Mammalian cell retention devices for stirred perfusion bioreactors

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Received 26 August 1998; accepted 26 August 1998

Key words: centrifuge, cross-flow filter, review, sedimentation, spin-filter, ultrasonic cell retention

Abstract

Within the spectrum of current applications for cell culture technologies, efficient large-scale mammalian cell production processes are typically carried out in stirred fed-batch or perfusion bioreactors. The specific aspects of each individual process that can be considered when determining the method of choice are presented. A major challenge for perfusion reactor design and operation is the reliability of the cell retention device. Current retention systems include cross-flow membrane filters, spin-filters, inclined settlers, continuous centrifuges and ultrasonic separators. The relative merits and limitations of these technologies for cell retention and their suitability for large-scale perfusion are discussed.

Introduction

Mammalian cell culture is important in biotechnology because many protein products require complex post-translational processing that is presently either difficult or impossible to achieve with bacteria, yeast or insect cells. The growing demand for these products has motivated the development of more efficient and reliable mammalian cell culture production technologies. Cell culture systems aim to provide optimal conditions for mammalian cell proliferation and protein production by controlling the cell environment (temperature, pH, osmolarity) and by supplying dissolved oxygen (DO), nutrients and growth factors at appropriate concentrations without exposing the cells to damaging conditions. Bioprocess selection is determined by economic as well as protein and cell line specific considerations, including the anticipated production needs and the dependence of product consistency on culture conditions. Changing culture conditions can influence the consistency of protein post-translational modifications (Maiorella et al., 1993; Marino et al., 1997). Thus, it is necessary to demonstrate consistent

process performance and product quality for approval by regulatory agencies.

Packed-cell bioreactors such as hollow-fiber and ceramic-matrix reactors attain high cell densities ($>10^8$ cells mL⁻¹) as a result of continuous medium perfusion of the immobilized cells. In particular, hollow-fiber bioreactors are commonly used to produce gram quantities of MABs (monoclonal antibodies) for diagnostic applications. High MAB concentrations ($\sim 1\text{--}10$ g L⁻¹) are attained due to ultrafiltration retention of the product (Jackson et al., 1996). However, because of the spatial heterogeneity inherent in these plug-flow reactors, scale-up is limited (Piret et al., 1991).

Stirred tank culture is readily scaleable and its relatively uniform environment greatly facilitates monitoring and control. Anchorage dependent cells are often grown in stirred tanks on suspended microcarriers for large-scale production. Non-anchorage dependent cells grow directly in suspension, simplifying the process in a number of ways (Arathoon and Birch, 1986). In stirred batch reactors (Figure 1), the pH, DO and temperature are controlled, but nutrient and metabolite concentrations change dramatically with time, resulting in decreasing growth and protein pro-

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Figure 1. Alternate stirred bioreactor processes. In stirred mammalian cell culture the temperature, pH, and dissolved oxygen level are controlled. A defined concentrated feed stream is added to fed-batch culture in response to the depletion of one or more nutrients. In perfusion, nutrients are continuously supplied and metabolites diluted, while cells are separated from the product stream and retained in the reactor.

duction. The cell density in batch generally reaches a maximum of around 10^6 cells mL^{-1} , resulting in a much lower volumetric productivity than in a packed-cell bioreactor. In fed-batch, the initial batch culture is supplemented with fresh medium in response to the depletion of one or more nutrients, such as glucose and glutamine (Glacken et al., 1986; Bibila and Robinson, 1995). Careful optimization of the feed formulation can extend the culture duration to approximately 3 weeks from 1 week for simple batch, and can result in an order of magnitude increase in final product concentration.

In continuous stirred or chemostat bioreactors, nutrients are continuously replenished and metabolites diluted, minimizing time-varying culture conditions. In stirred perfusion cultures, higher cell densities (over 10^7 cells mL^{-1}) can be achieved by separating the cells from the outflow stream, and retaining them in the reactor (Figure 1). In these cultures, the volumetric productivity can be almost two orders of magnitude higher than in batch and in some cases product concentrations are increased up to 5-fold (Reuveny et al., 1986; Hiller et al., 1993; Bodeker et al., 1994; Trampl et al., 1994). Thus, the required reactor volume for a given production rate can be reduced by approximately 100-fold. Because perfusion cultures can last up to 6 months or more, further economic benefits are accrued due to reduced labor requirements for reactor inoculation and turnaround.

Fed-batch operation is frequently selected over perfusion for its shorter duration in cases where replicate processes must be validated in a minimum time. However, perfusion processes can be acceptable under

such time-constrained circumstances by terminating the runs after as little as 1 month. Fed-batch is also often preferred to perfusion based on the potential for higher product concentration and yield on medium. This is especially true in cases where the cell line productivity is unstable, as fed-batch runs involve many fewer cell doublings. For these reasons, more effort has been directed toward fed-batch process development. However, perfusion may be preferred when temporal changes in culture conditions adversely affect product consistency or when the product is susceptible to degradation, because the mean product residence time is on the order of hours rather than days as in fed-batch (Kadouri and Spier, 1997). Perhaps most importantly, perfusion cultures are simpler to develop and operate than fed-batch in at least one respect: beyond the brief start-up period, only the single steady-state production phase of a perfusion process requires optimization, rather than the continuously changing dynamic culture and feeding of a fed-batch process. With the progression of the genomics initiative, increasing numbers of proteins will need to be produced rapidly, and will likely motivate the wide-spread use of simpler, higher productivity perfusion processing. The principal aspect of perfusion processing that can be more complex than fed-batch, particularly upon bioreactor scale-up, is the need for cell retention. This review will focus on the relative advantages and disadvantages of the various alternative technologies for perfusion bioreactor cell retention.

Cell retention devices

During a typical perfusion run inoculated at 10^5 cells mL^{-1} , the cell concentration will increase by two orders of magnitude or more over a span of approximately 10 days. As in any culture, some nonviable cells and debris will accumulate throughout the run due to apoptosis and necrosis (Mercille and Massie, 1994), and cell lysis will release intracellular macromolecules into the culture medium. These dynamic characteristics of long-term perfusion culture present a challenge to the robust operation of cell retention devices. Ideally, a cell retention device should operate satisfactorily for the required duration without replacement or maintenance in order to minimize additional risk of contamination. The device should not adversely affect cell viability or productivity and it should separate close to 100% of the viable cells from the effluent stream, regardless of the cell concentration, in order to maintain high viable cell concentration in the reactor. Finally, although the build-up of nonviable cells and debris can be mitigated by the use of a bleed stream (whereby the suspension is drawn directly from the reactor without cell separation), an ideal retention device should selectively retain viable cells while allowing the nonviable cells to pass through.

Table 1. Cell separation in retention devices. The cell attributes forming the basis for retention are indicated by check marks. Cell size only enhances the effect of one of the other factors when indicated by a bracketed check mark

Retention device	Basis for cell retention		
	Size	Density	Compressibility
Cross-flow filter	✓	×	×
Spin-filter	✓	×	×
Inclined settler	(✓)	✓	×
Centrifuge	(✓)	✓	×
Ultrasonic separation	(✓)	✓	✓

The majority of cell retention devices currently in use (Table 1) are adaptations of conventional particle-liquid separation methods and fall into two general categories: filtration and sedimentation devices. Spin-filters and cross-flow membrane filters retain cells on the basis of size. Sedimentation devices, such as inclined settlers and continuous centrifuges, take advantage of the difference in density between the cells and the medium to effect separation. Ultrasonic cell

separation is a more recently developed approach that retains cells on the basis of differences in both density and compressibility between the cells and the medium. There is currently no consensus as to the best retention device for mammalian cell perfusion in stirred suspension reactors due to their different inherent strengths and weaknesses. The following discussion examines recent progress in the development of cell retention devices for mammalian cell perfusion in stirred suspension bioreactors.

Filtration

To sustain long-term perfusion culture performance, filter design and operation must mitigate fouling of the filtration surface by cells, cellular debris and macromolecules (Esclade et al., 1991; Bowen and Gan, 1992; Kelly and Zydney, 1997). Fouling occurs as these components are concentrated at the filter surface by the permeate flux (flow through the filter), a process known as concentration polarization. Filter fouling not only limits the filtration rate, but may result in ultrafiltration retention of high molecular weight products. In general, filtration systems are designed such that the accumulation of cells and cellular material is limited by flow tangential to the filter surface, with the extent of accumulation controlled in large part by the fluid shear rate (Frenander and Jonsson, 1996). The hydrodynamic lift force acting on cells in the shear field may also play a role in reducing filter clogging by cells and debris (Belfort, 1988; Yabannavar et al., 1992).

Cross-flow filters

Hollow-fiber and flat-plate cartridges are suitable as cross-flow filters for cell retention in mammalian cell perfusion applications (Table 2). In these systems, the suspension is pumped to the external cartridge and is concentrated as it flows across a membrane. The concentrated stream is recycled to the reactor, while the cell-free permeate forms the effluent stream. The pressure drop between the inlet and recycle streams drives the cross-flow and determines the surface shear rate. The resulting transmembrane pressure difference is thus highest near the inlet and may even be reversed at the outlet, generating a non-uniform permeate flux (Van Reis et al., 1991).

Permeate flux has been established to be the most important parameter in determining the time to detrimental fouling (clogging) of cross-flow filters (Maiorella et al., 1991). Mean fluxes in the range of 30 to 50 $\text{L m}^{-2} \text{h}^{-1}$ are typically reported for the

Table 2. Cross-flow filter performance data in selected perfusion experiments

Membrane type, area and pore size	Reactor volume (L)	Maximum perfusion rate (L day ⁻¹)	Maximum permeate flux (L m ⁻² h ⁻¹)	Maximum total cell density (10 ⁶ cells mL ⁻¹)	Viability	Comments	Study
Hollow-fiber 0.2 μ m pore	1.0	1.3	0.5	18	>90%	perfusion lasted 32 days	Martin et al. (1989)
Flat plate 0.2 μ m pore	1.1	1.2	7.7	25	84%	perfusion halted after 9 days	Velez et al. (1989)
Flat plate 1 10 μ m pore	3.6	1.8	4.2	7	44%	filter replaced after 5 to 7 days	De la Broise et al. (1992)
Hollow-fiber 0.1 μ m pore	2.0	8.8	8.1	34	80%	perfusion lasted 22 days, initial growth rate same as in batch	Shi et al. (1992)
Hollow-fiber 0.2 μ m pore	1.0	4.0	2.8	14	70%	filter clogging at high flux, cell concentration	Zhang et al. (1993)
Flat plate 0.65 μ m pore	0.5	1.45	2.5	30	(NA)	perfusion lasted 33 days, growth rate 66% of batch	Kawahara et al. (1994)
Flat-plate 5 μ m pore	1.5	0.75	0.9	30	17%	filter replaced every 5 days	Mercille et al. (1994)

(NA) = Not available.

cross-flow filtration process during product harvest after batch culture (Maiorella et al., 1991; Van Reis et al., 1991). In successful perfusion experiments, reported fluxes were an order of magnitude lower due to the requirement for long-term operation (Table 2). Despite the lower fluxes, filter fouling remains a problem. The influence of membrane pore size, mean cell size, culture state, viability and surface shear rate on the maximum flux and on the operating life of the filter have been examined in order to optimize filter design and operation.

The majority of reported cross-flow filters use microporous membranes with 0.2 to 0.65 μ m pores. Such relatively small pores might be expected to clog more readily than larger pore filters. However, a 5 μ m pore flat-plate filter membrane required changing every 5 days during perfusion with a filter flux of 1 L m⁻² h⁻¹ (Mercille et al., 1994) while 2 and 10 μ m diameter pore membranes were replaced every 5 to 7 days during perfusion with a filter flux of 4 L m⁻² h⁻¹ (De la Broise et al., 1992). Thus, there is no clear advantage to these larger pore filters, especially considering the poor cell retention efficiency (<70%) of the 10 μ m membrane.

The maximum flux was shown to increase at higher surface shear rates, likely due to the reduced accumulation of cells and debris at the filter surface (Van Reis et al., 1991). However, the surface shear rate can be limited by the shear tolerance of the cells. Maiorella et al. (1991) reported that cells were damaged at wall shear rates over 6000 s⁻¹, while Zhang et al. (1993) observed a drop in culture viability at a shear rate of 1300 s⁻¹ during perfusion using a hollow-fiber cartridge. Higher shear rates require higher inlet pressures and thus also increase the transmembrane pressure difference at the filter inlet. At sufficiently high transmembrane pressure differences, cell lysis has been shown to occur when cells occlude pores (Forstrom et al., 1975). Susceptibility to lysis was a function of transmembrane pressure difference, shear rate, pore size and cell membrane tension (Zydney and Colton, 1984). Increasing the shear rate to cell damaging levels can therefore ultimately reduce the maximum perfusion flux, because reduced culture viability is typically accompanied by a lower mean cell diameter and significant levels of debris, both of which decrease the maximum flux (Maiorella et al., 1991). Furthermore, the small pore radius (0.2 to 0.65 μ m) of the majority

Figure 2. Spin-filter for cell retention in perfusion bioreactor. The effluent stream is withdrawn from the cell clarified interior of the cylindrical spin-filter. In this configuration, impeller and filter rotate independently.

of membranes results in the accumulation of nonviable cells and cell debris in the reactor, causing the maximum flux to decline with time during long-term operation.

One of the main advantages of cross-flow microfiltration for cell retention is that the cell free effluent facilitates integration with the purification process. Moreover, such external devices can be replaced or regenerated when fouling becomes a problem. However, replacement represents a contamination risk as well as additional expense and complications. Pumping of the cell suspension to the external device introduces further complexity and may also cause cell damage (Maiorella et al., 1991). There is little reported information about how cross-flow filters might be integrated into large-scale processes, but for scale-up it is clear that the mean perfusion flux should be kept constant by increasing the total membrane area in proportion to the required capacity.

Spin-filters

Himmelfarb et al. (1969) were the first to report the successful perfusion of mammalian cells in a stirred suspension bioreactor. Their cell retention device, referred to as a spin-filter (Figure 2), consisted of a cylindrical membrane rotating inside the bioreactor around the same centerline axis as the impeller. Spent culture medium was drawn from the culture volume

into the cylinder and then out of the reactor, while the cells were retained by the membrane. Spin-filters are distinct from cross-flow filters in two major respects: fluid flow relative to the filtration surface is produced by the rotation of the cylinder, and the filtration surface is typically inside the reactor. As a result, the transmembrane pressure drop can be selected independently of the cross-flow velocity, the retentate cell concentration is more uniform over the whole filter surface, and the cells remain in a controlled environment.

High cell concentration and productivity were achieved in early spin-filter perfusion experiments using a variety of designs (Himmelfarb et al., 1969; Tolbert et al., 1981; Reuveny et al., 1986). However, perfusion was restricted to 10 days, with filter clogging leading in some cases to premature termination of the cultures. Because replacement of internal units is impractical, spin-filters should be designed and operated to avoid clogging during longer-term continuous operations. As with cross-flow filters, the rate of filter fouling increases with perfusion flux and cell concentration (Deo et al., 1996). However, the operating life and maximum perfusion flux are also functions of filter material, effective filter pore size and spin-filter rotational speed. The selection of these parameters for spin-filter optimization has been examined by a number of investigators.

By using larger-pore screens, the operating life of spin-filters has been prolonged (Table 3). While a 5 μm pore filter clogged within 7 days (Reuveny et al., 1986), continuous perfusion reactors were operated without clogging for 11 days using a 25 μm pore stainless steel mesh spin-filter (Yabannavar et al., 1992) and for 49 days using a 15 μm pore filter (Deo et al., 1996).

The upper limit to filter pore size is determined by the cell retention efficiency. Using a 53 μm mesh, Varecka and Scheirer (1987) found that 99% of an aggregating cell line was retained in the reactor during short-term separation experiments. During perfusion of the non-aggregating NS-1 cells (mean size 14 μm), the retention of a 25 μm pore screen varied between 60% at 100 rpm and 97% when the filter was at rest (Yabannavar et al., 1992). This was explained by the increase in the convective fluid exchange between the reactor volume and the volume inside the spin-filter that occurs at increased rotational speed. The retention efficiency was lower when the filter was rotated because cells were entrained with the exchange fluid flow. In perfusion experiments with a 50 μm mesh

Table 3. Spin-filter performance data in selected perfusion experiments

Filter pore size and area	Reactor working volume (L)	Maximum perfusion rate (L day ⁻¹)	Maximum permeate flux (L m ⁻² h ⁻¹)	Maximum total cell density (10 ⁶ cells mL ⁻¹)	Viability	Comments	Study
5 μ m pore (NA)	0.55	1.36	(NA)	22	70%	filter clogged every 7 days, culture moved to new vessel	Reuveny et al. (1986)
25 μ m pore 470 cm ²	12	12	1.1	5.5	72%	perfusion lasted 14 days	Yabannavar et al. (1992)
Vortex flow filter 0.8 μ m pore 200 cm ²	1.35	0.67	1.4	80	5%	filter replaced after 43 days, perfusion lasted 74 days	Mercille et al. (1994)
25 μ m pore 2200 cm ²	175	87	1.6	4.4	65%	perfusion lasted 5 days (no fouling)	Yabannavar et al. (1994)
15 μ m pore (NA)	500	500	(NA)	10 (viable)	(NA)	30 day duration	Deo et al. (1996)
Vortex flow filter 10 μ m pore 200 cm ²	3	6	1.25	10	95%	perfusion lasted 60 days	Roth et al. (1997)

(NA) = Not available.

filter, there was no detectable retention of the NS-1 cells regardless of operating conditions (Yabannavar et al., 1992). The 50 μ m pores were too large to retain the 14 μ m cells on the basis of size, and other proposed mechanisms, such as the hydrodynamic lift and centrifugal forces, did not result in measurable retention.

Apart from extending operating life, large-pore spin-filters allow smaller, nonviable cells to pass more readily through the filter than viable cells, resulting in a selective retention of the viable population. A 25 μ m pore spin-filter demonstrated modest selective viable cell retention for a range of rotational speeds (Yabannavar et al., 1992). The selectivity for viable cells varied, depending on rotation rate, agitation rate and perfusion rate. The same group found no selective retention by a 25 μ m pore spin-filter in a scaled-up bioreactor, possibly due to the 2.5-fold higher permeate flux (Yabannavar et al., 1994).

The effect of filter material on fouling was examined by Avgerinos et al. (1990) for perfusion culture of an aggregating CHO cell line with aggregates between 200 and 600 μ m in diameter. They found that stainless steel mesh filters ranging in nominal pore size from 44 to 105 μ m became plugged within 11 to 21 days, with

substantial cell attachment observed on the mesh. No fouling was apparent after more than 50 days of perfusion using ETFE (ethylene-tetrafluoroethylene) filters. Esclade et al. (1991) reported that clogging occurred after 7 days during hybridoma perfusion culture with a 10 μ m pore stainless steel mesh filter, while a comparable 11 μ m pore polyamide screen filter did not clog under similar conditions. The high surface charge density of stainless steel relative to polymer materials may be responsible for the increased fouling (Esclade et al., 1991). Nevertheless, stainless steel filters remain attractive because they are durable and reusable.

Increasing the filter rotation rate has a direct effect on the maximum perfusion rate and operating life. In experiments using a 15 μ m pore stainless mesh spin-filter, the maximum perfusion rate before filter clogging was proportional to the rotational velocity squared (Deo et al., 1996). This dependence of clogging on rotational speed is also consistent with the observation that, in the event of mechanical failure, perfusion must be halted while repairs are made, or the stationary spin-filter will foul rapidly and irreversibly (Deo et al., 1996).

In typical spin-filters, fouling is reduced at high rpm due to the high shear rate generated by the filter

motion relative to the bulk fluid volume. In external vortex flow filters (Rebsamen et al., 1987; Mercille et al., 1994; Roth et al., 1997), fouling is reduced by stable Taylor-Couette vortices generated in the gap between concentric cylindrical surfaces due to their relative rotation. A $0.8\ \mu\text{m}$ pore vortex flow filter membrane was replaced after 45 days of perfusion culture (Mercille et al., 1994), while myeloma and CHO cell perfusion culture experiments lasted up to 60 days without clogging using a $10\ \mu\text{m}$ pore steel mesh vortex flow filter (Roth et al., 1997). Compared to conventional spin-filters, vortex flow filters appear to be less susceptible to fouling (Table 3).

The scale-up of spin-filter perfusion devices is based on analyses of the mechanisms controlling the perfusion rate and fouling. Yabannavar et al. (1994) assumed that filter clogging due to the permeate flux was counteracted by a hydrodynamic lift force produced by the spinning filter, similar to the force observed on particles near a wall in a laminar flow field (Belfort, 1988; Drew et al., 1991). Keeping the ratio of the lift force to the permeation drag constant, they showed that the fluid exchange through the filter on a reactor volume basis was maintained in a scaled-up 175 L bioreactor and that the retention efficiency was similar to that in the 12 L reactor during a 10 day perfusion experiment. The importance of hydrodynamic lift in preventing clogging of large pore spin-filters is not clear, as there was no detectable contribution of such a force to the cell retention efficiency of a $50\ \mu\text{m}$ pore filter. However, filter fouling will be reduced at higher shear rates due to the increased tangential drag forces preventing cell or debris deposition. Conserving the ratio of shear rate to permeation drag will result in an identical approach to scale-up because the shear rate at the filter surface has the same dependence on filter rpm as the hydrodynamic lift force (Favre and Thaler, 1992).

Deo et al. (1996) expressed the perfusion capacity as a function of rotation velocity, filter dimensions and cell concentration. At a constant rotation rate, the spin-filter perfusion capacity scaled with height and with diameter cubed. Using these relationships to determine dimensions and operating conditions, scale-up from 7 to 500 L was successful. Perfusion of the 500 L bioreactor, at a rate of one reactor volume per day, lasted 30 days at a cell concentration surpassing 10^7 cells mL^{-1} . The growth profile, cell concentration and antibody concentration were the same as in the 7 L bioreactor.

It should be noted that despite much recent progress in the design and operation of spin-filters, their reported permeate fluxes (Table 3) are of the same order as those in cross-flow filtration perfusion (Table 2). This is likely due to the more conservative approach required to ensure robust operation, since the filter cannot be easily replaced. In fact, the reported duration of most conventional spin-filter runs has been limited to one month.

Sedimentation

Sedimentation exploits the difference in density between the cells and the suspending medium by establishing a quiescent liquid volume in which the cells settle from suspension (Table 1). Failure due to clogging does not occur because the separation is not carried out by a physical barrier and selective retention of viable cells is possible because smaller nonviable cells have an approximately 2-fold lower sedimentation velocity relative to viable cells (Searles et al., 1994). The major challenge facing sedimentation-based separation is the slow sedimentation velocity of mammalian cells due to their small size (on the order of $10\ \mu\text{m}$) and low density (approximately 5% greater than that of the medium). This limits the maximum flow-rate and can result in long, potentially detrimental hold-up times in an unoxxygenated, unmixed environment.

Vertical sedimentation devices (Figure 3A) have been used by a number of researchers for laboratory scale perfusion culture at cell concentrations up to 1.1×10^7 cells mL^{-1} (Takazawa and Tokashiki, 1989; Shitani et al., 1991). During perfusion at a cell density of up to 5×10^6 cells mL^{-1} , Hülscher et al. (1992) showed that 100% of the viable cells and 65% of the nonviable cells were retained by sedimentation at an upward flow velocity of $1.5\ \text{cm h}^{-1}$. Above $1.5\ \text{cm h}^{-1}$, the viable separation efficiency declined rapidly. This upper limit to effluent rate is overcome in two ways. With inclined sedimentation devices, the sedimentation path and effective upward fluid velocity are reduced by positioning narrow sedimentation channels at an angle to the vertical (Figure 3B). In continuous centrifuges the sedimentation velocity is increased by imposing a centrifugal acceleration many times greater than the gravitational acceleration.

Inclined sedimentation

Using an inclined settler for mammalian cell perfusion in a 1 L reactor, Batt et al. (1990) reached cell concentrations up to 1×10^7 cells mL^{-1} at a maximum

Figure 3. Sedimentation devices for mammalian cell retention in perfusion bioreactors. In vertical sedimentation (A), cells settle against the upward flow of the effluent stream. The settling path is shortened in inclined sedimentation devices (B) by angling a narrow channel from the vertical. Cells flow back to the bioreactor along the lower face of the channel.

perfusion rate of 2.5 L day^{-1} during a 12 day culture (Table 4). The retention efficiency of viable cells was greater than 99% throughout. The corresponding mean flow velocity of 28 cm h^{-1} was 20-fold higher than the limiting velocity for vertical sedimentation (Hülscher et al., 1992). At a mean flow velocity of 80 cm h^{-1} , over 90% separation efficiency was attained with a 50% longer settler (Batt et al., 1990).

The size selectivity of inclined sedimentation has been exploited to stably maintain a mixed culture of yeast and bacteria (Davison et al., 1985) and to preferentially retain a population of larger plasmid-bearing bacteria over a faster growing non-expressing reverted population (Henry et al., 1990). Selective retention of viable mammalian cells was demonstrated by Batt et al. (1990) as a function of flow-rate, with the difference between viable and nonviable cell retention being up to 50% within a range of intermediate flow rates.

Although there is an obvious improvement in maximum flow-rate and mean fluid hold-up time compared to vertical sedimentation, the mean cell residence time in the settler is not well defined because the cells form a sediment layer that must return to the bioreactor against the drag of the effluent flow (Davis and Acrivos, 1985). Also, fouling of the inclined surface due to the relative quiescence at the wall may result in a build-up of debris and attached cells over time, hindering the long-term operation of the separation

device. Searles et al. (1994) counteracted this cell attachment by vibrating the sedimentation column and cooling it to 4°C . Using labeled cells, they examined the mean cell residence time in the inclined settler. At two different concentrations (2.8 and $5.4 \times 10^6 \text{ cells mL}^{-1}$), but comparable flow rates and settler configurations, the mean residence time was approximately 1.5 h for the cells that returned to the bioreactor. However, a large fraction of the labeled cells (36% of the total at the high cell concentration and 13% at the lower concentration), remained in the settler more than 4.3 h. When air was bubbled through the settler, these remaining cells were resuspended and the majority returned to the bioreactor within 30 min. There was no clear impact on cell viability from this prolonged period in unoxygenated medium, probably because the medium in the settler was cooled to 4°C thereby slowing down the cell metabolism. For susceptible cells, prolonged residence in an anoxic environment and temperature cycling from 37 to 4°C are causes for concern.

For design purposes, the maximum flow-rate for an inclined rectangular channel can be estimated as the product of the cell sedimentation velocity and the projected horizontal channel area (Davis and Acrivos, 1985). In practice the maximum measured velocity for total retention of viable cells was only approximately 50% of the predicted value (Batt et al., 1990) proba-

Table 4. Sedimentation, centrifugation and ultrasonic cell retention device performance data in selected perfusion experiments. Retention efficiency was greater than 95% in all cases

Type of retention device	Reactor working volume (L)	Maximum perfusion rate (L day ⁻¹)	Maximum total cell density (10 ⁶ cells mL ⁻¹)	Viability	Comments	Study
Inclined settler	1.5	2.5	22	45%	selective retention of viable cells	Batt et al. (1990)
Continuous centrifuge mechanical seals	10	20	10 (viable)	(NA)	36 day perfusion	Tokashiki et al. (1990)
Vertical settler	5.4	6.8	6	50%	20 day perfusion	Hülscher et al. (1992)
Continuous centrifuge mechanical seals	6	33	20	88%	12 day perfusion growth rate and cell specific production rate same as in batch	Jäger et al. (1992)
Continuous centrifuge mechanical seals	21.5	63	10	90%	15 day perfusion	Jäger et al. (1992)
Inclined settler 7 channels	5.4	22	8	50%	30 day perfusion, recycle loop cooled to 20 °C, no pumping of cell suspension	Stevens et al. (1994)
Inclined settler multiple channels	25	50	10	47%	24 day perfusion	Thompson et al. (1994)
Ultrasonic separator	1	4.5	60	34%	29 day perfusion	Trampler et al. (1994)
Continuous centrifuge directly coupled	1.5	(NA)	13	35%	15 day perfusion cell specific productivity lower than in batch	Johnson et al. (1996)

(NA) = Not available.

bly because the prediction assumes a plug flow profile rather than the true parabolic velocity profile within each channel. Additional deviation from the ideal capacity can be attributed to disruptive convective flows due to non-uniform heating or cooling.

Scale-up of inclined sedimentation involves increasing the projected settling surface area. Because viscous damping of disruptive convective flows varies inversely with the smallest dimension of the channel, multiple shorter, shallower channels are favored over a single long, deep channel of the same width. A multiple channel settler with an internal volume of 2.2 L was operated at up to 50 L day⁻¹ during a 24 day perfusion experiment (Thompson and Wilson, 1994). The cell concentration reached 5×10^6 cells mL⁻¹ and viable cell retention was greater than 95%. Stevens et al. (1994) described a 7-channel settler operated at up to 22 L day⁻¹ with a separation efficiency of greater than

95%. This settler was configured such that the recirculation flow could be established without pumping, by cooling the inlet stream to 20 °C. Despite the potential limitations of inclined sedimentation devices, they appear to have reached a reasonable degree of acceptance for large-scale perfusion operations.

Centrifugation

The challenge of implementing a continuous cell centrifuge for perfusion was initially overcome by using rotating mechanical seals between the rotor, and the inlet and outlet streams (Tokashiki et al., 1990). With such a system applied to perfusion (Table 4), virtually 100% separation was attained at a cell density of 2×10^7 cells mL⁻¹ and a perfusion rate of 33 L day⁻¹ for a 6 L working volume reactor (Jäger, 1992). Due to concerns about the effect of repeated centrifugation on cells, Tokashiki et al. (1990) demonstrated that two

daily 10 min exposures over a one week period to centrifugal forces up to 500 times the force of gravity did not measurably affect the growth rate or antibody productivity of a hybridoma cell line. During the 12 day perfusion run of Jäger (1992), the growth rate of the cells was unchanged relative to batch, and culture viability remained high even though the cells passed through the centrifuge up to 6 times per hour.

The fallibility of rotating seals (Tokashiki et al., 1990) prompted the development of a rotor assembly that could be directly coupled to the bioreactor (Ito et al., 1975; Hodgson, 1991). This was ingeniously accomplished using a U-shaped tubing bundle, where the U arm at the outer edge of the centrifuge rotates at half the rate of the arm at the axis of rotation, canceling the twisting of the tubing that would otherwise result. Johnson et al. (1996) demonstrated that the viable hybridoma cell concentration (1×10^7 cells mL^{-1}) achieved in continuous perfusion with this novel centrifuge design was comparable to that using filtration-based cell retention under optimal conditions. However, the antibody productivity was in all cases approximately 30% lower. An analysis of the oxygen uptake rate and cell residence time in the centrifuge suggested that the pelleted cells experienced periodic oxygen starvation. Limiting nutrients were also likely depleted. As the cells passed through the centrifuge approximately once per hour, it appears that the drop in MAb production could indeed be attributed to oxygen and nutrient starvation.

Centrifuges are mechanically complex systems, and although recent improvements have been made to the disposable elements (Johnson et al., 1996), the separation insert required monthly aseptic replacement during continuous operation (or approximately every 20 million revolutions). That said, the manufacturer rated capacity of 2400 L day^{-1} for a production scale continuous centrifuge is the greatest of any retention device available for mammalian cell perfusion.

Ultrasonic cell retention

Ultrasonic separation is based on the acoustic forces that act on suspended cells in a standing wave field due to differences in density and compressibility between the cells and the medium (Gor'kov, 1962). When exposed in batch to an ultrasonic field, mammalian cells were shown to aggregate rapidly within planes corresponding to the pressure nodes of the standing wave field (Kilburn et al., 1989; Pui et al., 1995). When the field was extinguished, the aggregates quickly settled

Figure 4. Ultrasonic cell separation device. The standing wave field in the resonator chamber aggregates and retains cells against the drag of the effluent flow. The aggregates settle out of the resonator, return to the bioreactor in the recycle stream where the cells are resuspended by the bioreactor agitator. Periodic shut-down of the power to the field and effluent pumps helps clarify the chamber.

out of suspension. Pui et al. (1995) demonstrated that there was no negative impact on cell viability, glucose uptake rate or antibody production resulting from exposure to ultrasonic standing waves at power inputs to the wave field up to 220 W L^{-1} . The viability was affected at 260 W L^{-1} likely due to cavitation in the standing wave. Cavitation is used in sonicators to disrupt microbial suspensions but is avoided in ultrasonic separators.

The application of ultrasonic cell separation to perfusion is attractive because it is not susceptible to fouling or mechanical failure, having no physical barrier or mechanical parts. Many implementations have been proposed for continuous ultrasonic separation where enriched and clarified streams are generated by concentrating the suspended particles on one side of the flow path (Benes et al., 1991; Whitworth et al., 1991; Frank et al., 1993). Trampler et al. (1994) successfully developed a cell retention device for perfusion culture by an alternate approach in which the cells were aggregated and retained in the standing wave field of an ultrasonic resonator, then returned to the bioreactor by sedimentation and dispersed by the impeller (Figure 4). Cell retention was greater than

97% throughout a one month culture, with total cell concentrations reaching 6×10^7 cells mL⁻¹ (Table 4). There was no detectable impact on cell viability or evidence of cell lysis due to the retention device.

Factors that influence the maximum flow-rate in ultrasonic cell separation are the input power and cell concentration. Doblhoff-Dier et al. (1994) demonstrated that a higher input power was required to maintain a constant separation efficiency at high flow-rates. This trend is explained by the increased ultrasonic force required to retain the aggregated cells against the fluid drag, and by the lower mean fluid residence time. As demonstrated in batch, the formation of aggregates is time-dependent (Kilburn et al., 1989; Pui et al., 1995). The maximum flow-rate for 90% separation efficiency was almost 3-fold higher at 5.5×10^6 than at 1×10^6 cells mL⁻¹ (Trampl et al., 1994). This effect is likely due to the increased total retention force on the larger aggregates formed at higher concentration (Woodside et al., 1997). Although the same trend was observed in batch (Kilburn et al., 1989), there is undoubtedly a separation efficiency maximum at some higher concentration.

Doblhoff-Dier et al. (1994) have reported the highest superficial velocities (720 cm h⁻¹) for ultrasonic cell separation, with greater than 90% separation during short-term retention experiments. This velocity is almost 500 times the sedimentation velocity and close to 10 times the highest reported rate for inclined sedimentation (Batt et al., 1990). Because ultrasonic separators are operated with periodic shut down of the power to the field and effluent pumps, cells and aggregates are allowed to settle back into the reactor, and mean cell hold-up times are greatly reduced relative to sedimentation devices. Selective retention of viable cells was investigated by Gaida et al. (1996), who demonstrated that the proportion of nonviable cells in the effluent was up to 30% greater than in the inlet stream.

To date, ultrasonic cell retention has been limited to small-scale perfusion. The recommended maximum flow rate for commercial ultrasonic separators is 50 L day⁻¹. Additional improvements in capacity are anticipated by further increasing resonator dimensions, and through optimization of the force distribution in the standing wave field (Woodside et al., 1998).

Conclusions

Both fed-batch and perfusion are capable of attaining high cell concentration, volumetric productivity and product concentration. Although fed-batch has been more widely adopted in recent years, there is no universal, optimum approach to efficient cell culture. Instead, particular aspects of each process need consideration to determine the method of choice. As outlined in this review, considerable progress has been made to overcome fouling, increase the separation capacity, and improve the reliability of cell retention devices, thus making large-scale perfusion an increasingly viable option for cell culture at any scale.

Acknowledgements

Steven Woodside was supported by Natural Sciences and Engineering Research Council (NSERC) and BC Science Council GREAT scholarships.

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