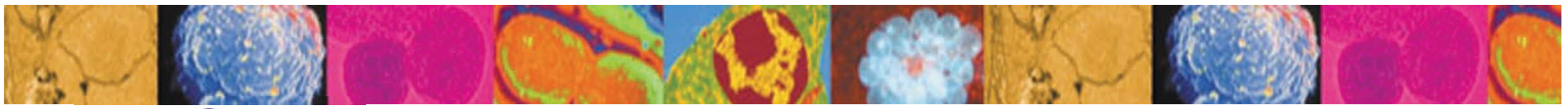

NCSRT

Works™ Optimization Procedure

*Secreted proteins from whole cells* with *SmartFlow*™ TFF

This *Secreted proteins from whole cells* WORKS™ Optimization Procedure is intended to isolate recombinant proteins secreted from microbial cultures including yeast and bacteria cultures.

This optimization procedure uses a high molecular weight ultrafiltration (UF) membrane that retains the cells and cell debris and allows the secreted protein to pass freely through the membrane. The passage characteristics of secreted proteins from microbial cells change with different buffers, temperatures, concentrations, and membranes. By examining the passage characteristics of the different UF membranes available in the appropriate process conditions, a well defined and executed process development study can identify the most efficient membrane and process conditions to achieve the required performance.

This optimization procedure starts with selecting the membrane module, membrane polymer, and pore size most likely to work based upon thousands of NCSRT trials. Once this module is selected, ranges in which to begin optimizing parameters such as membrane capacity, recirculation rate, and pressure are presented. Because of the variability in the products and processes using NCSRT's *SmartFlow* technology, we do not make specific process recommendations on parameters of temperature, pH, buffers, or other variables that may affect the separation process and the target product activity.

To learn how others have applied the *SmartFlow* filter technology to similar separations, please review the *Secreted proteins from whole cells* WORKS Case Study.

*Secreted proteins from whole cells* with *SmartFlow*™ TFF

Each parameter of the TFF process: product, membrane type, shear, pore size, temperature, concentration factor, pH, anti-foam, etc. may impact the phage passage through the membrane. This is why a systematic experimental plan must be developed and executed to optimize a concentration and diafiltration process.

Module and System Selection:

1) Select the *SmartFlow*™ filter module to evaluate. The selection requires specifying a combination of membrane type, channel height, and membrane area for a given module that will be tested.
   a) NCSRT has filtered thousands of solutions and therefore can provide several membrane chemistries and pore sizes that will likely work in the majority of cases. In general the pore size should be 5 to 10 times the size of the molecule to be passed through the membrane and one half to one third the size of a molecule to be retained.
   b) The combination of the channel height and the fluid velocity through the flow channel created by the recirculation pump produce a shear at the membrane surface. It is this shear that governs the separation performance and efficiency. Care must be taken in selecting and maintaining the shear at the membrane surface.
   c) The membrane area also affects the pump size required to achieve the necessary shear rates for a given separation.
2) Select the first membrane to test.
   a) Recommended starting membranes for isolation of secreted proteins from microbial cells are the 100 kD regeneratred cellulose (RC) and 500 kD modified polysulfone (MPS) membranes.
3) Select the channel height for the module.
   a) For the isolation of secreted proteins from microbial cells, a channel height between 0.75 and 1.5 mm is usual.
   b) In most cases a channel height of 0.75 mm is recommended because it will require the lowest recirculation rate (and thus the smallest pump) and produce the highest flux rate.
   c) Cases to use a higher channel height include:
      i) If cell aggregation is a occurring, the lower height channels may clog. If the channel is clogged by aggregates or process particles, the inlet pressure will increase dramatically and the permeate rate will decrease over a short period of time. This will occur usually in the first five minutes.
      ii) In cases where high solids (greater than 60% WCW) are desired, a channel height of 0.875 mm or above will be necessary.
4) Select the membrane area.
   a) The membrane area depends upon the batch size to be processed. For filtration process development trials, usually the smallest size membrane and thus the smallest batch size is desired.
   b) For cell harvests, an important parameter is the membrane capacity or LM ratio. The LM ratio is defined as the volume of starting material divided by the membrane area.
   c) The range of LM ratios for the isolation of secreted proteins from microbial cells we have observed varies from 30 to 250 LM.
      i) The typical starting ratio for mammalian cell harvest is 100 LM. See below for additional information on optimizing the LM ratio.
      ii) If a fermentation broth is being concentrated, the presence of antifoam is an important parameter that can impact the starting volume to membrane area ratio. Antifoam agents may significantly

reduce the permeate flux observed with ultrafiltration membranes. In the cases of fermentation broths containing antifoam, use a starting ratio of 30 LM.

iii) The minimum batch size is the system hold up volume times the concentration factor. For a continuous diafiltration, the minimum batch size is simply the hold up volume.

d) The membrane area needed is the batch size divided by the LM ratio.

5) Determine the linear velocity.

a) The typical velocity for the isolation of secreted proteins from microbial cultures is between 50 cm/sec and 150cm/sec.

b) The typical starting velocity for a process development run is 100 cm/sec.

i) The benefit of increasing the velocity may be an increased passage rate.

ii) Increasing velocity and transmembrane pressure (TMP) beyond an optimal level will decrease the passage of the secreted protein.

e) An increase in the velocity should be balanced by an increase in the flux rate or protein passage for the process to retain the same overall efficiency. The energy costs of running the pump at a higher velocity must be offset by savings on membranes to make increasing the shear rate efficient.

6) Calculate the flow rate needed to operate the selected module at the selected velocity using the *WORKS*™ Scale-UP LPM GPM spreadsheet. Ensure that a pump is available that can produce this flow rate at the needed pressure. If a suitable size pump in not available, consider either running a smaller trial or calling NCSRT to determine if a suitable size pump is available.

7) Use Table 1 to determine the module(s) part numbers for ordering.

Table 1: *SmartFlow*™ filter module part numbers

| Module Size | | | Channel Height | | Membrane polymer and pore size | |
|---|---|---|---|---|---|---|
| _____ - | | | ___ | | _____-_____ | |
| 74 | 100 ft² | Optisep 11000 | D | 0.5 mm | 5B-0100 | RC 100 kD |
| 72 | 50 ft² | Optisep 11000 | E | 0.75 mm | 1N-B100 | MPS 500 kD |
| 71 | 10 ft² | Optisep 11000 | G | 0.875 mm | | |
| 41 | 10ft² | Optisep 7000 | H | 1 mm | | |
| 40 | 5 ft² | Optisep 7000 | J | 1.5 mm | | |
| 52 | 2 ft² | Optisep 3000 | | | | |
| 51 | 1 ft² | Optisep 3000 | | | | |

Filter Operation:

1) After loading the filter modules and making all the connections, the first step is to perform a water and/or buffer rinse of the system directing the permeate to the waste.

2) After the rinse, direct the permeate line back to the retentate tank so no concentration occurs prior to establishing the desired velocity and performing the transmembrane pressure (TMP) optimization procedure.

IMPORTANT: Do not permit the permeate line to come in contact with the retentate fluid. This can contaminate the permeate pool in later samples.

3) Slowly increase the flow rate recirculation pump to the calculated rate from step 6 above.

4) Start with the backpressure at zero. The inlet pressure should be at least 5 psig (0.3 bar) and remain below

12 psig (0.8 bar). If the inlet pressure is above 12 psig (0.8 bar), the recirculation flow rate should be reduced such that the inlet pressure remains below 12 psig (0.8 bar). If the inlet pressure is less than 5 psig, then slight backpressure can be added until the inlet pressure increases to 5 psig. Table 2 provides typical TMP values for the different membranes used in *SmartFlow*™ TFF filter modules.

Table 2 Typical transmembrane pressure values for *SmartFlow* modules

| Membrane Pore Size | Transmembrane Pressure Starting Value PSIG (Bar) | Transmembrane Pressure Ranges PSIG (Bar) | Cell Harvest Inlet PSIG (Bar) Starting Value | Cell Harvest Outlet PSIG (Bar) |
|---|---|---|---|---|
| Ultrafiltration Membranes | | | | |
| 1 kDa | 75 (5) | 90 to 150 (6 to 10) | | |
| 5 kDa | 45 (3) | 60 to 90 | | |
| 10 kDa | 30 (2) | 45 to 90 (3 to 6) | | |
| 30 kDa | 15 (1) | 30 to 75 (2 to 5) | | |
| 100 kDa | 15 (1) | 20 to 60 (1.37 to 4) | 20 (1.37) | 12 (0.83) |
| 300 kDa | 10 (0.69) | 15 to 45 (1 to 3) | 20 (1.37) | 10 (0.69) |
| 500 kDa | 7.5 (0.5) | 10 to 30 (0.7 to 2) | 7.5 (0.5) | 0 |
| Microfiltration Membranes | | | | |
| 0.1µ | 2 (0.13) | 4 to 15 (0.27 to 1.0) | 4 (0.275) | 0 |
| 0.2µ | 2 (0.13) | 4 to 15 (0.27 to 1.0) | 4 (0.275) | 0 |
| 0.45µ | 2 (0.13) | 4 to 10 (0.27 to 0.69) | 4 (0.275) | 0 |
| 0.8µ | 1 (0.07) | 1 to 6 (0.07 to 0.41) | 2 (0.13) | 0 |
| 1.0µ | 1 (0.07) | 1 to 6 (0.07 to 0.41) | 2 (0.13) | 0 |
| 2.0µ | 1 (0.07) | 1 to 6 (0.07 to 0.41) | 2 (0.13) | 0 |
| 3.0µ | 1 (0.07) | 1 to 6 (0.07 to 0.41) | 2 (0.13) | 0 |

5) Begin Concentrating.
   a) Remove the permeate lines from the retentate tank and place them in the permeate vessel.
   b) Wait until about 5% of the starting retentate volume has passed through the membrane to the permeate to take the initial samples of the retentate and permeate.
   c) Take permeate and retentate samples when each additional concentration factor is reached.
   d) With each sample, record the permeate flow rate using a graduated cylinder, scale, or flow meter.
6) Once the desired concentration factor is reached, record the volume remaining in the retentate.
   a) The remaining volume in the retentate can be calculated by subtracting the permeate volume and retentate volume samples from the starting volumes.
   b) *Retentate Volume = Starting Volume – Permeate Volume – Retentate Sample Volume*
7) Diafiltration – the following describes the procedure for diafiltering the product 5x:
   a) Start to monitor the permeate volume with a graduated cylinder or scale.
   b) To start the diafiltration, add 5 to 15% of the starting retentate volume to the retentate tank.
   c) When the permeate volume has increased by the volume added in step b take a retentate sample from the retentate tank and a permeate sample directly from the permeate hose simultaneously. With each sample, record the permeate flow rate using a graduated cylinder, scale, or flow meter.
   d) Continue to add buffer at a rate equal to the permeate rate in aliquots equal to between 5 and 15%

of starting retentate value. Continue until 5 times the total starting volume has been added to the system.

e) Take samples from the permeate hose and retentate tank when each diafiltration factor is reached (i.e. take a sample when the permeate volume is equal to a multiple of the retentate volume such as 1X, 2X, etc.)

f) For other diafiltration factors, continue the process until the amount of diafiltration buffer equals number of desired diafiltration factor times the system volume recorded in step 6.

i) The theoretical recovery from a 3X diafiltration for a molecule with a 100% passage is 95%.

ii) Increasing the diafiltration factor will increase the yield especially when the target molecule has low passage. However, the cost of increasing the diafiltration volume is that the process time will be greater and a larger supply of buffer will be needed.

iii) Decreasing the diafiltration factor will decrease the yield. However, for molecules with high passage and low value, the small decrease in the yield may be worth the faster processing time and the saving on buffer.

Data Analysis:

Sample Analysis:

1) Check the permeate samples for cells.
2) Calculate the membrane flux rate or LMH (L/m²/h) by dividing the measured permeate flow rate at each sample by the membrane area.

$$LMH = Permeate\ Flow\ Rate\ (mL/min) * \frac{1L}{1000mL} * \frac{60\,min}{1hr} \div Membrane\ Area\ (m^2)$$

3) Calculate the instantaneous phage percent passage by dividing the permeate protein content by the retentate protein content and multiplying by 100.
4) Record the data on the Membrane Test Worksheet.

Process Optimization:

The procedure should be repeated under different process conditions to ensure that the optimized conditions are reached.

1) The important variables to optimize are the yield, membrane passage, and membrane flux rate.
2) An important parameter that effects the yield, passage, and flux rate for cell harvest is the membrane capacity or LM ratio.

a) Increasing the LM ratio decreases membrane performance, which increases processing time and decreases membrane costs. If membrane performance suffers greatly, then saving a little bit on membrane will not offset the costs in higher processing time.

b) Decreasing the LM ratio increases the membrane performance and increases membrane costs. Increasing membrane performance may decrease the processing time at a small incremental membrane cost, therefore decreasing total cost.

c) To find the optimal LM ratio:

i) If the current trial was too fast with very high yield, increase the LM ratio by starting with a larger volume of starting material.

ii) If the current trial was too slow or had a low yield, decrease the LM ratio by starting with a smaller volume of starting material.

3) The module used is an important optimization parameter. By changing the membrane chemistry or membrane type, optimized flux rates and passage may be found.

4) Using the same membrane, the velocity can be optimized by increasing and decreasing the velocity and measuring the effects on the membrane flux rate and passage. If an increase in the velocity results in a relatively large increase in the passage rate, then the savings in membrane cost will offset the increased energy consumption.

5) The concentration factor before starting diafiltration should also be optimized. The goal is to begin the diafiltration with the instantaneous passage of at least 50% to increase diafiltration efficiency.

After analysis of the data, select the best performing membrane. The best performing membrane will retain the cells, permit the desired protein to pass into the permeate and have high passage and permeate flux rates.

Conclusion:

This *SmartFlow*™ filter *Secreted proteins from whole l cells WORKS*™ Optimization Procedure provides a guideline for optimizing the application of NCSRT's *SmartFlow* filters. NCSRT has developed a simple step-by-step protocol that has been proven to deliver >90% product yield in the applications to which it has been applied. Along with the *Secreted proteins from whole cells WORKS* Protocol, NCSRT has developed a Scale-Up Component listing providing the part numbers and ordering information for the *SmartFlow* filter modules to execute the protocol at all process stages and volumes. To receive the complete application package, please request the *Secreted proteins from whole cells WORK*book.

NCSRT's *SmartFlow* filter technology….It *WORKS*.

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