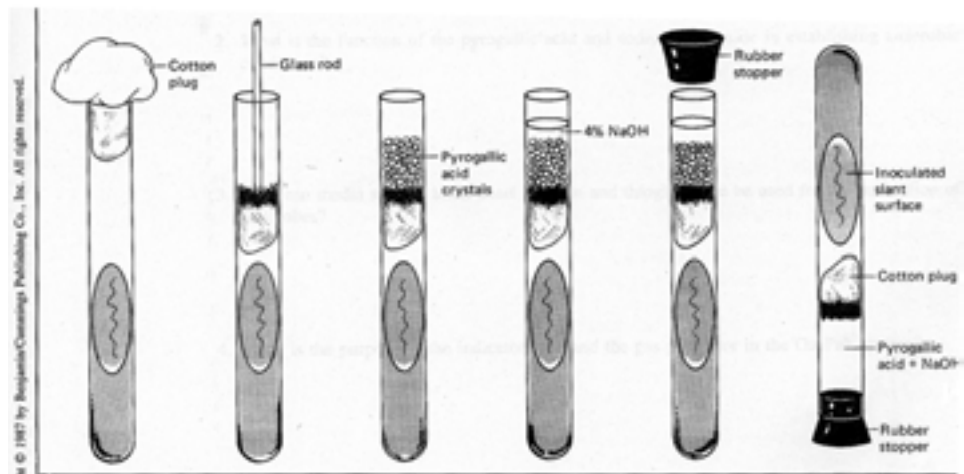


Experiment 4	Growth Conditions for strictly anaerobic Bacteria
Advisor	Christine Lehmann, Tel. (01) 63 48281, chleh@botinst.unizh.ch
Reading	Chapters in BBOM 9th: 5.12, 21.1. In BBOM 10th: 6.13, 24.1 BBOM: Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, 10th Edition, 2003. Prentice Hall.
Objectives	Learn to enrich for anaerobic bacteria.
Background	<p>Bacteria may be characterized based on their need for oxygen (O₂). Bacteria that absolutely require oxygen are obligate aerobes, whereas those that are unable to grow in the presence of free oxygen are called obligate anaerobes. Organisms that can switch between an aerobic and an anaerobic life style are called facultative anaerobes.</p> <hr/> <p>DESIGN OF A MICROBIAL ENRICHMENT MEDIUM FOR ANAEROBIC MICROORGANISMS</p> <p>The growth medium must meet the nutritional requirements of the bacteria (C,H,O,N,P,S etc. containing compounds) and establish the physico-chemical conditions necessary for growth: pH, absence of oxygen, redox level, etc. For a first enrichment from an environmental sample (soil, sediment, water, etc.), the medium can be undefined in terms of its composition; i.e. it may contain:</p> <ul style="list-style-type: none"> • an enzymatic hydrolysate of proteins (provides nitrogen, carbon and energy) • a carbohydrate (carbon and energy source) • various salts (inorganic nutrient and ion requirements) • yeast extract (provides vitamins and other supplements) • supplements (trace elements, amino acids, etc.) <p>The enrichment medium can selectively encourage growth of specific bacteria in mixed populations. In anaerobic enrichment media, the conditions must be inhibitory for aerobic bacteria and favor anaerobes. While facultative anaerobes can consume the oxygen before switching to an anaerobic metabolism, strict anaerobes cannot consume the oxygen in the medium, it may even be toxic for them. If we create an oxygen gradient within a culture tube, we will detect a whole diversity of aerobic and anaerobic bacteria each growing under its respectively optimal conditions.</p> <hr/> <p>REMOVAL OF O₂ FROM THE GROWTH MEDIUM</p> <p>Some possibilities are:</p> <ul style="list-style-type: none"> • When the medium is sterilized by autoclaving, the dissolved oxygen is 'driven out' of the liquid. Upon cooling, the headspace can be flushed or the liquid can be purged with an oxygen-free gas (N₂, Ar, He). Oxygen is not very soluble in water and it diffuses only slowly into the medium; that is, only the first few mm near the top of a tube will contain oxygen as long as it is not mixed with air. • From the gaseous phase of the culture vessel, O₂ is displaced by passing sterile O₂-free gas over it. • Addition of reducing agents which also lower the redox potential: e.g., sodium thioglycolate, titanium(III)citrate, cysteine, sodium sulfide, sodium dithionite. • Inoculation with a dense bacterial suspension (10% instead of 1%) still containing aerobes. Aerobic bacteria use up the oxygen usually faster than it can diffuse into the liquid unless one bubbles or shakes vigorously. • Addition of agar to the culture tubes further reduced O₂ diffusion. • The enrichment and the isolation of anaerobic bacteria by conventional plating methods and subsequent incubation in a closed container (anaerobic jar) without oxygen can be done if the microorganisms are not killed by exposure to O₂ during transfers.

- <http://www2.austin.cc.tx.us/microbugz/06anaerobicjar.html>
- Indicators used to assure anoxic conditions are **methylene blue** (used in anaerobic jars) or **resazurin** (used in media of low redox potential; e.g. methanogens). Sulfate reducing bacteria produce sulfide as a metabolite which keeps conditions anoxic.

FOR MORE O₂-SENSITIVE MICROORGANISMS, MORE FASTIDIOUS TECHNIQUES NEED TO BE APPLIED

Dilution shake culture (see Materials and Experimental Protocols)
Pyrogallol method (see below)



1. Inoculate a cotton-plugged tube containing a nutrient agar slant with a streak of a microorganism and label the tube with the name of the microorganism and the date of inoculation.
2. Ignite cotton plug by passing it through the flame of the Bunsen burner and push the burning cotton into the tube with a glass rod, the flame will be extinguished.
3. Add a few pyrogallol acid crystals to the tube.
4. Add 1 or 2 ml of 4 % NaOH and stopper tightly with a rubber stopper.
5. Make sure the NaOH will not flow through the cotton plug into the medium by inverting tube for incubation.

Hungate technique

This technique is used to isolate pure cultures of strictly anaerobic microorganisms.



Outline of the procedure: <http://bellcoglass.com/us/2047-16125.htm>

Crimped sealed anaerobic culture tube or bottle

Is very useful for studies which involve methane-producing bacteria that create high atmospheric pressures inside the tube.

Anoxic glove boxes allow one to work under an oxygen-free atmosphere. How gloveboxes function: <http://www.epa.gov/ada/overview/thumbnails/glovebox.htm>

For **pictures** on anaerobic techniques see also BBOM 9th, figures 5.22, 21.6.

	<p>PRACTICAL USE OF ANAEROBIC BACTERIA</p> <ul style="list-style-type: none"> • In medical and food microbiology • Industrial fermentations to produce acetone, butanol, etc. • Potential of anaerobic processes in bioremediation
<p>Practical Work</p>	<p>The students will learn:</p> <ul style="list-style-type: none"> • How to make a dilution shake culture for the growth of strictly anaerobic bacteria • How to observe anaerobic growth and report on it
<p>Materials and Experimental Protocols</p>	<p>DILUTION SHAKE CULTURE</p> <p>Growth medium for anaerobic agar shakes in g per liter medium (prepared) Dissolve in distilled H₂O: meat extract 1, yeast extract 2, peptone 5, NaCl 5, sodium thioglycolate 2, Resazurin 1 ml, distilled H₂O added to a final volume of 1 liter</p> <p>Resazurin Redox indicator (1000 x, prepared):</p> <ul style="list-style-type: none"> • Dissolve 11.5 mg Resazurin (MW 229.18) in 10 ml dH₂O (= 5 mM stock solution) and filter-sterilize • Usually 1 ml of this solution is added to 1 l of the medium (final conc. 5 µM) <hr/> <p>PROCEDURE FOR DILUTION SHAKE CULTURE</p> <ol style="list-style-type: none"> 1. Place 7 autoclaved 15 ml glass-tubes in a rack and label them 1-7 2. Fill each tube aseptically with 5.4 ml growth medium 3. Add 9 µl filter-sterilized resazurin stock solution to each tube 4. Add 0.6 ml inoculum (see below) to the first tube 5. Mix well and transfer 0.6 ml from the first tube to the second tube etc. (1:10 dilution steps). Do not add inoculum to tube 7 (control) 6. Add 3 ml of 2.4 % agar kept in a water bath at 45 °C to each tube 7. Close tubes tightly, mix well and let solidify at room temperature 8. Add „pyrogallol plug“ (see above) to each tube 9. Invert each tube for incubation (at 25°C in the dark for a few days) <div style="text-align: center;"> <p>10-fold serial dilution</p> <p>step 2 0.6 ml 0.6 ml 0.6 ml 0.6 ml 0.6 ml</p> <p>step 3 +3 ml +3 ml +3 ml +3 ml +3 ml</p> <p>step 1 5.4 ml 5.4 ml 5.4 ml 5.4 ml 5.4 ml</p> </div>

	<p style="text-align: center;">INOCULUM PREPARATION FOR ENRICHMENT OF <i>CLOSTRIDIA</i> AND OTHER SPORE-FORMING BACTERIA</p> <ol style="list-style-type: none"> 1. Prepare the inoculum suspension by adding 2 gram of soil to 8 ml of sterile saline solution in a screw-capped tube. Mix well, then keep the tube at 70°C in a waterbath for 10 minutes. Make sure that the soil suspension in the tube is completely immersed in the water bath. Cool the tube rapidly on ice to room temperature. 2. Pipette 1 ml of heat-treated, cooled inoculum into the first tube (see above).
<p>Laboratory Rules & Precautions</p>	<ul style="list-style-type: none"> • Some anaerobic bacteria produce H₂ or CH₄, which can cause flammable or explosive conditions. • Additionally CO₂ is produced by most anaerobes which will increase the internal pressure in the culture vessel. • Some anaerobes are potent toxin producer. Do not inhale! • Pyrogallic acid (1,2,3-Trihydroxybenzene-1,2,3-benzenetriol) can be absorbed through skin. Wear protective gloves, lab coat and safety goggles.
<p>Goals & Experiences gained</p>	<p>The work with anaerobic bacteria is more laborious than working with aerobes, but it enlarges the spectrum of microorganisms detected.</p>
<p>Timing</p>	<p>90 min</p>
<p>Reporting</p>	<p>Report your results in a table. Where do you detect bacteria in your tubes ? Count the colonies ! Pick a colony and prepare a microscope slide !</p>
<p>Questions</p>	<ol style="list-style-type: none"> 1. How can spore-forming bacteria be detected ? 2. Which types of microbes will grow anaerobically ? 3. Why do we add resazurin to the dilution shake culture ?