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Clarification of Animal Cell Culture Process Fluids Using Depth Microfiltration

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Large-scale culture of animal cells is becoming increasingly important for the production of complex recombinant molecules, such as erythropoietin and tissue plasminogen activator, and for numerous monoclonal antibodies and vaccines. Upstream aspects of such processes, such as cultivation of animal cells in various bioreactor configurations, have attracted intense research in the past decade. Quite remarkably, even though product recovery and purification accounts for up to 70% of a product's total manufacturing cost, alternative options for the various downstream steps in cell culture processes have received little critical analysis (1). This

and equipment maintenance, validation, cleaning, and sterilization are difficult. Finally, it is sometimes difficult to avoid aerosol generation in a large centrifugation operation, and processing times are typically long. Thus, for animal cell clarification, filtration has been accepted as a better alternative over centrifugation.

Numerous options are available for clarification of animal cell culture fluids, including tangential flow filtration (hollow-fiber systems), crossflow filtration (plate and frame), and flow-through depth filtration. Prefiltration of cell broths using one of these options can extend the life of downstream membrane filters that are used for removing submicron debris, endotoxin, viruses, and bacteria and the life of ultrafilters that are used for concentrating a product. A particular filtration alternative may be better than others depending upon the process and circumstances. For example, if a product is intracellular, and the cells are valuable, depth filtration may not always be the best option. However, in most mammalian cell culture applications, the product is secreted in the medium, and the supernatant is generally of interest. In such situations, any one of the filtration options described above should be feasible, and the process choice is governed by economics and by ease of validation and use.

Depth filtration. Bioprocess practitioners have long recognized the advantages of depth filtration, particularly the ease of use, the low initial cost of disposable depth filters, and the ease of validation when compared with centrifugation and tangential flow filtration devices. In many downstream

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Given the fragility and high value of cell-derived products, separation must be accomplished with minimum cell damage and loss of activity. Here, the authors investigate the feasibility of depth filtration for clarifying cell culture broths containing a secreted product. Using small-scale apparatus, they provide experimental data and suggest criteria for scale-up.

lack of interest has been primarily attributable to the high value of products derived from cell culture. Thus far, in an attempt to enter the market rapidly, manufacturers have chosen something that "works" rather than what is optimal. With the recent scrutiny over pricing of biologics, it is imperative to evaluate manufacturing options for these products with the same thorough technical and economical analysis that is customary of bulk products.

In this article we address the first unit operation in the downstream processing of cell culture broths: the removal of cells and cell debris from the supernatant containing the product. This separation and recovery step is called *clarification* and is a considerable challenge in the production of biological materials. Centrifugation and filtration are two unit operations employed for this purpose. Given the fragility and high value of cell-derived products, separation must be accomplished with minimum cell damage and loss of activity.

High-efficiency centrifugal separation of harvest fluids has yet to be fully mastered on a large scale. The small differences between the densities of animal cells, cell debris, and culture fluid make centrifugation inefficient for removing cells and subcellular debris under low shear conditions (2–3). Centrifugation involves high capital costs,

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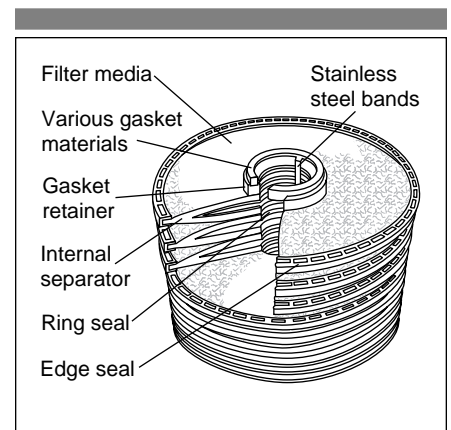


Figure 1. Cross-section of a Zeta Plus filter cartridge.

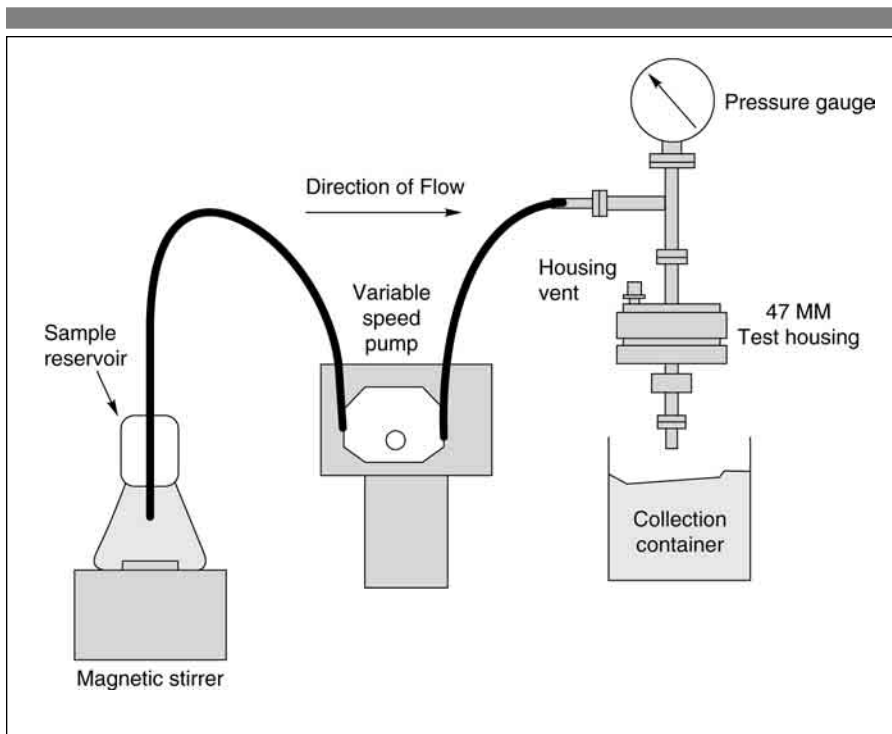


Figure 2. Small-scale experimental filtration setup.

purification operations, depth filter cartridges offer processing economies along with highly effective clarification of the feed streams. Depth filtration has been employed for numerous applications for the production of therapeutic and diagnostic biological products. Examples include clarification of plasma-derived biologicals (albumin, Factor VIII), clarification of animal cell culture broths, pyrogen removal, lipid removal, and protection of chromatography columns and sterile membrane filters (4). In spite of these studies, there is a lack of quantitative data to assess the utility and efficiency of these microfilters. Specifically, experimental data are unavailable that document the effect of levels and types of cell debris on the performance of depth filters and that describe the minimum pore size required to satisfactorily “clean” the stream at a minimum pressure drop. For this article we investigated the feasibility of depth filtration for clarification of cell culture broths containing a secreted product. Specifically, using small-scale apparatus we attempt to provide experimental data and suggest criteria for scale-up. Using these criteria, we successfully predicted the filter size used in a large-scale cGMP recovery operation

involving a secreted product from recombinant Chinese hamster ovary (rCHO) cells.

Materials and Methods

Cell culture. We performed small-scale experiments using CRL-1606 hybridoma cells producing anti-fibronectin monoclonal antibody. Cells were cultured routinely in 500-mL spinner flasks (Corning, Corning, NY) with 200-mL Iscove’s modified Dulbecco’s medium (Sigma, St. Louis, MO), supplemented with 5% dialyzed fetal bovine serum. The medium was also supplemented with 4 mM glutamine and 0.1% penicillin/streptomycin solution (Sigma). Cells were inoculated in the spinner flask from T-flasks (150 cm², Falcon brand, Becton Dickinson, Franklin Lakes, NJ) at an eightfold dilution and were grown to a final density of 2×10^6 cells/mL. We performed all filtration experiments at a cell density of 2×10^6 cells/mL. The average diameter of these cells was 14 μm.

Cell enumeration. We determined viable cell concentration using the trypan blue exclusion method. We mixed the cell suspension with an appropriate amount of 0.4% trypan blue solution with a two- to

fourfold dilution and placed the mixture in a hemacytometer. Cells were viewed at 200× magnification and counted. We used an Olympus model BH-2 microscope because of its phase contrast feature (Marcon Instruments Co., Norwood, MA). Viable cells appeared opaque and whole, whereas nonviable cells were blue.

Filter media and cartridge structure. The filters employed in these studies were manufactured at Cuno Filter Systems (Meriden, CT). The filter media is a composite structure composed of high-area filter aids embedded in a cellulosic fiber matrix. During the manufacturing process, molecules carrying a positive charge are chemically bonded to the matrix components, forming a permanent, interconnected, rigid filter sheet with positively charged electrokinetic capture sites. The resulting porous depth filter structure is a tortuous network of charge-enhanced flow channels capable of retaining cells, cellular debris, endotoxin, and submicronic contaminants to a level that mechanical screening alone cannot achieve. This manufacturing technique results in substantial contaminant capacity or long service life before clogging. The depth filters have a graded density — that is, a progressive change in the effective porosity of the media — and are fabricated as disposable cartridges or flatstock (sheet media).

Filter cartridges are fabricated from individual cells of media. The cells consist of two disks of media separated from each other so that liquid flows from the outside of the filter medium, into the space between the disks, and toward the core of the cell (Figure 1). The disks are secured at the outer periphery by injection-molded polypropylene and are spaced apart at the inner core with annular spacers between the cells. The cells are stacked together under compression in a cylindrical form and held together with stainless steel bands. Cartridges come in 8-, 12-, and 16-inch diameters with sizes ranging from 1 ft² to 37 ft² per cartridge. Cartridges of similar diameters can be combined in a housing to obtain the needed square footage required to achieve a desired flow rate or volume throughput. Filter cartridges are housed in totally enclosed, sanitary design stainless steel housings (Cuno).

Protein assay. We obtained total protein in the cell culture supernatants by Biuret assay. Samples were centrifuged at 1,000 rpm with an IEC Centra-4B centrifuge (International Equipment Co., Needham Heights, MA) for 10 minutes, and 0.5 mL of the supernatant was placed in a 15-mL centrifuge tube (Corning). Then, we added and mixed thoroughly 1.5 mL of Biuret reagent (1.5 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 6 g/L sodium potassium tartrate, and 30 g/L NaOH). After 30 minutes incubation at room temperature, absorbance was measured at 500 nm with a Perkin-Elmer Lambda-3 spectrophotometer (Norwalk, CT). Standards of bovine albumin (1–10 mg/mL) were used to obtain a standard curve.

Particle counting (debris quantitation). We obtained total particle counts using an electronic particle counter (model Z_F , Coulter Electronics, Miami, FL). Using a 100- μm aperture tube, we set the 1 \times amplification to 4, the 1 \times aperture current to 8, and the manometer to 500 mL (0.5 mL sample). We set the threshold dial to 5 for sample and to 1 for filtrates. We diluted cell suspensions in isotonic saline solution to bring the counts between 1,000 and 5,000 particles/count for best results. No dilution was necessary to obtain particle counts in this range for filtrates obtained after passing the cell suspensions through the filter device.

Antibody (IgG) assay. We employed an enzyme-linked immunosorbent assay (ELISA) to determine the titer of the monoclonal antifibronectin antibody. Cells were centrifuged for 10 minutes at 1,000 rpm with a Biofuge-A microcentrifuge prior to the antibody assay. We used Kappa mouse IgG1 (Sigma, catalog no. M9269) as a standard. First, 100 μL antigen solution (antibody to mouse IgG1, Sigma, catalog number M8770) was placed in a 96-well microtiter plate and incubated for one hour. After rinsing with washing buffer (2.5 mL amps Tween 20, 5 mL blocker bovine serum albumin [BSA] in phosphate-buffered saline [PBS] and one pack of BupH Dulbecco-PBS to 500 mL Milli-Q water, Sigma), 200 μL blocking buffer (10 mL blocker BSA in PBS in 100 mL BupH Dulbecco-PBS, Sigma) was added and incubated for another 30 minutes. After washing with buffer, 100 μL sample or standard solution was placed into

each well and incubated for one hour. After rinsing with washing buffer, 100 μL labeled secondary antibody was added and incubated for 30 minutes. The plate was then rinsed with washing buffer again and incubated with 100 μL washing buffer for another five minutes. Finally, 100 μL of enzyme substrate solution (ABTS substrate, catalog number 37615, Pierce Chemical Co., Rockford, IL) was added and incubated for 30 minutes. Absorbance was measured at a wavelength of 405 nm using a kinetic

microplate reader (Molecular Devices Corporation, Palo Alto, CA). We diluted samples 100- to 1,000-fold with blocking buffer before assay depending on the antibody concentrations.

Results and Discussion

Experimental setup. We performed all small-scale experiments with CRL-1606 hybridoma cells at a cell concentration of 2×10^6 cells/mL. Figure 2 shows the experimental setup. For each test, a 47-mm

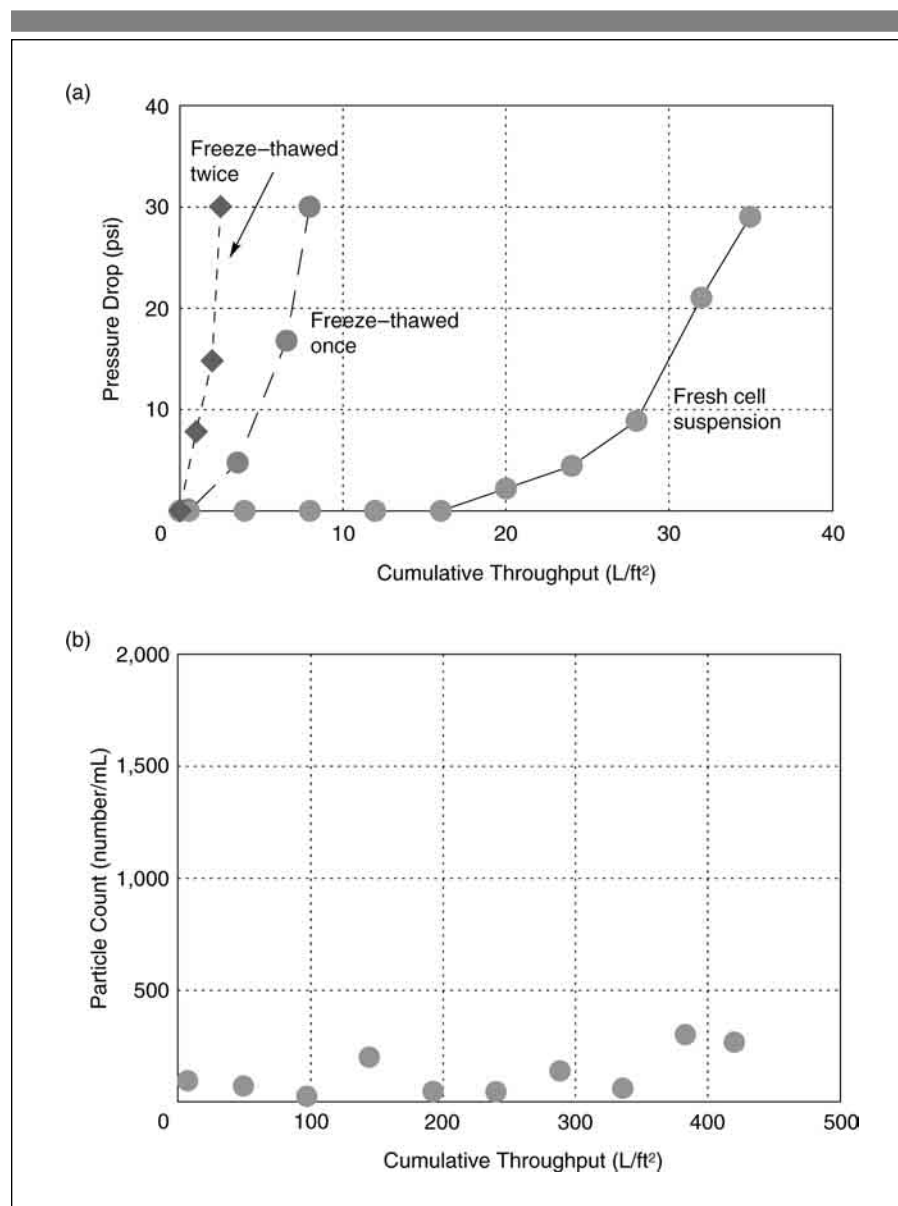


Figure 3. (a) Rate of increase in pressure drop across the filter is a function of debris level and debris type. A flux rate of 1 L/minute/ft² was used with a 10 SP filter pad. (b) Particle counts in the filtrate. The feed solution contained 2×10^6 cell/mL (fresh cells).

filter disk was sealed in an in-line 47-mm test housing (Cuno Filter Systems, P/N 70015-01A) with an effective filtration area of 13.5 cm². A variable speed Masterflex peristaltic pump fitted with standard silicone tubing (Cole-Parmer, Vernon Hills, IL) was used for the testing. The differential pressure was measured with a 0–30 psi test gauge (Ashcroft) connected to the inlet side of the

test housing. For each test, before connecting the pump to the test housing, we verified the desired flow rate using a stop watch and graduated cylinder with water.

We stirred the cell suspension constantly using a magnetic stir bar. The homogenous suspension of cells was pumped through the filter pad at a constant flow rate and the pressure drop measured periodically.

Samples of the filtrate were taken at regular intervals. The quality of the filtrate was quantified by counting the number of particles in the samples using a Coulter counter. In addition, IgG assays were performed to determine product losses.

Effect of debris levels on filtration performance.

Elucidating the sensitivity of filtration performance to the level of cell debris (number of particles/volume) and on debris quality (whole cells compared with fragmented cells) is important because of the vast difference in cell debris encountered in different cell culture operations. For example, a process in which the product is secreted and in which cell lysis is minimal can be expected to have a much lower debris level than in a vaccine operation in which the product is a virus that lyses cells. To study the effect of fragmented cells on filtration performance, we performed filtration experiments with fresh CRL1606 cells (with minimal lysis) and with cells that were frozen and thawed once or twice. The operation of freezing mammalian cells without cryopreservatives, such as dimethyl sulfoxide (DMSO), and subsequently thawing them results in extensive cell breakage. This technique is routinely used for small-scale mammalian cell breakage. By going through this operation once or twice, we hoped to increase the fraction of lysed cells in the broth and simulate situations that involve cell lysis.

A microscopic examination of fresh and freeze-thawed cells showed a remarkable difference in the quality of cell debris. Whereas the fresh cells appeared whole and round with little subcellular debris, freeze-thawed cell suspension was characterized by a large proportion of subcellular debris, few whole cells, and a large number of multicellular cell aggregates. In this regard, the size distribution of particles in the cell suspension was more varied in the freeze-thawed suspensions. Experiments with fresh cells and freeze-thawed suspensions using filter pads of a given pore structure (Cuno Filter Systems, grade 10 SP) showed dramatic differences in filtration performance. The data (Figure 3a) clearly indicate that for a given flow rate, a given filter type, and a given filter area, significantly more fresh cell suspension could be processed, compared with the

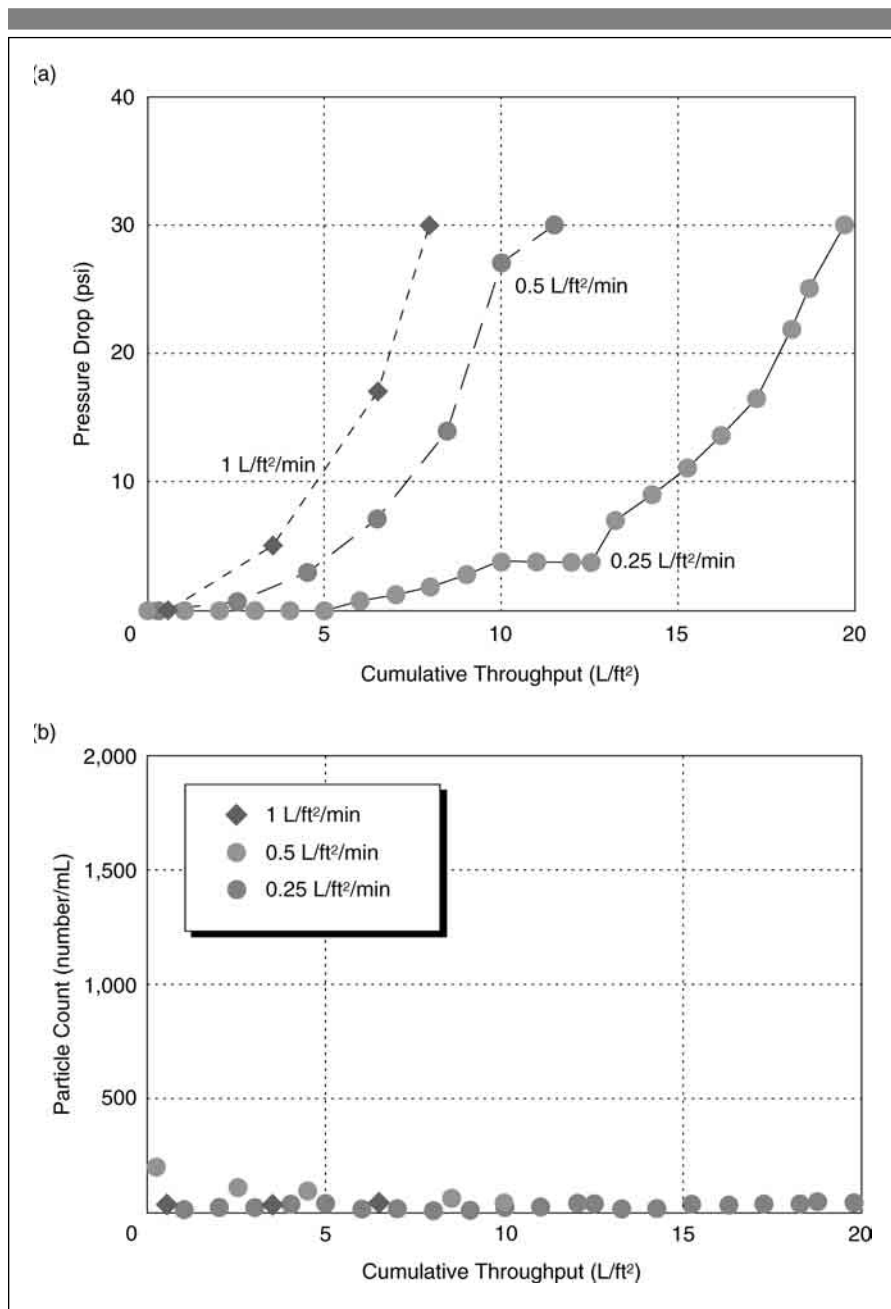


Figure 4. (a) Effect of flow rate on the rate of increase in pressure drop across the depth filter. A freeze-thawed cell suspension with 2×10^6 cell/mL was used as the feed solution. (b) Particle counts in the filtrate. Feed solution was a freeze-thawed cell suspension with a cell density of 2×10^6 cells/mL with a 10 SP filter pad.

freeze-thawed material, before the pressure drop increased to the high limit of 30 psi. For example, at a flow rate of 1 L/minute/ft², fourfold more fresh cell suspension could be processed compared with a suspension that was frozen and thawed once. We observed the same trend at other flow rates, as discussed later. This phenomenon was accentuated with cell suspensions that were freeze-thawed twice (Figure 3a). The quality of the filtrate, as judged by overall appearance and particle counts, seemed to be unaffected by the differences in debris levels. In all cases, the filtrate had a bright, shiny appearance, and the particle count was negligible (Figure 3b). Therefore, the differences in debris affected the rate at which the permeability of the filter changed with time, but did not affect the quality of the filtrate. This is not surprising given that the freeze-thawed material had large aggregates that could have plugged the pores on the outer surface of the depth filter pads and blinded them. Because of the nature of depth filtration, whole cells on the other hand were trapped on the surface and within the matrix of the filters.

Effect of flow rate on filtration. The goal of any filtration is to achieve maximum throughput at a minimum pressure drop in a reasonable time while maintaining a desired filtrate quality. To investigate how the filtration performance changed with flux, we passed the same cell suspension through a given filter type and filter area at different flux rates. We used a freeze-thawed cell suspension for this purpose with a 10 SP filter pad. Figure 4a shows the results of this experiment. The data clearly show that more material of a given quality can be processed at lower flux rates. This phenomenon has been described before (4) and can be explained by the fact that at higher flow rates, the depth filter is blinded. In other words, at higher flow rates the filter pores are plugged at the outer surface of the filter and the debris does not occupy the inside pores of the depth filter. This results in a situation similar to a dead-ended surface filter, and the advantages of a depth filter are lost. At lower flows, the debris percolates into deeper layers of the filter, resulting in lower pressure drops and higher throughputs. Figure 4b shows that the quality of the filtrate was once again

excellent, as judged by its bright, shiny appearance and very low particle counts per unit volume.

Effect of filter medium density. We obtained the results presented thus far using depth filter pads with medium density of 10 (designated 10 SP by the manufacturer). Filter medium density is inversely proportional to void volume. Results with the 10 SP filters were satisfactory in that suspensions with and without fragmented

cells could be processed with excellent filtrate quality. Because filter pads with a lower medium density can be expected to result in a lower pressure drop for the same throughput and cell suspension quality, the effects of filters with lower medium density were investigated. For this study we used filters with a lower medium density (50% of 10 SP and designated 05 SP). We used the same cell suspension that was used in experiments with the 10 SP pads. The data

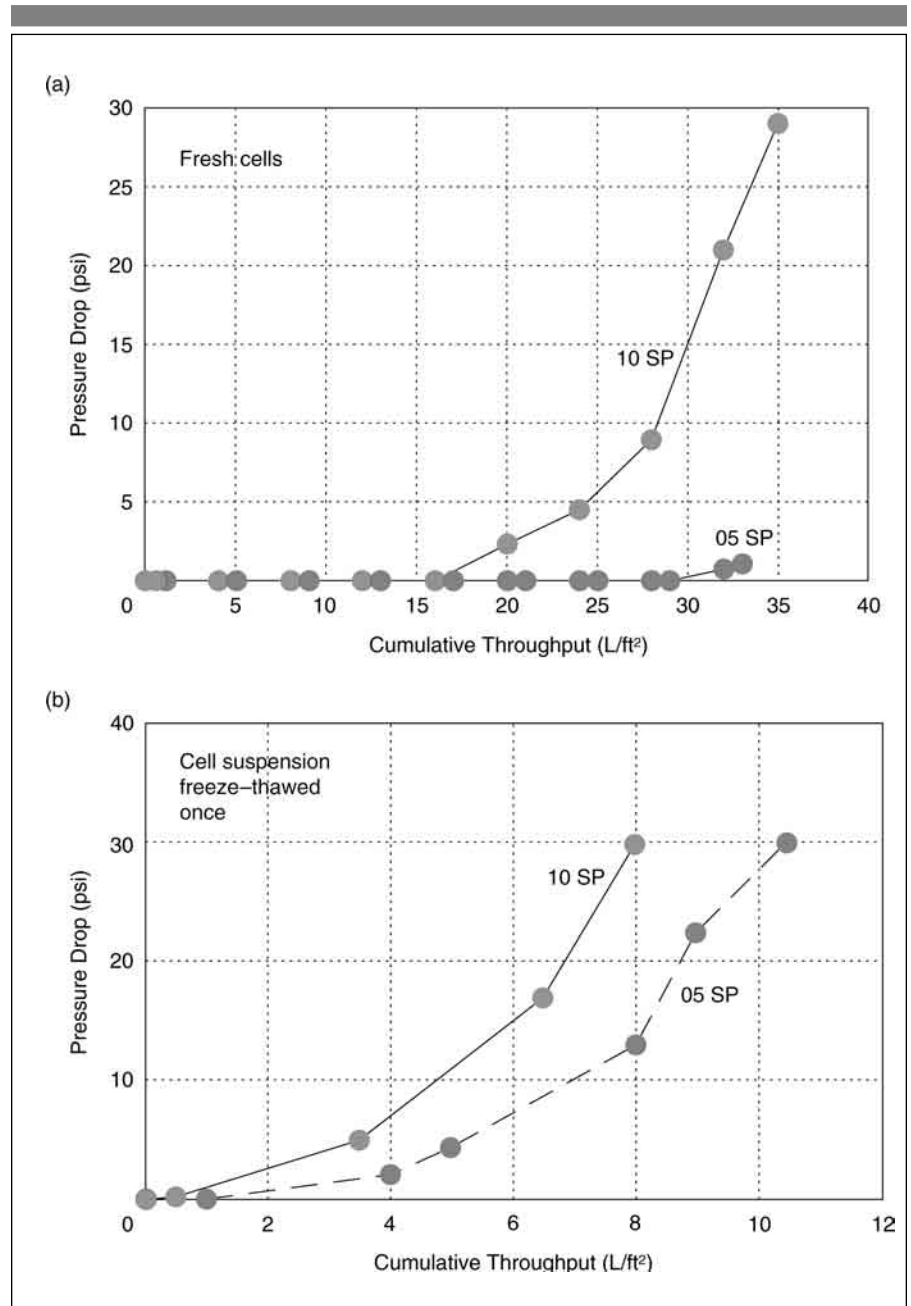


Figure 5. Effect of varying filter medium density on cumulative throughput. Throughput with both fresh cells (a) and cell suspension freeze-thawed once (b).

(Figure 5) indicate that for a given flux rate, higher throughputs can be obtained with the 05 SP filter than with the 10 SP filter with cell suspensions that are fresh and freeze-thawed once. More total material can be processed through a 05 SP filter using a

lower flux rate (Figure 6). This phenomenon is similar to that observed with the 10SP filters (Figure 4a). Quite remarkably, we found the quality of the filtrate in all these experiments with the 05 SP filters to be as good as that obtained with the 10 SP filters.

The appearance of the filtrates was bright and shiny red, and the particle counts never exceeded 200 per mL.

Effect of depth filtration on product titer.

The final and most critical factor in any downstream step is its effect on product titer. Therefore, we evaluated loss of product (IgG) in the present study for all filtration experiments. Total protein and IgG concentration in filtrate samples taken at regular intervals in the various filtration experiments were obtained and compared with the same in the starting material. Figure 7 shows the results of a typical experiment. Clearly, we saw no significant changes in either the total protein concentration or in the IgG concentration. This is interesting because of the possibility of product loss caused by adsorption on the matrix. We were concerned that the cationic surface chemistry of the filter matrix would cause an interaction between the antibody and the filter media.

Scale-up prediction.

We predicted the filter requirement for a large-scale depth filtration using the small-scale data, and compared it with an actual operation. 10 SP depth filters have been shown to clarify rCHO cell culture broths at the 1,200-L scale in a cGMP production facility (5). The cell broth contained 2×10^6 cells/mL, a density similar to the one we used in the small-scale experiments. Further, the product was secreted in the medium, and cells were intact and 85% viable at harvest. To predict the filter size required for this operation, we used small-scale experimental data obtained with fresh cells (Figure 3a) because the process feed (fresh cells) in the small-scale experiment closely matched that in the large-scale operation (intact cells with high viability). Further, the filter grade (10 SP) was the same in the two cases. From Figure 3a, it is clear that at a flux rate of 1 L/minute/ft², 450 L of process fluid containing fresh cells at a density of 2×10^6 cells/mL can be processed using a 12.5-ft² filter. Thus, using the flux rate as the scale-up parameter, 1,200 L of a similar process fluid can be processed using a 33-ft² filter, and the entire operation can be completed in 36 minutes. The actual operation was indeed performed using a 33-ft² filter (Zeta Plus 10 SP, Cuno) as we predicted. However, the large-scale operation was performed at a lower, more conservative flow rate, and the

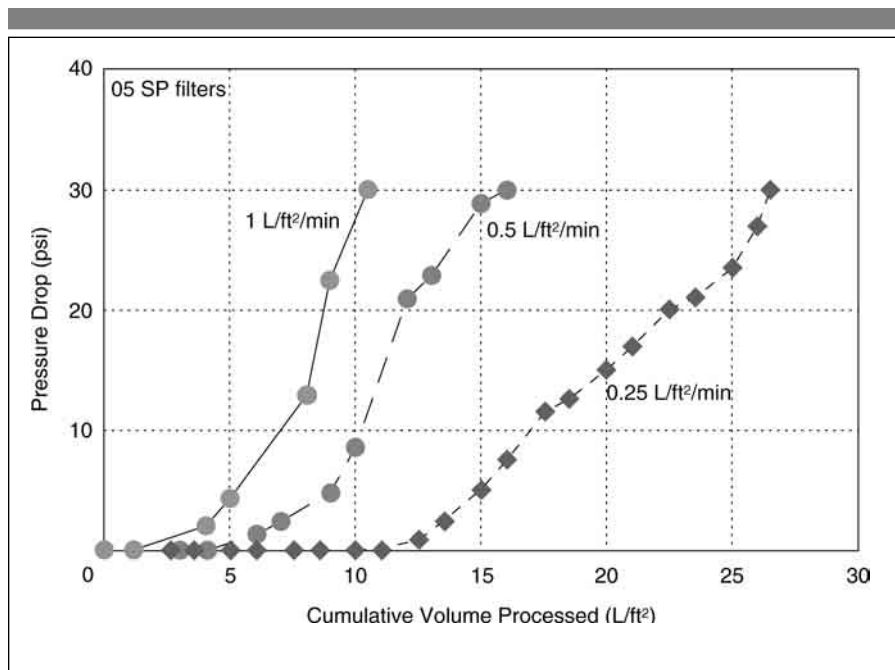


Figure 6. Rate of increase in pressure drop increases with higher flux rates. Filtration was performed with 05 SP filters with a freeze-thawed cell suspension containing 2×10^6 cells/mL.

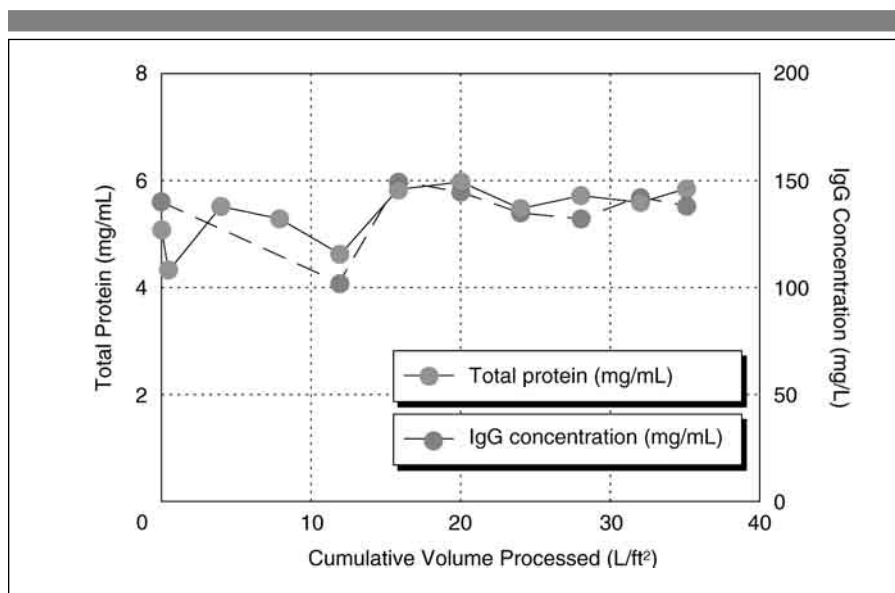


Figure 7. Changes in IgG (product) and total protein concentration in the filtrate during the course of filtration. A fresh suspension of cells containing 2×10^6 cells/mL was used as the feed solution with 10 SP filter.

entire batch was processed in 60 minutes instead of in 36 minutes as predicted (60% of maximum flow rate). Under these operating conditions, the filtration proceeded very well with excellent filtrate quality. This example illustrates that the flux, or cumulative volume processed per unit time per unit filter area (L/minute/ft²) can be used as a reasonable scale-up parameter. Further, this example demonstrates the utility of conducting small-scale experiments to predict large-scale filtration performance.

A Viable Technology

Several conclusions can be drawn from the data presented in this article. We have shown that depth filtration is a viable technology for clarifying cell culture broths with varied types and levels of debris. Quality of cell

debris dramatically affected filtration performance with respect to the total material processed per unit filter area. The quality of filtrate, as measured by the ratio of particles in the effluent compared with that in the feed, was unaffected by the quality of cell debris. We have further shown that the rate of increase of pressure drop across the filter was proportional to flow rate for the same feed solution. This implied that less material of the same quality can be processed at higher flow rates than can be processed at lower flow rates. Finally, filters with lower medium densities can be used to process the cell culture suspensions and obtain higher throughputs at the same flow rate with no loss in filtrate quality. We observed no significant loss in product concentration during the filtration. Using data from these small-scale experiments, a depth filtration

operation at the 1,200-L scale can be successfully predicted.

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