Sanitization of the Bio-Rad Process Chromatography Skid 00

Introduction

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

Equipment 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 demonstrate that Bio-Rad's Process Skid 00 can be completely sanitized after bacterial contamination. The wetted surfaces of the fluid path were contaminated with strains of *Bacillus subtilis* and *Serratia marcescens*, then tested for sterility after sanitization with 1 N NaOH.

Two sampling techniques were used. The first method consisted of sampling the effluent rinse solution. The second method included injecting culture media in the fluid path and allowing for growth of any remaining viable microorganisms. Opening the system and swab-testing was not considered in order to avoid false positive results.

Materials and Methods

Materials 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 16 hr. *S. marcescens* was grown for 16 hr and *B. subtilis* for 72 hr to allow it to sporulate.

Fig. 1. Bio-Rad process skid size 00.

Table 1. Material and equipment used in this work

System	Bio-Rad Process Chromatography Skid Size 00
Mobile phase	Deionized water 0.05 M sodium phosphate pH 7, sterile 1 N sodium hydroxide
Culture medium	Tryptone Soya (TS), sterilized by autoclaving
Bacteria	*Bacillus subtilis*, ATCC 6633 *Serratia marcescens*, ATCC 13880

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Sanitization Method

The method programmed for the sanitization process is outlined in Table 2. Designation of the valves and pumps is shown in Figure 2.

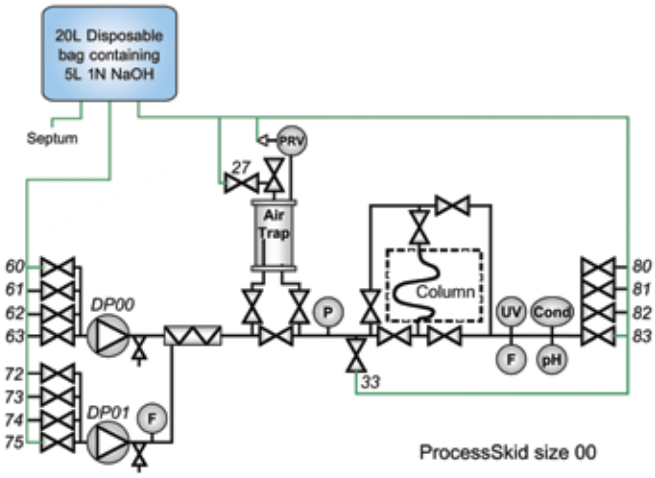

Fig 2. Schematic of Bio-Rad process chromatography skid 00.
For the bacterial challenge test the column was replaced by a flexible hose. **PRV**, pressure relief valve; **UV**, UV cell; **Cond**, conductivity meter; **pH**, pH probe; **F**, mass flow meter; **P**, pressure gauge.

Pre-Inoculation Sanitization

The entire fluid path of the system was initially primed with non-sterile water to remove air and any potential particles in the fluid path. The sanitization process was initiated with 1 N NaOH circulating in a closed loop via a stainless steel tank. After sanitization, the fluid paths of the skid were rinsed with 10 L of a 0.9% NaCl solution in 0.05 M phosphate buffer, pH 7.2. Because this preliminary sanitization was not critical, it was not evaluated for viable counts.

Inoculation

A mixture of 250 ml of *S. marcescens* (1 x 10^8 cfu/ml) and 250 ml of *B. subtilis* (1 x 10^8 cfu/ml) were injected using the two pumps at 20% of maximum speed through the process fluid path including the bubble trap and the column connections. The inlet valves (60–63 and 72–75) and the outlet valves (80–83, 27, and 33) were closed. All other valves in the fluid path were opened to allow the bacteria to invade the entire pathway. The skid was left for 12 hr at 25–27°C.

Post-Inoculation Sampling and Process Skid Sanitization

A sample was taken at the outlet of the skid to confirm contamination using the most probable number method, which yielded a value of 1.5 x 10^8 cfu/ml in the skid. The sanitization phase was then immediately initiated. The system was sanitized using the following steps:

- The sanitization method was performed with tap water to rinse the system
- The sanitization method was performed with 1 L of 2 N NaOH circulating in closed loop. The total holdup volume of the skid and flexible hoses is approximately 1 L; thus, the dilution led to an approximate final concentration of 1 N NaOH
- A sterile disposable bag containing 5 L of 1 N NaOH was connected to the system as shown in Figure 2. The sanitization method was executed again with NaOH circulating in a closed-loop using the sterile disposable bag instead of a tank. This minimized the number of connections that could have introduced external contamination

Neutralization and Sampling

After NaOH sanitization, the skid was rinsed with 10 L of 0.9% NaCl in 0.05 M phosphate buffer, pH 7.2 to neutralize the system. Three fractions (500 ml, 100 ml, and 1 ml) were collected from the skid outlet and mixed with the same volume of sterile TS broth. For a positive control, a 200 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 non-agitated at 25–27°C for 17 hr.

Table 2. Sanitization method

Step	Inlet #	Pump	Bubble Trap	Column	Outlet #	Flow Rate, L/hr	End of Step Volume, L
1	60	DP00	Through	Downflow	80 33 27	120 72	10 0.6
2	72	DP01	Through	Upflow	80 33 27	120 72	10 0.6
3	73, 61	DP00+01	Bypass	Bypass (50% each)	81 33 27	120 72	10 0.6
4	74, 62	DP00+01	Through	Downflow (50% each)	82 33 27	120 72	10 0.6
5	75, 63	DP00+01	Bypass	Upflow (50% each)	83 33 27	120 72	10 0.6
6	60, 72	DP00+01	Through	Bypass (50% each)	80 33 27 PRV (forced open)	120 72	10 0.6

In-Process Skid Incubation

Two liters of culture media were injected into the skid fluid path by the two pumps at 20% of maximum speed, through the bubble trap and the flexible hose (replacing the column). The skid was left for 19 hr at 25–27°C with the outlets and inlets closed and with the bubble trap inline and the column block in down flow mode (Figure 3).

Fig. 3. Incubation of bubble trap with TS broth.

Bacterial Generation Time

The bacterial incubation times were defined based on calculated generation times for the two bacterial strains. The generation time of *S. marcescens* in TS without agitation at 30°C is 33 min. The generation time of *B. subtilis* in the same media with agitation at 30°C is 30–40 min. Therefore, a 500 ml sample would need 29 generations for a single bacterium to reach 1 x 10^6 cfu/ml so that 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.

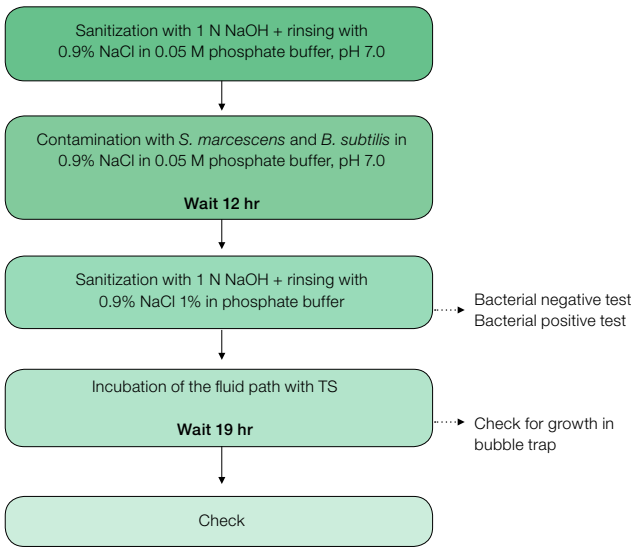

Fig 4. Summary of bacterial challenge test procedure.

In the case of the process skid containing 200 ml (holdup volume without flexible hoses, etc.), it takes 27 generations for a single bacteria to reach 1 x 10^6 cfu/ml, which corresponds to 16 hr at 30°C. A 19-hour incubation was used for revealing viable organisms (Figure 4).

Results and Discussion

After 17 hr, the positive control consisting of column buffer mixed with TS (1:1) and inoculated with bacteria, had a count of more than 1 x 10^8 cfu/ml, indicating that the process skid buffer could promote the growth of viable organisms. After 19 hr, the TS media samples (500 ml, 100 ml, and 1 ml) taken from the skid outlet, were still clear and free of growth, as confirmed by an A_{600} below 0.005. This indicates that the contamination level of viable B. subtilis or *S. marcescens* was less than 2 x 10^{-3} cfu/ml, demonstrating 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 process skid, also revealed that there was no growth after 19 hr as indicated by an A_{600} below 0.005

B. subtilis, an aerobic bacterium, will not grow in the anaerobic conditions of the fluid path. Therefore this test could only detect *S. marcescens*, which can grow in anaerobic conditions. Hence, the absence of growth after 19 hr proved that all of the wetted surfaces had been successfully sanitized against *S. marcescens*.

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

Conclusions

In this study we have demonstrated that the Bio-Rad Process Skid 00 has a sanitary design and that a successful cleaning procedure can be performed. This does not preclude users from testing the cleaning of the process system from contaminants under their own conditions. 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.

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