General Biology: Cells and Molecules Summer 2005

Lab 1: Sterile Technique: Isolating and Growing Pure Cultures

In working with microorganisms, we must have a sterile nutrient-containing medium in which to grow the organisms of interest. The term ***medium*** refers to anything in or on that we grow microorganisms (that piece of moldy bread at home is a solid medium, while the sour milk in the refrigerator is a liquid medium). A ***sterile medium*** is one that is free of all life forms and should in theory remain unchanged forever if just left alone and sealed.

To work with microorganisms, we must be able to ***transfer growing microorganisms*** from one pure culture to a new sterile medium without introducing any unwanted outside contaminants. This method of preventing unwanted microorganisms from gaining access is ***termed aseptic technique*** *(or sterile technique)*.

Part I: Aseptic Technique: The Theory and Practice

Culture Media

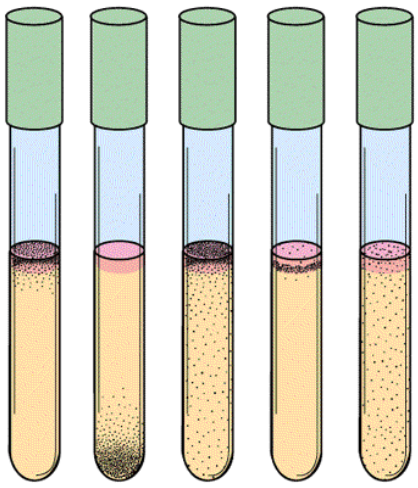
Types of media: Media can be divided into categories in two ways: by recipe (defined and complex) and by their final form (liquid or solid).

1) A defined medium is made of definite amounts of known materials. If we know an organism's growth requirements, we can make a mixture of specific salts and organic substrates adjusted to a suitable pH.
2) A complex medium**,** in contrast, is made with a variety of ill-defined materials such as broths, peptones, and tryptones. These generally are digested milk and meat products, so they are rich mixtures of amino acids, vitamins, and other organic components.

Defined media are generally as clear as water; complex media are usually yellow to brown in color, although sometimes added dyes make them red, green, or blue. Organisms often grow rather slowly in defined media; complex media are much richer in nutrients and support growth that is more rapid.

1) Microorganisms can be grown in a liquid medium contained in a flask or tube. All media are mostly water 98-99% water in fact-but a medium is said to be liquid only in contrast to solid media, which have the consistency of a gelatin because they contain a thickening agent. The thickening agent is usually agar**,** a sulfated polysaccharide derived from certain kelps.
2) A medium made with agar is generally called an agar; for instance, if agar is added to Hershey broth, the resulting medium will be called "Hershey agar". The principal advantage of a solid medium is that organisms can only move slowly across or through the medium and therefore stay in place. Thus, if a few bacteria are spread on the surface of an agar medium, each cell will grow into a distinct, isolated colony. In a liquid medium, all the cells will be mixed up, and we cannot separate one clone from another.

A typical complex nutrient containing broth medium such as Trypticase Soy Broth (TSB) contains substrates for microbial growth such as pancreatic digest of casein, papaic digest of soybean meal, sodium chloride, and water.



We can prepare media in a variety of containers to serve different purposes.

1) ***Slant tubes*** are tubes containing a nutrient medium plus a solidifying agent, agar-agar. The medium has been allowed to solidify at an angle in order to get a flat inoculating surface.
2) ***Stab tubes*** are tubes of hardened agar medium that are inoculated by "stabbing" the inoculum into the agar.
3) ***Agar plates*** are sterile petri plates that are aseptically filled with a molten sterile agar medium and allowed to solidify. Plates are

much less confining than slants and stabs and are commonly used in the culturing, separating, and counting of microorganisms.

4) ***Shake flasks*** are the general way that larger volumes (25 ml to 1 l) of cells are grown.

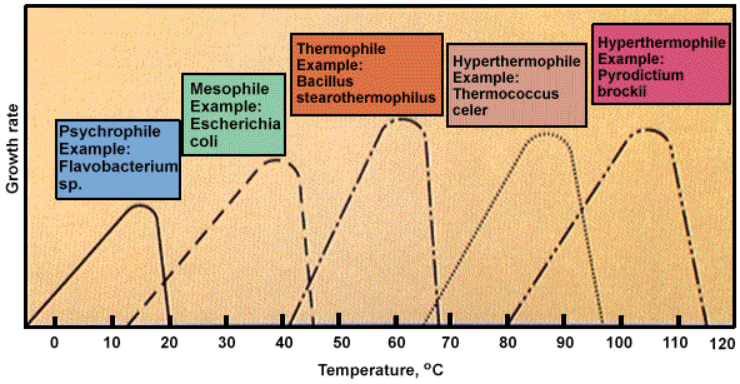
Oxygen requirements for Microorganisms

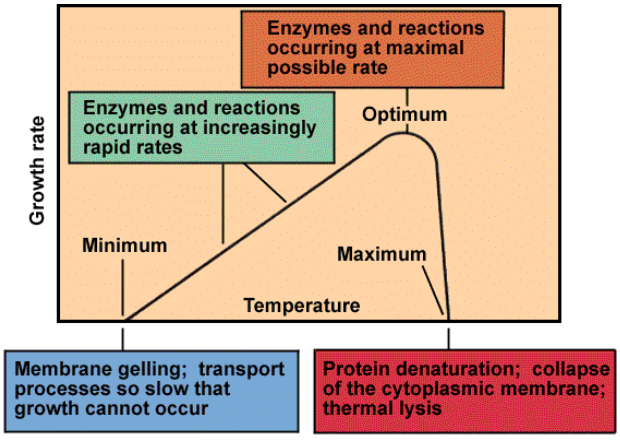
Microorganisms show a great deal of variation in their requirements for dioxygen. Most can be placed in one of the following groups (on figure from right to left):

1) Obligate aerobes: organisms that grow only in the presence of oxygen. They obtain energy from aerobic respiration.
2) Obligate anaerobes: organisms that grow only without oxygen and, in fact, oxygen inhibits or kills them. They obtain energy from anaerobic respiration or fermentation.
3) Facultative anaerobes: organisms that grow with or without oxygen, but generally better with oxygen. They obtain energy from aerobic respiration, anaerobic respiration, and fermentation. Most bacteria are facultative anaerobes.
4) Microaerophiles: organisms that require a low concentration of oxygen for growth. They obtain energy from aerobic respiration.
5) Aerotolerant anaerobes: like obligate anaerobes, cannot use oxygen for growth but they tolerate it fairly well. They obtain energy from fermentation.

Temperature requirements for Microorganisms

Microorganisms are divided into groups on the basis of their preferred range of temperature:





1) Psychrophiles are cold-loving organisms. Their optimum growth temperature is 0 ^{o}C to 15 ^{o}C with a maximum growth temperature of 20 ^{o}C.
2) Psychrotrophs can, like psychrophiles, grow at 0 ^{o}C and even lower but can also grow above 20 ^{o}C.
3) Mesophiles are organisms that grow best at moderate temperatures. Their optimum growth temperature is between 25 ^{o}C and 40 ^{o}C. Most bacteria you will use will be mesophilic.

4) Thermophiles are heat-loving organisms. Their optimum growth temperature is between 50 ^{o}C and 60 ^{o}C and a few can tolerate temperatures as high as 110 ^{o}C.

Observing cells on the macro level

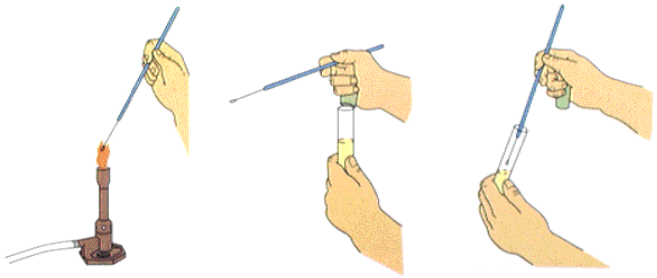
A colony is a visible mass of microorganisms growing on an agar surface and usually originating from a single cell if your technique is good. Different microorganisms will frequently produce colonies that differ in their morphological appearance (form, elevation, margin, surface, optical characteristics, and pigmentation). Probably the most visual characteristic is pigmentation (colour). Some microorganisms produce pigment during growth and are said to be chromogenic. Often, however, formation of pigment depends on environmental factors such as temperature, nutrients, pH and moisture. For example, *Serratia marcescens* produces a deep red pigment at 25 ^{o}C, but does not produce pigment at 37 ^{o}C.

Pigments can be divided into two basic types: water insoluble and water-soluble. If the pigment is water insoluble, as in the case of most chromogenic bacteria, it does not diffuse out of the organism. As a result, the colonies are pigmented but the agar remains the normal colour. If the pigment is water soluble (as in the case of *Pseudomonas aeruginosa*) it will diffuse out of the organism into the surrounding medium. Both the colonies and the agar will appear pigmented.

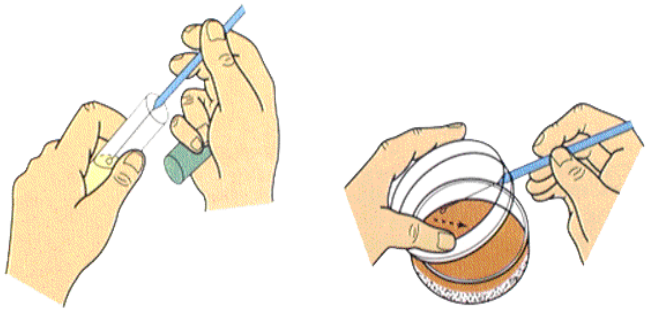
In liquid medium we can also observe cells. After incubation, growth (development of many cells from a few cells) may be observed as one or a combination of three forms:

1) ***Pellicle***: A mass of organisms is floating on top of the broth.
2) ***Turbidity***: The organisms appear as a general cloudiness throughout the broth.
3) ***Sediment***: A mass of organisms appears as a deposit at the bottom of the tube.

Sterile Technique Described: (Read this thoroughly before you go to the experimental procedure)



1) Sterilize the inoculating loop.
   a) The ***inoculating loop is best sterilized by heating it in the flame*** of a gas burner until the entire length of the wire becomes orange from the heat. In this way any organsims on the wire are incinerated.
   b) The ***loop is now sterile*** and you want it to remain that way, so ***never put a loop down once it is sterilized*** or it will become contaminated.
   c) Allow the loop to cool a few seconds (even though it looks cool it is still very hot, the more adventurous of you can check this with your fingers) to avoid killing the inoculum.
   d) ***I will demonstrate the best technique to you***.



2) Remove the inoculum.
   a) Hold the culture tube in one hand and in your other hand the sterilized inoculating loop as if it were a pencil.
   b) Remove the cap of the pure culture tube with the little finger of your loop hand. Never lay the cap down or it will become contaminated. This requires dexterity which over time you will develop.
   c) Very briefly "flame" the lip of the culture tube. This creates a convection current that carries air out of the tube and prevents any airborne contaminants from entering the tube.

d) Keeping the culture tube at an angle, insert the inoculating loop and remove a loopful of inoculum.
e) Again flame the lip of the culture tube. Replace the cap.
f) Removing inoculum from a plate culture (organisms growing on an agar surface in a petri plate): Sterilize the inoculating loop in the flame of a gas burner. Lift the lid of the petri dish and stab the loop into the agar away from any growth to cool the loop. Scrape off a small amount of the organisms and close the lid.

3) Transferring the Inoculum to the Sterile Medium.

a) Pick up the sterile broth tube and remove the cap with the little finger of your loop hand. Do not set the cap down.
b) Briefly flame the lip of the broth tube.
c) Place the loopful of inoculum into the broth, and withdraw the loop. Do not lay the loop down! Again flame the lip of the tube. Replace the cap.
d) Resterilize the loop by placing it in the flame until it is orange. Now you may lay the loop down until it is needed again.
e) Transferring the inoculum into a petri plate: Lift the petri dish up by the base. Streak the loop across the surface of the agar medium using a streak pattern demonstrated. Avoid digging into the agar by keeping the loop horizontal during streaking. Remove the loop and close the lid. Re-Sterilize the inoculating loop. In the future, every procedure in the lab will be done using similar aseptic technique.

Part II: Experimental Procedure (to be carried out in pairs)

1) In order to illustrate that microorganisms are all around us and to demonstrate the necessity for proper aseptic technique, contaminate two Trypticase Soy Agar plates as follows. Remove the lid from a TSB agar plate and expose the agar to the air for the duration of today's lab. At the end of the lab, replace the lid, label, and incubate it at room temperature. *Do this plate first*. Touch a doorknob or some other surface in the lab and then rub your fingers over the surface of the 2nd agar plate. Label and incubate at 37 ^{o}C. Wash you hands after this!

2) Aseptically inoculate one Trypticase Soy Broth (liquid) tube with *Bacillus subtilis*, one with *Escherichia coli*, one with *Micrococcus roseus*, and one with *Pseudomonas aeuroginosa*. Remember to label all tubes with a Sharpie type marker. Label everything with your initials and the date.

3) Aseptically inoculate one Trypticase Soy Agar plate with *Bacillus subtilis*, one with *Escherichia coli*, one with *Micrococcus roseus*, and one with *Pseudomonas aeuroginosa*. This procedure is termed streaking for isolation and has a diluting effect. The friction of the loop against the agar causes organisms to fall off the loop. Near the end of the streaking pattern, individual organisms become separated far enough apart on the agar surface to give rise to isolated single colonies after incubation.

4) Incubate all the tubes and plates at 37^{o}C in the following manner. Incubate the plates upside down (lid on the bottom) to prevent condensing water from falling down on the growing colonies and causing them to run together.

5) Streak a single TSA plate with the mixed culture provided at the front of the class.

Results that should be noted in your lab notebooks. (Nancy will save your cultures and you will have the opportunity to observe them).

1) Draw and describe the growth seen in each of the four broth cultures.
2) Compare colonies of the different organisms, elevation, margin (edge), surface, optical characteristics, and pigmentation.
3) Observe the results of the two "contamination" plates and note the differences in colony appearances.