White Paper: Recovering Lyophilized Anaerobes

Lyophilzation, or freeze drying, is used to preserve perishable materials, with minimal alteration. It makes storage, usually at room temperature, and transportation of the preserved material convenient. Lyophilization is regularly used for preserving and storing microorganisms. First, the cells are made into a dense suspension in a medium formulated for this purpose. The medium likely contains cryoprotectants. The suspended cells are distributed into vials or tubes. The suspension is frozen, a vacuum drawn, and heat is applied under controlled conditions to cause water to sublime. At the end of the process the vacuum is broken, nitrogen gas replaces the vacuum, and the tubes or vials sealed. The residual water content of lyophilized material is about 1-4%.

As you can surmise from the brief description, lyophilization is a complex process that includes many variables that can affect the outcome. These include microorganism, equipment, suspending medium, procedure used, and operator. Most results on lyophilization are presented anecdotally. Some lyophilized cells are reported to survive storage for decades; while for others survival is measured in months. Since freezing is a part of the process, some cell damage due to freezing is likely. What matters most to the end-user is that you can recover the target microorganism from the lyophile (refers to the freeze-dried microbe independent of form).

A source of quantified data on recovery of lyophilized microbes is from CAP Surveys (1). Lyophilized samples of microbes are sent to thousands of hospital laboratories for them to recover and identify the microbes. The laboratories do not know in advance the identity of these microbes. For the anaerobe challenge, a facultative microbe is included in the same lyophile incorporated into a swab. The hospital laboratories report their results back to CAP who collates and publish them for Survey participants who say that isolation and identification of anaerobes from the lyophile is significantly below that of the facultative microbe. Many times the anaerobe is missed. These observations attest to the difficulty in recovering and identifying anaerobes from lyophilized preparations.

Poor recovery of anaerobes from lyophiles is a common problem. Some anaerobes are very sensitive to the lyophilization process. In addition, the process for recovery of the lyophilized anaerobes needs to be taken into account, since those conditions can affect the viability of the restored microbe. They include the suspending diluent, exposure to oxygen during work-up, and other growth conditions, such as using PRAS media and reagents. Microbes that are damaged are more sensitive to restorative conditions than are healthy microbes.

It is not uncommon to find only a few colonies of an anaerobe on a plate from a restored anaerobe lyophile plated neat (without dilution). Consider that the original suspension contained 10^9 to 10^{10} cfu/ml, the loss due to lyophilization and recovery is very large, indeed. Factors contributing to this loss include the inherent sensitivity of the microbe to the lyophilization process, this differs from microbe to microbe, damage due to freezing, and response of the lyophilized microbes to the restorative process. It is the objective of this White Paper to provide methods for Recovering Lyophilized Anaerobes that takes these issues into account.

These methods utilize Oxyrase products to recover lyophilized anaerobes. Oxyrase, Inc. developed unique products to simplify working with anaerobes. They include products to isolate and grow anaerobes in broth, such as Oxyrase for Broth®. Oxyrase for Broth treated medium will generate an anaerobic environment repeatedly and will keep broth anaerobic for up to 16 days at 37C. Some plated media products are made in standard petri dishes and contain PRAS media (OxyPRAS Plus® plates). The OxyPRAS Plus plates retain their reduced condition even after 2 hours exposure to air, making working outside a chamber feasible. A special plate, the OxyPlate™, can be incubated in an air environment while it generates an anaerobic environment inside the plate. It can do this several times.

Unique packaging was developed to extend the longevity of Oxyrase anaerobic plate products during storage. For example, OxyPRAS Plus blood agar plates can be stored at cold temperatures for up to six months and meet specifications. Details about these products are found on the Oxyrase Web Site at www.oxyrase.com .

One property of Oxyrase that is particularly well suited to the recovery of lyophilized anaerobes is its impact on injured cells. This is best illustrated by going to the Oxyrase Web Site (www.oxyrase.com) and clicking on the panel that takes you to a page that lists results from Google Scholar searches. 'Oxyrase' is cited 1,140 times. 'Oxyrase and injured cells' is cited 250 times. Here you can peruse the many ways that Oxyrase was used in studies with injured cells.

Working with Lyophiles:

Lyophiles come in different forms. For some, the lyophile is in a swab. In another, the lyophile is in a dry powdery medium within a sealed tube. For others, the lyophile is incorporated into a dry pellet. Follow the directions provided by the vendor for preparing the lyophile for recovery (for example opening a sealed tube).

Transfer 1.0 ml. of Oxyrase for Broth (OB) to a sterile capped tube. Suspend the lyophile from a swab directly into the OB. Rotate the swab without creating a vortex or introducing air. Express the swab against the wall of the tube. Use the swab to spread a plate (blood agar OxyPRAS Plus or OxyPlate). Repeat as necessary.

In addition or alternatively, use a sterile loop to transfer and streak the plate (or quadrant streak?).

Assume the anaerobe lyophile is damaged. The next event, restoration, needs to be made in an environment devoid of oxygen, which is a damaging agent to anaerobes. Oxygen, if present, would further damage weakened cells. That is why OB is used in a concentrated form to provide that anaerobic environment and to maintain it when one introduces a swab that likely has oxygen trapped inside it.

If a diluent is provided and you want to use it, mix an equal volume of Oxyrase for Broth with the diluent. Incubate this mixture for 30 min. at room temperature before using it to dissolve and suspend the lyophile as above.

The diluent provided is likely to be oxygenated and would be a source of oxygen that could damage restored lyophiles.

If the lyophile is in a sealed tube, open appropriately then add the Oxyrase for Broth (or the diluent OB mix) to the opened tube with a bulb, transfer pipette. Resuspend and dissolve the lyophile by drawing the suspension into and out of the pipette forcefully, but without making bubbles or otherwise aerating the broth. Transfer to a sterile capped tube. Plate as described above.

Technique is important when working with anaerobes. Mixing is a potential source of reintroducing oxygen. Oxyrase makes it possible to do these operations on a bench top. When oxygen, in the head space of a tube dissolves into broth or liquid, Oxyrase rapidly removes it at the meniscus before it can damage the restored cells.

Keep and incubate the lyophile suspensions for possible further use.

Broth Cultures:

Prepare OxyPRAS BHI by adding one drop of OB for each ml of broth. Incubate at room temperature for 30 min. before use.

Inoculate the anaerobic broth tube with one or two drops of the suspended lyophile. Do not mix. Firmly tighten the cap. Incubate at 35 – 37C.

The most anaerobic location in the broth tube is at the bottom. Oxyrase removes oxygen progressively from the bottom of the tube to the top. Oxyrase keeps removing oxygen as it dissolves into the medium at the meniscus. Mixing a tube that is anaerobic destroys this relationship.

Media Choice:

Inoculate an aerobic blood agar plate, an OxyPRAS Brucella plate or an OxyPlate Schaedler Blood Agar for each lyophile. For broth culture, use an aerobic tube and an OB tube (OxyPRAS BHI).

Media and diluents should be made PRAS (see explanation on Oxyrase Web Site www.oxyrase.com) to prevent further damaging effects on the recovering anaerobe.

Incubation and Observation:

Incubate and observe at 24, 48, and 72 hours.

Incubation of OxyPRAS plates require a bag, jar, or chamber to establish anaerobic conditions. OxyPlates can be incubated in an air incubator without need for a bag or a jar. Sealed tubes made anaerobic with Oxyrase can be incubated in air.

Anaerobes may be found as small colonies, depending upon the particular microorganism.

Dealing with Mixed Lyophiles:

If the lyophile contains a facultative microbe in addition to an anaerobe, use AnaSelect® plates (OxyPRAS Plus or OxyPlate) and AnaSelect® for Broth. AnaSelect products were developed to allow anaerobes to grow while suppressing growth of facultative microbes. Add an AnaSelect plate and an AnaSelect Broth tube to the above media for each lyophile. See Web Site (www.oxyrase.com) for details on AnaSelect Media.

A note about working with the AnaSelect for broth culture: AnaSelect will retard facultative microbes. Staphylococcus and Streptococcus are particularly difficult to slow down. Look at the bottom of the tube. If you see turbidity at the bottom, use a bulb pipette to sample the bottom layer of broth. Put a drop onto a plate to incubate aerobically and one to incubate anaerobically. Observe AnaSelect tubes early (24 hrs.) rather than late (72 hrs.). Sample the tube even when no turbidity is apparent. Often, anaerobes may be present at levels below visible turbidity. Long-term incubation may provide time for the facultative microbe to break through the selective barrier, making isolation of the anaerobe difficult. When mixing broth tubes with anaerobes, swirl do not shake or agitate.

The combination of AnaSelect Oxyrase for Broth with an AnaSelect plate is a powerful selective combination which aids recovery of the anaerobe from mixed cultures.

Some anaerobes may form very small colonies on AnaSelect plates.

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White Paper No: 130515