Article

Why Mold Detection Method Matters

Mold detection is extremely important, so why do people use the wrong methods?

Protection of cleanroom environmental quality is a battle on 3 fronts, organisms from the air, organisms living on/in surfaces, and organisms from human activity/interventions in the manufacturing process. When organisms are found, investigations to determine the identification of the contaminant help to define the possible route of entry into the facility. Of the organisms likely to be encountered, the molds create a heightened level of concern due to their ability to sporulate and quickly spread. Molds can also grow inside dry walls and are difficult to detect and remove.

Unfortunately, efforts to release pharmaceutical products faster and more cost-effectively have often undercut the efficacy of mold detection.

Examples include:

- Streamlined environmental monitoring (EM) incubation strategies that risk under-reporting many species of mold and yeast.

- Media choices that leave critical coverage gaps in mold detection.

Given that the struggle between corporate profit and product quality will only continue in years to come, let's review some potential pitfalls that leave many companies exposed to mold risks with poor quality risk management and potential contamination events.

Traditional vs Rapid Methods

Traditionally two media types were used for the detection of environmental organisms present, TSA for bacteria and SDA for yeasts and molds and two temperatures 30-35°C and 20-25°C. To add specificity for mold, chloramphenicol was added to the SDA media to inhibit bacterial growth (EuPh 2.6.12, now removed to harmonize to USP <61). The media formulations for EM were also combined with neutralizing chemicals to inactivate disinfectants used to sanitize the facility. The disinfectant residue could be picked up during surface or air sampling to be deposited on the media surface, rehydrate and generate a false negative. As companies focused on the financial bottom line for testing, the rationalization was made that one TSA based media plate could recover all the "critical" organisms found in their facilities. To optimize the recovery of organism species with TSA, early strategies used serial incubations to maximally recover bacteria and molds, usually low (20-25°C) for 5 days followed by 3 days at high (30-35°C). The inverse schema was also used but was not as common. In a survey performed by the PDA in 2017 61% of the respondents used low to high incubation, while the remainder were divided 12% high to low serial and 26% at a single temperature (mostly 30-35°C).

With the development of commercial pressure to release product faster and the evolution of rapid microbial methods (RMM) many companies reviewed their EM incubation strategy with a goal to reduce incubation times further. Initially changing TSA based media from a serial incubation with a

5–14-day total incubation to a single temperature incubation at 30-35°C with a 3–5-day release. With the introduction of automated RMM an incubation time of ≤3 days can be used. During these developments a wide range of incubation temperatures have been evaluated using single temperatures at 22.5°C, 25.0°C, 27.5°C, 30°C and 32.5°C as the set point depending on the company preferences. In all cases the change in incubation media, temperature, and time were all validated for recovery of equivalent numbers of organisms using both pharmacopeial and in-house collection species. The selection process for the in-house test organisms can lead to false confidence in the test if suitable test organisms are not chosen that would highlight sensitivity to incubation conditions.

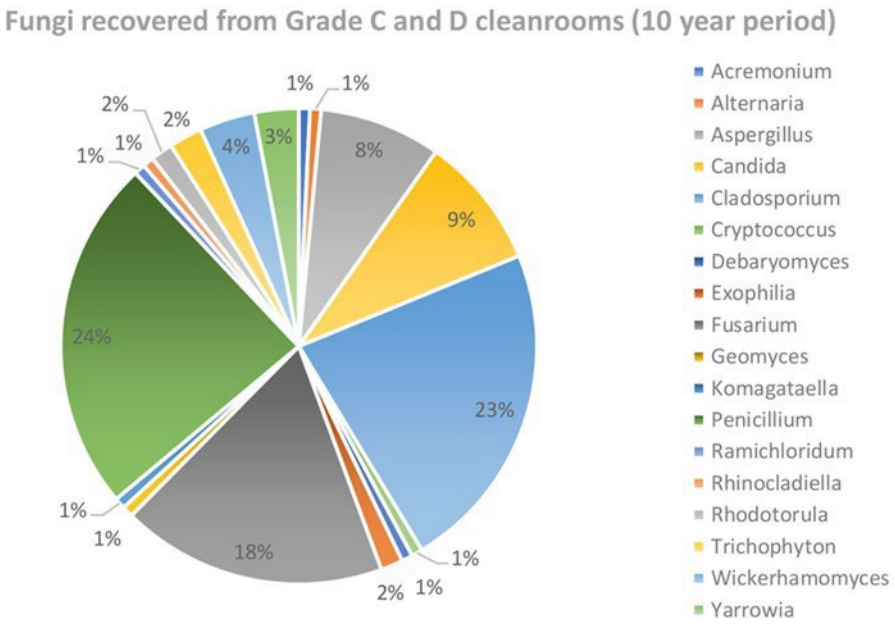

Figure 1 – Shows the reported frequency of the mold species found in manufacturing suites over a 10-year period incubated on TSA based media at 20°C - 25°C, followed by 30°C - 35°C. Sandle et al.

Avoiding Self-Inflicted Risks

Frequently, papers describing changes to EM incubation strategy include comments to under recovery of mold.

> *"All fungal recoveries, with the exception of Cladosporium spp., were superior at 20–25°C incubation. Recovery at 30–35°C was very poor for all fungi and yeasts"*. Symonds et al.
>
> *"recovery of moulds was found to be highly inefficient at 30 –35°C compared to lower incubation temperatures. This deficiency could not be rectified by subsequent incubation at 20 – 25°C". Gordon et al.*

In each case, under recovery was ascribed to certain mold species being intolerant of temperatures above 30°C.

Over time it has become clear that there are many species of mold and yeast that are not only temperature sensitive in the classically defined 30-35°C temperature range, but also at lower temperatures, 22-28°C. Table 1 shows mold species and the optimum temperature for growth. Those in red do not grow at 32.5°C.

"Optimal" Growth Temp				
22°C	22°C to 28°C	28°C	28°C to 32°C	32°C
Alternaria sp EM	*Penicillium notatum*	*Penicillium citrinum*	*Trichoderma asperellum*	*Curvularia hominis*
Alternaria species	*Aspergillus versicolor*	*Curvularia verruculosa*	*Fusarium keratoplasticum*	*Aspergillus fumigatus*
Arthrinium arundinis	*Penicillium Camemberti*	*Chaetomium globosum*	*Aspergillus flavus*	*Aspergillus terreus*
Aspergillus basiliensis	*Penicillium verhogenii*	*Curvularia pallescens*	*Curvularia lunata*	*Curvularia pseudobrachyspors*
Cladosporium herbarum	*Penicillium rubens*	*Chaetomium indicum*		*Hamigera insecttiola*
Penicillium roqufortii	*Penicillium chrysogenum*			*Cladosporium cladosporoides*
Exophiala xenobiotica	*Aspergillus caesiellus*			*Tricophyton interdigitale*
Exophiala lecanii-cori	*Aurobasidium pullulans*			*Tricophyton rubrum*
Ramichloridium ancep	*Epicoccum nigram*			*Phialemonium obovatum*
Geomyces pannorum	*Saccharomyces cerevisiae*			*Acremonium chrysogenum*
Geomyces asperulatus				*Aspergillus niger*
Penecillium olsonii				

Table 1. Molds grown on TSA based EM media containing neutralizer (Lecithin and Polysorbate) at temperatures from 22°C to 32.5°C. Optimum incubation temperature defined by larger colony size and faster speed of growth.

Validation of the EM method is heavily influenced by the natural variability inherent to microbiology. Organism distribution in air or across surfaces both in terms of numbers and species is varied. When comparing numbers or variances, small numbers of a species can get lost amongst a larger number of other species and the difference is not detectable by the statistics. Hence a few molds, growing at 22.5°C in the low to high serial incubation strategy, may not grow at 32°C in a single temperature study but their absence is not detectable by the statistics and the methods are defined as equivalent.

The incubator used for incubation can skew the data; most companies use the pharmacopeial range during temperature mapping of 32.5±2.5°C. The temperature cutoff for some molds is very sharp so they grow at 31.0°C but are killed at 33.0°C. That range can be found during a temperature map so depending where in the incubator the plates are placed, the mold may or may not be detected.

A general approach recommended for EM is to run TSA at a single temperature 30-35°C but to also run SDA incubated at 20-25°C at intervals (the sampling interval relevant to the risk of mold contamination), Gordon et al.

For in process bioburden testing of sterile products the EMA guideline on the sterilization of the medicinal product, active substance, etc. calls out the use of TSA @ 30-35°C for 5 days. This is separate to the nonsterile release in USP Ch <62> where pharmacopeia call out the use of TSA at 30-35°C and SDA media at 20-25°C. The temperature sensitive molds on EM plates are also sensitive when run on the bioburden TSA format using filtration on a membrane. The question then becomes "Is there a quality risk difference between EM and bioburden testing in regard to mold detection capability?". With sterile manufacturing a temperature sensitive mold may be missed in the EM testing but could be caught with in process testing on the SDA media. If only TSA at 30-35°C was used, the organism would need to be

captured by the sterility test. The reliability of the sterility test to detect a low-level contaminant has been the topic of much discussion over the years as batch sizes increase, but test sample numbers do not.

Recent presentations at PDA have shown that some RMM sterility tests, BacT Alert, England et al, may not be able to detect some mold species using the standard consumables but require an extra media canister. Similarly, a bioburden release test performed without SDA could miss a mold contaminant.

With the ongoing battle between corporate profit (faster/cheaper) and product quality, a solid microbiological risk assessment focusing on the accuracy, specificity, and robustness of the analytical methods employed should be performed. The assessment should cover EM through in-process and final release testing.

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