HANDLING AND CULTIVATION OF ANAEROBES

Oxygen, microbial metabolism and growth conditions

On the basis of oxygen tolerance, microorganisms can be classified in four groups: strict aerobes, strict anaerobes, aerotolerant anaerobes and facultative anaerobes.

Growth of microorganisms in the laboratory does not only depend on the presence of oxygen but also on its amount. There are aerobes that are unable to grow in atmospheric oxygen pressure, but need less of this gas. Others require the presence of gases other than oxygen, in concentrations differing from atmospheric air.

Strict **aerobes** always grow under **aerobic** conditions. Aerobic bacteria grow well on the agar surface of plates and slants. As oxygen is relatively insoluble in water, it can be limited in liquid cultures, and therefore special precautions must be taken to supply and dissolve oxygen in the medium during growth of aerobes. Sometimes shaking is not sufficient and air has to be forced through the liquid.

All aerobes preserved at the CECT are inoculated on agar medium on Petri dishes and incubated in standard microbiological incubators. Tubes of 16 by 160 mm are filled with 5 ml broth, inoculated with the culture and, unless otherwise indicated, incubated statically. If greater volumes are necessary, 100 ml flasks are filled with 20 ml broth and incubated in a shaker incubator (rpm indicated in the strain catalogue sheet).

Microaerophiles are aerobes that grow with lower oxygen concentrations than those in atmospheric air. There are microaerophiles such as *Campylobacter*, *Helicobacter*, and *Spirillum* that grow only under hypoxic conditions (from 1 to 10% O_2), whereas others exhibit microaerophilic behaviour only under certain conditions, for example the species of *Azospirillum* when growing under N_2-fixing conditions.

Different strategies for microaerophilic culture are available. At the CECT, we use either a candle jar or, preferably, gas generating sachets inside single-use re-sealable pouches, such as the GasPak™ EZ Gas Generating Pouch Systems by BD. The latter is indicated in the strain data sheet as "Atmospheric needs: microaerophilic generating system".

Capnophiles are aerobes that require, or thrive on, high CO_2 levels. Such microorganisms succeed in the presence of concentrations of 3 to 20% of CO_2. Some examples are *Haemophilus influenza* and *Neisseria gonorrhoea.*

This kind of bacteria is cultured in the appropriate medium and incubated with gas generating sachets inside single-use re-sealable pouches, such as the GasPak™ EZ Gas Generating Pouch Systems by BD, or in a CO2 incubator. The choice of one or the other option is indicated in the strain data sheet.

Facultative anaerobes are microorganisms able to grow in the presence or absence of oxygen, depending on the nutrients and culture conditions.

Unless otherwise indicated, facultative anaerobic strains preserved at the CECT are cultured and incubated in the laboratory following the same recommendations as those given for strict aerobes microorganisms.

Microorganisms unable to respire oxygen are **anaerobes**. **Aerotolerant anaerobes** grow on the surface of agar plates with low amounts of oxygen, whereas **obligate** (strict) **anaerobes** die in contact with the air, or their growth is inhibited, if they are exposed to such environment. Oxygen present in the liquid medium can be removed by boiling or by flushing with an oxygen-free gas, but this may not be sufficient to permit the growth of obligate anaerobes. Some anaerobes require not only the absence of oxygen but also a low oxidation-reduction potential (Eh) in the medium (Eh ≤ -200 mV). Thus, it is indispensable to include a reducing agent to lower and balance the Eh ratio of the medium.

All anaerobes preserved at the CECT are aerotolerant anaerobes, which can be exposed to oxygen briefly. They can be handled under aerobic conditions but several recommendations should be followed. Please, read carefully the "General techniques for preparation of culture media and handling of aerotolerant anaerobes (including many clostridia)" and the "General techniques for routine culture of fastidious anaerobes" specified below.

General techniques for preparation of culture media and handling of aerotolerant anaerobes (including many clostridia)

All these procedures can be performed in air by taking some precautions to prevent excessive exposure of media and cultures to oxygen.

- Use always freshly prepared culture media. Avoid storage of media for prolonged periods. If storage is necessary, boil the liquid culture medium (broth) for 10 minutes to extract oxygen and cool it in an ice water bath before inoculation.
- In broth with resazurin, if the top part of the tube is pink, boil and cool the medium before inoculation.
- Autoclave the media without the reducing agent and add the agent from a sterile stock solution after the medium has cooled to about 45ºC.
- Prepare and dispense liquid medium in containers that provide a small surface to volume ratio, e.g. tubes of 16 by 160 mm filled with 9 ml broth.
- Prepare liquid medium slightly viscous by adding small amounts of agar (0.05 to 0.1%), in order to reduce convection currents.
- When using screw cap vials fill to the half and sterilize them by autoclaving. Afterwards and while still warm, fill them to the neck with the same sterilized medium. These vials are excellent for prolonged incubation of anaerobes that do not produce gas, e.g. photosynthetic bacteria.
- Avoid bubbles when inoculating broth.
- Pour a thick layer of mineral oil on the surface of inoculated media to avoid diffusion of oxygen into the liquid broth. Anoxic conditions can also be achieved by pouring 2% agar.
- Vegetative cells of aerotolerant anaerobes may be spread on agar medium in Petri dish if the dish is subsequently incubated in a jar or a chamber free of oxygen. The double-layer agar technique can also be used.
- Incubate tubes and plates in an appropriate atmosphere (follow the indications stated in the *Atmospheric needs* section in the strain data sheet).

General techniques for routine culture of fastidious anaerobes

The following techniques are used for the culture of fastidious anaerobic bacteria. They are based on anaerobic culture methods described by Hungate (1950). These methods make possible to transfer and culture fastidious anaerobic organisms relatively routinely.

- **Production of anaerobic media:**

For fastidious anaerobes media must be reduced at the time of inoculation. These media are produced by boiling the medium containing resazurin in an Erlenmeyer flask. The media are boiled until colorless to resazurin and then bubbled with 100% N2 gas, which is introduced through a 16g x 2" needle blunt end standard hub. Then, cysteine is added and the pH adjusted if necessary. The medium is bubbled again with N2 gas and dispense under the same gas atmosphere into anoxic Hungate-type tube. Sterilization is done in autoclave at 120 ºC for 20 minutes, and other components such as cellobiose and vitamins are aseptically added sterilized by filtration.

- **Anaerobic transfers**

Inoculations of fastidious anaerobes are made through the rubber stopper using a 21g x 1 1/2" hypodermic needle attached to a 5-20 ml sterile syringe.

Examples of anaerobes preserved at the CECT

Genera of Lactic Acid Bacteria (LAB) are aerotolerant anaerobes that use to grow well under aerobic, microaerophilic or anaerobic conditions. Most species of ***Lactobacillus, Lactococcus, Leuconostoc, Oenococcus*** and ***Weissella*** are able to grow under aerobic conditions. Other species, however, grow only under microaerophilic conditions. This means that deep cultures, for example in freshly prepared (or boiled) liquid media, provide adequate conditions to obtain good growth, whereas the same inoculum on Petri plates will not show growth. Only few species require incubation under specific anaerobic conditions. If so, they are detailed in the corresponding datasheet for each strain in the CECT on-line catalogue.

Species of ***Streptococcus*** may grow under aerobic conditions, but some strains require additional CO2 for growth (e.g. incubator at 5% CO2). Ensure growth following the recommended conditions stated in the strain catalogue sheet.

Species of ***Enterococcus*** are facultative anaerobes that have a fermentative metabolism. They may grow under aerobic conditions, but some species require CO_2. Strain specific recommended growth conditions are detailed in each catalogue sheet.

Staphylococcus is a genus of facultative anaerobic bacteria. Although most of the species grow well under aerobic conditions, some strains need microaerophilic conditions to develop colonies on a Petri plate. These cases are registered in the CECT catalogue.

Species of ***Bifidobacterium*** are aerotolerant anaerobic bacteria. Strains preserved at the CECT

are always incubated under anaerobic conditions using gas generating sachets.

Most species of the genus ***Clostridium*** are obligate anaerobes, although tolerance to oxygen varies widely. Some species can grow but do not sporulate in atmospheric oxygen pressure. Species of *Clostridium* preserved at the CECT are handled in air by taking precautions to prevent excessive exposure of media and cultures to oxygen. Plates of *Clostridium* strains are always incubated in pouches with gas generating sachets. Broth tubes are successfully incubated in a CO_2 incubator with 5% pressure of CO_2 for most of the strains.

There are many strict anaerobes preserved at the CECT that only grow in the laboratory following the **Hungate technique** described above. Species of *Herbinix*, *Bacteroides*, *Hungateiclostridium* ... are examples of these bacteria.

Examples of microaerophilic/capnophilic bacteria preserved at the CECT

Campylobacter is the best represented genus of microaerophilic bacteria at the CECT. Species of Campylobacter grow well under microaerobic conditions with 5-15% O_2 and more than 6% CO_2. At the CECT we use the GasPak EZ Campy Pouch System from BD to produce this atmosphere in the laboratory.

Other species that require the same incubating conditions are those of *Helicobacter*.

Species of *Haemophylus* and *Neisseria* grow well if incubated in an incubator with 5% CO_2.

Glossary

Aerobic bacteria are species capable to grow at full oxygen tensions (atmosphere air contains 78% nitrogen, 21% oxygen, 0.9% argon, 0.04% carbon dioxide, and small amounts of other gases).

Microaerophiles are aerobes that can grow with oxygen when it is present at levels lower than in atmospheric air (usually 1-10% oxygen).

Capnophiles grow at (or are stimulated by) elevated levels of CO_2, ranging from 5 to 20%.

Facultative anaerobes are microorganisms able to grow either with or without oxygen, depending on the nutrients and culture conditions.

Anaerobes are microorganisms that are not able to use oxygen for growth. **Aerotolerant anaerobes** can tolerate oxygen and grow in its presence although they do not use it, whereas **obligate anaerobes** are inhibited or killed by oxygen.

Reference: Brock Biology of Microorganisms, 14th Edition by Madigan et al. Pearson, Inc.