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This article presents strategies to improve bioreactions by reducing contaminations by adventitious agents.

Simple Strategies to Improve Bioprocess Pure Culture Processing

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Bioreaction and fermentation processes of all scales – from 100,000 liter vessels to small development reactors – require pure culture for their successful, productive operation. In this article the term "pure culture" and "pure culture capability" will be used instead of "sterile" or "sterility assurance" to acknowledge that bioreactions are, by nature, the process of growing large populations of helpful microorganisms or cells as opposed to the more unwanted varieties. Contamination by adventitious agents costs time, money, and lost productivity; moreover, contaminants and their sources can be very difficult to locate and eliminate.

Many elements of pure culture bioprocess design and operation are straightforward, often even a matter of common sense. Most modern facilities have pure culture capability built into their design, including latest technology and control systems, and correct operating procedures to ensure sterility is achieved and pure culture maintained. However, these controls will become less effective over time, due to deterioration, obsolescence, loss of experience, process changes, and personnel turnover. Sometimes, many years of solid performance can lead to a false sense of stability, which leads to complacence (or a lack of attention to pure culture capability requirements) inevitably followed by one or more Foreign Growth (FG) episodes.

Ultimately, a process with robust pure culture capability is the goal of both technical support staff as well as management. It is clearly beneficial to *proactively* manage pure culture design, capability, and practices before problems occur rather than create unintentional sterility experts out of your support staff as they struggle with root cause investigations instead of more productive efforts.

Contained below are a number of strategies, illustrative case studies, and techniques to help maintain or improve pure culture capabilities of traditional bioprocess reactors and fermentation processes. They are targeted squarely for the practitioner in their simplicity, and based on many years of managing pure culture operations at many scales. The focus of this article is on preventing *bacterial* contaminations in traditional fermentation systems; much of the material applies to protection of *any* bioreactor systems from *any* adventitious agent. It is up to you to understand your system and process and to appropriately apply the principles outlined in order to meet the requirements of your process and business. Higher potential risk from longer growth times, longer inoculum trains, and lack of selective agents (e.g., antibiotics) can be offset through the use of disposables, newer equipment, and more stringent environmental and raw material controls.

The focus of this work is divided into four basic sections: 1. design aspects for sterile operations; 2. common contamination root causes; 3. troubleshooting FG events; and 4. applying rigorous microbiology to better understand and improve pure culture. Additionally, several examples and case studies are included to illustrate and emphasize concepts.

Facility Design and Sterilization Best Practices

The scope of this article is for scientists and engineers supporting existing bioreactor processes. However, a short survey of current sterile design best practices will be helpful to improve or troubleshoot your axenic process. It is fundamental to realize that *prevention* of FG is the most important factor to long-term successful pure culture performance.

Sterilize in Place (SIP) System Design Considerations for Sterile Operations

Design for optimal sterilization is covered in many texts and articles.[1,2] Main points are essentially as follows:

- Using steam, quick heat up of all points in the sterile boundary to 121.1°C (121.1°C is the United States Pharmacopeia (USP) standard sterilization temperature with a minimum moist heat sterilization requirement of 15 minutes.)[3]
- free drainage of condensate
- easy displacement of air
- replace collapsing steam with sterile air (collapsing steam creates vacuum, which must be avoided at all costs)

These, and other factors to consider are described below:

Sterile Boundary – process piping connected to sterilized equipment must be sterilized up to and through the closest valve which isolates the sterile from the non-sterile system. Another way to isolate the sterile system is through an appropriate 0.2 micron-rated sterilizing grade filter. Other sterile boundaries include vessel walls themselves, mechanical seals (subject to pressure gradient), feed nozzles, internal cooling coils, rupture discs, sterilizing filters, steam traps, exhaust lines, and o-rings (elastomers) on instrument ports. See a more detailed discussion of the sterile boundary, located in the next section on contamination root causes.

Disposables – disposable bioreactors and attachments are gaining in popularity because they can reduce risk of cross-contamination between cell culture batches, while providing flexibility, minimizing turnaround time, reducing cleaning costs, and easing validation restrictions. Additionally, disposables typically have fewer connections (sterile boundary points) than fixed reactors, which provide incrementally better FG protection. (Obviously, due to scale of disposables, this may not be feasible for large scale fermentation operations.) As bioreactor demands increase, the tradeoff between flexibility afforded by disposables will be outweighed by increasing costs and will justify more traditional fixed bioreactor systems. Increasing titers will continue to shift this tradeoff toward disposables.[4,5,6]

Steam – steam must not be superheated or diluted. First, a quick steam refresher: saturated steam is steam at its boiling point for a corresponding pressure. This differs from *superheated* steam, which is steam heated to a temperature *higher* than the boiling point at a given pressure. One reason superheated steam is undesirable for bioreactor sterilization is because it has further to cool in order for it to transfer its heat of vaporization, making it less efficient than saturated steam. Relatedly, *diluted* steam is steam that has air or other gases mixed in the vapor, which can be observed by steam temperatures that are lower than expected for a given pressure or higher observed pressure for a given temperature on the saturated steam curve.

Condensate – since condensate forms throughout steam sterilization cycles, systems must be designed for quick and complete drainage to a low point where a steam trap is installed.

Air Removal – air must be completely displaced by saturated steam for sterilization to be effective. Air must be either pulled by vacuum or displaced effectively by the steam itself. Typically, air is discharged through a sterilizing filter.

Cold Spots – temperature measurement must include the coldest spot in the system to ensure that all points are held above sterilization temperatures. Redundant temperature measurement is essential to verify sterilization temperatures are maintained.

Equipment Drains – all areas in process equipment must be totally drainable. Ideally, all equipment surfaces should drain toward one common bottom outlet (each drain point is a potential cold spot). A steam trap should be installed to remove condensate in this outlet.

Setup: During an investigation of a recurring Foreign Growth (FG) contamination series on a large microbial fermentor, experienced operators noted that the equipment SIP dynamics had shifted in the past few weeks. Specifically, the system was reaching correct sterilization temperatures, but was at a higher pressure in order to meet the required temperature. Additionally, FG contamination events were intermittent and also seemed to correlate with high level or "foam out" events in the system.

Resolution: Detailed investigation found that a routine automation change on some unrelated control parameters inadvertently and subtly altered sterilization logic. The SIP logic change resulted in a minor delay in the cycling of certain valves that feed steam into the vessel's Vapor Liquid Separator (VLS), a small, separate vessel above the main fermentor. The delay in introducing steam caused air to remain trapped in the VLS, essentially "insulating" the VLS vessel from sterilization temperatures due to steam dilution. Foreign microbes, escaping proper sterilization conditions at these points, were allowed to gain a foothold on the VLS surfaces (presumably in cracks, crevices, corners, etc.). When foam or high level reached these contaminated surfaces, some refluxed back into the fermentor, carrying FG with it that eventually caused the tanks to become rife with foreign growth.

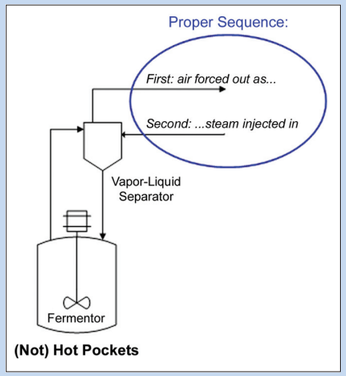

Figure 1. Displacing air with steam during fermentor SIP.

Lessons Learned:

- The shift in temperature vs. pressure observed by the operators was an indicator of trapped air in the system, because as the air dilutes that steam and a higher pressure is needed to reach the sterilization temperature.
- All process indicators showed that the equipment was reaching sterilization temperatures, but the increased air in the VLS tank created an insulated pocket and this localized area was not reaching correct temperature.
- Always need to be vigilant on unintended consequences of changes, as well as pay close attention to changes in the process. Nobody knows the process better than the operators, and this experience can be a valuable troubleshooting resource.

Table A. Case study – (not) hot pockets.

Pure Culture Bioprocessing

Setup: Microbial fermentor experienced sporadic foreign growths, especially common during periods of high tank volume or "foam outs." Extensive investigation into source of contamination did not reveal any sterile boundary flaws except a very minor one: the insulation around the top of the Vapor Liquid Separator (VLS) had been removed for repair work some time before, and was not replaced.

Resolution: Replacing the insulation decreased the frequency of foreign growth events, but did not eliminate them. Further investigation revealed that the VLS had a stationary Clean-In-Place (CIP) spray ring with spray holes drilled on the side of the ring, not the bottom, so that the cleaning solution was ejected horizontally outward from the spray ring instead of vertically downward.

Consequently, the spray ring was not free draining and always had a heel of water lying stagnant within, where environmental bacteria could gain a foothold between runs. Sterilization process in the upper portion of the VLS was adequate for surface sterilization, but was not as effective in sterilizing the heel of water when the insulation was removed and was occasionally ineffective even with the insulation in place. When the process broth or foam refluxed to the upper portion of the VLS, some of the foreign bacteria would be swept back to the main reactor, causing contamination.

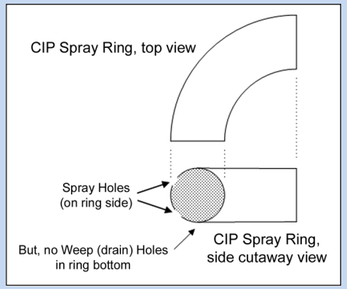

Figure 2. Schematic sketch of an internal CIP spray ring with no drain hole.

Lesson Learned: An effective CIP design, the spray ring was not a good aseptic design for fermentation. Besides assuring that criteria for vapor liquid separation, cleanability, and surface sterilization be considered throughout the VLS qualification, good concepts for pure culture design should have been considered as well.

Table B. Case study – hidden spray ring design flaws.

Piping and Pipe Slopes – process piping to be sterilized should be configured to completely drain back into the equipment if possible, minimizing the number of separate drain points. Sterilized piping should be sloped to eliminate holdup points. Slopes need to be much greater if against the direction of steam flow. Never branch a line from the bottom because it could promote condensate buildup. A key quality of pipe insulation is the ability to wick moisture and freely drain so that it won't retain leaks (wet insulation is less effective and provides potential cold spots in steam seals).

Elastomers – o-rings, gaskets, and such are often a critical element of the sterile boundary simply because they have no backup in case of failure. Thus, they need to be designed with the optimum material of construction for conditions of the bioreactions (which usually means the temperature exposure from SIP) and replaced on a set frequency rather than be allowed to run to failure.

Valves – clearly, not all valves are equal in sterile services. For valves sterilized through, diaphragm valves with high temperature-rated diaphragms are better than ball valves because of the difficulty cleaning behind the ball. However, ball valves typically hold up better in steam services. For diaphragm valves, care must be taken to control steam temperature, flow, and differential pressure across the diaphragms in order to prolong service. Use of condensate seals as opposed to steam seals also can prolong valve elastomer life.

Trade-Off between In-Line and Off-Line Monitoring Devices – in some cases, the number of required in-line monitoring devices can be reduced by using external lab sampling or indirect relationships between key operating parameters. The appropriate number and location of analytical instruments and in-process checks must be reconciled against: 1. capital and operating cost constraints; and 2. pure culture concerns, due to the fact that an increasing number of instrument ports raises FG risk to the bioreactor.

Dead Legs – the piping configuration of the sterile system is one of the most critical attributes that contributes to maintaining a system free of FG. Avoiding so called "dead legs" is crucial. Basically, a dead leg is defined as a one-way system, typically on the end or a branch of a piping distribution system, which results in a process hold-up area that is difficult to clean and sterilize. The ASME BPE standard suggests that bioprocessing systems, such as fermentation, along with other bioprocesses, should be designed with a target L/D ratio of 2:1. L is defined as the length of the dead leg extension measured from the ID wall normal to the flow pattern. D is diameter of the dead leg extension. This tight ratio ensures that process piping can effectively complete SIP and CIP cycles without concern of building up cleaning solution or condensate which could contribute to sterilization failures and FG events.

Agitator Shaft Seals – double-mechanical seals are standard. The key idea is to ensure that seals in pure culture operations are lubricated with a sterile fluid, such as steam or clean condensate.

Setup: A new state-of-the-art bioprocess facility shortly after start-up was experiencing foreign growth events in a particular bioreactor. Swabbing of locations was used to determine hiding spots of the contaminating organism. Removal and swabbing of the pH probe ports on the bioreactor found the organism of interest lingering behind the pH probe o-rings (i.e., outside the sterile boundary).

Resolution: The o-rings on the pH probes had a small nick or deformation that allowed media from the vessel to migrate into the nonsterile region behind the probe o-ring. This area outside the o-ring would not reach sterilization temperatures, providing a place for foreign agents to reside and proliferate on the media. Contamination of the fermentor occurred when the invading microbes migrated backward through the defect into the axenic contents of the fermentor.

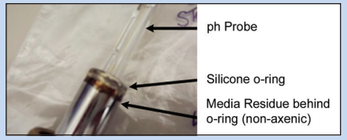

Figure 3. Internal pH probe with media leaking past o-ring.

Lesson Learned: The practice of routine o-ring replacement was instituted and the material of construction was optimized to minimize swelling and deterioration through SIP cycling. Additionally, the o-ring groove design was optimized to further reduce leak-by.

Table C. Case study – elastomer headache.

State of the art sterile design equipment and components are often costly, and typically, only able to be justified by facilities that produce high-value specialty chemicals and pharmaceuticals. These include large-pipe diameter diaphragm and sanitary valves, sanitary tubing, specialized aseptic fittings, removable components and instruments, automated SIP/CIP systems, and ultra pure water for process use. Still, reliable pure culture capability can be achieved with lower cost designs; for example, standard industrial valves with welded pipe connections, more rigorous SIP/CIP procedures, and valve/piping preventative maintenance are all cost effective ways to improve pure culture capability. (It is worth noting that validation of processes using lower cost designs can be more difficult and maintenance costs higher.)

Contamination Root Causes

A Foreign Growth (FG) is essentially a failure: 1. of the process to either kill all adventitious organisms at the start through the sterilization processes; or 2. to successfully keep the process isolated from outside invaders. Or, more simply put, you didn't kill them or keep them out. Furthermore, the system fault can be grouped into one (or several) of the following:

- Design and Sterile Boundary
- Equipment
- Human Error [Procedures, Execution]

Design and Sterile Boundary Faults

As previously discussed, the sterile boundary is defined as the point in your system where you plan to maintain an environment free of foreign microorganisms. Any breach of (or migration across) sterile boundary has potential to bring FG into the system. Therefore, the first goal in protecting your system from FG is to know your boundary.

You need to understand not only every element of the sterile boundary, but also how the boundary's sterility might change over time. An imperfect sterile boundary condition – for example, a small crack in a weld – might maintain sterility if the pressure differential is always favorable, but if the pressure equalizes or if vacuum were to develop in the process at some point, even for an instant, then sterility will no longer be maintained. So thorough process understanding is essential, including microbial challenges in and around the boundary and how and when process interacts with boundary points (steam, feeds, process, gases, etc.).

The most common sterile boundary failure is, of course, a leak. Leaks in a sterile system provide a route for bacterial contaminants to enter your process, a nutrient-rich, climate controlled environment reserved for your chosen cell line. Foreign bacteria can either "ride in" or "grow in." "Ride in" is where the contaminant is present in a feed or gas and carried into the process. "Grow in" is where the contaminant finds a fault in the system, proliferates at a leak point, and eventually migrates into and through the leak point, sometimes even against a flow gradient.

Leaks and defects are a natural consequence of inevitable system decay, so identifying and eliminating leaks in your process is a continuous challenge. A proactive leak detection program where systems are periodically inspected and leaks repaired prior to operation is essential for successful pure culture capability. System pressure checks and light gas (hydrogen, helium) checks are standard tools to check for leaks in the sterile boundary, but have limitations. For example, if a line leaks upstream of a valve at the reactor (referred to as a "near-to" valve), a pressure check won't find the leak since the sterile boundary is the "near-to" valve. Also, some defects do not become detectable until after the extreme heating and cooling cycles of SIP, at which point a pressure check may already have been completed. So these tools must be combined with other proactive efforts to detect leaks, from thorough preventative maintenance to as simple as a detailed visual and hands-on inspection.

Sometimes leaks are simple to find by inspection. Figure 5 is a picture of a leaky valve (the valve handle cannot be seen due to the angle of the photograph) in a sterile fermentation dextrose feed line. A messy leak such as this may not result in an immediate FG, but nonetheless should be repaired as soon as detected. An organizational tradition that encourages frequent visual and hands-on inspections, as well as vigilance and wariness of all leaks, is consistent with long-term, foreign-growth free operation.

Setup: In a microbial fermentation facility, a system started to have repeat contamination events associated with one seed vessel.

The seed vessel employed two rupture disks in series on vent line from Vapor-Liquid Separator (VLS). Rupture discs had burst disc indicators to alarm in case of vessel over pressurization. There were no burst alarms and by visual inspection, rupture discs appeared to be intact.

Resolution: Over many years, both rupture discs on the VLS had developed pin-hole leaks which allowed non-sterile moisture and environmental microbes from the top side of the discs to reflux into the vessel and cause foreign growth events. This was confirmed by sampling and bioburden assays.

The rupture disc design was non-optimum, and consisted of a membrane sandwiched between a stainless steel upper and lower piece, where the membrane provided a sealing surface for the disc. As the system underwent many heat cycles from SIP, the stainless sections had worn small holes in the membrane, creating a breach in the boundary.

Moreover, the placement of the rupture discs was poor. A breached disc would allow non-sterile material to directly fall back into the process.

Finally, the additional failure mode of the system which made it difficult to troubleshoot was the burst disc alarming mechanism. The burst disc indicator was not showing that the disc had a failure (as it was not a rupture of the disc); therefore, the operations staff initially did not realize that there had been a rupture disc issue.

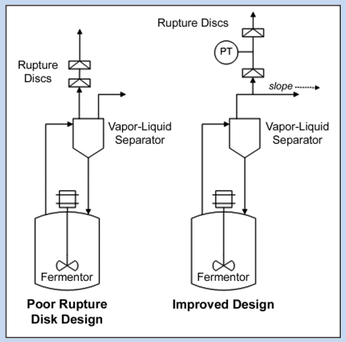

Figure 4. Poor rupture disc design and the improvement.

Lessons Learned:

- A new design was installed (solid piece of stainless steel) that was less likely to develop leaks from SIP cycling.
- If possible, it is better to have the rupture disc located in the exhaust system and orientated in a manner that if the disc were to leak, contaminants could not enter the vessel.
- The recommendation out of the investigation was to install a pressure transmitter between the two rupture discs to determine, based on pressure, if the main near-to disc had lost integrity.

Table D. Case study – poor rupture disc design.

Figure 5. A leaking dextrose hand valve.

Equipment Faults

Equipment faults as a source of foreign organisms may simply be the mechanism by which a sterile boundary leak develops, such as a sterilizing filter flaw, a weld defect, or an imperfect o-ring. In addition, as equipment ages, faults and defects begin to arise that could compromise sterility. Also, even though sterilization and cleaning was qualified/validated for the equipment at one point, over time the system might decay in subtle ways, creating equipment defects that could alter the dynamics of the system to create sterilization issues. Simple illustrations: debris in a spray ball that could alter cleaning patterns in a vessel and allow for media hold-up or a weld defect could harbor pockets of unsterilized FG.

Small patches of media that accumulates and builds up in the vessel, either from poor cleaning, incomplete draining, or exposed seams, seals, defects, etc., will over time, become insulating and prevent heat penetration during SIP. Eventually, this will become a spot to harbor foreign microorganisms.

Consider the case of bolted and screwed connections in a bioreactor as a source of media buildup. Bolted connections are less prevalent in newer vessels, but still exist in many bioreactors, especially legacy fermentors. Bolts, screws, and washers will occasionally and unpredictably loosen from repeated heating and cooling cycles, creating pockets and crevices for environmental bacterial contaminant to fester. Over time, media or biofilm buildup will create insulated pockets and allow colonies of foreign bacteria to survive sterilization, which eventually contaminate the vessel.

The solution is to remove as many bolted connections as possible – replace with welded connections – and for those that remain, institute a periodic inspection cycle to remove, clean, and replace worn out connectors with new ones.

However, with the transition to more welded connections, weld integrity becomes the new point of emphasis in the discussion of FG prevention.

Weld defects can have the same material hold-up implications as bolted connections by allowing bacterial contaminants a place to fester and perpetuate in a system. This is especially true when a weld defect is in a location where it can become hard to sterilize. When welding stainless steel, it is critical to maintain temperature of the weld material with heating and pace of the weld both impacting weld integrity. Welding too fast, for example, can place waves in the weld material, which create pockets where material can migrate or corrosion can take hold, or worse, be inaccessible to SIP steam. Temperature of the weld is critical at overlap points between two welds, especially at the end of a welding pass. If the weld at an overlap becomes cool, an open pocket at the seam of the weld can result. An example where this defect can be found is when there is a circular or square pad welded onto a vessel wall with the end of the weld overlapping the starting point. Figure 7 is a photo of a welded pad in an older fermentation vessel which contained weld defects that may have contributed to FG problems.

When locations and welds like the one shown in Figure 7 exist, the best approach is to grind away all suspect areas,

Setup: A large legacy fermentor had been experiencing intermittent foreign growth events. Investigation included a detailed internal inspection to locate potential defects where contaminants could be held up in the system. Inspectors noted that the fermentor had a threaded nozzle on the inside of the vessel that was plugged and no longer in use.

Resolution: This particular threaded plug was located on the internal head space of the fermentor. The cavity space on the top side of the plug, just past the sterile boundary, was filled with standing moisture, grime, and oil that had seeped from the top of the fermentor agitator gear box over a long time. After years of SIP temperature cycling, the threaded plug was found to be leaking a minute amount, intermittently introducing contaminates – both microbial and traditional – into the fermentor's axenic contents. Incidentally, the leak was not detected by routine pressure checks because it was so small.

Figure 6. Leaking internal threaded connection in legacy fermentor.

In order to remedy this issue, the threaded plug was welded closed and vented caps were placed on the atmospheric side of the nozzle. The vented cap would prevent the accumulation of oil and dirt from building up again inside the unused head space nozzle.

Table E. Case study – threaded connection space invaders.

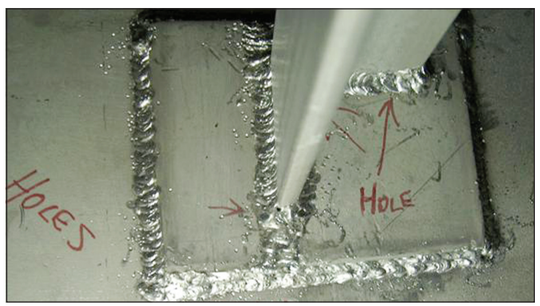

Figure 7. Bioreactor internal welds displaying defects.

re-weld, and finish the weld to a smooth finish. This finishing of the weld to a smooth surface will significantly reduce the opportunity to hold up material around the weld and also be easier to inspect.

Human Error [Procedures, Execution]

No matter how much a process is automated, human intervention is still essential for all pharmaceutical manufacturing. In one sense, every FG root cause can be traced back to human error: from error in design, to errors in maintenance, operation, procedures, handling non-routine events, negligence, or even sabotage. The following section illustrates a few key issues associated with human decision-making, judgment, and operator technique that could affect pure culture operations.

Preventative Maintenance

Preventative Maintenance (PM) is clearly essential to maintaining reliability, safety, and pure culture capability of biotech manufacturing facilities (regardless of the age of the equipment). However, PM work itself is only half of the strength, the other half is designing an effective PM *schedule*: understanding the systems enough to determine both **what** needs to be done, and also the correct **frequency** of when it needs to be completed. Unfortunately, to make the optimal decisions, different technical expertise is often needed for different types of equipment. And if that weren't complicated enough, the pressure to minimize *non-urgent* maintenance costs (by definition, *preventative* maintenance is always non-urgent) is usually prevalent. It is beyond the scope of this work to cover the correct PM work and frequency for all sterile barriers, but here are a few examples to illustrate the importance of the concept:

- Steam Traps: a steam trap will no longer be an effective sterile barrier if it is either malfunctioning or set-up incorrectly (i.e., bypassed). Therefore, all critical steam traps need to be on some kind of PM plan. And, considering the cost of a "run to failure" maintenance strategy for your sterile boundary, the steam trap "PM" may actually need to specify *replacing* the trap at regular intervals. Traps have been known to malfunction if they go unused for an extended period of time, so if your equipment is idled, pay special attention to the traps upon restart (you may even want to run a sterile hold test after long periods of facility idle time).

  Most importantly, it should be the responsibility of the operators to inspect critical sterile boundary traps to ensure they are set-up properly, and then temperature check (via adjacent temperature sensors or even a temperature stick) the trap at the proper location to ensure that it is currently functioning. This obviously can't be done on a PM since it needs to be done for every run so it needs to be spelled out in the batch record or on a separate checklist.

- Valves: the diaphragm in diaphragm valves will wear out over time so it is essential to inspect and replace them on a periodic basis. The exact timing should be based on the frequency of use and exposure to high temperatures, etc.

  Likewise, the ball surface or socket in ball valves also can develop defects that could harbor FG. A ball valve will typically stand up to harsher services longer than a diaphragm valve so that will need to be factored into the PM schedule.

- Vessel: fermentors and bio-reactor internals should be inspected regularly by experienced sterility experts (in addition to vessel experts) to ensure that they remain free of corrosion and defects that could cause media hold-up and eventually lead to a FG.

- Elastomers: elastomers used in and around fermentors to seal connections should be replaced regularly to ensure they don't wear or crack in service. Again, a "run-to-failure" strategy is generally not advised since they are a key element in the sterile boundary, as described above.

- Miscellaneous connections: some preventative activities will be unique to each process. For example, on the piping manifold external to a large-scale fermentor, there was a threaded nipple connection for the occasional addition via a portable inoculum vessel (similar to a threaded hose connection port you have at home). The nipple was normally sealed with a screwed plug and kept under steam block. However, the connection went for many years without being used, and eventually the screwed connection loosened to the point where it would no longer hold pressure. During a standard sterilization process on the vessel, steam collapsed near the connection, and outside, non-sterile air was drawn into the tank post sterilization and caused a contamination. To prevent recurrence, a start-up checklist was developed to include ensuring the nipple was tightened, among other sterility checks.

Operator Technique

In an ideal world, procedures and batch records would be completely objective and able to be followed in a standard, repeatable way, every time. However, in the real world, some operational steps require a certain manual technique gained through experience or coaching to be performed optimally.

An example where good operational technique is required would be the process of transitioning from deadheaded steam to sterile feed in a pipe, such as manually filling a sterilized feed header. After closing all steam traps, while the header is still pressurized, the steam must be closed *while* (or *immediately* before) the feed header is being opened to fill the line. Technique (or a precise automation sequence) is critical because the steam must *not* be given a chance to collapse (creating trapped vacuum) and the header must remain pressurized with steam or feed at all times.

Changes

GMP operations require a formal change control process to ensure that product Safety, Identity, Strength, Purity, and Quality (SISPQ) and process safety are not negatively impacted by process or equipment alterations. For bioprocess operations, it is equally important to carefully scrutinize pure culture impact, both intended and unintended, with just as much emphasis as process safety and SISPQ.

Contamination Investigation and Recovery

If you have supported bioprocesses for very long, you have experienced a FG contamination in your tenure. And if you have been unlucky enough to deal with multiple contaminations, you know that each event is wholly unique. Unfortunately, the investigation into the root cause can be similarly unique with no way to predict what factors and conditions might be significant. Indeed, sometimes events outside of your control or changes external to your process facility could be key causal factors in an eventual contamination (see Table F for a case study illustrating this fact).

Nonetheless, even though a comprehensive investigation and recovery guide cannot be developed to cover every FG incident, there is enough in common with any FG investigation that a general strategy can be formed.

The first step in troubleshooting a FG event is to determine if there are any abnormalities observed in process operation. Adverse trends can often suggest where the FG event originated. Conversely, FG or sterile boundary flaws also can be non-detectable by continuous process monitoring measurements. The worst luck of all is to have a system that fails intermittently or in some non-repeatable pattern. The next section will spell out investigation points that have yielded success in troubleshooting FG events.

The challenge of the investigation is to find and fix the design/sterile boundary, equipment, or human factor faults. Table G provides a sample checklist for attacking a FG investigation systematically. A discussion of the key actions contained in the checklist follows. Remember, just because something has worked or been maintained correctly in the past is *no guarantee* that it is *not* an issue now.

Setup: Process air is fed to a large fermentation facility from a plant utility at a separate location. As a part of the compression process, the air is heated to potentially sterilizing temperatures. It is then cooled at the utility facility by large shell and tube heat exchangers with chlorinated tower water.

The fermentation facility began to experience significantly higher rates of foreign growth. The entire process and sterility controls were checked and rechecked for vulnerabilities, but nothing was found that suggests root cause for increase in foreign growth incidents.

Resolution: Finally, the utility department discovered that the air cooling shell and tube heat exchanger has developed a large leak in the tubes, leading to a significant amount of non-sterile tower water sparging into process air. Compounding this problem was the fact that the air filter design for the fermentors was such that free water can promote channeling through the filters as well as alter key electrostatic capabilities of the filter that aid in bioburden removal.

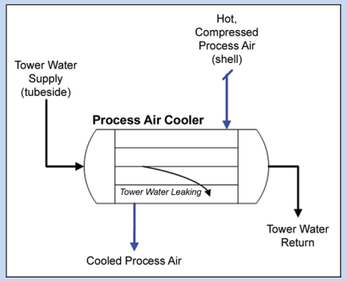

Figure 8. Compressed air cooler with a tower water leak.

The only clue that the fermentation support staff could have used was the dew point meters on their process air. But these were not checked during the intense phase of the investigation. (Admittedly, if the supply air had been sampled, increased bioburden might have been detected, but this action is normally not employed because of the lack of a bioburden baseline or control data for the non-sterile supply).

The real issue in this case was a lack of a preventative maintenance plan on the heat exchanger tubes. They were inadvertently set up to "run to failure;" however, production consequences of this failure were either not fully understood or likely never considered at all.

Lesson Learned: Besides the obvious lesson of looking outside facility boundaries for root causes of poor performance, it is the realization that you need to keep bioburden challenges down around process sterile boundaries. After all, for example, no filter – even a well-designed HEPA – is 100% effective.

Table F. Case study – process air challenge – literally.

Time is of the Essence

If you are able to detect a FG while your process is in operation (as opposed to a post-production analytical contamination test), it is critical to inspect the "on-run" condition of the process, including feed tanks, seed vessels, bioreactor/fermentor, headers, valves, and so on. You are looking for any set-up faults, unusual observations, leaks or other upsets, process alarms, cold spots, or visual faults.

Go for Data

The next step is generally to capture as much data as possible about the process and FG, such as:

- age of FG
- pattern of FG
- any recent changes or unusual observations
- identity of FG
- history of vessels
- recent audits/inspections/Environmental Monitoring (EM) data
- utility upsets
- foaming issues

Widen the Search

To continue to widen the data search, investigate automation and process profiles from your data visualization system, including batch plots, sterilization temperatures, control valve positions, back pressures, feed flows and timing, any process interventions, or unusual previous metabolic trends.

Performing post pressure checks or more sensitive checks with light gases (hydrogen or helium) will help to locate leaks that may have appeared at SIP or on-run. An important note: pure hydrogen should never be used as trace gas. A standard industrial grade mix of five percent hydrogen in nitrogen is used for modern leak detecting. This mix is inexpensive, non-flammable (per ISO standard #10156), easily available, and still holds the important features needed for using hydrogen as trace gas.

Reviewing the batch record and procedures for comments/remarks, as well as interviewing operations personnel associated with the run, can help uncover any unusual execution

- ☐ If possible, as soon as foreign growth is detected, examine on-run condition of process (vessels, feed tanks, headers, etc.)
  - ✓ Valves set-up properly
  - ✓ Steam traps set-up properly and sufficiently hot
  - ✓ Leaks
- ☐ Isolate and identify foreign organisms
- ☐ Gather relevant process data, including tank/process history
- ☐ Check automation/computer profiles of feed tanks, inoculum, and fermentor...
  - ✓ Batch plots
  - ✓ SIP temperatures, including temperature control valve positions
  - ✓ Backpressures
  - ✓ Feeds and timing, including feed control valve position if continuously feeding
  - ✓ Other process interventions
- ☐ Review manufacturing batch record and procedures for observations/remarks
- ☐ Track recent history of facility (environmental monitoring, cleaning, etc.)
- ☐ Note any equipment or process changes
- ☐ Identify recent maintenance that has been performed on the system; check work notes for observations
- ☐ Check for recent process upsets or deviations
- ☐ Interview operations personnel who set-up and monitored process
- ☐ Integrity check and inspect any process air filters
- ☐ Leak check tanks, valves, flanges, and piping
- ☐ Check calibration on temperature probes
- ☐ Inspect pH, DO, etc., probes (install new probes if applicable)
- ☐ Inspect rupture discs
- ☐ Internal vessel inspection
  - ✓ Obvious visual defects – initial inspection
  - ✓ Other tank defects – conduct a very thorough examination of the tank walls and interior hardware
  - ✓ Agitator shaft and seal areas
  - ✓ In a tank with older welds: visual inspection, dye penetrant check, flame check, X-ray examination
  - ✓ If the tank has internal coils, pressure, or leak test
  - ✓ If the tank has internal bolted connections, inspect them for hold-up
  - ✓ Swab suspect areas and test for organism of interest
- ☐ Perform SIP cycle, check all areas within the sterile boundaries to ensure areas are heating up to target temperatures, utilizing probes, temperature sticks, IR technology, etc.
- ☐ Carefully consider changes or shifts to processes and facilities outside of your immediate control (air, water, utilities, media, etc.)
- ☐ Brainstorm other less likely scenarios with investigation team; follow-up and check off items
- ☐ Formulate "return to service" strategy

Table G. Foreign growth investigation checklist.

issues. Tracking recent facility data (such as EM and cleaning records), compiling recent equipment and process changes, and reviewing recent maintenance work (check work notes if available) all help to identify where the facility or its design might be migrating from the original design capability.

Get Hands-on

Walk the system; look for anything out of the ordinary; including: leaks, cold spots, steam traps set up incorrectly, incorrect connections, etc. Inspect and integrity check air filters. Remove (and check calibration on) temperature probes, pH, DO, etc., probes to inspect the probe and o-ring/gasket and groove.

Time to Open Up

If efforts to locate the root cause from the above actions are not successful, a more thorough internal inspection process may be warranted. Internal inspections should include any of the following relevant checks: obvious visual defects, subtle defects detected by a very thorough examination of all wall and internals surfaces, agitator/shaft vulnerabilities, weld defects or hold-ups (typically older tanks), coil leaks (if any tank has internal coils, they always need to be inspected, and should be on a regular inspection program), and bolted connection defects (again, typically older vessels).

Experimental

This is hypothesis and scenario testing. For instance, if the sterilization process is suspected, conduct sterilization runs in which as much of the area within the sterile boundary can be checked to ensure it is meeting the minimum temperature. This can be as sophisticated as temperature mapping or Infra Red (IR) sensing technology to the low-tech temperature stick check of lines. Investigative media holds are another example.

As the investigation proceeds, microbiological data on the adventitious agent could be essential to understanding the FG and its root cause. The discussion below will provide details on how understanding the microbiology of the FG agent will aid the investigation.

Microbiology Investigation

To begin, you must understand the sensitivity and limitations of your FG control strategy and test methods. This understanding is crucial to evaluating the impact to process quality and understanding how and at what point the FG may have been introduced into the process. Some critical factors associated with a control system include sample frequency, test volume, testing media, test method, risk of false positives, confirmation testing, retest/resample options, and so on.

By definition, FG testing is screening for the presence of a small population of unknown organisms within a high background of known organisms; thus, it is essential to have a method for isolating the foreign organism so it can be identified and evaluated. Selective broths and agars are indispensable in this activity. Streaking for isolation onto non-selective agar from a test plate or tube may be sufficient to gain isolated colonies for identification. However, odds are greatly increased if selective agars are employed to inhibit the growth of the production culture and/or stimulate the growth of the potential contaminant. This is illustrated in Figures 9 and 10.

Once the foreign organism is isolated, appropriate identification testing should be completed. Biochemical and/or genetic ID methods are useful in comparing one isolated organism to another (i.e., from a different location or an earlier FG event) to confirm a potential common source. Confirmation of the contaminating organism as genetically identical within the limits of the method may be helpful in focusing the investigation on a common system or alternatively, focusing the investigation on independent root causes.

Studies to determine phenotypic characteristics for carbon/nitrogen utilization and growth rate in the production medium, as well as media for a "media hold study," should be initiated concurrent with ID of the isolated organism.

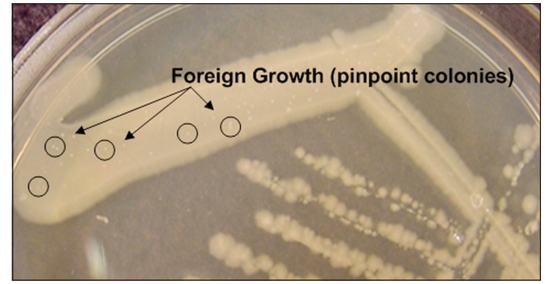

Figure 9. Non-selective agar plate. Note visible foreign growth (pinpoint colonies) mixed within the lawn of production microbial cells.

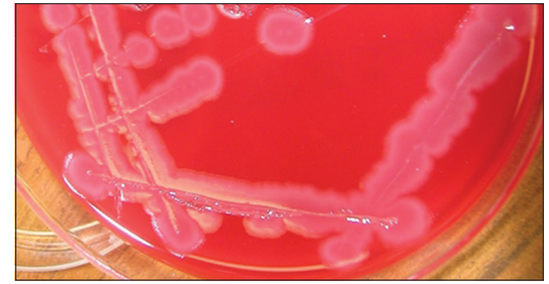

Figure 10. Selective Columbia CNA agar plate with Bacillus foreign growth (pinpoint colonies from Figure 9) isolated from Gram negative production cells.

(**Media hold** refers to a "sterile" test run of the fermentor and associated systems utilizing a suitable nutrient medium capable of supporting growth of likely foreign organisms.) Phenotypic characteristic studies will facilitate hypotheses about the potential source of the organism and potential time of ingress. They also provide a foundation for determining an appropriate media hold strategy.

If antibiotics are included in the production medium for plasmid selective pressure or are being produced by the culture determining the Minimum Inhibitory Concentration (MIC) for the FG organism is again beneficial in theorizing potential root cause and time of ingress.

Caution should be taken when considering non-routine testing of systems that have no baseline data for comparison. In this situation, to avoid erroneous data or interpretations, additional work may be required to demonstrate recovery of various organisms and to 'qualify/validate' the test method.

Fit for Purpose Strategy

The ideal scenario, but unfortunately not realistic, is to have a bioreaction system that could be sterilized and remain free of FG for an infinite period of time. Further, a decision to initiate media holds to confirm process capability following a FG event is not trivial since it may have a huge impact on your ability to return the equipment to service. Therefore, it makes sense to define a "Fit for Purpose" timeframe based on the process being run, and then design your media hold test strategy to demonstrate system capability within this timeframe. Again, it is worth noting that the discussion below is derived from a traditional microbial fermentation process. However, the principles can be applied to any bioreactor system by adjusting for specific process and business requirements.

Proper Fit For Purpose definition of your equipment scope, medium, end time, sampling/testing strategy, and number of repetitions is crucial to maintaining operational flexibility. For instance, it is recommended to define end time based on your process cycle time (or alternatively, the longest process time performed in that equipment set), plus an appropriate safety factor.

Safety factor can be represented by both additional hold time and testing sensitivity. The longer you hold your medium, the more likely to detect FG if it is present. Also, the larger volume tested, the more likely you are to detect FG. An appropriate combination of these factors should allow you to obtain a suitable safety factor that can be agreed upon by both technical and quality partners.

For more rigorous sterility challenges, such as mammalian cell cultures, a fit for purpose study might be focused specifically on maintaining sterility for the required process time plus an additional hold time as a safety factor (granted, for very long perfusion-type bioreactions, even maintaining a sterility test for the length of the process might not be a practical restart condition). For shorter microbial fermentations overcoming a specific bacterial contamination, a different approach can be employed. For example, a process takes 24 hours from SIP to harvest and typically tests 50 µL (5×10^{-5} L) of pre-harvest broth. A FG is detected and analyzed to have a one-hour doubling time. A media hold is designed to test 5mL (5×10^{-3} L) of broth and extend hold time by six hours to a total of 30 hours. This provides a 100X ($5 \times 10^{-3}/5 \times 10^{-5}$) sensitivity increase from volume and a 64X (six additional hours equals six doubling times = 2^6) increase from hold time for a total safety factor of $100 \times 64 = 6{,}400$X.

Growth testing of each batch of sterile hold medium with suitable FG organism(s) is required to confirm the validity of your hold medium. At the very least, this should include the current organism of interest and may include previous FG organisms and/or USP organisms.

If the investigation has not yielded a root cause, a more extensive, *investigative* media hold can be a beneficial tool to uncover the source of the contamination. However, this should be initiated only after establishing your Fit for Purpose acceptance criteria in order to avoid getting operationally limited by unreasonable expectations. A typical Fit for Purpose hold strategy may include all feeds added at the start of the hold period to demonstrate that the system, as a whole, can remain free of detectable FG for the required Fit for Purpose time frame. In contrast, an investigational media hold may separate these feed additions by an appropriate hold time to allow for identification of a contaminant source. The amount of each feed added should be based on an adequate volume representative of the process. The hold time between additions needs to be long enough to reasonably detect a contaminant if

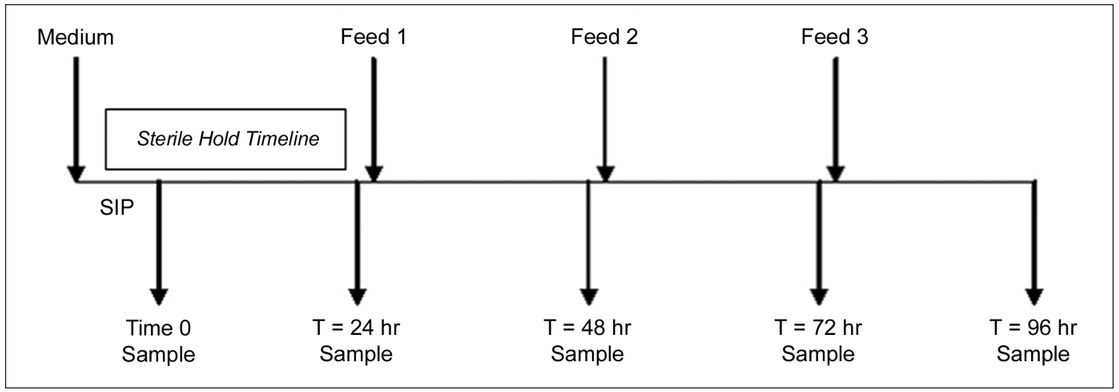

Figure 11. Timeline for investigative media hold detailing feed and sample times.

introduced. Again, you can increase your test volume in order to increase test sensitivity and reduce the time interval. See Figure 11 as an example of an investigative media hold based on a 24-hour addition interval.

Conclusion

FG events in fermentation processes are a significant cause of factory loss and reduced productivity, to what degree depending on the process being run and the tolerance for FG. Natural product processes may suffer a loss of productivity or a shift in factor ratio as a result of FG but the broth may still be harvestable. Most microbial and cell culture processes have a zero tolerance for FG so any detectable FG represents a complete loss.

As the biotech industry matures improvements will be made in the following arenas: reactions will become more concentrated as titers and specific activities increase, disposable systems will become more prevalent as smaller amounts of the therapeutic compound are required, operational excellence will continue to improve as companies become more skilled at running bioreactions (or outsource them to specialized third party contractors), and equipment will perform more efficiently – and more reliably – through engineered improvements. As a company evolves on these fronts so does their competitive advantage in the biotech industry.

Correspondingly, as bioreactions become more concentrated and reactors more specialized, the need to improve pure culture capability becomes more critical to achieve this competitive advantage. Basic fundamentals that are well-understood and practiced for decades are implemented to achieve successful operations with low contamination rates. Why then do contaminations continue to plague bioreactions even as technology improves and the industry becomes more mature? A recent study found that the contamination rate in production-scale bioreactors is still over 2%.[17] The answer is it takes more than good sterile design to eliminate contaminations; human factors and system decay, combined with adventitious agents that continuously and relentlessly probe every possible and improbable vulnerability, are all invariably working against your pure culture operation.

In this work we have illustrated the basics of sterile design and sterilization. We have detailed through examples and case studies the importance of building an organizational tradition where bioreactor staff are always vigilant regarding system decay, changes in equipment or procedures, and who are dedicated to preventative measures to keep systems performing at peak. Moreover, when contaminations occur, we describe how effective and comprehensive investigations are managed, including the microbiological elements required to minimize future recontamination by the same adventitious agent.

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