Isolation of Proteins from Fermentation Broth of high Cell Density with a new Cross Flow Filtration

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1 Introduction

P. pastoris and E. coli are commonly used hosts for recombinant product expression because of their ability to produce large quantities of recombinant protein quickly and economically. P. pastoris can express proteins with correct primary, secondary and tertiary structure and post-translational modifications such as glycolysation, proteolytic processing and disulfide bond formation. E. coli are readily transformed and can express large quantities of proteins quickly with correct primary structure in the soluble or insoluble forms. These attributes ensure that each host will remain central in recombinant protein expression of the future.[1-2] During the past twenty-five years, regulatory requirements have directed industry to use modern technology to improve process purity and control. In the same period, the recombinant protein industry has become focused on process efficiency, speed, yield and cost. Centrifugation has remained, essentially unchanged since the mid1950s, however recent advances in cross flow filtration (CFF) technologies have facilitated improvements in process purity, control, efficiency, speed, yield and cost.[3-10] Traditionally, CFF development focused on membrane flux rates, trans-membrane pressures, water flux recovery and target/contaminant passage. Optimal modern CFF development is focused on obtaining peak module flow performance throughout traditional development stages as well as traditional parameters.[10-13] Development efforts utilizing optimal modern CFF development methodologies consistently achieve process goals in the laboratory through commercial production scale. Appropriate membrane materials, design, module flow characteristics, system cleaning and sanitization technologies will enable the next generation of CFF processes to quickly and efficiently perform fine separations with consistent yields in excess of 90%.[11]

The new and innovative OXIPURE™ micro-reactor is a convenient cold sanitization technology. OXIPURE sachets are incubated in water to generate in situ chlorine dioxide. The technology is designed for sanitary equipment that is heat sensitive, such as filtration systems. Chlorine dioxide is a water-soluble oxidizing gas that sanitizes in water and air over a wide pH range. Chlorine dioxide has a history of over thirty years of effective sanitization in the water treatment, brewing, beverage and processed food industries. These industries report effective use of chlorine dioxide in removal of biofilms, reduction of endotoxin and equipment sanitization.[14-19]

Here, we present an assessment of CFF membrane and module performance in both P. pastoris and E. coli expression systems. In addition, we present data on the use of OXIPURE technology for bacterial contamination and endotoxin reduction. Two universities and a large international biotechnology company independently conducted the P. pastoris and E. coli CFF and OXIPURE experiments. The results from P. pastoris, E. coli and OXIPURE are presented separately in this paper.

2 Materials and Methods

2.1 P. pastoris, Experiments using Microfiltration/Diafiltration

The objective of the P. pastoris harvest operation was to provide an example of the process flux measured in liters per meter^2 hour (LMH) and clean water flux recovery expected from a typical P. pastoris microfiltration/diafiltration operation using common filter/module systems.

The P. pastoris cell culture broth was composed of a genetically engineered GS115, pHIL-D4 prepared per methods described by Stratton et al.[2] The final cell suspension was grown to an OD_{600} of 210 with a wet cell weight of approximately 25% and a working volume of 60L. Six to eight liters of broth was aliquoted and chilled to below 15°C during CFF operations. Two liters of deionized water were used to wash each liter of broth aliquoted.

The experimental design and objectives were communicated to the filter/system vendors who provided optimal membrane and module parameters. The membranes/systems used are provided (Table 1). Recirculation rate and

trans-membrane pressure were adjusted to obtain and maintain linear velocity based upon recommendations provided by the filter manufacturers. The operating conditions required to maintain the optimal linear velocities and membrane pressure ascribed by the manufacturer were calculated and operations were controlled to setpoint (Table 2).

Table 1 Membrane Systems Used for P. pastoris Microfiltration/Diafiltration

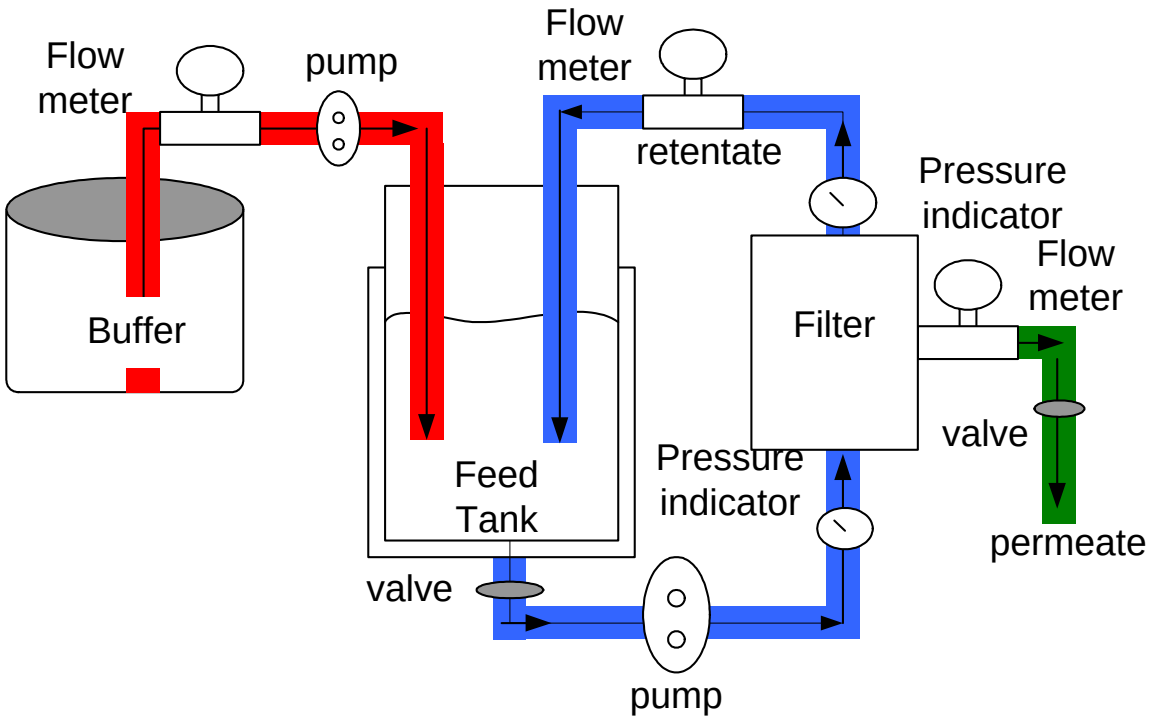
Manufacturer	Membrane material	Pore size	Area
NCSRT OPTISEP® 3000 with Pall membrane	Polyethersulfone (PES)	0.2	0.17m^2
Pall CENTRASETTE®	Polyethersulfone (PES)	0.16	0.45m^2
Sartorius Sartocon®	Crosslinked Cellulose	0.2	0.6m^2
Millipore Pellicon®	PLCXK Regenerated Cellulose (RC)	0.2	0.5m^2
Millipore Prostak®	GVPP Polyvinylidenfluoride (PVDF)	0.22	0.9m^2

Table 2 Average Operating Parameters during P. pastoris Microfiltration/Diafiltration

Membrane system	Inlet pressure (bar)	Outlet pressure (bar)	Pump Setting (Hz)	Recirculation rate (LPM)	Linear velocity (m/s)
NCSRT OPTISEP PES	1.66	0	46	40.1	3.0
Pall CENTRASETTE PES	1.72	0	21	4.9	1.0
Sartorius Sartocon Cellulose	1.86	0	20	2.7	0.5
Millipore Pellicon RC	1.86	0	24	2.0	0.4
Millipore Prostak PVDF	2.28	2	48	35.9	4.0

The P. pastoris CFF system configuration is provided in Figure 1. Data on retentate rate, recirculation rate, temperature, trans-membrane pressure, LMH, diafiltration rate, feed tank volume, permeate volume and pre-use and post use/cleaning water flux rates were documented and evaluated.

Figure 1 P. pastoris CFF Schematic



2.2 E. coli Constant Volume Harvest, Experiments using Ultrafiltration

The objective of the E. coli constant volume harvest operations were to provide an example of the sustained maximum flux and average process flux expected from a typical harvest. Since the expressed protein was not secreted, protein passage was not assessed.

The E. coli cell culture broth was composed of a genetically engineered K-12, prepared per propriety methods (unpublished). The final cell suspension had an OD_{600} of 322 with a dry percent solid of 16.2% and a working volume of 39.6L. The experimental design used frozen cell broth to create worst case fouling conditions. Frozen cell broth was thawed over 48 hours in a 2-8ºC refrigerator. Six liters of broth was aliquoted and controlled at ≤15°C during CFF operations.

The experimental design and objectives were communicated to Pall and NCSRT, who performed all operations on site. The membrane/system details are shown in Table 3. The process was operated in constant volume recirculation by routing permeate and retentate lines to the feed tank. Constant volume recirculation enables collection of extended time course data and creates worst-case membrane fouling conditions. Recirculation rates and trans-membrane pressures were adjusted to maintain optimal linear velocities. The operating conditions required to maintain the optimal linear velocities and membrane pressure ascribed by the manufacturer were calculated and operations were controlled to setpoint (Table 4).

Table 3 Membrane Systems Evaluated for E. coli Harvesting.

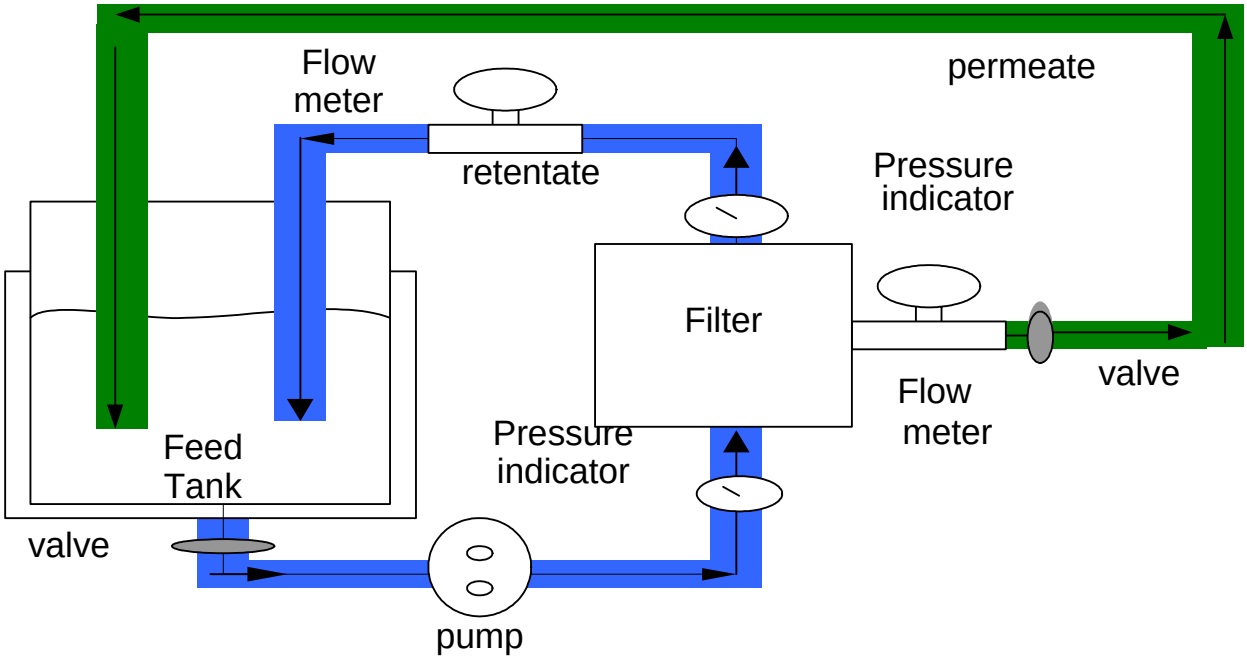
System manufacturer	Membrane material	Pore size	Area
Pall Centramate	Omega Polyethersulfone (PES) open channel	300 kD	0.19m^2
Pall Centramate	Alpha Polyethersulfone (PES) Suspended screen	10 kD	0.09m^2
NCSRT OPTISEP 800 with Nadir membrane	Regenerated Cellulose (RC)	100 kD	0.03m^2
NCSRT OPTISEP 800 with Pall membrane	Polysulfone (PS)	0.01	0.03m^2
NCSRT OPTISEP 800 with Pall membrane	Polysulfone (PS)	0.02	0.03m^2

Table 4 Average operating conditions for E. coli harvest

Membrane system	Inlet pressure (bar)	Outlet pressure (bar)	Recirculation rate (LPM)
Centramate Omega PES	0.28	0.14	2.5
Centramate Alpha PES	2.90	0.00	0.9
NCSRT OPTISEP RC 100	1.66	0.34	14.1
NCSRT OPTISEP PS 0.01µ	2.07	0.41	14.1
NCSRT OPTISEP PS 0.02µ	1.93	0.34	14.1

The E. coli CFF system configuration is depicted in Figure 2. Data on retentate rate, recirculation rate, temperature, trans-membrane pressure, LMH, diafiltration rate, feed tank volume and permeate volume were documented and evaluated.

Figure 2 E. coli CFF Schematic



2.3 OXIPURE Viability Influence

OXIPURE is a micro-reactor sachet that generates chlorine dioxide when combined with water. As the concentration of chlorine dioxide increases, the concentration in the vapour phase rises according to Henry's law, Equation 1, ensuring complete closed vessel sanitization. Figure 4 depicts the chemical composition of chlorine dioxide. Figure 5 depicts the experimental design for sanitization of a bacterial contaminated water storage tank. Before treatment, the tank was swabbed below the liquid line, at the liquid line and above the liquid line. A 4-gram sachet of OXIPURE was added to a 50L tank, which generated 50 ppm of chlorine dioxide. The total generation and exposure time was 60 minutes. The chlorine dioxide was consumed in a quenching reaction with sodium bisulfite, which ended the sanitization treatment. Post treatment swabs were taken at the same locations. .

Figure 4 Chlorine dioxides

Equation 1

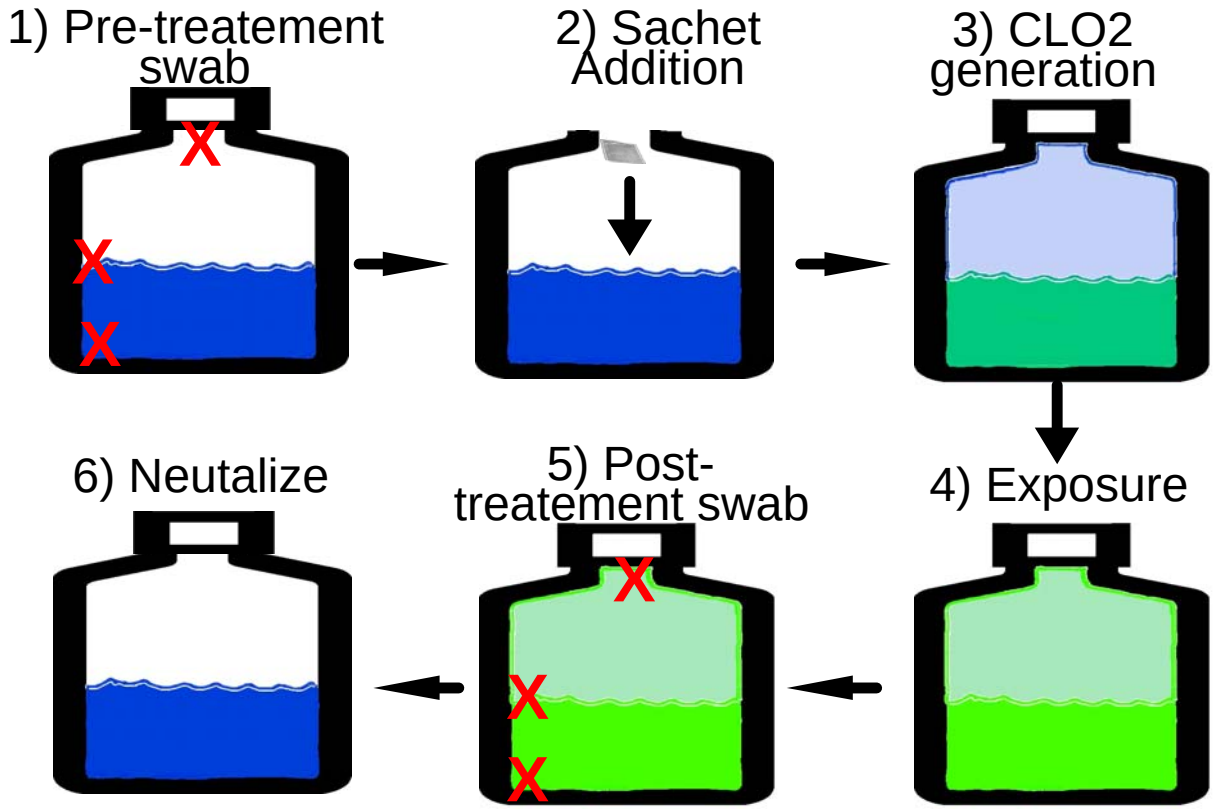
$C_{gas}=K_h * P_{gas}$

C_{gas} is the concentration of gas in air

K_h is Henry's constant

P_{gas} is the partial pressure of the gas

Figure 5 Chlorine Dioxide Generation/Exposure Setup



2.4 OXIPURE Endotoxin Influence

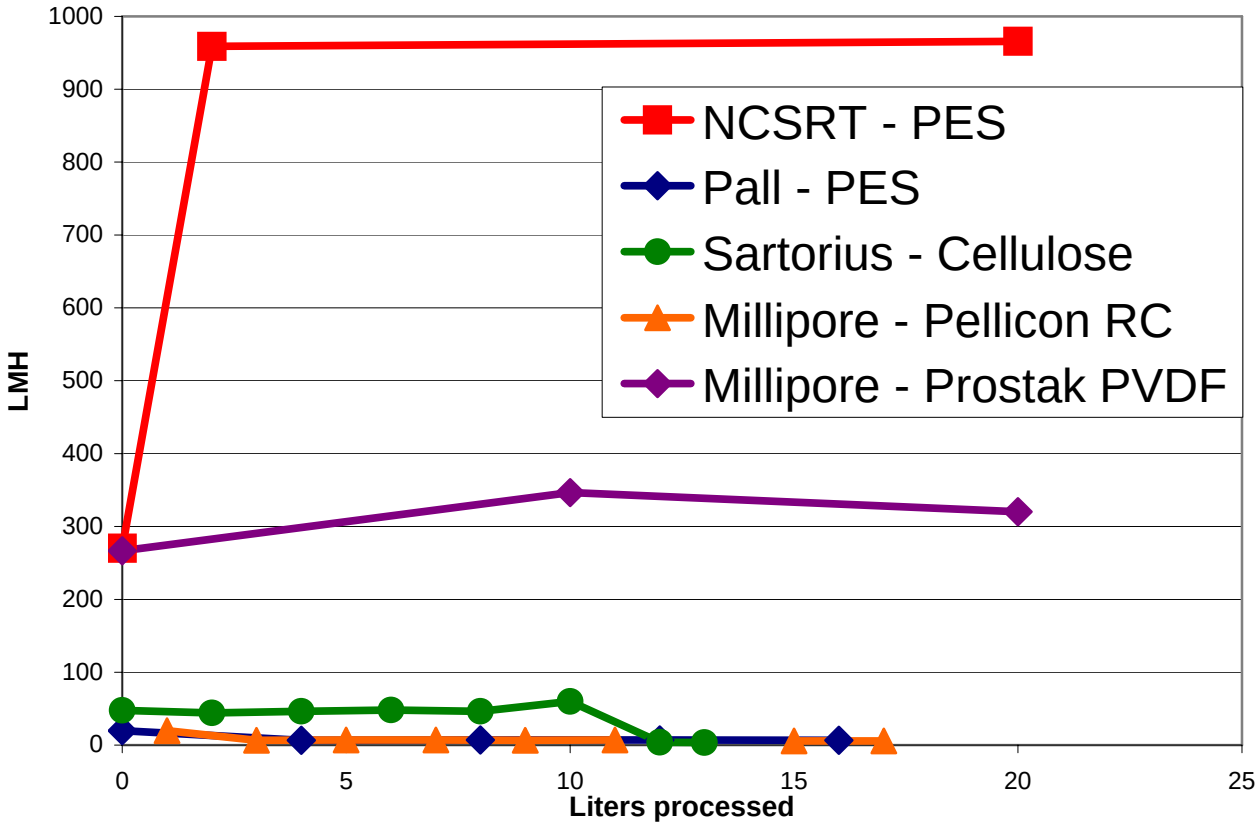
A known concentration of endotoxin was diluted from 1.0 to 0.03 and incubated with 0.66 ppm of chlorine dioxide for two minutes. The chlorine dioxide was consumed in a quenching reaction with sodium bisulfite which ended the sanitization treatment

3 Results and Discussion

3.1 P. pastoris, Experiments using Microfiltration/Diafiltration

Yeast based processes present harvesting difficulties because of high cell densities, temperature control requirements and sensitivity of secreted product to shear and oxidation. Production scale centrifugation is of limited value because of low yields, high shear, high temperature and foaming. In addition, post-centrifugation filtration is typically required. Several filtration modular systems were assessed for their ability to separate P. pastoris from conditioned media while controlling temperature, shear and foaming. Figure 6 presents the P. pastoris microfiltration/diafiltration flux against liters of diafiltered buffer processed. Despite similarity in membrane type, pore size and operating conditions, the NCSRT OPTISEP module with Pall PES performed 2.8 fold more efficiently than the Millipore PVDF/Prostak system. The NCSRT OPTISEP module with Pall PES system filtered over 20 fold more efficiently than all other membrane/modules systems tested. The NCSRT OPTISEP PES module and the Pall PES cassette system used enable a direct comparison of the effect of flow path over the membrane. This comparison indicates that module flow differences result in a 48 fold more efficient flux with the NCSRT OPTISEP module. Within the experiments assessed, this efficiency difference suggests that flow over the membrane surface is a dominant parameter in process flux. The critical factor of fluid flow over the membrane indicated by the P. pastoris data is supported by Schlegel[10,] Stratton[11] and Mallubhotla[12] who suggest that membrane flow may be the single most important parameter in membrane system selection.

Figure 6: P. pastoris Microfiltration/Diafiltration Flux Versus Liters Processed



Clean water flux recovery is an indicator of membrane cleaning effectiveness, with the higher percent recovery corresponding to a cleaner membrane. Additionally, flux recovery provides an indication of filter longevity, suggesting that a blinded membrane is not likely to be effectively cleaned. Table 5 details the pre and post use clean water flux rates observed. The NCSRT OPTISEP with Pall PES module and the Millipore Pellicon RC system achieved nearly 100% water recovery rates, which suggest that both systems were able to sweep away debris that might otherwise blind membrane pores. This data implies that these systems were more likely to have maintained consistent performance beyond the process duration tested. A flux comparison of the NCSRT OPTISEP module with PES indicates the sustained filtration rate of the NCSRT OPTISEP PES module was 38 fold higher than the Millipore Pellicon RC system (Figure 6). Comparing the water flux recovery of the NCSRT OPTISEP module with Pall PES membrane to the Pall PES system indicates a 2.1 fold higher flux recovery with the former, which is not attributable to membrane difference or operating conditions. These data further supports Schlegel[10], Stratton[11] and Mallubhotla[12] who suggest that feed flow over the membrane surface is the dominant factor in selecting a membrane system. Despite achieving flux rates of nearly 400 LMH, the Millipore Prostak had a clean water flux recovery of 13%. The low clean water flux recovery suggests that the Prostak may have been adversely impacted by processing and may not have been capable of sustaining constant flux rates beyond the durations tested.

Table 5 P. pastoris Clean Water Flux Recovery

Manufacturer	Flux before processing (LMH)	Flux after cleaning (LMH)	Percent Membrane Recovery
NCSRT OPTISEP with Pall PES	1510	1402	93%
Pall CENTRASETTE PES	612	186	30%
Sartorius Sartocon Cellulose	299	N/A*	N/A*
Millipore Pellicon RC	431	419	97%
Millipore Prostak PVDF	572	73	13%

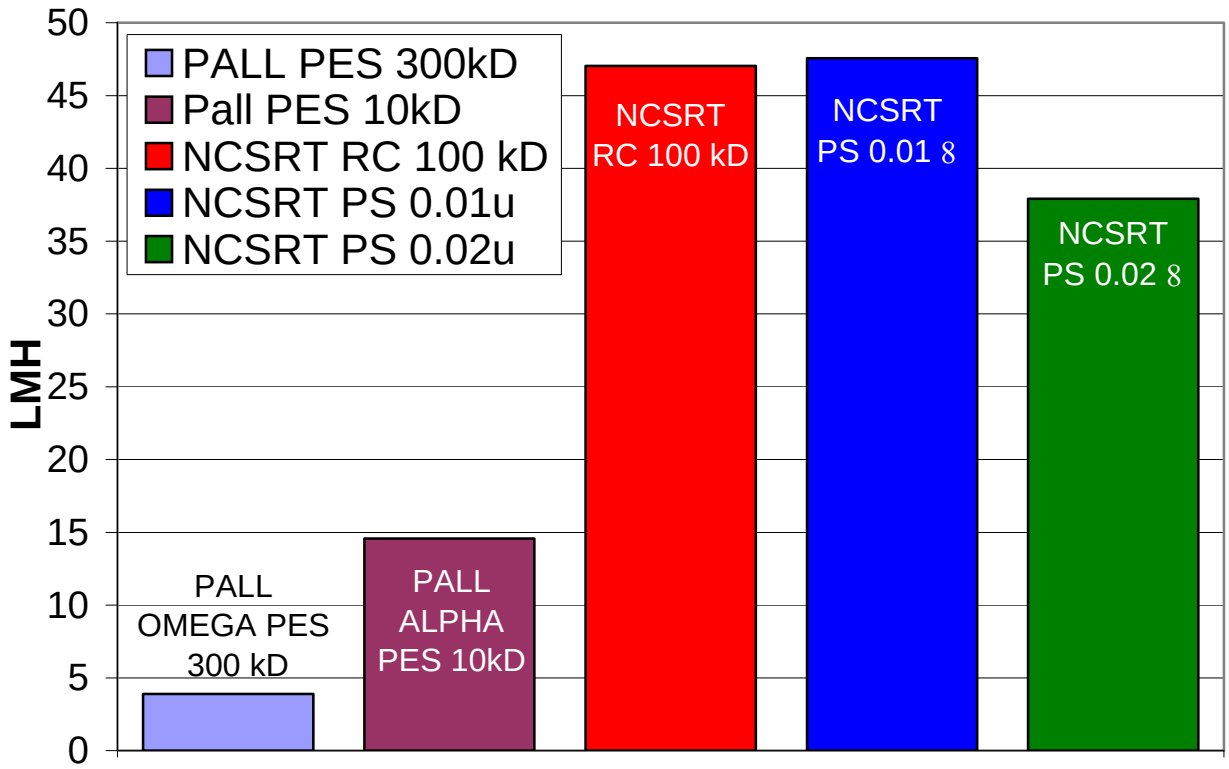
*Not applicable: Post cleaning flux data were not obtained because this membrane incapable of sufficient flux to perform cleaning operations.

3.2 E. coli Cell Harvesting, Experiments using ultrafiltration

Despite the potential of filtration for fine protein fractionation, improved contaminant removal, improved and simplified control and lower capital costs, centrifugation of E. coli remains the primary cell harvest operation employed. This is mainly due to concerns over maintaining sustainable and high flux rates. A large international biotechnology company performed a worst case E. coli harvest challenge. The experimental design generated worst case operating conditions by utilizing material with high levels of antifoam and one freeze thaw cycle. This design was intended to challenge the capabilities of the systems assessed. If sustained high flux CFF were achievable under these challenge conditions, then CFF would be the unit operation of choice for cell harvest operations.

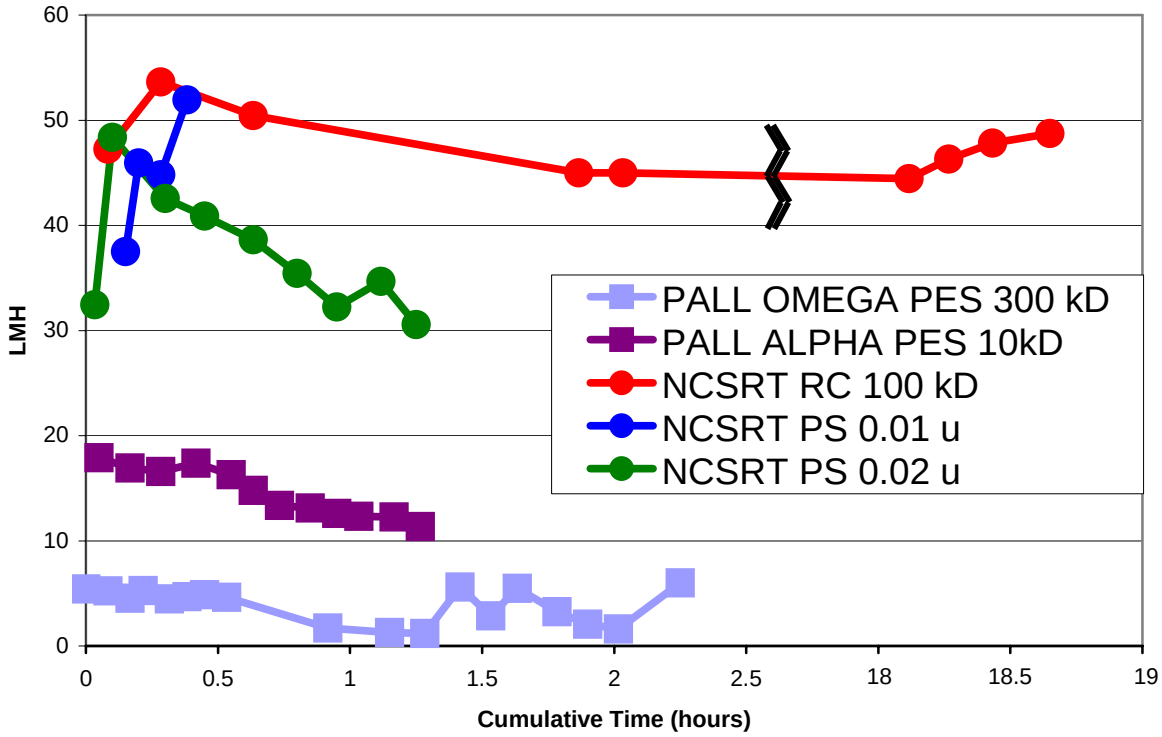
Despite similarities in membrane type, pore size and operating conditions, the NCSRT OPTISEP modules with PS 0.01µ and RC-100kD performed over 3 fold more efficiently than the Pall Alpha PES and over 12 fold more efficiently than the Pall Omega PES (Figure 7). A comparison of the two NCSRT OPTISEP PS membranes indicates the smaller pore size performed 47% more efficiently, which is consistent with the improvements Stratton et al.[19] reported when using ultrafiltration membranes to process E. coli. Care should be exercised when comparing the alpha and omega membranes as the former is optimized for resistance to antifoam, which was added to the culture during growth and production phase. Therefore, the observed difference between the alpha and omega membranes may be attributable to some combination of pore size, feed flow and membrane chemistry differences. A comparison of the NCSRT OPTISEP RC 100kD membrane and the NCSRT OPTISEP PS 0.01µ membrane indicates that both performed comparably.

Figure 7 Average Process Flux during E. coli Harvest



The NCSRT OPTISEP RC 100KD was allowed to operate in recirculation mode for over 18 hours, which demonstrates sustained flux rates of 45 to 55 LMH during worst case conditions (Figure 8). Within the experiments performed, the data suggests that the NCSRT OPTISEP RC100 is capable of sustaining high flux rates through at least 18 hours of processing. Both the NCSRT OPTISEP RC 100KD and NCSRT OPTISEP PS 0.01µ achieved the goal of sustained high flux rates sought by the biotechnology company.

Figure 8 Process flux versus process time for E. coli harvest



The major reason for the better performance of the Optisep Cassettes is their improved fluid dynamic design. Using Optisep microfiltration in a first loop followed by Optisep ultrafiltration in a second loop allows a continuous and highly automated process with improved flux and product yield.

3.3 OXIPURE Viability and Endotoxin Influence

The data for OXIPURE treatment of a contaminated water storage tank with a combined 60-minute generation and exposure time is shown in Table 6. As measured by plate viability, all sampled locations throughout the contaminated vessel were reduced to zero contaminants. This data indicates that, in a closed system with viabilities up to $7X10^3$ CFU, OXIPURE represents an effective bioreduction step. Experimental work by Tanner[14], Finch[15], Lindsay[16], Brown[17], Gates[18] and Masschelein[19] support and extend this experimental data across multiple bacterial species and provide comparison of common sanitizers.

Table 6 Viability Pre and Post OXIPURE Treatment

Swab location	Pre treatment viability (CFU)	Post treatment viability (CFU)	Viability reduction
Above liquid level	1.79×10^3	0	100%
Liquid level	1.01×10^3	0	100%
Below liquid level	7.26×10^3	0	100%

Endotoxin levels were also reduced after OXIPURE treatment (Table 7). In all cases, endotoxin was reduced to below measurable levels. This data indicates that OXIPURE can reduce endotoxin levels at or below one EU/mL.

Table 7 Endotoxin Pre and post OXIPURE treatment

Starting level (EU/mL)	Post treatment (EU/mL)*
1.0	<0.03
0.5	<0.03
0.125	<0.03
0.06	<0.03
0.03	<0.03

*Assay sensitivity 0.03 EU/mL

The bioreduction capabilities of OXIPURE suggest that OXIPURE may have application for bioreduction of CFF systems. Potential uses for OXIPURE are both pre and post use bioreduction.

4.0 Conclusion

During the CFF conditions examined, both P. pastoris and E. coli CFF achieved sustained high flux rates, simplified processing, improved process control, lower shear and higher yield than centrifugation harvest technologies and achieved all processing objectives. Under the conditions examined CFF system flow over the membrane surface appears to be the dominating factor when choosing CFF systems. NCSRT OPTISEP modules were 2.5-48 fold more efficient in processing P. pastoris and E. coli compared to all other systems evaluated. In addition to improved flux, NCSRT OPTISEP modules demonstrated high clean water flux recovery, which suggests that NCSRT OPTISEP modules are readily cleanable. When using optimized conditions, NCSRT OPTISEP modules were able to achieve sustained filtration rate of over 900 and 45 LMH for P. pastoris and E. coli respectively and within the range of conditions tested was the system of choice with any membrane.

Using Optisep microfiltration simultaneously with Optisep ultrafiltration, coupled in two consecutive loops, it is possible to design a continuous process which allows to take advantage of the Optisep cassette's improved fluid dynamic characteristics in both stages leading to higher flux and higher yield.

Within the conditions examined, OXIPURE effectively reduced both bioburden and endotoxin. Additional experimentation would be required to determine the effectiveness of OXIPURE with NCSRT CFF systems. However, an examination of the indirect evidence suggests coupling the two systems may enable cross flow filtration operations to achieve high levels of bioreduction before and after processing. Effective process performance, cleanability and sanitization would enable CFF to exceed both regulatory and scientific requirements.

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