Cytotechnology **44:** 1–14, 2004.

Long-term continuous production of monoclonal antibody by hybridoma cells immobilized in a fibrous-bed bioreactor

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Received 19 December 2002; accepted in revised form 24 August 2003

Key words: Fibrous-bed bioreactor, Hybridoma, Monoclonal antibody

Abstract

The kinetics and long-term stability of continuous production of monoclonal antibody IgG2b by hybridoma HD-24 cells immobilized in a fibrous-bed bioreactor (FBB) were studied for a period of ~8 months. The cells were immobilized in the fibrous bed by surface attachment of cells and entrapment of large cell clumps in the void space of the fibrous matrix. A high viable cell density of 1.01×10^8/ml was attained in the bioreactor, which was about 63 times higher than those in conventional T-flask and spinner flask cultures. The continuous FBB produced IgG at a concentration of ~0.5 g/l, with reactor productivity of ~7 mg/h·l, which was about 23 times higher than those from conventional T-flask and spinner flask cultures. The IgG concentration can be further increased to ~0.67 g/l by using higher feed (glucose and glutamine) concentrations and running the reactor at a recycle batch or fed-batch mode. The long-term performance of this bioreactor was also evaluated. For a period of 36 days monitored, the MAb produced in the continuous well-mixed bioreactor at 50 h retention time (0.02/h dilution rate) was maintained at a steady concentration level of ~0.3 g/l with less than 8% drift. At the end of the study, it was found that ~25% of the cells were strongly attached to the fiber surfaces and the other ~75% entrapped or weakly immobilized in the fibrous matrix. The strongly attached cells had a high viability of ~90%, compared to ~75% for cells weakly immobilized and only ~1.4% for freely suspended cells, suggesting that the fibrous matrix preferentially retained and protected the viable (productive) cells. The FBB thus was able to maintain its long-term productivity because nonviable and dead cells were continuously washed off from the fibrous matrix. The high MAb concentration and production rate and excellent stability for continuous long-term production obtained in this study compare favorably to other bioreactor studies reported in the literature. The reactor performance can be further improved by providing better pH and aeration controls at higher feed concentrations. The FBB is easy to operate and scale-up, and thus can be used economically for industrial production of MAb.

Introduction

Monoclonal antibodies (MAb) are widely used as diagnostic reagents, *in vivo* imaging agents, and for therapeutic purposes (Bibila and Robinson 1995), with an estimated annual sale of ~$4 billion in the US in 1998. Production of MAb by *in vitro* culturing hybridoma cells have been studied for several decades (Köhler and Milstein 1975). Cells producing MAbs are usually cultivated in suspension cell cultures on a batch or perfusion mode (Lambert et al. 1987; Backer et al. 1988). However, animal cells are sensitive to hydrodynamic shear force. The concentration of viable cells attained in suspension cultures is usually low, 10^6 cells/ml (Katinger 1987; Altshuler et al. 1987; Ramirez

and Mutharasan 1990; Hayter et al. 1992; Jan et al. 1997; Cherlet and Marc 2002). Consequently, MAb concentration (usually less than 100 mg/l) and volumetric productivity (usually 20–70 mg/l·day) are also low as compared to those from *in vivo* cultivation in mouse or rabbit ascites (Stoll et al. 1995; Jackson et al. 1996).

More recently, several perfusion systems, some with cell recycle, have been developed to improve cell density (10^7 cells/ml) and MAb productivity (up to 150 mg/l·day) in suspension cultures (Batt et al. 1990; Broise et al. 1992; Hülscher et al. 1992; Linardos et al. 1992; Lu et al. 1995; Bierau et al. 1998; Yang et al. 2000). Also, shear damage to cells can be minimized and higher cell density and MAb production can be obtained with an improved impeller design (Shi et al. 1992). However, scale-up of these suspension culture systems to industrial scale is difficult because of the complexity in fluid hydrodynamics and shear damage to cells in these suspension bioreactor systems.

The damaging effect of fluid-mechanical forces on cells can be reduced by cell immobilization (Lee and Palsson 1990; Nikolai and Hu 1992; Pörtner et al. 1997). Various immobilization techniques have been studied, including entrapping cells in agarose (Nilsson et al. 1983; Cadic et al. 1992), gelatin, and alginate beads (Sinacore 1984; Lee et al. 1991), in hollow fibers (Tharakan and Chu 1986; Altshuler et al. 1987; Brotherton and Chau 1995; Gramer and Poeschl 2000; Valdes et al. 2001; Gramer and Briton 2002), between two membrane sheets (Klement et al. 1987; Scheirer 1988), in membrane-bound capsules (Rupp 1985), and by cell adhesion to and entrapment in fibers (Chiou et al. 1991; Wang et al. 1992). Immobilized cell reactors can be easily perfused to receive a continuous supply of fresh culture medium, extending the productive lifetime of the cells and increasing the cell concentration obtained in the reactor. High cell densities of 10^7–10^8 cells/ml can thus be easily achieved in immobilized cell cultures and MAb productivity can be increased by more than 20-fold as compared to suspension cultures. Other advantages of cell immobilization include cell reuse, prevention of cell washout, and providing a favorable microenvironment for cell growth (Emery et al. 1987). Also, products from such bioreactors are free from cells, thus reducing the burden on downstream processing.

Among all immobilized animal cell bioreactors studied, fiber-bed bioreactors (FBB) seem to have the greatest potential for commercial use (Chiou et al. 1991; Junker et al. 1993). As compared to microcarriers, fibers are inexpensive materials to use for cell adhesion. The fiber bed has a high porosity (>90%), a high specific surface area (40 m^2/l) for cell adhesion, good mass transfers, a relatively low pressure drop, and a relatively low hydrodynamic shear field (Perry and Wang 1989), and gives high cell density (10^8 cells/ml) and productivity for most animal cell cultures reported in the literature (Wang et al. 1992; Chen et al. 2002). However, there are limitations associated with the packed fiber-bed and other immobilized cell bioreactors. Long-term use and scale-up of these bioreactors can be problematic. For example, MAb production usually starts to decline after several weeks of cultivation (Wang et al. 1992). This production decline was attributed to cell leaking and washout due to the destruction of the particle beads used for cell immobilization (Cadic et al. 1992), poor mass transfer caused by a build-up of cell biomass, and cell degeneration and accumulation of nonviable cells over long-term operation in membrane and fiber bioreactors (Broise et al. 1992). The conventional packed FBB also may suffer from severe clogging and channeling over long-term use.

To address the scale-up and long-term operation issues associated with conventional packed-bed immobilized cell bioreactors, we have developed a new, structured, FBB, which contains a spiral wound fibrous sheet with spaces between wound layers to facilitate medium flow in the fibrous bed (Lewis and Yang 1992). The spaces between fiber sheet layers are designed as free-flow channels for gas, liquid, and solids, which also allow continuous cell regeneration in the fibrous bed. This bioreactor has been successfully used in several microbial fermentations, including recombinant GM-CSF production with yeasts (Yang and Shu 1996) and carboxylic acids production with bacteria (Yang et al. 1992, 1994; Silva and Yang 1995). More than 10-fold increase in productivity with up to 1 year stable continuous operation has been obtained in these fermentations using the spiral wound FBB.

The goal of this study was to evaluate the FBB for long-term continuous MAb production. In this work, the production of immunoglobulin IgG2b by

mouse hybridoma cells (HD-24) immobilized in the FBB was studied and compared with cultures in static T-flasks and spinner flasks. The MAb production level and viable cell concentration achieved in various culture systems were compared to assess the efficiency of various bioreactor systems studied. Cell morphology and cell distribution in the fibrous matrix were also studied using scanning electron microscopy and confocal laser microscopy. The effects of cell immobilization to fibers on cell viability and MAb productivity were also studied and are discussed in this article. This work is the first study demonstrating that the FBB originally developed for microbial fermentations also work well for animal cell cultures.

Materials and methods

Cell line and culture medium

The mouse hybridoma HD-24 cell line was maintained in T-25 culture flasks in a CO_2 incubator (Napco E series, Model 302) at 37 °C. The culture medium consisted of 90% (v/v) Dulbecco's Modified Eagle's (DME) medium (Sigma Chemical Co., MO), which contained 4 mM glutamine and 4.5 g/l glucose as carbon and energy sources, 10% (v/v) fetal bovine serum (Cell Culture Laboratories), and 60 μg/ml gentamicin (Whittaker). The medium was sterilized by filtration through a 0.22-μm medium filter.

Batch culture kinetic studies

The kinetics of cell growth and MAb production in suspension culture was first studied in T-125 T-flasks, each containing 25 ml of the medium, and a 500-ml spinner flask (Bellco), containing 200 ml of the medium, at 37 °C. Appropriate amounts of cells were seeded into the T-flask and spinner flask to give an initial cell concentration of 3.2×10^5 cells/ml in the culture medium. These flasks were incubated in a CO_2 incubator at 37 °C. The spinner flask was agitated at a stirring speed of 70 rpm. Samples (1 ml) were taken every 12 h and were frozen for future analysis of MAb, glucose, lactate, and glutamine immediately after the viable cell number had been counted.

Fibrous-bed bioreactor

Selection of fibrous materials

Five different fabric sheet materials were used to test the attachment condition of hybridoma cells. They were: 100% polyester, 100% cotton, 50% polyester/50% cotton, 100% fiber glass, and 50% polyester/50% rayon. The fabric materials were cut to small pieces (1×1 cm), autoclaved, and placed in a 24-well culture plate. Cells with final concentration of 3×10^5 cells/ml were inoculated into each well and the medium was changed every 72 h. After 7 days, cell attachment to these fibrous materials was examined using a scanning electron microscope (SEM). The 100% polyester fibrous material was chosen for use as the packing material for the FBB in this study due to its better property for cell attachment. The cell density on 100% polyester was the highest among all materials studied (Zhu 1995). Cell attachment on 100% cotton and 50% polyester/50% rayon was also good, but 100% glass fiber and 50% polyester/50% cotton had relatively poor attraction for cells.

Bioreactor construction

The FBB was made from a jacketed glass column (4.5 cm inner diameter $\times$ 19 cm length) containing a spiral wound fibrous sheet ($10 \times 100 \times 0.1$ cm) as the fibrous bed packed in the bioreactor on top of 1″ thick 1/4″ glass beads, which served to evenly distribute the liquid medium. The reactor had a working (liquid) volume of about 200 ml. A pH probe, a dissolved oxygen (DO) probe, an inoculation port, and a sampling port were installed on the top of the column reactor. The reactor was maintained at a constant temperature by circulating water at 37 °C through the water jacket of the column reactor. Figure 1 shows the schematic diagram of the FBB operated as a recirculating-type (well-mixed) reactor. A 2-l spinner flask (containing 100 ml liquid) was installed in the recirculation loop. Fresh medium was continuously fed into the bioreactor with a peristaltic pump, while another pump recirculated medium through the recirculation flask at a high flow rate (80 ml/min) to provide a near well-mixed condition in the reactor. Sterilized air containing 5% CO_2 was used to flush the surface of the liquid in the recirculation flask to supply oxygen to the medium and also to balance the pH in the bioreactor. The effluent was collected

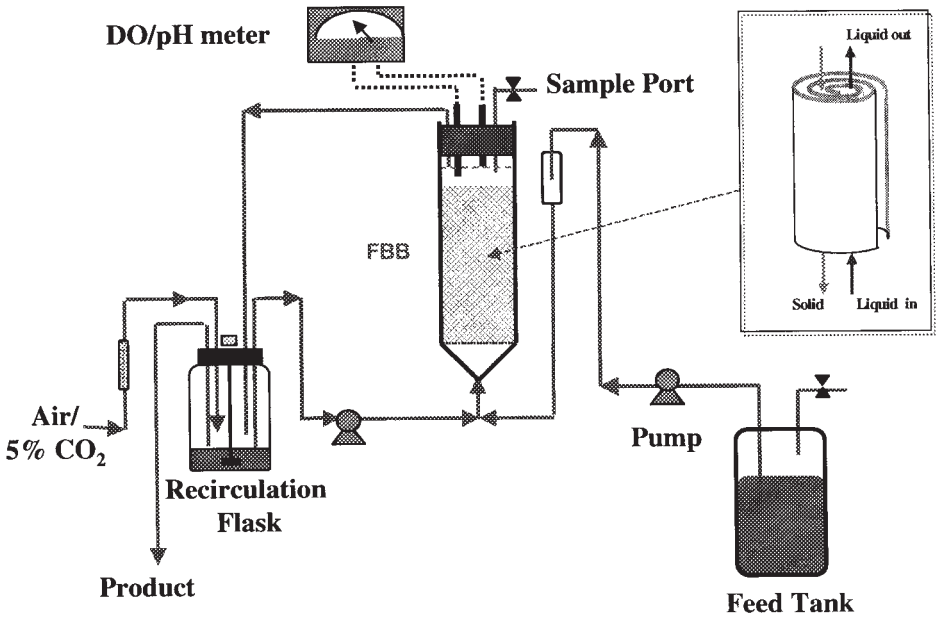

Figure 1. Schematic diagram of the continuous FBB with medium recirculation used in this study. The inset shows the spiral wound configuration of the fibrous bed with built-in flow channels allowing free flows of liquid and solids (cells).

from the spinner flask overflow caused by the pressure built-up inside the flask.

Bioreactor start-up

The entire bioreactor system, including pH and DO probes, medium tank, spinner flask, and all tubing and connections, was autoclaved for 1 h at 121 °C, 15 psig twice at a 24-h interval. The filter-sterilized medium was pumped into the reactor, and the temperature of the water jacket was maintained at 37 °C. Suspended cells in several T-flasks were collected by centrifugation. They were then injected from the inoculation port into the bioreactor using a 10 ml syringe to give an initial cell concentration of 2×10^5 cells/ml in the bioreactor. The medium recirculation rate was set at 20 ml/min to ensure that the cells were evenly distributed in the fibrous bed. No fresh medium was fed into the bioreactor until the pH had dropped to 6.8 or the DO dropped to 15%. The medium feed rate was 0.2 ml/min initially, and was later gradually increased to 0.8 ml/min. The bioreactor reached pseudo-steady-state conditions in 20–30 days, as determined from stable outlet glucose and lactate concentrations. At this time, the bioreactor also reached a high cell density and was ready for use in kinetic and long-term stability studies.

Bioreactor kinetic studies

The kinetics of MAb production in the FBB was studied under continuous, well-mixed conditions at various retention times (up to 120 h) for a period of about 3 months (95 days). At each feed rate, effluent samples were taken after the bioreactor had reached pseudo-steady state, which usually happened after 2–4 reactor volumes of the feed had been passed through. Samples were frozen immediately after pH measurements and stored at −20 °C for future analysis of MAb, glucose, lactate, and glutamine concentrations. Between every two retention time studies, the bioreactor was fed at ~20 ml/h (~15 h retention time) for several days to restore the reactor condition and to minimize any adverse effect caused by operation at a long retention time (>50 h).

After completing the study under well-mixed conditions, the bioreactor was maintained at a fixed retention time of ~20 h for about 40 days. The same reactor was then reconfigured to a

single-pass bioreactor system. The bioreactor was converted to a single-pass bioreactor by: (1) reconnecting the feed from the medium tank to the recirculation spinner flask, and (2) disconnecting the outlet from the reactor to the recirculation spinner flask. The liquid velocity in the single-pass bioreactor was low (<3 cm/h), and the flow condition in the bioreactor was close to plug-flow (PF). The bioreactor kinetics under PF condition was studied at various feed rates for a period of ~35 days. After that, the reactor was changed back to well-mixed conditions for long-term stability study.

Bioreactor long-term stability study

The long-term stability of the FBB was tested under continuous, well-mixed conditions, at a fixed retention time of 50 h for a period of 37 days. The pH in the system was maintained at 7.0, and the DO level was kept at 30% saturation. Samples were withdrawn daily and frozen (−20 °C) for future analysis after viable cell number had been counted.

Fed-batch culture

The bioreactor was also studied under fed-batch conditions, after completing the long-term stability study. The continuous feed was turned off and the spent medium in the bioreactor was drained and replaced with fresh medium. After 48 h, 5 ml of sterilized Dulbecco's phosphate buffer saline (PBS) containing 180 g/l glucose and 160 mM glutamine was injected into the bioreactor to bring the glucose concentration back to ~4.5 g/l and glutamine to ~4 mM. The addition of glucose and glutamine was repeated again after another 48 h. Samples were taken from the bioreactor at 12-h intervals to measure pH, glucose, glutamine, lactate, and MAb concentrations.

Determination of immobilized cell density

At the end of the bioreactor study, the immobilized cells in the FBB was removed by washing the cells off the fibrous matrix with PBS containing 0.5% trypsin. Five pieces of matrix samples (2 × 5 cm) were cut off from the fibrous matrix. The weakly bound cells were removed by dipping the fibrous matrix 10 times in PBS with known volume. The resulting cell suspension was then counted for total cell number and viability. The fibrous matrix was then scrubbed hard to wash off the remaining cells, which were counted as strongly-bound cells. The cell number and viability of the freely suspended cells present in the bioreactor bulk fluid were also counted.

Scanning electron and confocal microscopies

Fibrous matrix samples (1 × 1 cm) taken from various locations in the fibrous bed at the end of the bioreactor study were examined for cell morphology and immobilization conditions using SEM and confocal microscope. The preparation of SEM samples followed the procedures previously described (Silva and Yang 1995). SEM photos were taken with JOEL-Model 820 SEM. For confocal microscopic samples, cells on the fibrous matrix were first fixed in 2.5% formaldehyde solution for 2 h and then stained by immersing in a 40-μg/l propidium iodide (PI) solution for 1.5 h. Cell images at various depths in the fibrous matrix were taken using a Bio-Rad MRC 600 confocal microscope.

Analytical methods

Cell density and viability

The cell suspension was diluted with PBS containing 0.1% (w/v) trypan blue. After staining with 0.1% (w/v) trypan blue, the dead cells appeared blue, while viable cells remained colorless, round, and bright under the light microscope. Both total and viable cell numbers were counted on a hemocytometer, and cell viability was estimated from the ratio of viable cell count to total cell count.

Glucose, lactate, glutamine, and MAb concentrations

The concentrations of glucose and lactate were measured by using high performance liquid chromatography (HPLC) and a glucose/lactate analyzer (YSI 2000, Yellow Spring Instrument). The glutamine concentration was determined using an enzyme assay kit (Boehringer Mannheim). The concentration of MAb was analyzed using a sandwich enzyme-linked immunosorbent assay (ELISA). Rabbit anti-mouse IgG (Pierce, Rockford, IL) was coated onto 96-well microplates (Corning,

Cambridge, MA) in carbonate/bicarbonate buffer (pH 9.6). After stored overnight at 4 °C, the microplates were blocked with 1% BSA (Sigma, St. Louis, MO) in PBS containing 0.05% Tween 20 (Pierce, Rockford, IL) for 1 h at 37 °C. Diluted standard IgG (Sigma, St. Louis, MO) and sample solutions were then added in duplicate to the plates and incubated for 2 h at 37 °C. Subsequently, HRP-conjugated rabbit anti-mouse IgG (Pierce, Rockford, IL) was added and incubated for another 1 h at 37 °C followed by reaction with TMB substrate (Pierce, Rockford, IL). The absorbance at 450 nm was determined after stopping the reaction with 1 M H_2SO_4. The duplicate readings were averaged. A standard curve was derived for each plate from the readings of the standard IgG. The concentration of IgG in the samples were calculated by referring to the standard curve. In addition, each incubation step mentioned above was followed by washing with PBS containing 0.05% Tween 20.

Results and discussion

Batch suspension cultures

Typical kinetics of batch suspension cultures of hybridoma HD-24 cells are shown in Figure 2. For the static T-flask culture, there was a long lag phase of ~36 h and the culture took about 144 h to reach a maximum viable cell density of 1.6×10^6 cells/ml and MAb concentration of 43.6 mg/l (Figure 2A). The substrate glucose and glutamine went down to 0.114 g/l and 0.11 mM, respectively, and lactate increased to 2.89 g/l at the end of the batch culture. The overall productivity of MAb was ~0.3 mg/l·h. A similar kinetics was found with the spinner flask culture (Figure 2B). However, there was significant cell death and the viable cell density decreased dramatically after 108 h, indicating severe shear damage of cells caused by agitation in the spinner flask. The maximum MAb concentration was 56.8 mg/l at 192 h, and the overall productivity of MAb was 0.30 mg/l·h. The glucose and glutamine concentrations decreased to 0.121 g/l and 0.17 mM, respectively, at 144 h. In these suspension cultures, cell yield was 3.9×10^5 cells/g glucose consumed during the exponential phase, and the lactate yield from glucose during the entire culturing period was 0.64 g lactate/g glucose consumed. Regardless of the initial cell concentration, the maximum viable cell concentration obtained was $\sim 1.6 \times 10^6$ cells/ml. It was also found that MAb production was not directly proportional to glucose or glutamine consumption.

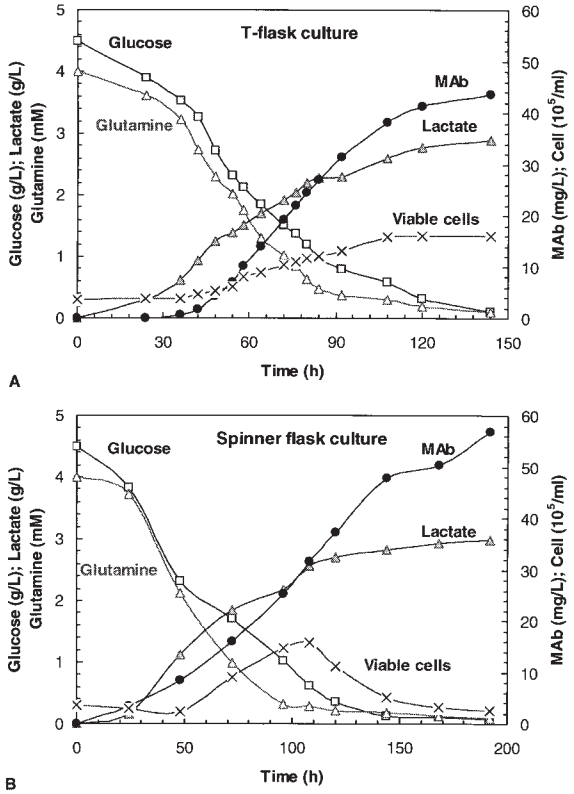

Figure 2. Kinetics of MAb production by hybridoma cells in suspension cultures. (A) Static T-flask; (B) Spinner flask.

It is clear that the suspension cultures in static T-flasks and spinner flasks had relatively poor MAb production partially due to the low viable cell density. Similar results have also been reported by various researchers. For example, Dalili and Ollis (1990) cultured hybridoma cell line MRC OX-19 in 100-ml T-flasks for 170 h, and the highest cell density obtained was 2×10^6 cells/ml while the MAb concentration was only 21 mg/l.

Fibrous-bed bioreactor

Continuous cultures

The pseudo-steady-state performance for the continuous FBB operated at various retention times is

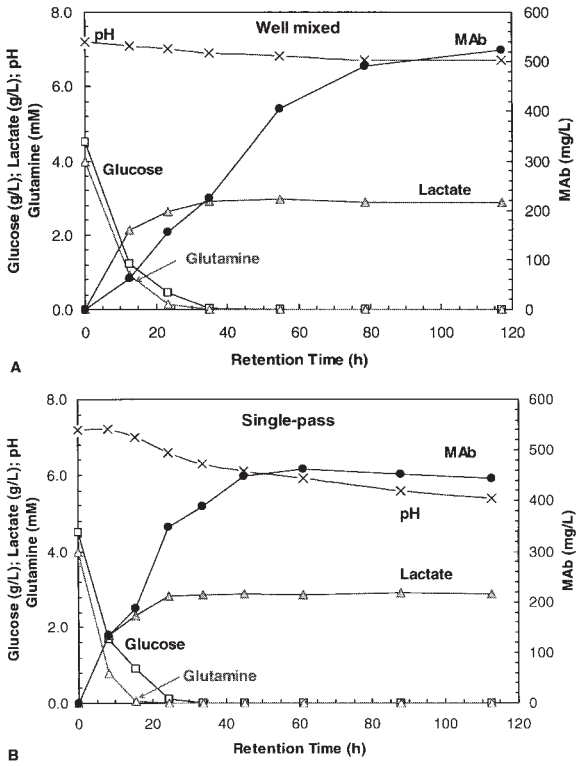

Figure 3. Kinetics of MAb production by hybridoma cells in the fibrous-bed bioreactor. (A) Reactor operated under well-mixed conditions or as a CSTR; (B) Single-pass or PF reactor.

shown in Figure 3. For the continuous well-mixed bioreactor, the highest MAb concentration attained was 522.4 mg/l at the retention time of 117.2 h (Figure 3A). The concentrations of glucose and glutamine decreased to zero at retention time 54.7 and 34.9 h, respectively. However, the depletion of these substrates did not stop MAb production to a higher level, although the MAb production rate decreased after glucose was depleted. Also, the reactor pH decreased from 7.2 to 6.7 due to the accumulation of lactic acid in the medium. For the FBB operated under PF conditions, initially MAb production was faster but then slowed to a halt at retention time of 61.25 h due to pH limitation (Figure 3B). The pH in the PF bioreactor could not be adjusted, and therefore, as the retention time increased, a pH gradient developed along the length of the bioreactor due to the formation of lactic acid. The effluent pH decreased from 7.2 to below 6.0 when the retention time was 45 h. The low pH in the bioreactor adversely

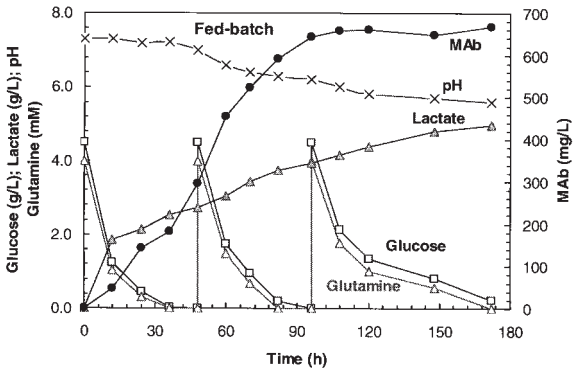

Figure 4. Kinetics of MAb production by hybridoma cells in the FBB operated at the fed-batch mode.

affected the cell physiology and inhibited MAb production. Consequently, the highest MAb concentration was only 461.2 mg/l obtained at 61.25 h retention time, and further increasing the retention time reduced MAb production. MAb production at higher retention times might also be limited by the depletion of substrates. Glucose was almost depleted at 33.8 h retention time and glutamine depletion occurred at 24.7 h retention time.

Fed-batch culture

In order to produce MAb at a maximum concentration, the FBB was operated under fed-batch conditions with pulse additions of concentrated glucose and glutamine solution to the medium. As shown in Figure 4, MAb production continued to reach a high concentration of 668.9 mg/l at ~108 h, but then stopped probably because of the accumulation of toxic metabolites (lactate and ammonia) and the low pH (pH <6 at time >110 h) in the culture medium.

Long-term stability

Figure 5 shows the long-term stability of the continuous FBB. As can be seen in this figure, the concentrations of MAb, glucose, and glutamine in the bioreactor effluent remained almost unchanged during the entire 37-day continuous operation at 50 h retention time under well-mixed conditions. The concentration of MAb in day 37 decreased by only 8.0% as compared with that in day one. The effluent cell density also remained at about the same level at all times. A total amount of ~1.55 g MAb was produced by the 200-mL FBB in 37 days.

Table 1. Cell number, viability, and percentage of suspended and immobilized cells in the FBB.

	Total cell		Viable cell		
	Cell number (10^8)	Percent total (%)	Cell number (10^8)	Percent total (%)	Viability (%)
Suspended cells in fluid	5.85 ± 0.65	2.2	0.08 ± 0.02	0.04	~1.4
Immobilized cells in fibrous matrix:					
Weakly attached	201.5 ± 6.5	76.5	151.5 ± 14.9	75.1	~75.2
Strongly attached	56.1 ± 3.9	21.3	50.2 ± 3.8	24.9	~89.5
Total in the bioreactor	263.45 ± 11.05		201.78 ± 18.72		~76.6

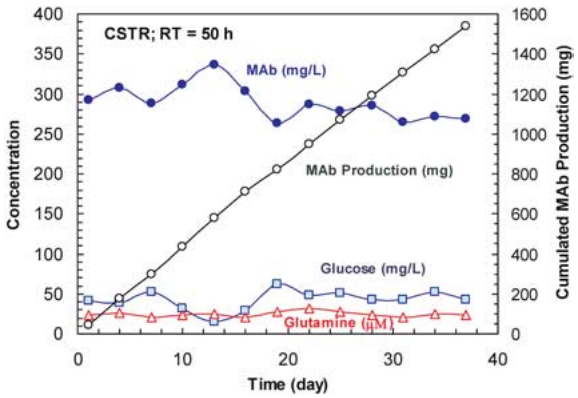

Figure 5. Long-term stability of the continuous FBB operated as a CSTR at a retention time of 50 h.

The average reactor productivity was ~8.73 mg/l·h, and the average MAb concentration produced in the medium was ~300 mg/l. It is noted that before this experiment was conducted, the FBB had been operated for more than 6 months under various operating conditions without suffering from any operation problems. The reactor productivity at the beginning of the long-term stability study was consistent with the MAb level obtained in the beginning for the continuous well-mixed reactor study at the same retention time of 50 h (see Figure 3A). It is clear that the FBB has a good stability for long-term continuous operation for MAb production.

Cell density and viability in the fibrous-bed bioreactor

The total cell density and viable cell density in the bioreactor at the end of this study were found to be 1.32×10^8 and 1.01×10^8 cells/ml, respectively. Thus, the overall cell viability in the bioreactor was ~77% even after more than 7 months operation. As shown in Table 1, more than 97% of the total cells were immobilized cells present in the fibrous matrix and almost all suspended cells present in the bioreactor bulk fluid were dead cells. This indicated that cell immobilization in the fibrous bed had protected cells well from shear damage. It also indicated that dead cells were preferentially washed out from the fibrous matrix, avoiding the accumulation of dead cells in the fibrous bed and allowing the bioreactor to be renewed continuously in its cell population and thus to maintain stable long-term production of MAb. In a separate experiment done with static T-flasks containing fibrous matrices, it was found that almost all cells strongly attached to fibers were viable cells whereas suspended cells in the medium had a significantly lower viability (<80%) after culturing for 13 days, suggesting that dead and/or nonviable cells had relatively poor adhesion to fibers and thus would not be immobilized (retained) in the fibrous matrix under continuous flow conditions. The ability to continuously dislodge dead cells from the fibrous matrix is an advantage for the FBB over hollow-fiber membrane bioreactors, in which all cells (both viable and dead) are confined within the tight membrane, resulting in culture degeneration over extended operation period. It should be noted that the high shear environment in the recirculation loop would kill cells coming off the fibrous matrix, which also contributed to the low viability of the suspended cells in the bioreactor.

As seen under SEM, cells either attached to the surface of the fibers or were entrapped in the interstitial spaces of the fibrous matrices (Figure 6). According to Needham et al. (1991), five distinct cell morphologies can be identified for hybridoma

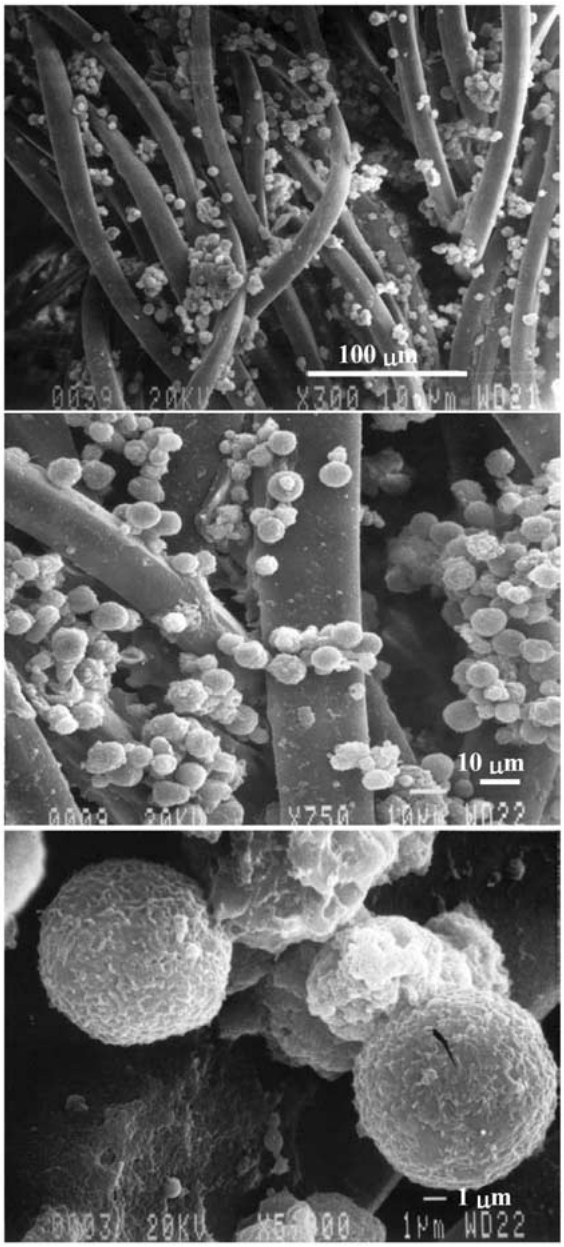

Figure 6. Scanning electron micrographs of hybridoma cells immobilized in the fibrous matrix.

cultures: (i) daughter cells in early G_1 phase as small smooth spheres; (ii) cells at G_2 phase as large, rough spheres; (iii) cells at late G_1 or S phase as irregular shapes; (iv) cells at M phase as large smooth spheres with patterned interior; (v) cells at cytokinesis of M phase as patterned, spherical cells undergoing cell division. The morphologies of hybridoma cells at various stages of the cell cycle were found under the SEM, indicating that cells cultured in the FBB were healthy and proliferating well.

Figure 7 shows the confocal microscopic pictures of stained hybridoma cells located at different depths in the fibrous matrix. There were relatively few cells at the surface of the fibrous matrix, indicating that the hydrodynamic shear forces at the interfaces had removed some cells from surface of the fibrous matrix. Slightly below the surface, shear forces and mass transport resistance were small, and the matrix structure provided an ideal environment for cells to grow and immobilize. Thus, a relatively large number of cells were found at a depth of 100–200 μm. Further into the matrix, transport of substrates and toxic metabolites became relatively difficult and inefficient. Thus, the cell number decreased with an increase in the matrix depth from the surface. However, the actual decline in cell number with depth might not be as significant as is seen in these photos, which only show stained cells. It was found that cells in the deeper parts of the fibrous matrix were not stained properly. Even at the center of the fibrous matrix, a large number of cells were found, but they were not stained and thus could not be clearly seen in these photos. A longer time for PI staining of the cell-matrix samples might be necessary to view all viable cells inside the matrix (Chen et al. 2002).

Comparison of MAb production in various culture systems

Figure 8 compares the production of MAb in the FBB under different operating modes. Under the well-mixed (CSTR) condition, the MAb productivity reached a maximum of ~7.4 mg/l·h with a MAb concentration of 404.8 mg/l at 54.7 h retention time. The MAb productivity then decreased, probably due to substrate depletion and inhibition by high lactate concentration. Compared to the well-mixed bioreactor, the PF reactor gave higher MAb production rates at lower retention times. The PF reactor productivity decreased from 16.4 mg/l·h at 8.23 h retention time (with 134.9 mg/l MAb) to 9.89 mg/l·h at 45.26 h retention time (with 447.8 mg/l MAb). For the fed-batch culture, productivities were similar to those for CSTR but lower than PF at culture (retention) times lower than 60 h. However, at longer culture times, the

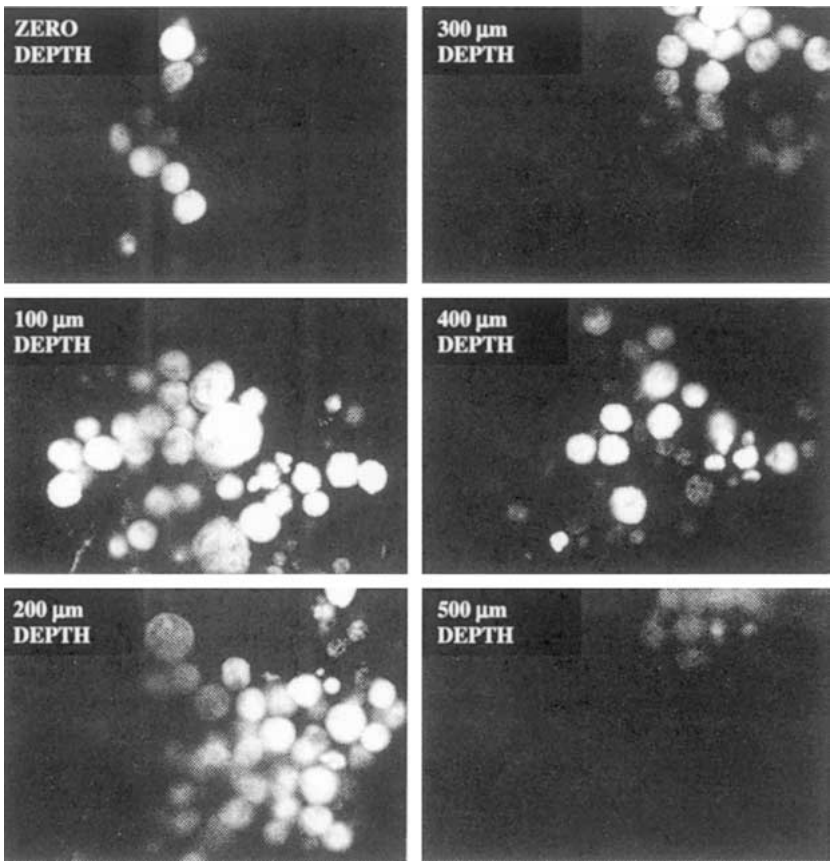

Figure 7. Confocal microscopic images of PI-stained hybridoma cells at different depths of the fibrous matrix.

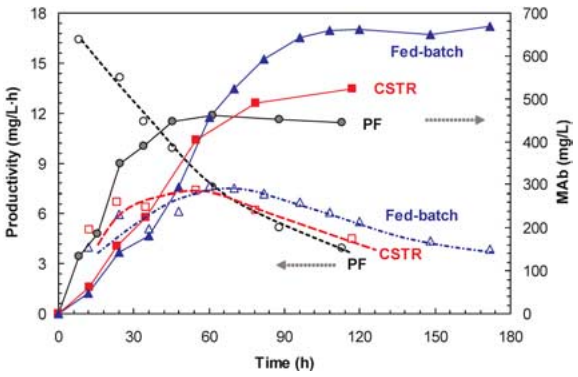

Figure 8. Comparison of MAb production by hybridoma cells in the FBB operated under various conditions (CSTR: well-mixed; PF: Single-pass). The open symbols connected with the dashed lines are volumetric productivity (left Y-axis). The solid symbols connected with the solid lines are MAb concentration (right Y-axis). The time on the X-axis is the retention time for PF and CSTR, and culture time for the fed-batch culture.

fed-batch culture gave higher productivity and MAb concentration. The optimal operating mode for the FBB is thus dependent on the desirable MAb concentration to be produced. It should be mentioned that the productivity in the fed-batch culture could have been higher. The bioreactor had been operated at a high retention time of 50 h for a period of 36 days, and the fed-batch culture was conducted immediately after the long-term stability study without allowing the reactor to recover completely to its optimal condition. It is also noted that the volumetric productivity reported here is based on the total liquid medium volume (300 ml) in the reactor system. The reactor productivity based on the FBB volume (200 ml) would be 50% higher.

Compared to conventional T-flask and spinner flask cultures, the FBB gave a much higher MAb

Table 2. Comparison of various culture systems for MAb production by hybridoma HD-24.

Culture system	Final MAb concentration (mg/l)	MAb productivity (mg/l·h)[#]	Viable cell density (#cell/ml)	Sp. MAb productivity (10^{-7} mg/h·cell)
Suspension cultures:				
Static T-flask	43.64	0.3–0.6	1.60×10^6	~3.75
Spinner flask	56.78	0.3–0.44	1.57×10^6	~2.8
Fibrous-bed reactor:			1.01×10^8	
Well-mixed	522.4	4.5–7.6		~0.75
Plug-flow	461.2	8.0–16.4		~1.62
Fed-batch	668.9	4.0–7.5		~0.74

[#] The volumetric productivity is based on total liquid medium volume in the culture. The FBB volume was only 2/3 of the total liquid volume in the culture system.

production because of the higher viable cell density attained in the fibrous bed. The maximum cell density, productivity, and highest MAb concentration in various culture systems are summarized and compared in Table 2. It can be seen that the viable cell density in the FBB was more than 60 times of that in T-flasks and spinner flasks, and the reactor productivity as well as the highest MAb concentration produced in the medium were more than 10-fold higher in the FBB. It is noted that about 10 times more MAb was produced in the FBB than in the suspension cultures even though the same medium composition was used in all culture systems studied, indicating a higher MAb yield from the medium by cells cultured in the FBB. It should be noted that the lactate yield from glucose in the FBB was similar to that in the suspended culture, ~0.64 g/g glucose consumed. In a separate static T-flask experiment, it was found that MAb yield from T-flasks containing fibers was ~10% higher than that from control (without fibers). It is thus clear that more MAb can be produced by the immobilized cells in the fibrous matrix than by the suspended cells from the same amount of the medium used in the culture. The increased MAb yield may be attributed to the immobilization effect on cell cycle, which is known to affect MAb production (Ramirez and Mutharasan 1990; Simpson et al. 1999). Culturing cells in the three dimensional fibrous matrix was found to have profound effects on cell morphology, cell size, cell cycle, and apoptosis, which in turn affected protein production (Luo 2002; Chen et al. 2003).

However, the specific cell productivity in the FBB was less than that in the static T-flask and spinner flask. This could be attributed to the high MAb and lactate concentrations in the FBB. It is known that high concentrations of lactate and ammonia are toxic or inhibitory to MAb production (Hansen and Emborg 1994; Matsumura et al. 1995; Chen et al. 2001). The relatively low specific productivity in the well-mixed bioreactor also indicated the significance of product (metabolite) inhibition. Also, there might be significant nutrient limitation for the immobilized cells in the fibrous matrix, which in turn would limit cell growth and MAb production rate. It is very likely that under the high-cell-density condition, cells inside the matrix were deprived of oxygen (Luo 2002). Although the DO level in the bulk medium was kept above 30%, the actual DO level inside the matrix would have been much lower since oxygen transfer into the cell matrix was limited by diffusion. Thus, the viable cells in the fibrous bed could not function as well as those in flask cultures because of the limited availability of the substrates in the bioreactor. There might be some mass transfer limitations when there were high concentrations of cell biomass built-up in the bioreactor.

The high MAb concentration and production rate and excellent stability for continuous long-term production obtained in the FBB also compare favorably to other bioreactor studies reported in the literature (Wang et al. 1992; Fong et al. 1997; Bierau et al. 1998; Yang et al. 2000). The FBB reactor performance can be further improved by providing better pH and aeration controls at higher feed concentrations (Ozturk and Palsson 1990, 1991). MAb production also can be greatly enhanced by using a better medium design and feeding strategy (Xie and Wang 1996) and continuous perfusion to remove toxic metabolic byproducts (Banik and Heath 1995; Wen et al. 2000).

Conclusions

The hybridoma HD-24 cells were successfully cultured in the FBB for long-term continuous production of MAb. The viable cell density reached 1.01×10^8 cells/ml in the bioreactor and the volumetric productivity of MAb was more than 10-fold of those in spinner and static flasks. The superior performance from the FBB, as compared to suspension cultures, can be attributed to cell immobilization in the fibrous matrix, which protects cells from shear damage and provides a conducive environment for cell growth and MAb production. The continuous operation of the bioreactor for ~8 months and the long-term stability test demonstrated that the productivity of the bioreactor was stable for continuous operation for a long period of time, partially due to its ability to wash off nonviable and dead cells from the fibrous matrix. The reactor performance may be further improved if mass transfer, particularly oxygen transfer, in the fibrous matrix can be enhanced and the toxic metabolites can be removed by continuous perfusion.

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