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Short communication

Use of hollow fiber systems for rapid and direct scale up of antibody production from hybridoma cell lines cultured in CL-1000 flasks using BD Cell MAb medium

Michael J. Gramer[1,*], Jodi Maas[1] and Michael M. Lieberman[2]
*[1]BioVest International, Inc., 8500 Evergreen Boulevard, Minneapolis, MN 55433, USA; [2]Hawaii Biotech, Inc. 99-193 Aiea Heights Dr., suite 200 Aiea, HI 96701, USA; *Author for correspondence (e-mail: mlieberman@hibiotech.com; phone: 763-786-0302; fax: 763-786-0915)*

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Abstract

The combination of BD Cell MAb medium with the CL-1000 flask is increasingly being used to generate a few hundred milligram of antibody for early stage research projects. Cells are inoculated at 2 million per ml, and the antibody is harvested after 15 days or when the antibody concentration reaches above 10 mg ml^{-1}, whichever comes first. Currently, there is no means to scale up beyond this production level using this technology. In this study, we evaluated hollow fiber technology as the scale up alternative. The hollow fiber system was run in batch mode to mimic the method used for the CL-1000 with BD MAb medium. The FL–NS murine hybridoma cell line was simultaneously inoculated at 2 million cells per ml in a CL-1000 and the Maximizer hollow fiber bioreactor system, a 21-fold theoretical scale up over the CL-1000. The Maximizer produced 23-fold more antibody, very close to the expected theoretical amount. However, production was complete after 9 days in the Maximizer, while the CL-1000 required the full 15 days for production. In summary, these results demonstrate successful scale up of antibody production from the CL-1000 to a hollow fiber system.

Introduction

Substantial process development efforts for biologicals are more often being delayed to later in the clinical development process. A number of factors have contributed to this shift. One factor is that lead protein candidates are being generated at an ever increasing rate; resources to produce and evaluate these candidates have not kept pace. Another factor is the concept of well-characterized biologicals. The acceptance of analytical characterization for product equivalence allows less emphasis to be placed on maintaining a constant process while progressing through the early stages of clinical development. A typical scenario is that a lead candidate is developed, and brute force production methods are used to generate enough product for animal testing and a Phase I clinical study. In the meantime, as more data are generated to assess clinical efficacy, more process development is performed. Between Phase I and Phase III, there are often wholesale changes in the cell line, medium, and production systems used to produce a therapeutic candidate.

Monoclonal antibodies account for a large portion of the biological products in development. In 2002, 75 of the 371 biological products in clinical development were monoclonal antibodies (PhRMA Report 2002), and countless other monoclonal antibodies are being developed and used for research, pre-clinical and *in vitro* diagnostic applications. Two recent innovations have the potential

for enabling accelerated time to production and subsequent evaluation of monoclonal antibodies. The first innovation is BD Cell MAb medium. This unique medium provides a 3–10 fold increase in antibody titer from hybridomas compared to traditional media, thereby substantially reducing the process development effort necessary to produce sufficient amounts of early phase clinical material (Fritchman 2000). The second innovation is the CL-1000 production flask. This unique flask provides high density cell culture by sandwiching a cell growth compartment between a gassing membrane for oxygenation and an ultrafiltration membrane for medium exchange (Trebak et al. 1999; Wolf and DeSutter 1999). This static culture device is easy to use and provides the antibody in a concentrated form. BD Cell MAb medium and the CL-1000 in combination are very useful for producing a few hundred milligram of concentrated protein for research purposes. However, at present, scale up beyond a few hundred milligram using this production system is not possible, since larger CL flasks are not available and would be difficult to manufacture.

The primary purpose of this study was to evaluate the potential for using a hollow fiber bioreactor as a scale up alternative for the CL-1000. The CL-1000 is a static culture device, while hollow fiber systems are usually operated in perfusion mode. However, in theory the hollow fiber bioreactor can be operated similarly to the CL-1000, and therefore, should provide similar performance. To test this hypothesis, the performance of a hybridoma cell line was evaluated in the CL-1000 versus a Maximizer hollow fiber bioreactor system, a 21-fold theoretical scale up. If scale up is successful, material produced from these runs will be useful for pre-clinical studies and the results will allow planning for clinical scale production.

Materials and methods

Cells and medium

The FL–NS murine hybridoma cell line was used for this study. This cell line secretes an IgG antibody specific for the Dengue virus. BD Cell MAb Medium Animal Component Free was used for this study (BD Biosciences). An additional 2 mM L-glutamine was added to the medium (HyQ L-glutamine 200 mM 100×). Cells were routinely propagated in T-flasks or roller bottles in a humidified incubator at 5% CO_2 and 37 °C.

CL-1000 Protocol

The optimal protocol for the CL-1000 (BD Biosciences) was previously determined as follows. Pre-warm one liter of medium to 37 °C. Add 25 ml of this medium to the nutrient compartment (non cell side) of the CL-1000. Pellet cells and resuspend them in fresh medium at a nominal concentration of 2 million per ml. Inoculate 15 ml of this resuspension in the cell compartment of the CL-1000. Add the remainder of the 1-l bottle (960 ml) to the nutrient compartment, and transfer the flask to an incubator at 37 °C and 5% CO_2. Take a 1-ml sample every three days from the nutrient compartment starting on day 3 for determination of glucose and lactate concentrations. Take a 0.25-ml sample from the cell compartment (after mixing five times using a 10-ml pipette) every 3 days starting on day 6 for cell counts and antibody concentration. Harvest the antibody from the cell compartment after 15 days, or when the cells are crashing and the antibody titer is above 10 mg ml^{-1} (whichever comes first).

Hollow fiber system

The Maximizer hollow fiber bioreactor system was used for this study (BioVest International). A standard Maximizer cultureware set (Cat No 600068-221) was placed in the instrument. The Maximizer cultureware set contained two 2.1 m^2 10 kDa nominal MWCO bioreactors (35 kD absolute MWCO). Typical operating procedures used for this system in perfusion mode have been published (Hirschel and Gruenberg 1988; Gramer et al. 1999). For this study, the systems were used in batch mode to mimic the CL-1000 flask (Figure 1).

Hollow fiber bioreactor preparation

Before use, the hollow fiber system was flushed with 10 l of medium. After flushing, the system was placed in pre-inoculation mode with temperature controlled at 37 °C and CO_2 on gas setting #10 (which is about 5% CO_2 to mimic the atmosphere of the incubator housing the CL-1000). The IC

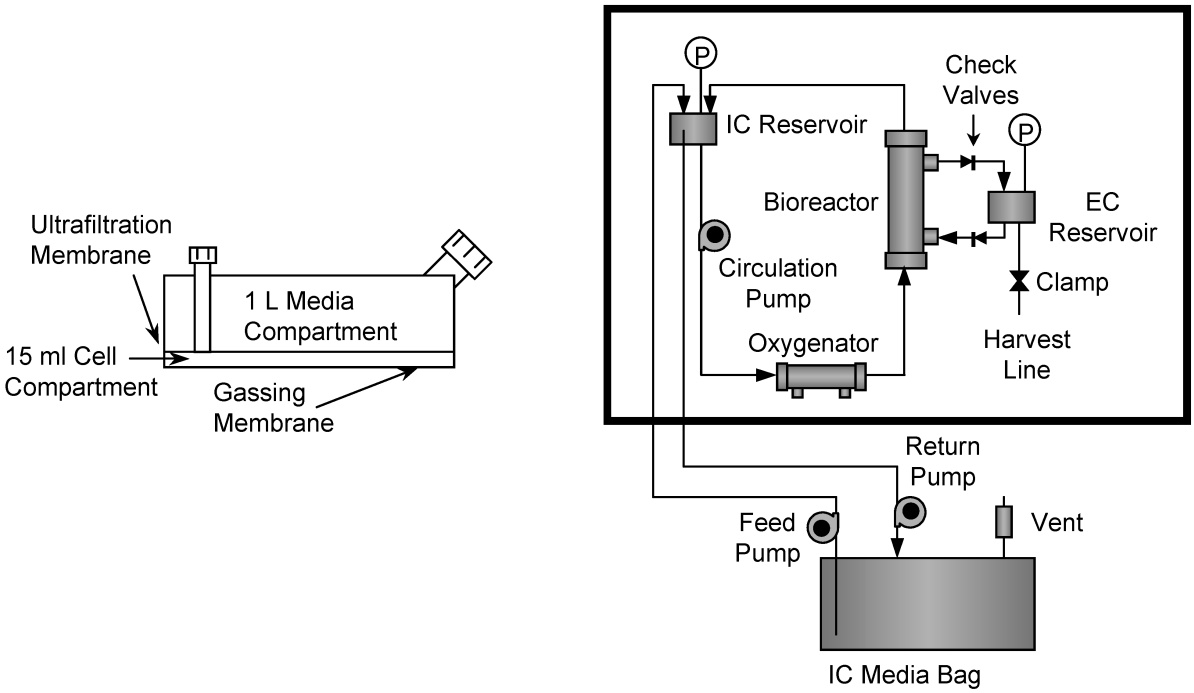


Figure 1. Comparison of CL-1000 (left) and hollow fiber system (right).

(intracapillary, non cell side) circulation pump was initiated on the Maximizer at 500 ml min^{-1}. The Maximizer in-line pH probe, located between the gas- exchange cartridge and the bioreactor in the IC circuit, was then calibrated.

Scale up factor

Scale up from the CL-1000 to the Maximizer was based on the cell compartment EC (extracapillary) space, which was 160 ml in each hollow fiber bioreactor. With two bioreactors, the Maximizer was a 21-fold scale up from the CL-1000. Based on this factor, 21 l of medium was used for the Maximizer IC circuit. The IC medium was continually recirculated from a bag, to the instrument and back to the bag at 800 ml h^{-1}. The Maximizer IC circulation rate was increased from 500 ml min^{-1} to 1000 ml min^{-1} on day 3.

Inoculation

The hollow fiber system was inoculated with the same pool of cells as the CL-1000 flask. The pool was pelleted and resuspended at 6.4 million viable cells per ml. A 50-ml quantity of these cells was injected into each hollow fiber bioreactor to provide an inoculation density of 2 million per ml EC for the Maximizer. The inoculum pool was then diluted to 2 million per ml for inoculation of 15 ml into the CL-1000 flask.

Maximizer EC cycling

Just before inoculation, the EC chamber media level was set to the 'LOW' point, which is 108 ml in the Maximizer, and the EC return clamp was shut. The EC return clamp was opened on day 3, and cycling was initiated on day 5 in the Maximizer. The cycling volume was 60 ml. As a result, the EC chamber volume fluctuated from 108 ml to 168 ml in the Maximizer. The cycle time was 30 min for the rise and 30 min for the fall.

Maximizer IC outflow pump rate

The IC outflow media pump on the hollow fiber system is designed to sip from the IC chamber. The outflow pump rate is set faster than the inflow rate in order to insure a constant system volume. As

a result of the faster rate, the outflow pump frequently takes up air in addition to medium. Usually, the IC media feed and IC outflow waste pumps are hooked to different reservoirs. However, in this case, the two pumps were hooked to the same reservoir. To deal with this modification, a bag with several ports was used (Bioprocess containers from Hyclone). The IC media feed line was hooked to a fitting on the bag that had an extension going to the bottom of the bag. The IC waste line was hooked to a fitting on the top of the bag. Since the outflow is run faster than the inflow, the bag tended to fill with air. To minimize this, the outflow pump was turned down to match the inflow pump as closely as possible.

Maximizer harvest

At the end of the run, antibody was harvested from the instrument by repeated collection of medium through a line on the bottom of the EC chamber. The instrument was kept running as normal (temperature and pH control, circulation, etc.), and the line between the EC chamber and the bottom of the bioreactor was clamped off. The EC chamber was placed in a rise mode and filled to about 250 ml. The system was then placed in drain mode, and the harvest in the EC chamber was collected through a line from the bottom of the chamber. This procedure was repeated six times. The harvests were clarified by centrifugation and sterile filtration (Sartobran P 0.2 μm cellulose acetate filters from Sartorius).

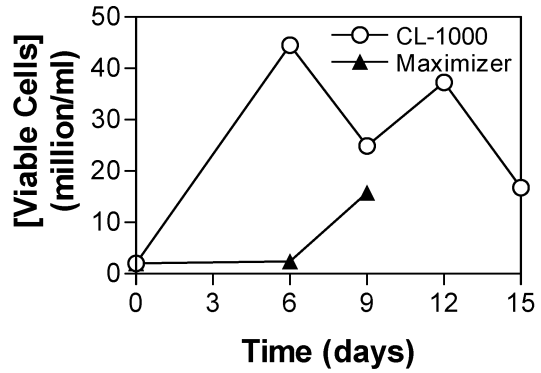
Assays

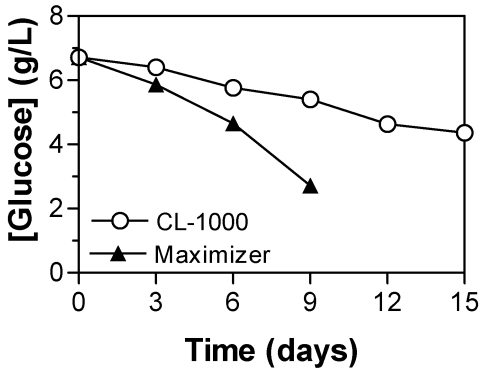
Samples for cell density, cell viability, and antibody titer were taken from the cell compartment on the CL-1000 and the EC circuit for the Maximizer. Cell densities were determined using a hemacytometer with trypan blue. Antibody concentrations were determined by ELISA using a goat anti-mouse IgG H + L capture antibody (Sigma P/N 8642) and a goat anti-mouse IgG H + L HRP conjugate (Kirkegaard and Perry P/N 474–1806); color was developed with ABTS, and purified mouse IgG was used as a standard (Sigma I-5381). Samples for glucose and lactate concentrations and off-line DO (dissolved oxygen) and pH determinations were taken from the nutrient compartment for the CL-1000 and from the IC circuit for the Maximizer. Glucose and lactate were determined using a YSI 2700 select bioanalyzer. DO and pH were determined with a blood–gas analyzer (AVL critical care analyzer).

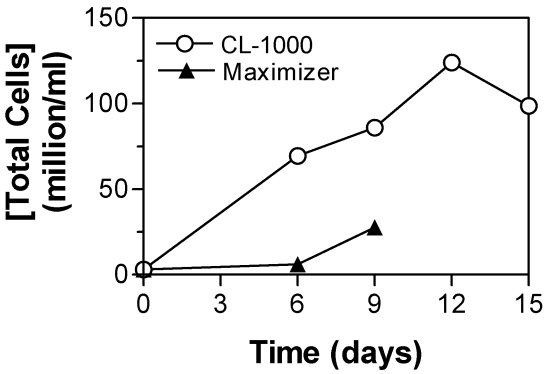
Results

HF procedure to mimic the CL-1000

In the CL-1000, the cells are placed in a 15-ml compartment at 2 million ml^{-1} with a gas-permeable membrane on the bottom and an ultrafiltration membrane on top to separate cells from the media compartment (Figure 1). The balance of 1 l of medium (985 ml) is placed in the media compartment, and the CL-1000 is placed in a humidified incubator at 5% CO_2 and 37 °C. Antibody is recovered from the 15-ml compartment after 15 days or when the antibody concentration is above 10 mg ml^{-1}, whichever comes first. The hollow fiber bioreactor procedure is designed to mimic this CL-1000 protocol. Hollow fiber bioreactors are placed in parallel in the flow circuit (Figure 1; only one bioreactor is shown). Cells are placed outside the fibers in the cell compartment (160 ml per bioreactor), providing a 21-fold scale up of the CL-1000 based on amount of cell culture space. The hollow fibers in the bioreactor are ultrafiltration membranes, essentially equivalent to the ultrafiltration membrane on top of the CL-1000. Medium is circulated from the IC reservoir through the gas-exchange cartridge, through the inside of the fibers, and back to the IC reservoir at 500–1000 ml min^{-1}. A 5% CO_2/air mixture is passed through the gas exchange cartridge to mimic the gas mixture in the incubator. Oxygen and CO_2 dissolved in the media are transported across the hollow fibers to and from the cells. The entire circulation loop is essentially replacing the gas-permeable membrane on the bottom of the CL-1000. Medium is pumped between the IC circulation loop and medium bag (21 l at 800 ml h^{-1}) to continually mix the non cell side media; this loop is a surrogate for the 985-ml medium compartment. The Maximizer uses a patented process to keep the cell side medium mixed up, resulting in enhanced production (Gramer et al. 1999). Medium is ultrafiltered between the IC and EC circuits by pressurizing the reservoirs to transfer 60 ml every 30 min. (check







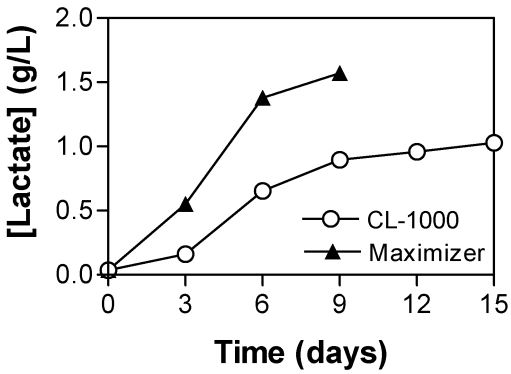


Figure 2. Viable and total cell density. The FL-NS cell line was inoculated at 2 million per ml in the CL-1000 and the Maximizer. The Maximizer was harvested on day 9 and the CL-1000 was harvested on day 15.

Figure 3. Glucose and lactate concentrations corresponding to Figure 2.

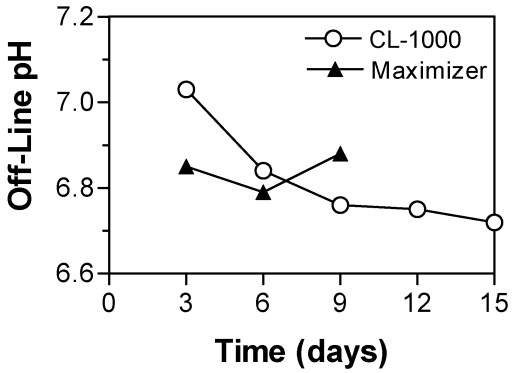
valves insure that medium enters the top of the reservoir and leaves through the bottom); there is no analog of this process in the CL-1000. The entire flow circuit except for the bag is kept in a built-in incubator at 37 °C in the hollow fiber instrument. Antibody is harvested in a concentrated form from the EC chamber through the harvest line at the end of the run.

Cell growth comparison

The FL–NS cell line was inoculated at 2 million viable cells per ml in both the CL-1000 and the Maximizer. In the CL-1000, the cell density can be determined quantitatively; the viable cell density peaked on day 6 at 45 million per ml and was still near 20 million per ml by harvest on day 15 (Figure 2). The total cell density continued to rise to over 120 million per ml by day 12 in the CL-1000 (Figure 2), resulting in an overall viability near 20% on day 15. In the Maximizer, cells cannot be sampled quantitatively. Cells are mostly retained in the bioreactor until the bioreactor completely fills with cells. Thereafter, cells begin to slough off into the EC circuit. This is apparent on day 9, where the cell density was flat between days 0–6 with a sharp increase on day 9. Still, the circulating cell density was lower in the Maximizer; the viability of circulating cells remained near 50%.

Glucose and lactate concentration

Since cells are difficult to sample in the hollow fiber instrument, the glucose and lactate concentrations are perhaps more useful for comparing the production systems. Glucose uptake and lactate production proceeded at a faster rate in the hollow fiber system compared to the CL-1000 flask (Figure 3). These data suggest a faster increase in the viable



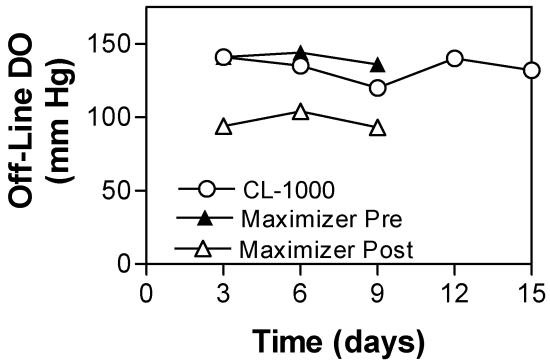


Figure 4. pH and DO corresponding to Figure 2. Pre and post refer to DO entering and leaving the bioreactor respectively.

cell density in the hollow fiber system. Upon harvest, the medium appeared more spent in the hollow fiber system (lower glucose and higher lactate concentrations).

pH

The initial pH in the absence of cells in both the CL-1000 and the hollow fiber system was just above pH 7. The pH then dropped faster in the hollow fiber system, presumably due to the accelerated production of lactate (Figure 4). On day 5, the Maximizer in-line pH probe read pH 6.62. According to literature from the supplier of BD medium, the optimal pH is 6.8. Because the pH was well below 6.8, the gas number of the Maximizer was reduced from gas 10 (5% CO_2) to gas 1 (0% CO_2). The pH increased to about 6.8 and remained near there for the rest of the run. The pH in the CL-1000 flask eventually dropped below 6.8 and finally reached pH 6.72, but no CO_2

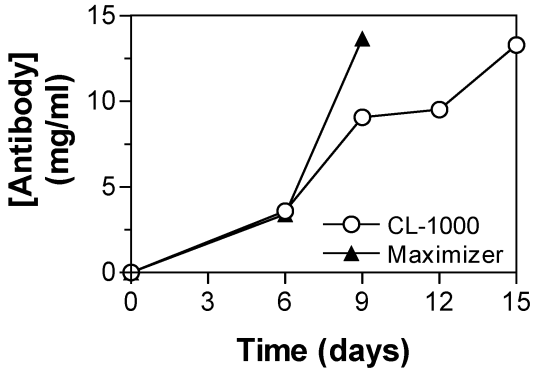


Figure 5. Antibody concentrations corresponding to Figure 2.

adjustments were made to the CL-1000 incubator. This difference in pH control in addition to accelerated initial cell metabolism in the hollow fiber system may partially explain why the medium was more spent at the end of the run when considering glucose and lactate. Low pH has a tendency to reduce lactate formation, and decreasing the CO_2 concentration in the hollow fiber instruments might have allowed for more lactate production from glucose (Miller et al. 1988).

DO

The oxygen uptake rate (OUR) can be a good indicator of viable cell density in a hollow fiber system (Gramer and Poeschl 1998; Gramer and Britton 2002). The OUR in a hollow fiber system is calculated from the difference in pre and post DO times the circulation rate. The pre DO remained relatively constant, as the gas-exchange cartridge was able to saturate the circulating medium with oxygen near 140 mm Hg (Figure 4). The post DO was below 100 mm Hg on day 6 and remained there for the rest of the run. These results suggest that by the end of the run, there was still a healthy viable cell population remaining in the hollow fiber system. The DO data for CL-1000 flasks are also presented, but these data have little meaning since oxygenation occurs through the lower membrane of the flask.

Antibody concentration

As seen for the glucose and lactate concentrations, antibody production was accelerated in the hollow fiber system relative to the CL-1000 flask (Figure 5).

Table 1. Harvest results for FL–NS.

FL–NS Parameters	Production device	
	CL-1000	Maximizer
Harvest volume (ml)	15	1250
Harvest concentration (mg ml^{-1})	15.0	4.12
Total harvest (mg)	225	5150
Medium used (l)	1	21
Efficiency (mg l^{-1})	225	245
Scale up factor	1	23

The antibody concentration reached well over 10 mg ml^{-1} in the Maximizer by day 9. As a result, antibody was harvested from the Maximizer on day 9. The antibody concentration in the CL-1000 flask reached a similar value as the Maximizer; however, this concentration was not achieved until day 15.

Scale up factor

While antibody concentration is useful for comparison, in the final analysis total antibody produced is key to determining performance. The initial total volume of the cell side compartment was known, but this volume changes in each system due to several factors. In the CL-1000, the high cell density displaces some of the volume, and the total cell side volume tends to fluctuate between 10 and 20 ml depending on handling. In the Maximizer, cells grow to very high density, again displacing volume. In addition, the EC circuit adds extra volume. The CL-1000 cell compartment can be easily recovered quantitatively. In the Maximizer, repeated cycles were necessary to extract the antibody from the circuit, resulting in dilution upon harvest. The volume, concentration, and total amount of antibody harvested after extraction and cell removal are shown in Table 1. The concentration of antibody remaining in the hollow fiber system after extraction was about 0.15 mg ml^{-1}, indicating that antibody recovery was high (estimated near 99%). As shown in Table 1, the scale up factor for the Maximizer was 23, which was very close to the theoretical value of 21.

Discussion

This work demonstrates a quick and effective method for the rapid scale up and production of antibodies from hybridomas. Cells are adapted to BD Cell MAb medium and inoculated in a CL-1000 flask for production of a few hundred milligrams of antibody for initial research purposes. For production of larger quantities, a hollow fiber system is used. Performance of the hollow fiber system is estimated by linear extrapolation based on the cell compartment space. For example, the largest off-the-shelf hollow fiber system is the XCellerator, which operates 20 160-ml bioreactors simultaneously. Extrapolation of results for the cell line evaluated here suggests the yearly production potential of about 2 kg yr^{-1} from this system without further optimization.

The primary difference between the CL-1000 and the hollow fiber system was that growth and production were accelerated in the hollow fiber system. While results were shown here for only one cell line, similar conclusions were drawn from a second cell line (data not shown). The reason for accelerated growth is not entirely clear, but at least three possibilities exist. First, nutrient exchange in the hollow fiber system (including medium and oxygen) is likely to be much better due to medium circulation on both the cell side and non cell side compartments. Second, on the average, cells are much closer to a nutrient source in the hollow fiber system (fibers on the average of 100 μm apart) compared to the CL-1000 (membranes 1500 μm apart). Theoretical calculations based on oxygen consumption suggest that cells should be no further than 100 μm from a nutrient source for optimal high density growth (Piret and Cooney 1991). Third, the CO_2 level in the hollow fiber system was reduced, but was not altered in the CL-1000. It is possible that this procedure allowed for accelerated medium consumption by more effectively neutralizing the lactic acid in the hollow fiber systems.

Another alternative would have been to scale up production in a stirred-tank system. However, it was apparent that this cell line did not grow well in roller bottles. It is possible that the extra shear provided by the roller bottles is a problem when using this low protein medium. The hollow fiber system is a low shear system, whereas the shear in stirred tanks may be problematic. In addition, hollow fiber systems more closely mimic the CL-1000, with ultra-high density cell growth and concentrated antibody harvests.

Because the goal was to simulate the CL-1000, the hollow fiber system was run in batch mode instead of the usual perfusion mode. It is interesting to note that oxygen uptake was still very strong at the end of the run in the hollow fiber system. It is possible that production could be increased by using more media with the same flow path, either in a larger initial batch, a semi-batch or a perfusion mode; these possibilities are left for further investigation.

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