Endotoxin control in the process development lab: from upstream bacterial fermentation to downstream purification processes

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Abstract
The use of *E. Coli* in the production of biologics leads to high endotoxin levels in the material passed from upstream fermentation to downstream purification. There are several challenges in removing that endotoxin to meet FDA standards, including endotoxin's stability over a wide pH range and the small size of endotoxin molecules. In this work, we test commercially available products marketed at removing endotoxin and evaluate them for endotoxin reduction and yield. The products tested are Mustang Q membranes, Sartobind STIC PA membranes, Proteus endotoxin removal spin columns, and a Triton X-100 protocol. All products decreased the endotoxin levels of the samples tested while producing good yield, but scalability, ease of use, and product purity vary. We also simulate the endotoxin level of a process development lab environment where bacterial fermentations are regularly performed to measure the baseline lab endotoxin level and generally find a low level of endotoxin in the lab environment. Finally, we test common NaOH cleaning procedures to quantify the reduction in endotoxin that may be achieved in a real-life working lab environment.

Introduction and Background

Although *E. Coli* has proven itself an important, if not vital, player in the production of biopharmaceuticals, its use in upstream fermentation does not come without challenges and costs for downstream purification. At the start of purification, clarified cell lysate may have an endotoxin level greater than 1 billion EU/mL. By the time the material becomes a final formulated drug product, its endotoxin level must be reduced many orders of magnitude to meet the FDA standard of 5 EU/kg, the threshold pyrogen level for humans.

However, the small size of individual endotoxin molecules makes removal by filtration difficult for in-process buffers and solutions or impossible if the target molecule is larger than individual endotoxins. When endotoxin is present at higher concentrations, it can micellize into aggregates with molecular weights as high as 1 MDa and interact with other molecules, becoming entangled with the molecule of interest. Endotoxin molecules are also highly pH and heat resistant, requiring prolonged exposure to strong acids or bases or nonstandard autoclave procedures. Given these well-known challenges of endotoxin removal, we test the endotoxin reduction capabilities of several processes – anion exchange membranes, surfactant-based protocols, and endotoxin binding resin. Additionally, we evaluate cleaning procedures for their effectiveness at depyrogenation and quantify the endotoxin level of a process development lab environment

where bacterial fermentations are regularly performed to determine strategies to mitigate endotoxin cross contamination in the process development lab.

Materials

The material tested was an enzymatic protein produced by *E. Coli*. The endotoxin removal strategies were obtained from the following sources: Mustang Q (MQ) membrane capsules from Cytiva, Sartobind STIC PA (primary amine) anion exchange membranes from Sartorius, Triton X-100 from Sigma Aldrich, and Proteus endotoxin removal spin columns from Bio-Rad. All endotoxin testing was performed using the Endosafe nexgen-PTS and Endosafe LAL cartridges purchased from Charles River Laboratories.

Methods and Results

Environmental endotoxin baseline: The endotoxin level of the lab environment was quantified by placing sterile petri dishes filled with 20 mL of endotoxin-free water in locations around the upstream and downstream labs. The upstream and downstream labs are separated by a hallway and multiple doors, and both labs commonly handle material from bacterial fermentations although no fermentation was in process when samples were placed unless otherwise noted. During the test, lab personnel were allowed to move around the lab and perform experiments with no restrictions except to avoid direct contact with the samples. After 1 day, the samples were tested for endotoxin. This procedure was meant to simulate a purification process where the material would be found in various aqueous solutions and could be exposed to the lab environment for up to a day (ex. a column that elutes overnight into an uncovered fractionator).

Even though the samples were collected while no bacterial fermentation processes were occurring, a low level of environmental endotoxin was measured for all samples except for a closed room in the downstream lab (Table 1). The samples evaporated at different rates, showing variations in air flow, temperature, and humidity throughout the labs. Two samples evaporated completely, so 20 mL of endotoxin-free water was added to the petri dish and that water was then tested for endotoxin.

Samples from the upstream lab and waste room had slightly (30-40%) higher endotoxin levels than the DSP main lab, which is consistent with both spaces generally handling material with a higher quantity and concentration of endotoxin. The sample from the enclosed room in the downstream lab was kept in the room with the door closed and no personnel entered the room until the end of the test to collect the sample. That sample returned an endotoxin level below the test limit of 0.05 EU/mL, indicating that simple physical separation and minimizing traffic can be effective to reduce endotoxin. The other sample that contained minimal endotoxin (< 0.05 EU/mL) was inside a biosafety cabinet (BSC) in the upstream lab. If a fully separate lab is not available, working inside a BSC can provide an alternative way to minimize endotoxin.

Table 1: Environmental endotoxin levels

Sample	Final volume (mL)	Endotoxin (EU/mL)	Endotoxin (EU)
Downstream main lab	4.2	0.301	1.26
Downstream side lab (door closed)	12.2	< 0.05	< 0.61
Upstream main lab	11.5	0.155	1.78
Upstream fermentation	(20)	0.206	4.12
Biosafety cabinet	(20)	< 0.05	< 1
Waste room	13.0	0.125	1.623

(volume in parenthesis indicates sample fully evaporated and had water added to test the endotoxin level)

The last sample tested was placed next to a 1-L fermenter while a test fermentation process was ongoing on a separate day from when the other samples were collected. The endotoxin level of this sample was 2-3 times higher than the other lab samples. During fermentation, although the bioreactor is closed, endotoxin can be aerosolized and introduced into the lab environment. Overall, these results show that endotoxin molecules may persist even after active fermentation and purification activities and contribute to a baseline endotoxin level in the lab. However, for material produced by bacterial fermentation, the environmental endotoxin level is negligible compared to the endotoxin already in the material during capture and early purification. Later in the purification process, during late stage polishing and formulation, the environmental endotoxin level may be significant compared to endotoxin remaining in the material.

Cleaning protocols: Higher concentrations (> 0.5 M) of NaOH are commonly used in cleaning procedures since they are able to deactivate endotoxin. We quantified the endotoxin reduction of several cleaning protocols using 0.5 M NaOH at room temperature to evaluate their effectiveness at depyrogenating TFF tubing and chromatography systems. New TFF tubing was first tested by passing endotoxin-free water through a length of tubing and testing the endotoxin level of the outflow after several minutes. This sample had an endotoxin level < 0.05 EU/mL, confirming that the starting endotoxin level of tubing used in our lab is low.

We then tested the effect of cleaning a sample TFF system by measuring the endotoxin level before and after a 30-minute cleaning with 0.5 M NaOH at a low flow rate. The TFF system was set up with pressure sensors, hose barbs, gaskets, and tubing that were previously used for a capture TFF process. After the initial endotoxin reading, 0.5 M NaOH was flowed through the system slowly for 30 minutes, then the system was flushed with water until the pH dropped to ~7

and the endotoxin level post-cleaning was tested. A 35% reduction in endotoxin level was achieved (see Table 2).

Table 2: Endotoxin reduction achieved by cleaning procedures

Sample	Initial endotoxin (EU/mL)	Final endotoxin (EU/mL)	Endotoxin reduction
MasterFlex PharmMed BPT tubing (new)	N/A	< 0.05	N/A
MasterFlex PharmMed BPT tubing (used) (30 min cleaning)	0.136	0.089	35%
AKTA (30 min cleaning)	0.486	0.322	34%
AKTA (30 min cleaning w/flow)	2.88	2.37	18%
AKTA (300 min cleaning)	2.37	0.630	73%

We next looked at AKTA Explorer systems that had been used for various capture and polishing columns and trialed three different cleaning protocols – 1) 30 minutes of contact time, 2) 30 minutes of contact time with a low flow rate, and 3) 300 minutes (5 hours) of contact time. Following contact with NaOH, the AKTA was flushed with water until the conductivity decreased, then water from an outlet was tested for endotoxin. In all cases, the endotoxin level post-cleaning was lower than pre-cleaning (Table 2). The endotoxin reduction varied depending on the instrument and starting endotoxin level. Having a low flow rate did not improve the endotoxin reduction in this case, but the AKTA tested had been heavily used for bacterial purifications and was inconsistently cleaned, potentially making the endotoxin more difficult to remove. Our results indicate that longer contact times are effective at increasing depyrogenation.

Endotoxin reduction: Four different products were tested for their endotoxin removal capabilities. As above, endotoxin levels were quantified with the Endosafe nexgen-PTS. Yield was calculated based on absorbance readings at 280 nm (A280) measured on a NanoDrop One.

The Mustang Q (MQ) and Sartobind STIC PA (STIC) experiments were performed using an AKTA Pilot or Explorer as appropriate for the process scale. The membranes underwent a sanitization and equilibration procedure before the product was flowed through the capsules and followed by a flush with equilibration buffer. Flowthrough was collected starting when the A280 began to rise until the absorbance decreased back to a baseline.

The Triton X-100 protocol involved an initial incubation step with a surfactant-spiked buffer, repeated concentration and dilution cycles with the spiked buffer to remove endotoxin, then a

second set of concentrations and dilutions with unspiked buffer to remove the Triton X-100. Concentration was done in Amicon centrifugal filter units in 15-minute increments at 3000 rpm (1860 *g*) and 20°C.

The Proteus endotoxin removal spin columns were used following the provided instructions. After equilibrating the column, the material to be purified was mixed with the resin and left for 2 hours at room temperature. The column was then centrifuged as instructed at 750 *g* for 10 min and the flowthrough was tested for protein concentration and endotoxin.

All methods demonstrated some efficacy at removing endotoxin from the starting material, ranging from 43-99.9% of endotoxin removed (Table 3). In general, the percentage of endotoxin removed was higher for material starting at higher levels of endotoxin. This may be because as endotoxin levels decrease, the endotoxin molecules are more tightly entangled or interact more strongly with the target molecule and become increasingly challenging to remove.

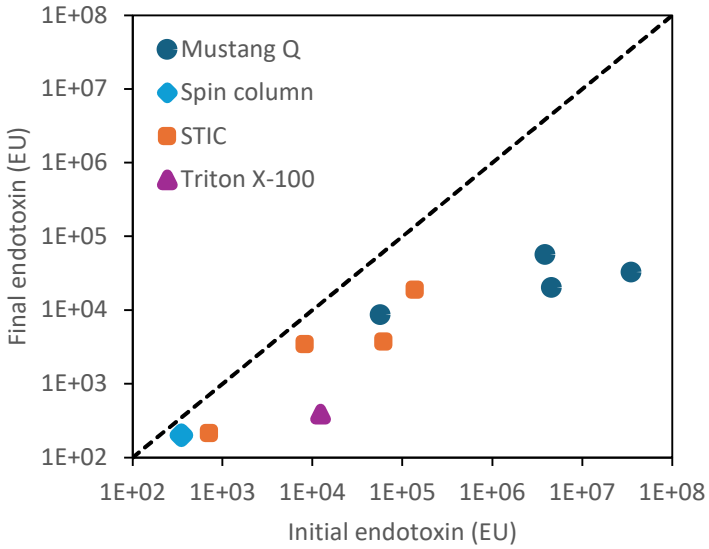

Figure 1: Final endotoxin plotted against initial endotoxin

Figures 1 and 2 graphically show the data in Table 3. In Figure 1, the final endotoxin is plotted against initial endotoxin and points under the dashed line are data points for experiments where endotoxin decreased. As the initial endotoxin increases, the vertical distance between the points and the dashed line increases, showing the higher percentage of endotoxin removed. In Figure 2, the log endotoxin reduction is higher when the starting amount of endotoxin is higher.

Table 4 shows the yield results. Most yields were relatively high, averaging 87%, though the yield was unable to be quantified for the Triton X-100 protocol due to absorbance at 280 nm from Triton that was not removed from the final product. This result indicates that the endotoxin removal products could decrease endotoxin while allowing most of the target molecule to be recovered.

The MQ and STIC processes both performed well and were effective at removing endotoxin while maintaining yield. The two membranes are scalable, either by adding more membranes in

series or increasing the size of the membrane. Both membranes can also easily by incorporated into a downstream purification process as an additional polishing column step.

The Triton X-100 protocol as performed here is not scalable and was somewhat labor-intensive and time-consuming. For scale-up it would need to be converted to or incorporated into a TFF process, and the shear sensitivity of the target molecule would need to be considered. The yield results showed that the procedure needs optimization to completely remove surfactant from the final product, and we recommend also considering other, more environmentally friendly surfactants.

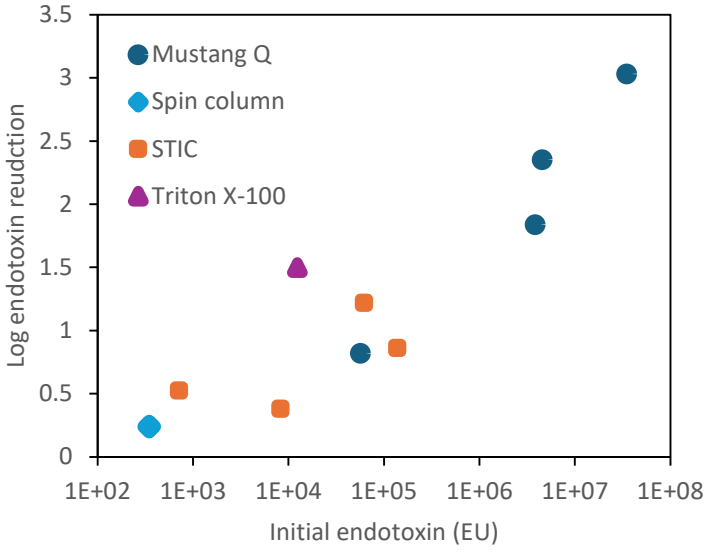

Figure 2: Log endotoxin reduction

The Proteus spin columns were simple and fast to use but are not scalable, so we recommend them only for small-scale development work if a particular test requires low-endotoxin material. The spin columns performed the worst at removing endotoxin. However, the starting material already had one of the lowest endotoxin levels of the samples tested and we should also note that the sample we used was frozen. The manufacturer recommends not allowing the sample to freeze before processing through the spin column because any aggregation caused by the freeze/thaw cycle makes the endotoxin harder to remove.

Conclusions

The use of bacterial fermentation to produce biopharmaceuticals can contribute to the overall endotoxin level of a process development lab. However, when compared to the endotoxin present in cell lysate from a bacterial fermentation, the environmental endotoxin level is negligible. Late in the purification process when endotoxin levels have greatly decreased, the product can be moved to a separate room away from bacterial material or a BSC to minimize endotoxin in the final product.

Although endotoxin is generally heat and pH resistant, it is susceptible to NaOH. When we tested various 0.5 M NaOH cleaning protocols, we found that all were effective at reducing endotoxin levels in equipment following use with bacterial-based materials, with greater reduction coming with increased contact time.

For the protein studied here, all endotoxin reduction methods were effective at removing endotoxin while maintaining good yields. We would not recommend the Triton X-100 surfactant-based protocol, however, since it was not easily separated from the final product, although the endotoxin level was reduced. Given its environmental toxicity and ban in the EU, we suggest other methods or a different surfactant, and the protocol would still have to be optimized to make sure no residual surfactant is found in the product. The spin column was somewhat effective and could be a good strategy for the process development scale if the endotoxin level of a final product needed to be lowered slightly for testing, but it is not scalable. The other two methods (MQ, STIC) were both effective and scalable, though would not be appropriate as used above for a negatively charged molecule. Overall, determining which method is most suitable for endotoxin reduction of a target molecule is heavily dependent on the particular molecule's characteristics, as well as factors including ease of use, operation time, cost, and scalability.

Table 3: Endotoxin reduction capabilities of tested procedures

Sample	Initial volume (mL)	Initial endotoxin (EU/mL)	Initial endotoxin (EU)	Final volume (mL)	Final endotoxin (EU/mL)	Final endotoxin (EU)	Endotoxin reduction
MQ (10 mL)	77	738	56800	143	60.4	8640	85%
MQ (60 mL)	553	6970	3850000	1130	50	56300	99%
MQ (2 x 60 mL)	3850	9090	35000000	4820	6.78	32700	99.9%
MQ (260 mL)	15800	287	4540000	17300	1.17	20200	99.6%
Spin column	11.5	30.3	348	11.9	16.8	200	43%
STIC (1 x 1 mL)	103	6.91	712	130	1.64	213	70%
STIC (2 x 1 mL)	275	< 500	< 13800	300	62.9	18900	< 86%
STIC (3 x 1 mL)	307	26.8	8230	350	9.77	3420	58%
STIC (4 x 1 mL)	1580	39.1	61700	1580	2.37	3740	94%
Triton X-100	36	342	12300	2.7	144	389	97%

Table 4: Protein recovery yield results for endotoxin reduction procedures

Sample	Initial volume (mL)	Initial conc. (mg/mL)	Initial protein (mg)	Final volume (mL)	Final conc. (mg/mL)	Final protein (mg)	Yield
MQ (10 mL)	77	4.0	306	143	2.0	281	92%
MQ (60 mL)	553	3.8	2110	1130	1.4	1570	75%
MQ (2 x 60 mL)	3850	0.82	3160	4820	0.63	3040	96%
MQ (260 mL)	15800	0.85	13400	17300	0.75	12900	96%
Spin column	11	4.4	48.5	11.9	3.8	45.5	94%
STIC (1 x 1 mL)	103	2.6	265	130	1.6	212	80%
STIC (2 x 1 mL)	275	1.3	358	300	0.84	253	71%
STIC (3 x 1 mL)	307	4.2	1470	350	3.4	1200	82%
STIC (4 x 1 mL)	1580	7.1	11300	1580	7.0	11000	98%
Triton X-100	36	0.78	28.1	2.7	N/A	N/A	N/A

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