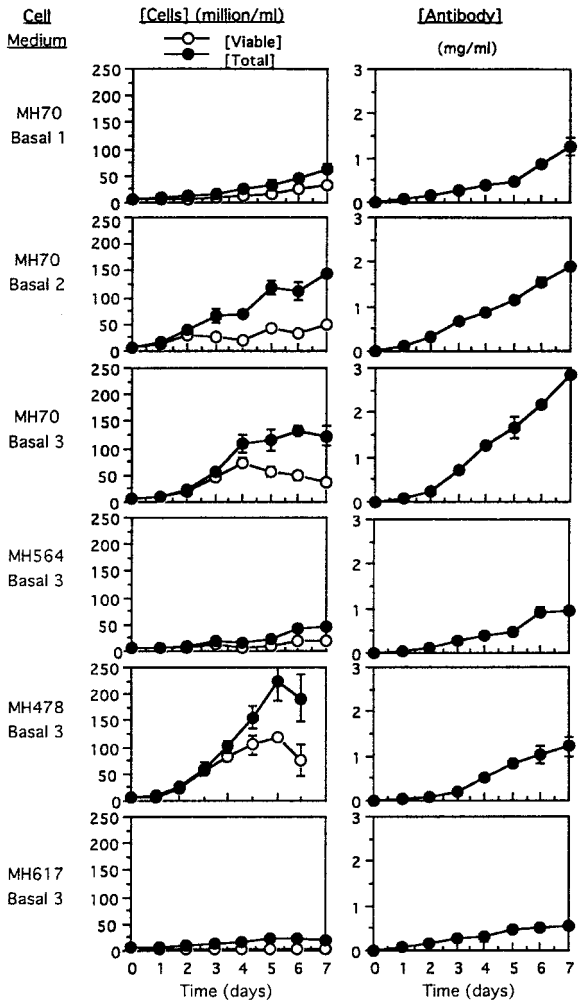

Figure 2. Growth and antibody concentration profiles for cells grown in micro hollow fiber bioreactors inoculated at the normal density of 5×10^6 ml^{-1}. Data shown are the average and standard deviation of triplicate determinations. Some of the error bars are obstructed by the size of the data points shown.

cells in Medium 2, MH564 cells, and MH617 cells produced some additional antibody in the death phase.

Micro bioreactor normal inoculation density growth curves

Growth curves in the micro bioreactors seeded at the normal inoculation density of 5×10^6 ml^{-1} were more varied than that seen in the T-flasks (Figure 2). MH617 cells essentially died over the 7 days of culture. MH70 cells in Medium 1 and MH564 cells demonstrated a prolonged lag phase but eventual growth. MH70 cells in Medium 2 demonstrated rapid initial growth, but after 2 days the viable cell density began to fluctuate. Growth of MH70 cells in medium 3 and MH478 cells more closely resembled a T-flask growth curve with an initial exponential growth phase followed by a death phase. Despite the varied growth kinetics, antibody production was linear with the integral of viable cells throughout each 7-day experiment (data not shown).

Micro bioreactor high inoculation density growth curves

Growth curves in the micro bioreactors seeded at an elevated inoculation density of 5×10^7 ml^{-1} are shown

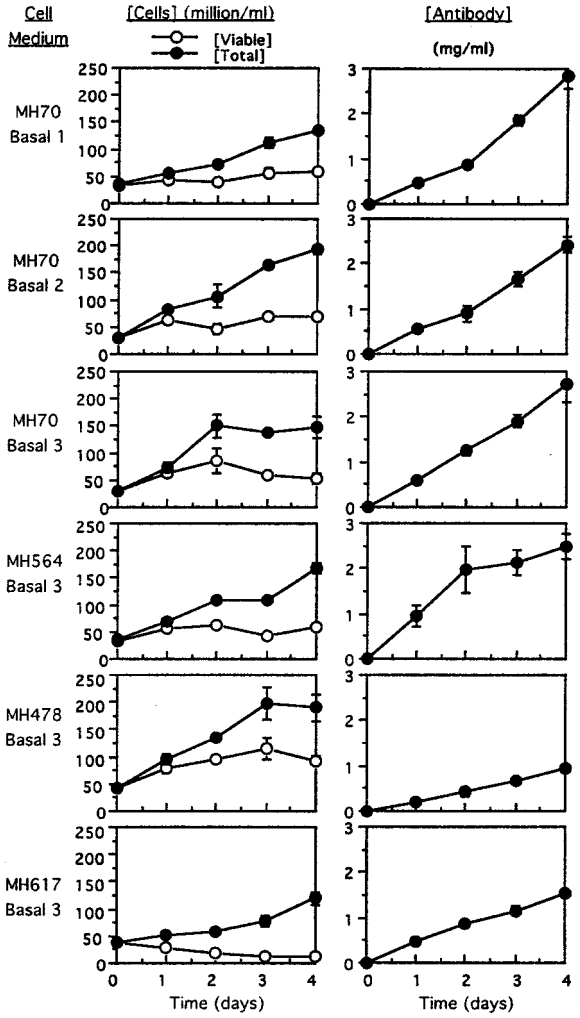

Figure 3. Growth and antibody concentration profiles for cells grown in micro hollow fiber bioreactors inoculated at the high inoculation density of 5×10^7 ml^{-1}. Data shown are the average and standard deviation of triplicate determinations. Some of the error bars are obstructed by the size of the data points shown.

in Figure 3. The MH617 viable cell density slowly declined over the 4-day experiment. For every other case, there was little change in the viable cell density which remained between about $0.5–1 \times 10^8$ viable cells per ml. The total cell densities continued to increase in most cases, and the antibody production was again linear against a plot of integrated viable cell density (data not shown).

Cell-specific rates

The range of apparent cell doubling times in T-flasks varied from about 11–16 h (Figure 4). There was a much wider range in the apparent doubling times in

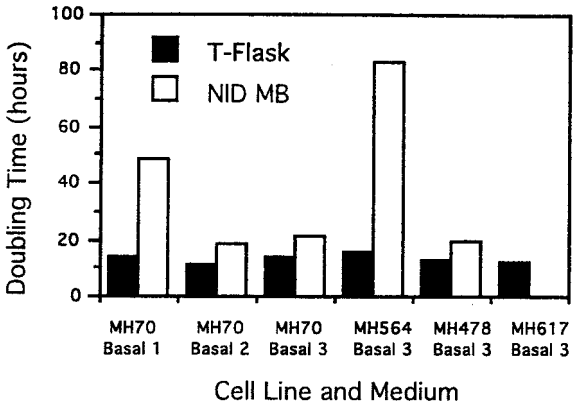

Figure 4. Comparison of doubling times for cells grown in T-flasks and in micro bioreactors at the normal inoculation density (NID) of 5×10^6 ml^{-1}. A doubling time for MH617 in micro bioreactors is not defined since the viable cell density decreased after inoculation.

micro bioreactors which varied from about to 19 to 83 h when inoculated at the normal cell density (Figure 4). There was no correlation between the growth rates of cells in the two different systems, except that the apparent doubling time was always higher in a micro bioreactor relative to that in the corresponding T-flask. True cell doubling times (taking into account cell death rates) were still substantially higher in micro bioreactors (data not shown).

The cell-specific glucose consumption rates were in most cases higher in T-flasks than in micro bioreactors (Figure 5). When comparing antibody production rates, there appeared to be more variability between the cell lines than between the different growth conditions for each cell line suggesting that the specific antibody production rate depends more strongly on the cell line than the culture conditions (Figure 5).

Large scale hollow fiber bioreactor

The cell density cannot be directly quantified in a traditional large scale hollow fiber bioreactor. However, results from the micro bioreactor data suggest the glucose uptake rate (GUR) is a good first approximation of cell density since the cell-specific rates of glucose consumption were similar throughout each experiment for both the normal and high density inoculation conditions (Figure 4). Results for the 30-day instrument runs are shown in Figure 6. Each profile of GUR shows a growth phase followed by a stationary production phase where antibody production is essentially linear through the end of the experiment. Based on the GURs in Figure 6 and the cell-specific gluc-

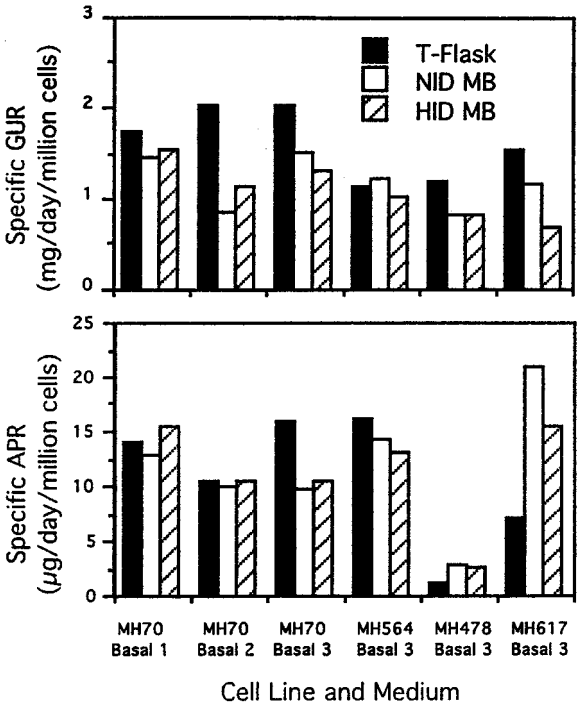

Figure 5. Comparison of the cell-specific glucose uptake rates (GUR) and antibody production rates (APR) for cells grown in T-flasks and in the micro bioreactor at the normal inoculation density (NID) of 5×10^6 ml^{-1} and a high inoculation density (HID) of 5×10^7 ml^{-1}.

ose consumption rates in Figure 5, cell densities in the Maximizers reached an estimated 2–4 $\times$ 10^8 viable cells ml^{-1}.

Growth phase comparison

The rate of growth in a T-flask (Figure 1) clearly does not correlate with the initial rate of growth in a hollow fiber system (Figure 6). However, there appears to be a much better correlation between the initial growth rate in the micro bioreactor (Figure 2) and the Maximizer (Figure 6). To further explore this relationship, the GUR for the first 7 days of culture in the Maximizer is compared that in the micro bioreactor in Figures 7 and 8. The GUR is normalized on bioreactor cell culture volume; 0.2 ml for the micro bioreactor and 100 ml for the Maximizer. Figure 7 shows a remarkable correlation between growth for one cell line (MH70) in the three different media where Medium 3 clearly supports better growth than Medium 1 or 2. The normalized GUR increased nearly identically when comparing the micro bioreactor and the Maximizer. Interestingly, the normalized GUR for MH70 in

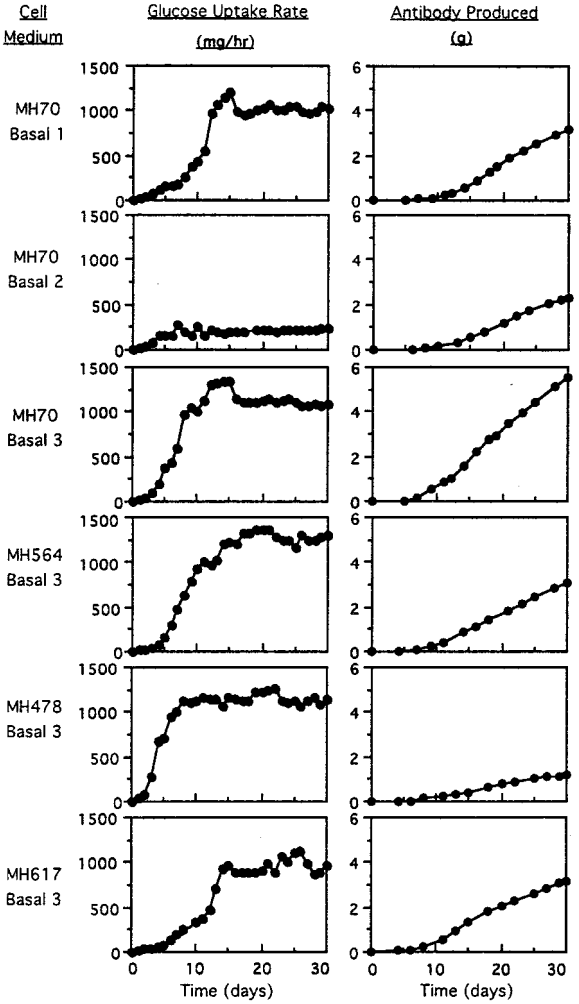

Figure 6. Glucose uptake rate (GUR) and total amount of antibody produced for cell growth in the Maximizer. Cells were inoculated at 5×10^6 ml^{-1}.

Medium 3 plateaued in the micro bioreactor on day 4 and thereafter, while the normalized GUR for the Maximizer continued to increase. This is likely due to medium limitations in the micro bioreactor; the micro bioreactor was limited to 4.6 ml per day per 0.2 ml of cell culture space (a ratio of 23:1) while the maximum pump rate in the large scale system provided 9.6 l per day for a 100-ml cell culture space (a 96:1 ratio). As a result, the cells were provided 4.2 times more medium per cell culture space in the Maximizer relative to the micro bioreactor. Figure 8 compares the normalized GUR for the four different cell lines using Medium 3. There was a reasonable correlation where the two fastest growing cell lines in the Maximizer were also the fastest growing cell lines in the micro bioreactor.

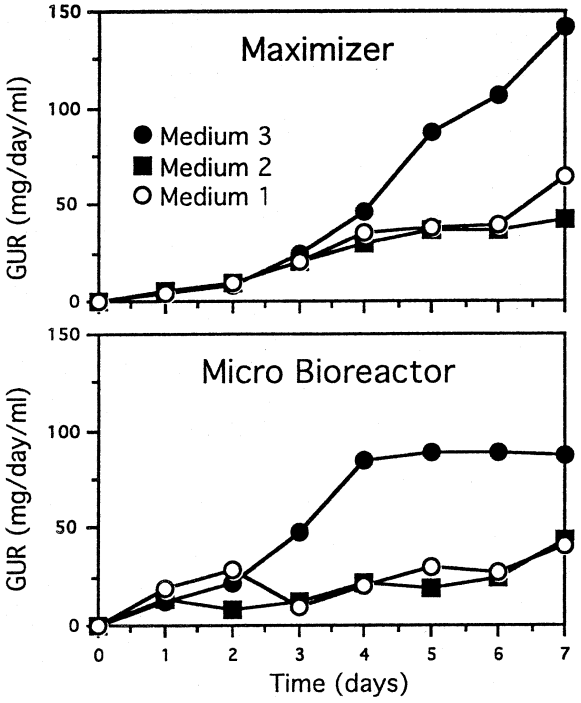

Figure 7. Volumetric glucose uptake rate (GUR) in the Maximizer and the micro bioreactor for MH70 cells inoculated at 5×10^6 ml^{-1} in three different media.

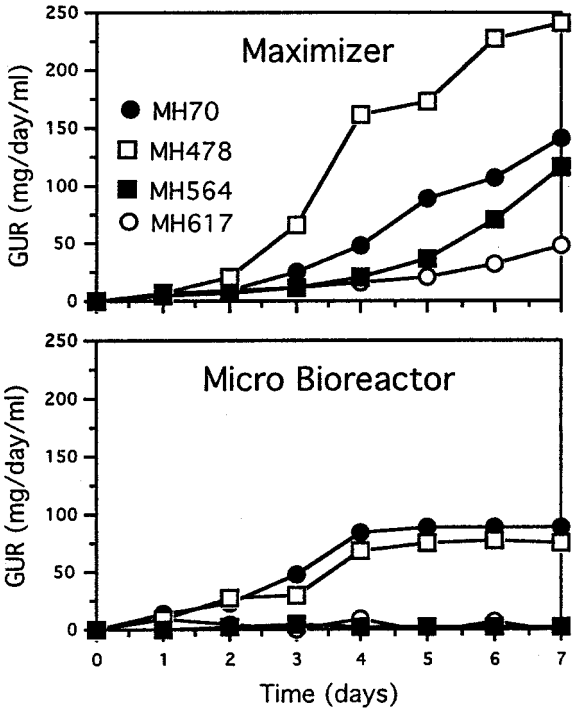

Figure 8. Volumetric glucose uptake rate (GUR) in the Maximizer and the micro bioreactor for cells inoculated at 5×10^6 ml^{-1} in medium 3.

The enhanced cell growth in the Maximizer after day 3 for MH564 and MH478 cells might be a result of initiating the EC feed which supplies additional serum components (Gramer and Poeschl, 1998). No additional serum was added to the micro bioreactors. As in Figure 7, there appeared to be medium limitations in the micro bioreactor for the faster growing cell lines (MH478 and MH70).

Production phase comparisons

Figure 9 compares the maximum antibody titer in a T-flask to the steady-state production level in a Maximizer. For MH70 with three different media (top graph), it is clear that the T-flask data have no predictive value. For the four different cell lines with one medium, there was a better correlation. Perhaps the better correlation is the result of using a better medium (Medium 3) which appears more optimal for growth of murine hybridomas in a hollow fiber system. Interestingly, the two cell lines which grew well in the micro bioreactor in Medium 3 (MH478 and MH70) had better productivity in the Maximizer than might be expected based on the best fit line from T-flask data (Figure 9). Similarly, the two slowest growing cell lines (MH546 and MH617) in the micro bioreactor had a lower productivity in the Maximizer than might be expected based on the T-flask data best fit line.

In Figure 10, the steady-state antibody production rate in the Maximizer is compared to the antibody production rate in a micro bioreactor when inoculated at high density. For more direct comparison, both values are normalized on the volume of the cell growth compartment. There was a generally good correlation in productivity, with the best fit line demonstrating that the normalized antibody production rate was about 3 times higher in the Maximizer than in the micro bioreactor. This may be due to the fact that the cells are provided up to four times more medium per day (on a normalized basis) in the Maximizer relative to the micro bioreactor.

Discussion

Very little work has been published regarding practical approaches to the optimization of hollow fiber bioreactors. In the history of hollow fiber bioreactor research and development, much attention has been given to mathematical or experimental analysis of a

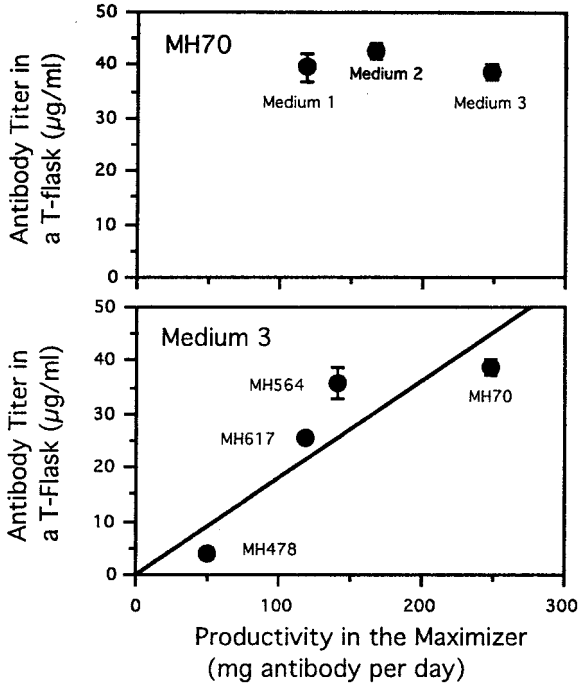

Figure 9. Antibody titer in a T-flask vs. antibody productivity in the Maximizer at steady-state. The best fit line shown was forced through the origin.

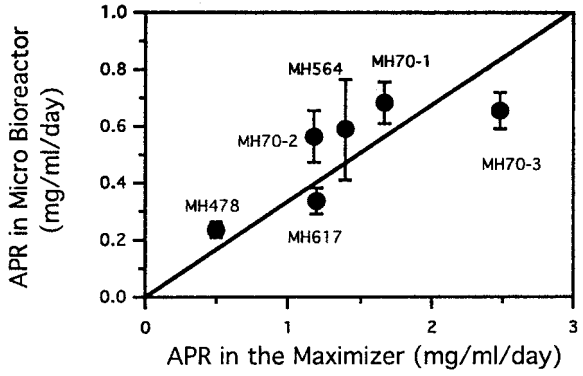

Figure 10. Volumetric antibody production rate (APR) in the Maximizer compared to that in the micro bioreactor inoculated at 5×10^7 ml^{-1}. MH70-1, -2, and -3 refer to the MH70 cell line grown in the three different basal media, while all the rest were grown in media 3. The best fit line shown was forced through the origin.

limiting nutrient or of how the fluid flow distribution affects nutrients availability (Brotherton and Chau, 1996; Patkar et al., 1995). This has been useful to provide guidelines for design parameters such as the surface area to volume ratio. However, cell growth in a hollow fiber system is too complex to be dependent on a single limiting nutrient. One reason for the narrow scope of research is that not enough is known about cellular metabolism to create an accurate model which can be applied generally. Another reason is that a good model system for experimental research has not been available. Standard hollow fiber bioreactor systems are too large, too expensive, or too cumbersome. In addition, the concentration and viability of cells cannot be directly examined in a standard hollow fiber bioreactor, resulting in data which can be difficult to interpret.

In this article, a novel micro hollow fiber bioreactor was assessed as a research tool by comparing cell growth in the micro bioreactor to that in a T-flask and the AcuSyst-Maximizer, a large scale industrial hollow fiber bioreactor system. The data demonstrate that T-flasks do not provide the proper environment when considering media or cell line development in large scale hollow fiber system. The cell growth rates and antibody titers found in a T-flask were generally not predictive of that in the Maximizer. This is not surprising given the complexity of cellular metabolism. Large molecular weight medium components and autocrine factors will be concentrated on the cell side of the membrane while small molecular weight components will be diluted across the fiber membrane.

In contrast, the micro hollow fiber bioreactor generally correlated well with the Maximizer. This was especially true when comparing initial growth rates. However, it was more difficult to accurately project the long-term steady state productivity of the Maximizer. When inoculated at 5×10^6 ml^{-1}, a growth period of 7 days was not sufficient in some cases to reach a maximum steady cell density in the micro hollow fiber bioreactor. The experiment could be extended longer than 7 days, however there is no turnover (feed or harvest) of cell side medium in the micro bioreactor. This may lead to inaccurate results in the micro bioreactor since in some cases the growth rates increased in the Maximizer shortly after the initiation of cell side feeding and harvesting on day 3.

Inoculation of cells at 5×10^7 ml^{-1} in the micro bioreactor provided some indication of the expected final antibody productivity in the Maximizer. The volumetric antibody productivity was about 3 times higher in the Maximizer relative to the micro bioreactor. This might have been due to the fact that the Maximizer pump rates provided 4.2 times more basal medium on a volumetric basis. One potential problem of high density inoculation to simulate the Maximizer production phase is that the cell population inoculated at the beginning of the culture might not be representative of the cell population that grows up in the

bioractor over a period of weeks. Perhaps a better way to examine productivity at the high cell density is to inoculate cells somewhere between 5×10^6 and 5×10^7 per ml; this will require some cell growth, but also will allow the cells to reach a maximum density in less time.

The Maximizer also provided process controls not available in the micro bioreactor. These include pH control, continuous EC and IC feeding, and cycling to mix the cell side media components. Despite the large differences between operation of the micro bioreactor and the Maximizer, the microbioreactor provided good guidelines as to what to expect in the Maximizer. At this point, it is not clear which of these parameters would be critical to examine to provide more quantitative relationships between the micro and industrial scale hollow fiber bioreactor systems. However, a general examination of the data would suggest that optimal conditions in the Maximizer are reached by finding conditions in the micro bioreactor which support fast cell growth and a high viable cell density. This approach to optimization with the micro bioreactor has led to 1.7 to 8-fold decrease in the cost of antibody production from various cell lines in a hollow fiber system (manuscript in preparation).

Future efforts will be aimed at determining specifically what limits or affects the performance of cells in a hollow fiber system. Potential candidates are process control parameters such as pH (Ozturk et al., 1991), temperature (Chuppa et al., 1997), and continuous vs. intermittent feed (Palsson et al., 1993). Other parameters include oxygenation (Piret and Cooney, 1990), basal nutrients (Banik and Heath, 1996), growth factors (Richards et al., 1998), waste product build up (Ozturk et al., 1992), and/or autocrine factors (Kidwell, 1989). Knowledge of these limiting factors will allow more directed use of the micro bioreactor as an optimization tool for large scale hollow fiber bioreactor systems.

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