Effects of pH and Ionic Conditions on Microfiltration of Mammalian cells: Combined Permeate Flux enhancement and mAb Purification Capabilities

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Introduction

The recent drive for generating increased titer cell culture processes at large-scale for producing therapeutic proteins has required bioreactors to operate at high cell densities (>$1x10^7$) introducing elevated levels of nucleic acids, host cell proteins, and more complex media and nutrient feed components. Consequently, these complex harvest streams have placed stronger demands on both cell harvesting operations and downstream purification chromatography for removing cells, cell debris, and high levels of impurities. The harvesting of monoclonal antibodies (mAb) and recombinant proteins from manufacturing-scale bioreactors containing mammalian cells is usually performed using either filtration or centrifugation with the former typically operated with microfiltration membranes in a crossflow-mode. Traditional downstream processing of therapeutic proteins has been designed to place nearly all of the purification capabilities on chromatography steps with the clarification steps designed exclusively for cell and cell debris removal.

Previous work has already demonstrated the control of fermentation broth pH during the production of antibiotics and biochemicals from bacteria to precipitate the microorganism and to remove contaminants. Studies have also briefly demonstrated the feasibility of this technique in mammalian systems. However, there is no clear understanding of the coupled effects of harvest conditions such as pH, ionic strength, and composition on the product quality attributes and microfiltration (MF) operational performance used to process mammalian cells in the production of recombinant proteins. The objective of this work is to present a framework for describing the performance and optimization of microfiltration systems for both mammalian cell and impurity removal using harvest feed titration data and microfiltration process data to illustrate the key phenomena governing the behavior of these systems.

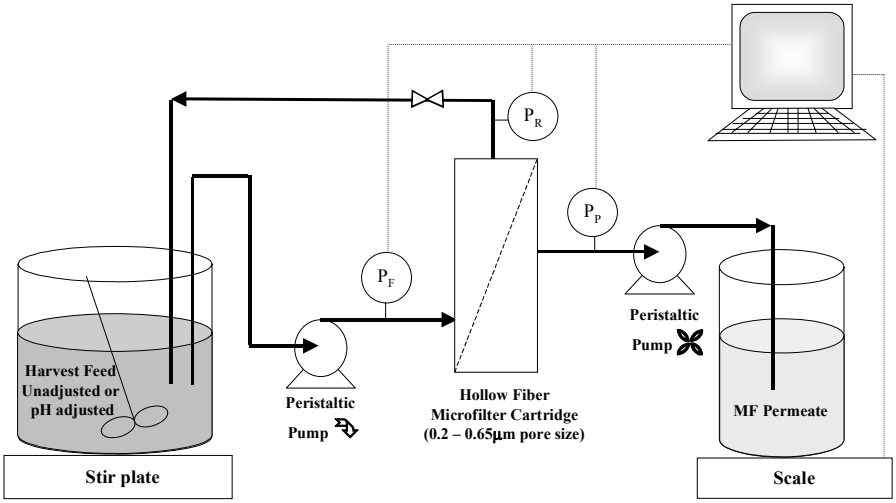
Materials and Methods

Bioreactor Harvest Adjustment/Settling Experiments
Settling experiments were performed on various unclarified harvest media compositions containing protein products such as IgG1 and IgG4 monoclonal antibodies, and fusion proteins produced using mammalian cells. The settling experiments were performed on harvest streams adjusted to pHs ranging from 7 to 4 using citric or acetic acid stock solutions and adjusted with various alkaline and transition metal ions. Following

predetermined settling times samples of the supernatant were taken and measured for turbidity level, product titer, and impurity amounts.

Clarification Experiments

Harvest clarification experiments were performed using microfiltration hollow fiber modules with 0.65µm pore size (GE Healthcare) in Tangential filtration mode. Experiments were performed at various scales ranging from 0.2 –2L (bench) to 200L (Pilot) and 2000L (manufacturing scale). All clarification experiments were run in simple batch mode at 2 to 8 °C. Bench scale development work was performed using CFP-6-D-MM01A cartridges (24cm^2) and CFP-6-D-5A cartridges (1600 cm^2). The CFP-6-D-MM01A cartridges utilized FilterTec peristaltic pumps manufactured by Scilog (Middleton, WI) to drive the recirculation flow and control filter shear rates, and Masterflex L/S peristaltic pump (Cole-Parmer, IL) to control the permeate flux. The CFP-6-D-5A cartridges utilized a Masterflex pump to drive the recirculation flow and the permeate flux. A schematic of the lab-scale system is shown in Figure 1. Biogen Idec Analytical Development performed assays, including Titer by Protein A/G and DNA by quantitative PCR. Cell density and viabilities were measured with the Innovatis Cedex AS20 (Bielefeld, Germany), and turbidity was measured with the Hach 2100AN Turbidimeter (Loveland, CO).



- Constant Retentate or Recirculation Flowrate establish with Pump
- Constant Permeate Flowrate establish with Pump

$$Transmembrane\ \Pr essure = \left(\frac{P_F + P_R}{2} \right) - P_P$$

Figure 1: Schematic of the Microfiltration Lab-scale Setup used for 24cm^2 and 460cm^2 area filter experiments

Harvest Settling Results and Analysis

The effect of pH adjustment on the turbidity of harvest medium supernatant for various media compositions is shown in Figure 1. The harvest feed total cell densities and percent viabilities ranged from 10^6 to 10^7 cell/ml and 40% to 90% viable cells, respectively. The data was generated by pH adjusting aliquots of harvest feed to the specified pH using 25% v/v acetic acid, allowing the flocculation and settling of the cellular mass to occur over a 24hr period and measuring the turbidity (degree of clarity) of the supernatant. The data in Figure 1 is generated from different harvest streams containing extracellular recombinant proteins such as monoclonal antibodies and fusion proteins. The data demonstrates a general reduction in supernatant turbidity as the media composition pH decreases from ~ pH 7 to pH 4 indicating a clearer supernatant at lower pH. This behavior is a direct result of pH-induced flocculation of the cells and cell debris, which causes increased settling rates and amounts of these entities. Limited experimental work has demonstrated that flocculation of cells may expand the operational robustness of the clarification step.

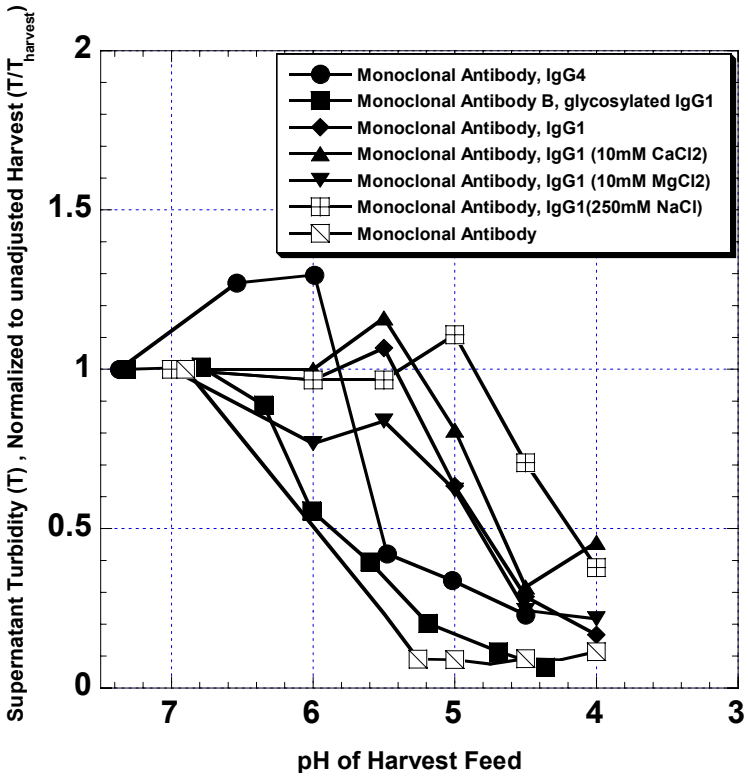


Figure 2: **Plot of supernatant turbidity of various pH adjusted harvest samples normalized to the unadjusted harvest supernatant turbidity. For the data shown here, the unadjusted harvest media pH ranged from 6.9 to 7.4. Data is shown for various harvest streams containing different protein products**

Figure 3 shows the effects of harvest pH adjustment and presence of different divalent cations on product protein titer in the extracellular harvest media. The titer data is shown

for an IgG1 monoclonal antibody and a fusion protein normalized to the titer measured under unadjusted harvest conditions. The antibody data shows a slight drop in cell-free protein concentration as harvest pH drops from 7 to 5 with a significant titer decrease at pH 4. At pH 5 the presence of alkaline metal ions increases the amount of antibody by 6-8% in solution from the pH adjusted harvest control. The same behavior is shown with the fusion protein harvest using a divalent transition metal ion; however, the titer recovery is more pronounced (15% increase in protein titer). In summary, the data in Figure 3 demonstrate the competing effects of harvest pH reduction and the presence of divalent cations on protein titer loss and recovery, respectively. The reduction in harvest pH brings about protein precipitation and/or protein co-precipitation with the flocculated cells and cell debris resulting in loss of soluble product. However, the addition of divalent cations at these low pH values significantly reduce or eliminate protein precipitation resulting in improved recoveries. It is believed the divalent cations selectively interact with the histidine regions of the proteins potentially shielding undesired attractive electrostatic interactions of the desired protein with the negatively charged cell flocs.

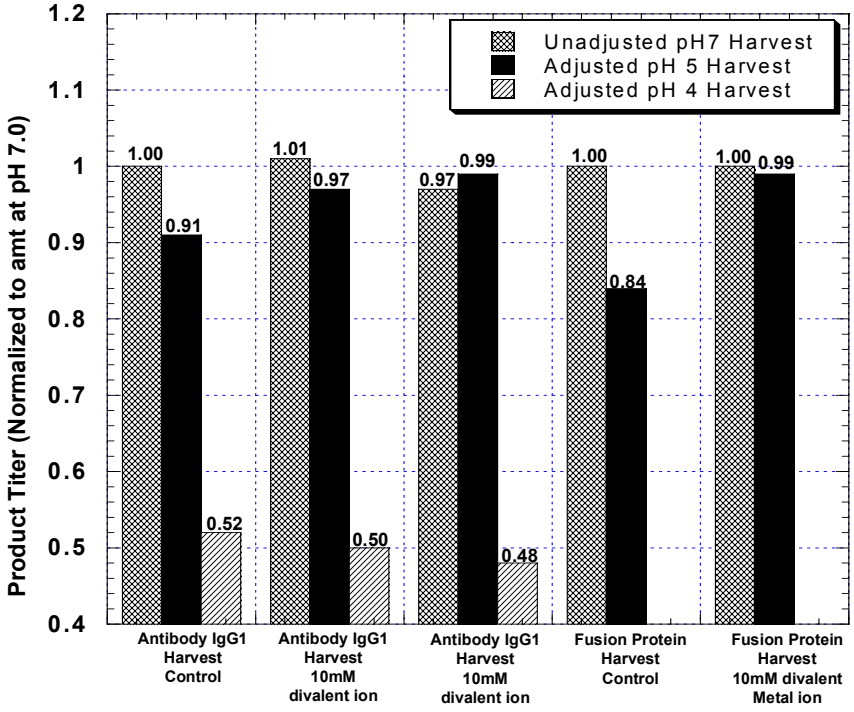


Figure 3: **Plot of protein titer in cell-free supernatant at three harvest pH values and various divalent cations. The titer data for the monoclonal antibody and fusion protein are normalized to their titer at unadjusted harvest conditions.**

Figure 4 shows the effects of harvest pH adjustment on DNA contaminant reduction in the cell-free supernatant. The DNA contaminant levels are measured at two pH values (pH 7.0 and 4.7) for three different harvest streams containing antibodies.

For all antibodies shown in Figure 4, the adjustment of harvest pH from ~7.0 to 4.7 effectively brings about precipitation of DNA causing the cell-free harvest stream DNA impurity levels to drop, significantly from 1.5 to 3 logs.

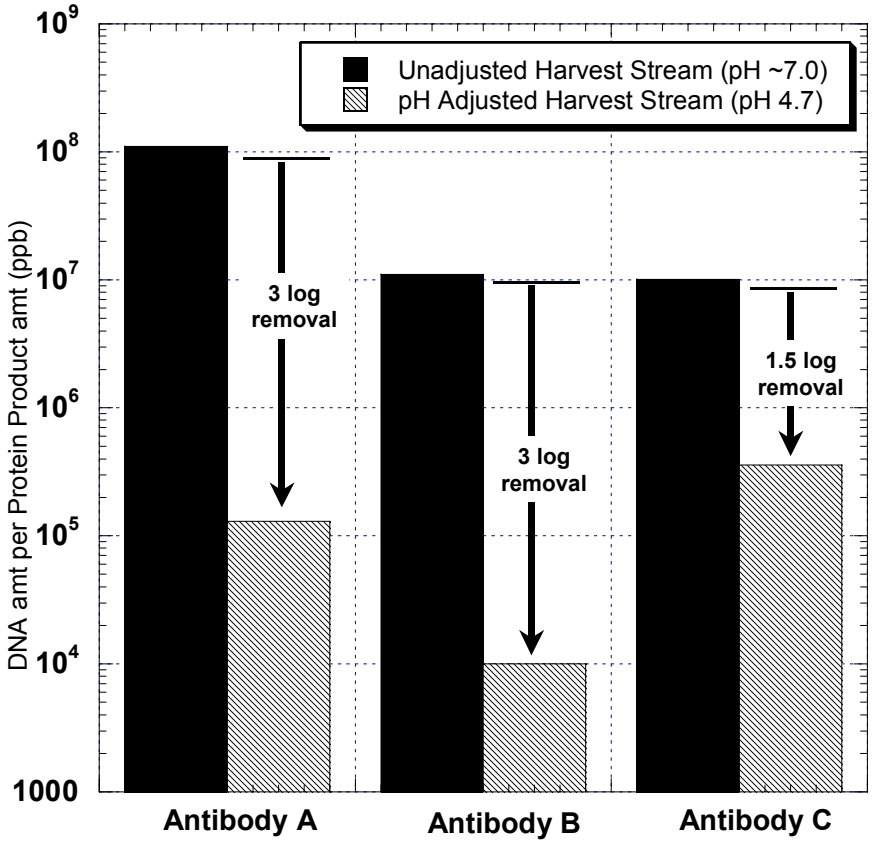


Figure 4: **Plot of DNA levels (ppb: amount of DNA per kg of product protein) in the cell-free harvest medium at pH 7.0 and 4.7. Data is shown for three different monoclonal antibodies.**

Overall, the data in Figures 2, 3, and 4 demonstrate how the harvest pH adjustment can dramatically impact clarity or turbidity of the supernate and how harvest acidification can selectively precipitate proteinacious materials. Although these studies provide direction as to how one may want modify the biochemical property of the Bioreactor harvest stream, the harvest condition evaluation also needs to be coupled with the operational design of a large scale clarification system to ensure operational feasibility while optimizing overall desired product recovery, impurity removal. The subsequent discussion summarizes the results and analysis of various microfiltration experiments performed at different scales using both unadjusted and adjusted harvest streams, in an attempt to better understand the coupled effects of Harvest feed conditions on filter operational performance.

MF Clarification Results and Analysis

Microfiltration transmembrane pressure (TMP) during concentration and diafiltration phases for seven separate experiments is shown in Figure 5. The TMP is plotted as a function of cumulative volume processed through the microporous membrane for harvest streams ranging from pH 4.5 to 7.0 and viabilities ranging from 41% to 88%. All filtration experiments were operating under the same flow conditions of permeate flux (15LMH) and recirculation rate (4000sec^{-1}). The filled circles in Figure 5 show the TMP data generated using unadjusted Harvest feed, whereas the open symbols represent pH ≤ 5.3 adjusted harvest streams. The graph shows lowering the harvest pH to a range of 4.7-5.3 results in a significantly smaller TMP increase throughout the concentration and diafiltration steps relative to the unadjusted harvest feed. For example, the ΔTMP change throughout the 7x concentration step is only 2.5 psi compared to approximately 20psi for the unadjusted runs. In addition, the data in Figure 5 indicate TMP behavior is insensitive to reduced % viability at low pH. The data indicates that lowering the pH of the harvest streams allows for reduced resistance at constant permeate flux or reduced fouling of the MF filters (as indicated by the lower TMP at high loading). This in turn allows for a more robust Microfiltration operation and increased filter capacity.

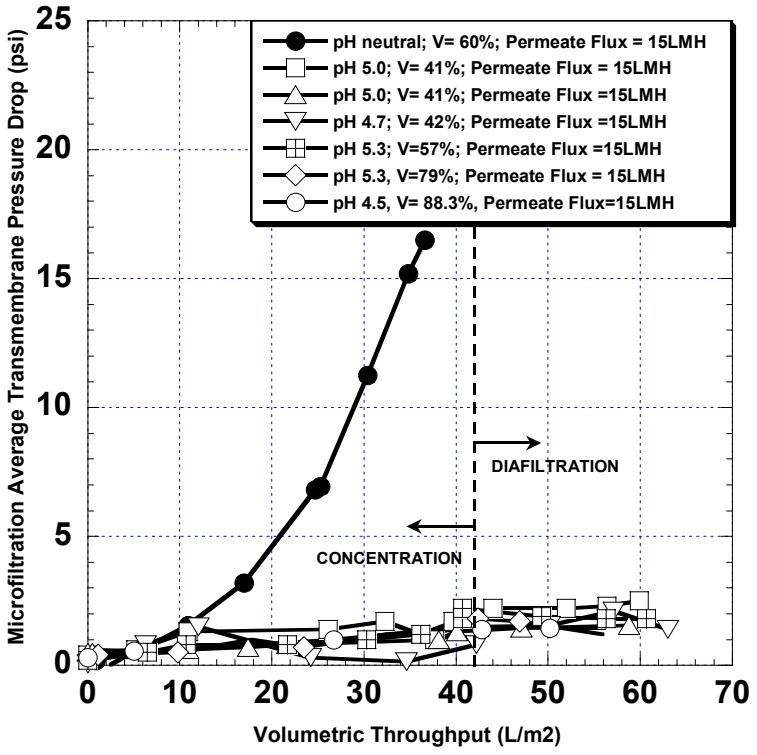


Figure 5: **Plot of Microfiltration Transmembrane pressure as a function of volumetric loading (L/m2) throughout the concentration and diafiltration phases of clarification. Data is shown for various % viabilities and adjusted harvest pH**

Figure 6 shows data for the instantaneous protein product filter rejection as a function of cumulative volume processed through the microporous membrane throughout the

concentration and diafiltration phases. The filter rejection is determined by measuring the retentate and permeate titers at various time intervals and calculating the rejection coefficient as shown in Figure 6. Data is shown for four separate MF experiments: the runs shown by the open and filled circles represent harvest streams that have been pH 5.0 adjusted including the addition of 10mM $CoCl_2$; and runs shown by the open and filled triangles represent unadjusted harvest streams with no presence of $CoCl_2$. The data shows the MF retention coefficient is lower for pH adjusted harvest feed containing 10mM $CoCl_2$ for all loading ratios studied. For example, at typical large-scale loading ratios of 60-70L/m^2, the calculated rejection coefficients are ~30% compared to nearly complete rejection (90-100%) for the runs that were not pH adjusted. Also shown in Figure 6 are overall protein recoveries from each microfiltration run. Clearly, the runs containing the Co2+ divalent ions show complete recovery of the desired protein as compared to the 20% yield loss with the runs using unadjusted harvest feed.

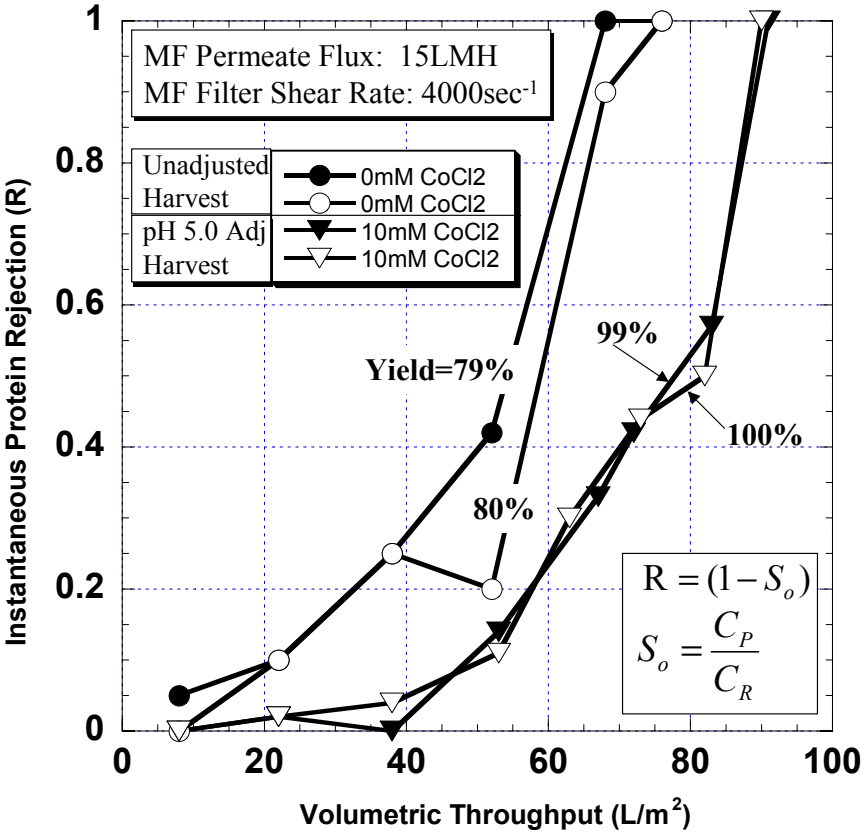


Figure 6: **Microfiltration filter instantaneous protein retention as a function of volumetric loading (L/m2). The vertical dashed line represents the start of the diafiltration phase. MF experiments are performed at Permeate flux =15LMH, Recirculation shear rate = 4000sec-1; and pH 5.0 adjusted harvest w/ 25% acetic acid.**

The data in Figures 4 and 6 clearly demonstrate how the presence of metal cations in the pH adjusted harvest stream significantly improve protein product recoveries in the Feed stream and the Microfiltration operation.

Conclusions

In summary, the data and analysis presented here provided a clearer understanding of effects of harvest solution conditions such as pH and ionic composition on harvest feed quality attributes and subsequent microfiltration performance. The data demonstrates significant drop in supernatant turbidity below pH 5.0 irrespective of ionic conditions indicating effective cell and cell debris precipitation at this pH range. In addition, cellular DNA levels reduced by 3 orders of magnitude in the supernatant at pH of 4.7 indicating selective precipitation of this impurity from the extracellular protein product. Corresponding MF clarification of the conditioned media under the same adjusted feed conditions showed significantly improved flux performance and reduced transmembrane pressure drops along with similar reductions in permeate DNA levels. Overall, the offline settling experimental results provide an appropriate framework for the design and optimization of cross-flow microfiltration systems for processing mammalian cell culture broths for production of therapeutic mAbs.