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Application Note

Influence of Environmental Sample Pre-Storage on Counting Results

Environmental monitoring using culture media in the form of settle and contact plates is a crucial part of aseptic manufacturing. Sampling is generally carried out in cleanrooms and then transferred to an outside lab space for incubation and evaluation of the results. The manufacturing process does not always allow for immediate transfer of samples to the incubator space. Therefore, the samples may be pre-stored at ambient room temperature or in refrigerators until the official incubation is started.

Objective

This study investigates the influence of different interim storage conditions with regards to the following:

- Growth of selected microorganisms
- *In situ* surface sampling on naturally contaminated surfaces

Gamma-irradiated casein soya bean digest agar (TSA) with 4 neutralizers was chosen as the test media (TSA w. LTHThio Contact ICR+; Cat. No. 146783).

Interim storage conditions were chosen based on different lab practices and are listed below:

1. The sample is directly incubated at 30 °C to 35 °C after surface sampling or inoculation
2. The sample is stored at room temperature for 16 to 18 hours after surface sampling or inoculation and then incubated at 30 °C to 35 °C
3. The sample is stored at 4 °C to 8 °C for 2 hours after surface sampling or inoculation and then incubated at 30 °C to 35 °C
4. The sample is stored at 4 °C to 8 °C for 6 hours after surface sampling or inoculation and then incubated at 30 °C to 35 °C
5. The sample is stored at 4 °C to 8 °C for 16 to 18 hours after surface sampling or inoculation and then incubated at 30 °C to 35 °C
6. The sample is stored at 4 °C to 8 °C for >55 hours after inoculation and then incubated at 30 °C to 35 °C

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Method and Results

Part One: Influence of interim storage on selected microorganisms

The test strains were selected based on their general occurrence in cleanrooms (3), their relationship to the human skin microbiome (2) and on recommendations for growth promotion tests on casein soya bean digest agar acc. to US and European Pharmacopoeia (1, 4). The selected strains and incubation conditions after sampling and direct storage conditions are listed in Table 1.

Table 1: Selected test strains and incubation conditions

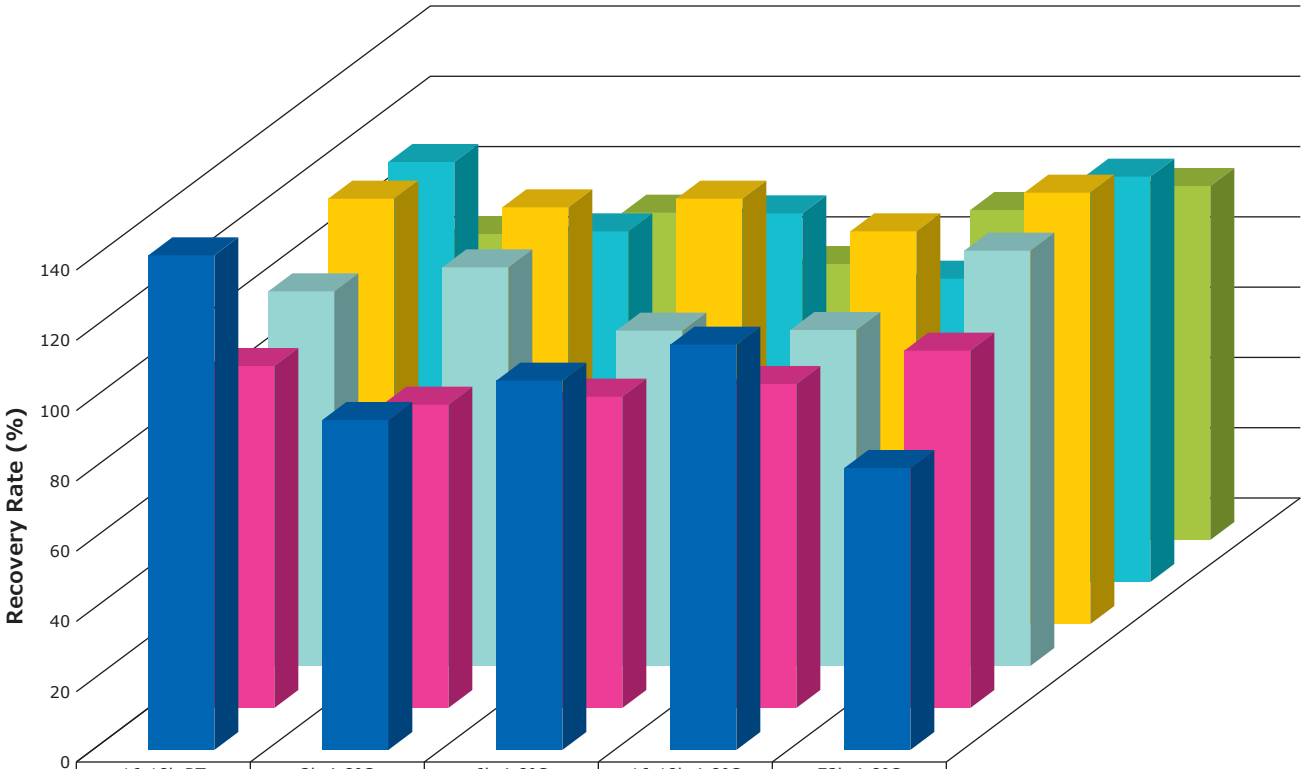
Test Strain	ATCC® Number	Direct incubation conditions (excluding interim storage conditions)
Staphylococcus aureus	6538	Aerobic; 24 hours at 30-35 °C
Kocuria rhizophila	9341	Aerobic; 48 hours at 30-35 °C
Staphylococcus epidermidis	14990	Aerobic; 48 hours at 30-35 °C
Cutibacterium acnes (formerly *Propionobacterium acnes*)	6919	Anaerobic; 10 days at 30-35 °C
Corynebacterium striatum	7094	Aerobic; 48 hours at 30-35 °C
Escherichia coli	25922	Aerobic; 24 hours at 30-35 °C
Penicillium commune	10428	Aerobic; 5 days at 30-35 °C
Surface Samples		Aerobic; 3 days at 30-35 °C

An inoculum of fresh overnight cultures was prepared and diluted to achieve approx. 10 to 120 CFU to be inoculated on the surface of the test plates. Each test condition was performed in two independent test runs with a five-fold repetition in each run. For the interim storage condition of >55 hours at 4 °C to 8 °C only one test run was performed. The recovery rates were calculated to the average CFU on the plates without interim storage.

Recovery Rate = (CFU – test) × 100 / (CFU – direct-incubation)

Recovery rates of the two independent rounds demonstrated the same trends. Therefore figure 1 and 2 show only the results for one test round.

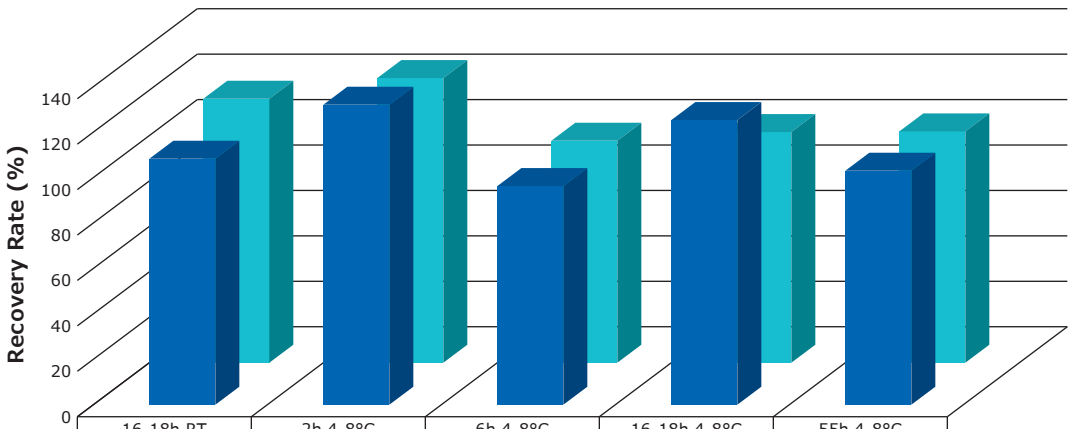
The results for the aerobic test strains are indicated in figure 1.



	16-18h RT	2h 4-8°C	6h 4-8°C	16-18h 4-8°C	72h 4-8°C
S. aureus	144	93	105	115	80
S. epidermidis	96	86	88	91	101
C. striatum	106	112	94	95	117
K. rhizophila	121	118	120	111	122
P. commune	119	99	105	86	116
E. coli	86	92	78	93	100

Figure 1: Recovery Rates (%) of selected microorganisms after each interim storage condition compared to direct incubation

In addition, the interim storage for *Cutibacterium acnes* was performed both anaerobically and aerobically. The results for this test strains are indicated in figure 2.



	16-18h RT	2h 4-8°C	6h 4-8°C	16-18h 4-8°C	55h 4-8°C
aerobic interim storage	108	131	95	124	102
anaerobic interim storage	113	123	96	100	100

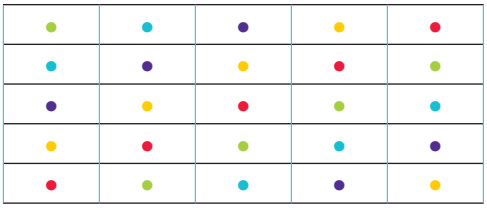
Figure 2: Recovery Rates (%) of *Cutibacterium acnes* after each interim storage condition compared to direct incubation

Part Two: In situ samples

Surface samples were taken from a non-classified laboratory environment in 20 rows and 5 positions per row using a TSA w. LTHThio Contact ICR+ contact plate (article 146783). The sampling pattern is demonstrated in table 2. These plates were incubated for 3 days at 30 °C to 35 °C following the interim storage conditions. Finally, the colonies were counted and a comparison was made for each of the interim storage conditions. Please find the various interim storage conditions listed below:

- The sample is directly incubated at 30 °C to 35 °C after surface sampling
- The sample is stored at room temperature for 16 to 18 hours after surface sampling and then incubated at 30 °C to 35 °C
- The sample is stored at 4 °C to 8 °C for 2 hours after surface sampling and then incubated at 30 °C to 35 °C
- The sample is stored at 4 °C to 8 °C for 6 hours after surface sampling and then incubated at 30 °C to 35 °C
- The sample is stored at 4 °C to 8 °C for 16 to 18 hours after surface sampling and then incubated at 30 °C to 35 °C

Table 2: Sampling pattern for the first 5 rows (row 6 to 20 will repeat this pattern every 5 rows)



The results are indicated in figure 3. The average of all test samples is comparable for most interim storage conditions with 10 or 11 CFU. A longer interim storage for 16 to 18 hours at cool temperatures resulted in a lower recovery rate with an average of 6 CFU. This is also indicated in figure 3 for all test results per interim condition. It seems that cool storage conditions might reduce the recovery of some microorganisms, which do not grow properly afterwards in incubation.

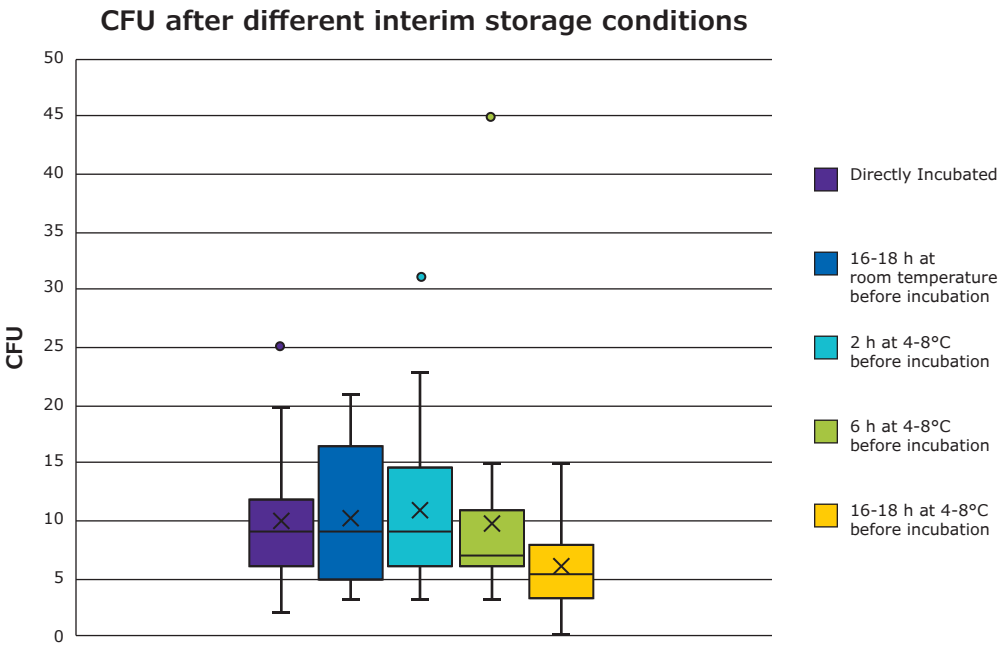


Figure 3: Recovery of microorganisms at each interim storage temperature

Summary and Conclusion

This study showed that there was no influence on the recovery rate of the selected microorganisms *Staphylococcus aureus*, *Kocuria rhizophila*, *Staphylococcus epidermidis*, *Cutibacterium acnes*, *Corynebacterium striatum*, *Escherichia coli* and *Penicillium commune* when subjected to different pre-storage conditions prior to standard incubation. All recovery rates were between 70% and 200%.

The data presented in this study revealed no influence on the growth promoting properties of the samples if they were stored at room temperature for up to 18 hours or placed in cool storage for up to 6 hours. A slightly decreased recovery rate was obtained for samples which were pre-stored for longer than 6 hours at cool temperatures before incubation took place. Storage at room temperature after sampling and before official incubation seems to be the preferable choice, because this temperature will already support growth of a variety of microorganisms. But on the other hand, the presence of molds might bear the risk of overgrowing if exposed to prolonged storage at room temperature.

The slight difference between the direct inoculation study (Part I) and the *in situ* study (Part II) to test pre-storage of 16-18h at 4-8 °C might be explained by the different composition of the *in situ* microflora.

Additional studies would be necessary to verify the difference.

Literature

1. European Pharmacopoeia 9.8 (2019): 2.6.12. Microbial examination of non-sterile products: Microbial Enumeration Tests
2. Grice E. A. and Segre J. A. (2011) The Skin Microbiome. Nature reviews Microbiology 9, 244-253
3. Sandle T. (2011) A Review of Cleanroom Microflora: Types, Trends and Patterns. PDA J Pharm Sci and Tech Vol 65, No 4
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