PROCESS-SCALE CHROMATOGRAPHY

Sanitization of a Packed Bed in the VERDOT Ips² InPlace™ Process Chromatography Column

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Introduction

Process chromatography columns must provide a very high level of cleanability to meet the requirements of various regulatory agencies. The efficiency of the column cleaning process of must satisfy regulations, norms, and standards, such as cGMP, Norm ISO 13408, FDA 21-211.67, or USP 32 NF27.

Columns used for the downstream processing of biopharmaceutical ingredients must be regularly cleaned in place, typically by using a sanitizing agent. Sodium hydroxide (NaOH) is a commonly used sanitizing agent because of its efficiency against all living organisms, low cost, availability, ease of use, and compatibility with most materials used on biopharmaceutical hardware.

In this application note, we show using two different tests, that VERDOT Ips²'s InPlace columns (Figure 1) can be safely sanitized after bacterial contamination. A VERDOT Ips² InPlace column packed with Macro-Prep® High S chromatography media was contaminated with strains of Bacillus subtilis and Serratia marcescens, then tested for sterility after sanitization with 1 N NaOH using a sensitive and accurate method.

Two sampling techniques were used. The first method consisted of sampling the effluent rinse solution. The second method included resuspending the packed bed by injecting one column volume of culture media and allowing for growth of any remaining viable organisms.

Materials and Methods

Material and equipment used in this work are listed in Table 1.

Preparation of Bacterial Suspensions

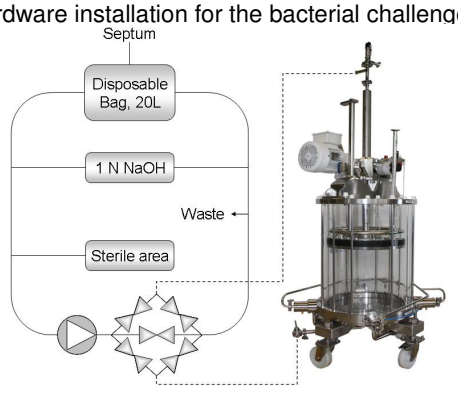
Bacillus subtilis (ATCC 6633) was chosen as a model organism for gram-positive bacteria and for its ability to resist harsh sanitizing conditions in the spore state. *Serratia marcescens* (ATCC 13880) was chosen as a model organism for gram-negative bacteria and for its capability to grow rapidly under anaerobic conditions. Bacterial cultures were aerobically grown in Tryptone Soya (TS) broth at 30°C for 16hr. *S. marcescens* was grown for 16 hr and *B. subtilis* for 72hr to allow it to sporulate.

Fig.1. VERDOT Ips² InPlace column

Table 1. Materials and equipment used in the experiments

Column	VERDOT Ips² InPlace column (D446 x H600 mm) with acrylic tube
Additional equipment	VERDOT Ips² media transfer device 50 (MTD50), manual process skid (1000 LPH), 20 L disposable bag, 50 L stainless steel 316 L tank (Figure 2).
Chromatography media	18 L Macro-Prep High S from Bio-Rad Lab.
Mobile phase	Deionized water 1 M sodium phosphate pH 7, sterile 1N Sodium hydroxide 37% hydrochloric acid (pH neutralization)
Culture medium	Tryptone Soya (TS), sterilized by autoclaving
Bacteria	*Bacillus subtilis*, ATCC 6633 *Serratia marcescens*, ATCC 13880

Fig. 2 Hardware installation for the bacterial challenge test



Slurry Preparation and Column Packing

The column was filled with 1 N NaOH with the adaptor positioned at a height of 200 mm. It was then sanitized by upflow circulation of 2.5 CV of 1 N NaOH at 200 cm/hr, followed by downflow of 2.5 CV at 200 cm/hr. The slurry transfer line was also sanitized by circulation of 1 N NaOH through the media transfer device (MTD) at 20 L/min for 15 min. This preliminary sanitization is not critical, therefore it was not evaluated for viable counts.

A 50% (v/v) Macro-Prep High S slurry in 0.1 N NaOH was injected into the column with the MTD. Once transferred, the slurry valves were closed and flushed with 1 N NaOH. The media was packed in pure axial packing mode with a piston speed of 200 cm/hr. When the piston was 1 cm above the consolidated bed, the speed was reduced to 50 cm/hr and the piston moved down to compress the bed to a final packed bed height of 103 mm, which corresponds to a compression factor of 1.1.

Column Sanitization before Inoculation

The packed column was sanitized with 1 N NaOH using the following steps:

1. 2.5 CV downflow at 100 cm/hr; to adjust for the dilution with the 0.1 N NaOH solution of the packed bed, 30% NaOH was added into the 1 N NaOH tank
2. 2.5 CV upflow at 100 cm/hr
3. Downflow at 200 cm/hr while the adaptor was raised 80 mm above the packed bed at a speed of 100 cm/h for sanitizing the seal of the adaptor
4. 2.5 CV downflow at 300 cm/hr
5. Downflow at 100 cm/hr while the adaptor was lowered down to the settled bed at a speed of 100 cm/hr

The process skid was then stopped and the adaptor was lowered to the original packed bed height at 50 cm/h. The pH was slowly adjusted with 30% HCl while maintaining closed loop circulation until the pH reached a stable value of 7.3.

Column Contamination

A mixture of 250 ml of *S. marcescens* and 250 ml of *B. subtilis*, each at 1.5 x 108 cfu/ml was injected at low speed through the column in upflow. The column was left for 12 hr with the isolation valves closed. A sample was then taken at the bottom connection of the column for quantitation by the most probable number method, which yielded a value of 9.2 x 106 cfu/ml for *B. subtilis* and 2.4 x 109 cfu/ml for *S. marcescens* in the column.

Column Sanitization

The column was sanitized with 1 N NaOH using the following steps:

1. 5 CV downflow at 200 cm/h; the first 8 L of effluent were sent to waste, then the liquid was recycled through the NaOH tank
2. 5 CV upflow at 200 cm/hr
3. Downflow at 200 cm/hr while the adaptor was raised 80 mm above the packed bed at the speed of 100 cm/hr for sanitizing the seal of the adaptor
4. 5 CV downflow at 200 cm/hr
5. Downflow at 100 cm/hr while the adaptor was lowered down to the consolidated bed at a speed of 100 cm/hr

The process skid was then stopped and the adaptor was lowered to the original packed bed height. During this final compression the speed of the piston was 50 cm/hr.

Neutralization and Sampling

After NaOH sanitization, the skid and the column were rinsed with 20 L of sterile deionized water. Following this, 15 L of sterile deionized water and 2 L of 1 M sterile phosphate buffer, pH 6.9 were injected into the process system.

During this operation, the fluid exiting the skid was sent to a disposable bag. Since the void volume of the complete circuit is approximately 20 L, a dilution of the phosphate buffer to 0.1 M resulted.

The phosphate buffer was circulated in a closed loop through the entire fluid path with the exception of the bubble trap which was in bypass mode. The pH was measured and slowly adjusted with 30% HCl while maintaining closed loop circulation until the pH reached a stable value of 7.0.

Three fractions (500 ml, 100 ml, 1 ml) were collected and mixed with the same volume of sterile TS broth, and incubated for 33 hr at 30°C under continuous agitation.

For a positive control, a 250 ml fraction was also collected, mixed with the same volume of sterile TS broth and inoculated with 100 cfu of each bacterium into an Erlenmeyer flask. The flask was left without agitation at room temperature for 33 hr.

Incubation of Chromatography Bacterial Growth Media

TS (7.5 L) was injected in the column in downflow while the piston was raised at 30 cm cm/hr. Then an additional 12.5 L of culture media were injected in upflow at the same speed for resuspending the chromatography media in the column and allowing any surviving bacteria to grow (Figure 3). The column was left at room temperature for 33 hr with the isolation valves closed.

Fig. 3. Resuspension of chromatography media with TS broth.

The incubation time was defined based on calculated generation times for the two bacterial strains. The generation time of *S. marcescens* in TS without agitation at 30°C was 33 min. The generation time of *B. subtilis* in the same media with agitation at 30°C was 30–40 min. Therefore, a 500 ml sample would need 29 generations for a single bacterium to reach 1 x 106 cfu/ml where it can be visually detected. This amounts to 16 hr for *S. marcescens* and 19 hr for *B. subtilis* at 30°C in TS under agitation.

In the case of the column containing 36 L of TS and chromatography media, it takes 35 generations for a single bacterium to reach 1 x 106 cfu/ml, which corresponds to 19 hr at 30°C. Hence, a 33 hr incubation was deemed sufficient for revealing viable organisms.

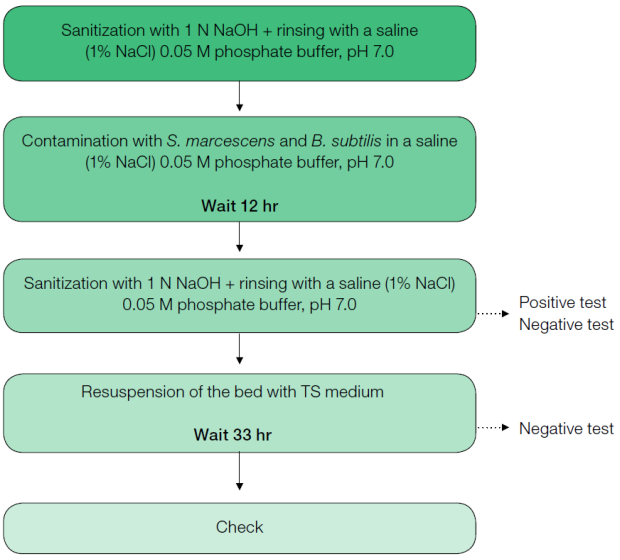


Fig. 4. Summary of bacterial challenge test procedure.

Results and Discussion

After 33 hr, the positive control, made of column buffer mixed 1:1 with TS and inoculated with bacteria, had a count of more than 1 x 1010 cfu/ml (from a starting count of 0.8 cfu/ml), indicating that the column buffer could promote the growth of viable organisms.

Two different tests were used to assess the efficiency of the sanitization with NaOH. Column buffer samples mixed with TS remained clear and free of growth after 33 hr of incubation, as indicated by an A 600 below 0.005. This showed that the contamination level of the sanitized column by viable *B. subtilis* or *S. marcescens* was less than 2 x 10–3 cfu/ml, indicating that the sanitization procedure led to at least a 12 log reduction in contamination.

The second test, consisting of direct incubation of growth medium in the column, also revealed that there was no growth after 33 hr as indicated by an absorption at 600 nm below 0.005.

B. subtilis, an aerobic bacterium, will not grow in the anaerobic conditions of in-column incubation. Therefore the in-column growth test could only detect *S. marcescens,* which can grow in anaerobic conditions. Hence, the absence of growth after 33 hr proved that all of the column and chromatography media surfaces had been successfully sterilized against *S. marcescens*.

The two tests can be qualified as orthogonal as the effluent sampling method provided lower sensitivity but was compatible with aerobic microorganism growth. On the other hand, the method with direct in-column incubation provided high sensitivity but showed lower compatibility with aerobic microorganisms growth.

Conclusion

In this study we have demonstrated that the VERDOT Ips² InPlace column has a sanitary design and that a successful cleaning procedure can be performed using this column. This also applies to VERDOT Ips²® EasyPack™ columns, which use the same product fluid path design.

This work also showed the advantages of the orthogonal sampling method used, which can provide high sensitivity as well as reduce the need for a sterile environment to only the area around the single injection point. Additionally, tests using a packed bed that require opening the column and rinsing the top piston prior to sampling introduce an additional cleaning step, which could lead to false negatives.

Acknowledgements

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