BACTERIOLOGICAL TECHNIQUES
UNIT 2

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Pure Culture Techniques

- Culture – Act of cultivating microorganisms
- Mixed Culture
- Pure Culture

Why Pure Culture Is Important?

- Desire of getting one type of Organisms
- Spontaneous mutation rate is low
- Pure Culture clone is 99.99% identical

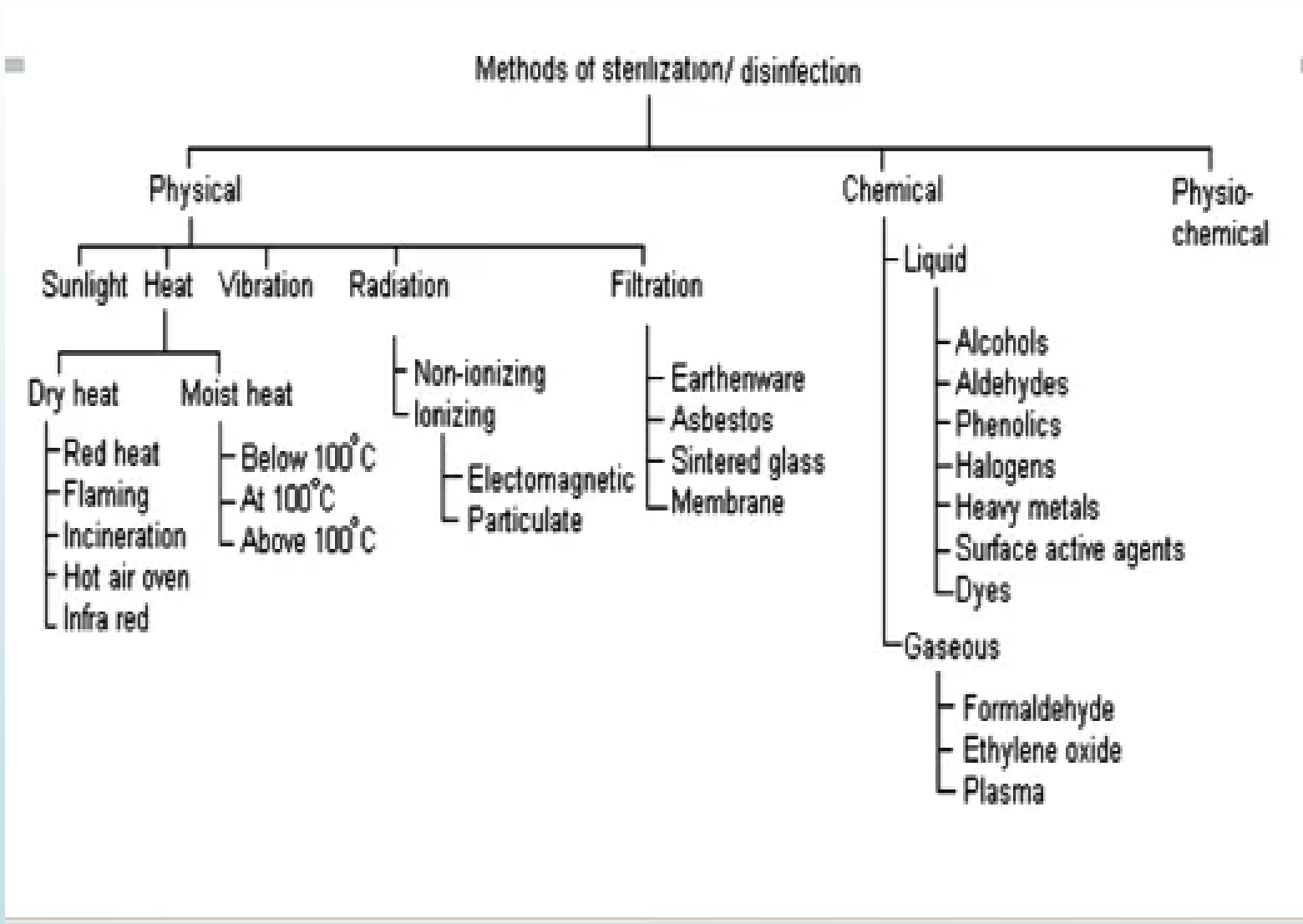
Discovery Of Agar

Agar was discovered around 1658 by Minoya Tarozaemon in Japan

Sterilization

- Autoclaving
- Filtration
- Dry Heat Sterilization
- Bunsen Burner Flame
- Biological Safety Cabinet(BSC)

i.e Laminar Airflow[HEPA Filters – High Efficiency Particulate Air Filters]



Autoclaving

Autoclaves provide a physical method for disinfection and sterilization. They work with a combination of steam, pressure and time. Autoclaves operate at high temperature and pressure in order to kill microorganisms and spores. Maintain a temperature of 121° C for at least 15 minutes by using saturated steam under at least 15 psi of pressure.

An autoclave is a machine that provides a physical method of sterilization by killing bacteria and even spores present in the material put inside of the vessel using steam under pressure.

- Autoclave sterilizes the materials by heating them up to a particular temperature for a specific period of time.
- The autoclave is also called a steam sterilizer that is commonly used in healthcare facilities and industries for various purposes.
- The autoclave is considered a more effective method of sterilization as it is based on moist heat sterilization.

Autoclaving Procedure

•The autoclave works on the principle of moist heat sterilization where steam under pressure is used to sterilize the material present inside the chamber.

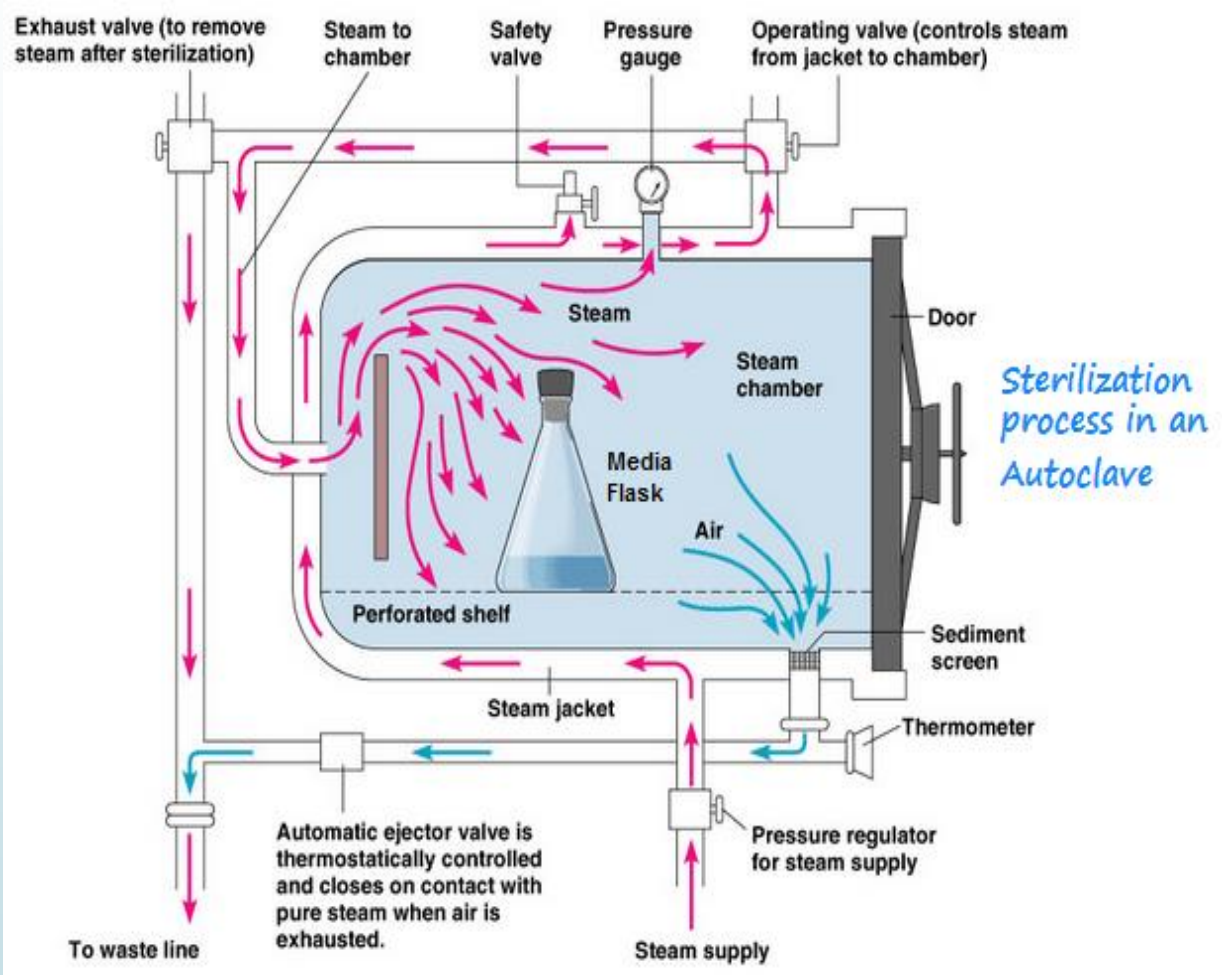
•The high pressure increases the boiling point of water and thus helps achieve a higher temperature for sterilization.

•Water usually boils at 100°C under normal atmospheric pressure (760 mm of Hg); however, the boiling point of water increases if the pressure is to be increased.

•Similarly, the high pressure also facilitates the rapid penetration of heat into deeper parts of the material, and moisture present in the steam causes the coagulation of proteins causing an irreversible loss of function and activity of microbes.

•This principle is employed in an autoclave where the water boils at 121°C at the pressure of 15 psi or 775 mm of Hg.

•When this steam comes in contact on the surface, it kills the microbes by giving off latent heat.



•The condensed liquid ensures the moist killing of the microbes.

•Once the sterilization phase is completed (which depends on the level of contamination of material inside), the pressure is released from the inside of the chamber through the whistle.

Autoclaving

AUTOCLAVE-COMPATIBLE MATERIALS	AUTOCLAVE-INCOMPATIBLE MATERIALS
Tissue Culture Flasks	Acids, bases and organic solvent
Surgical Instruments	Chlorides, sulphates
Glassware	Seawater
Pipette tips	Chlorine, hypochlorite, bleach
Media Solutions	Non-stainless steel
Animal food and bedding	Polystyrene(PS)
Waste	Polyethylene(PE)
Polypropylene (Secondary containers)	Low density (LDPE) and High density polyethylene(HDPE)
Stainless steel	Polyurethane
Gloves	

Isolation of Pure Cultures

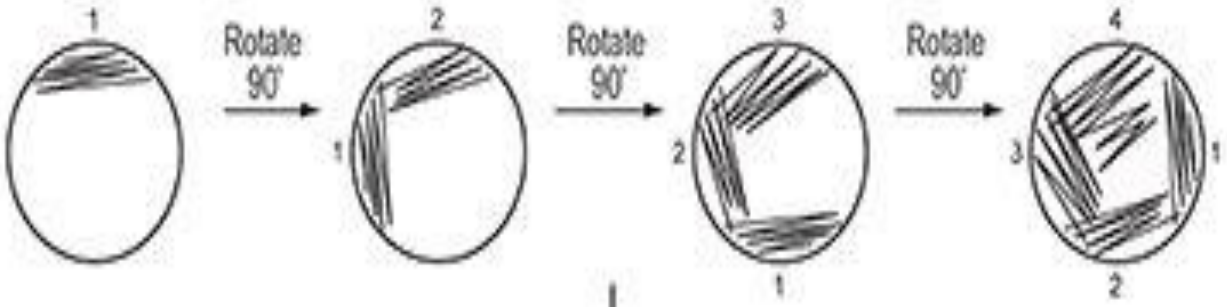
- **Streak Plate Method**
- **Spread Plate Method**
- **Serial Dilution Method**
- **Pour Plate Method**

Composition of Nutrient Agar Media

1. Peptone (partially digested protein)
2. Beef Extract
3. Sodium chloride
4. Agar (2%)
5. Water

A

Quadrant Method
Streak Pattern:

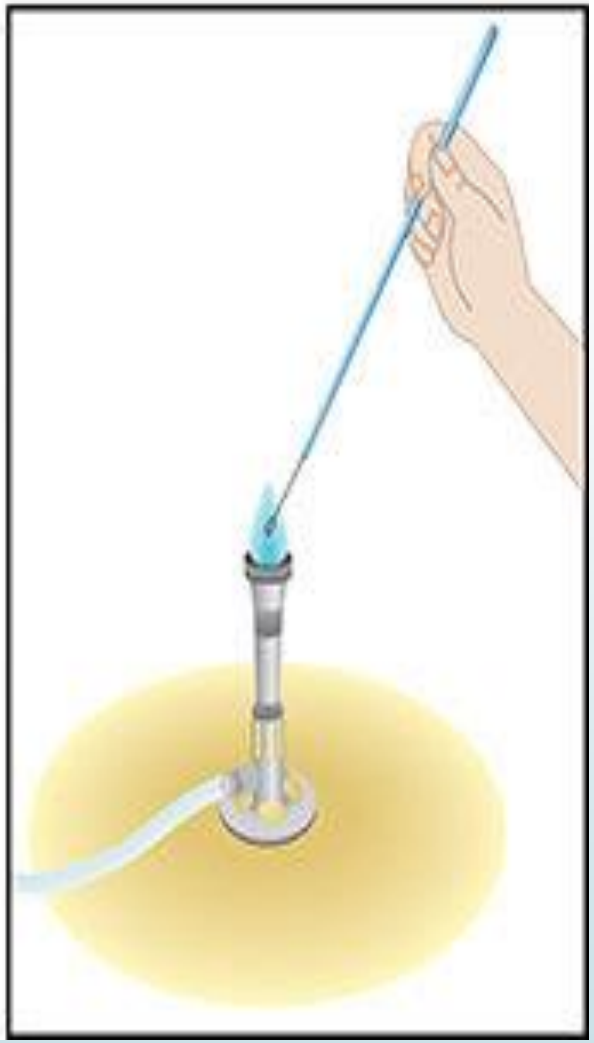


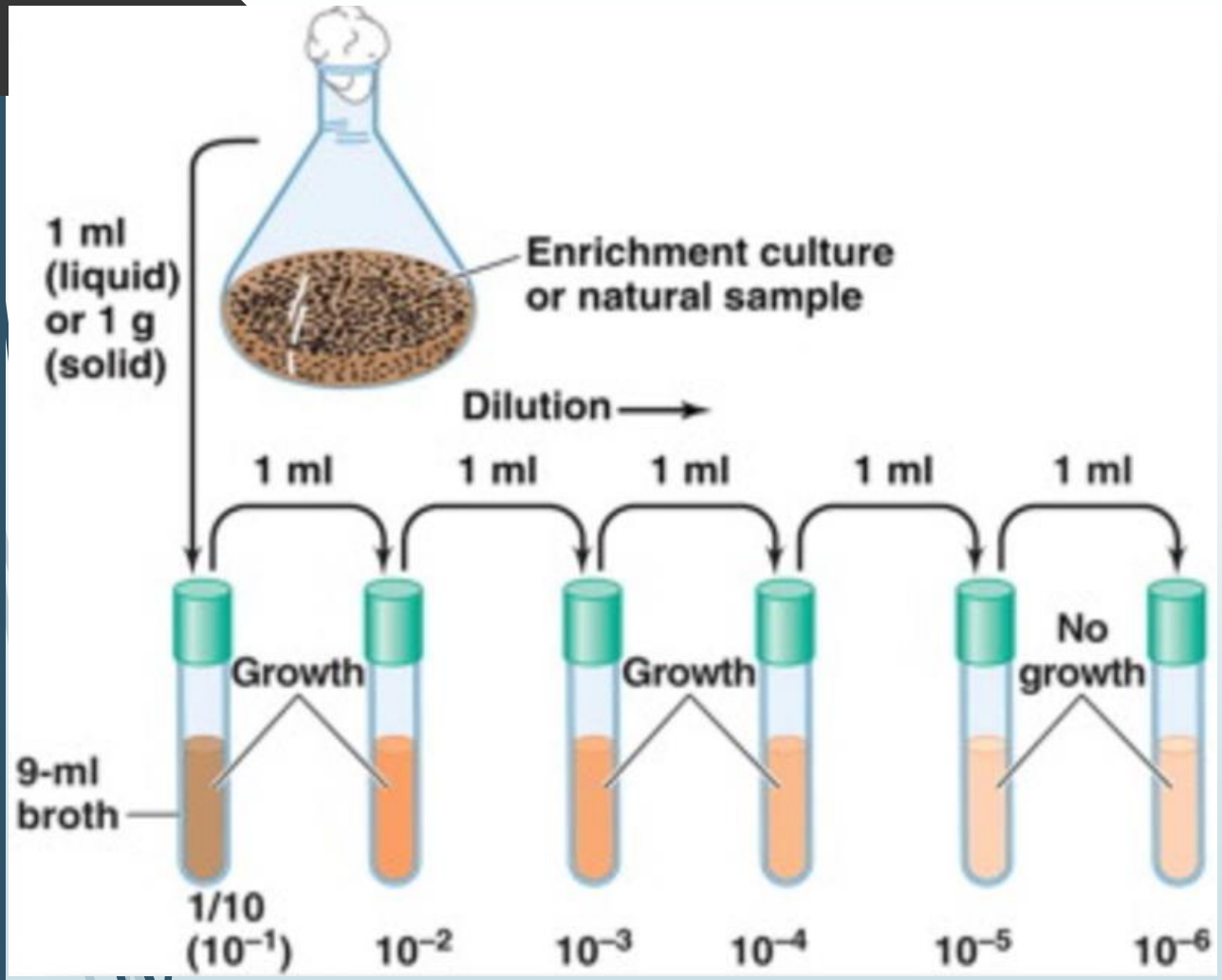
Incubation

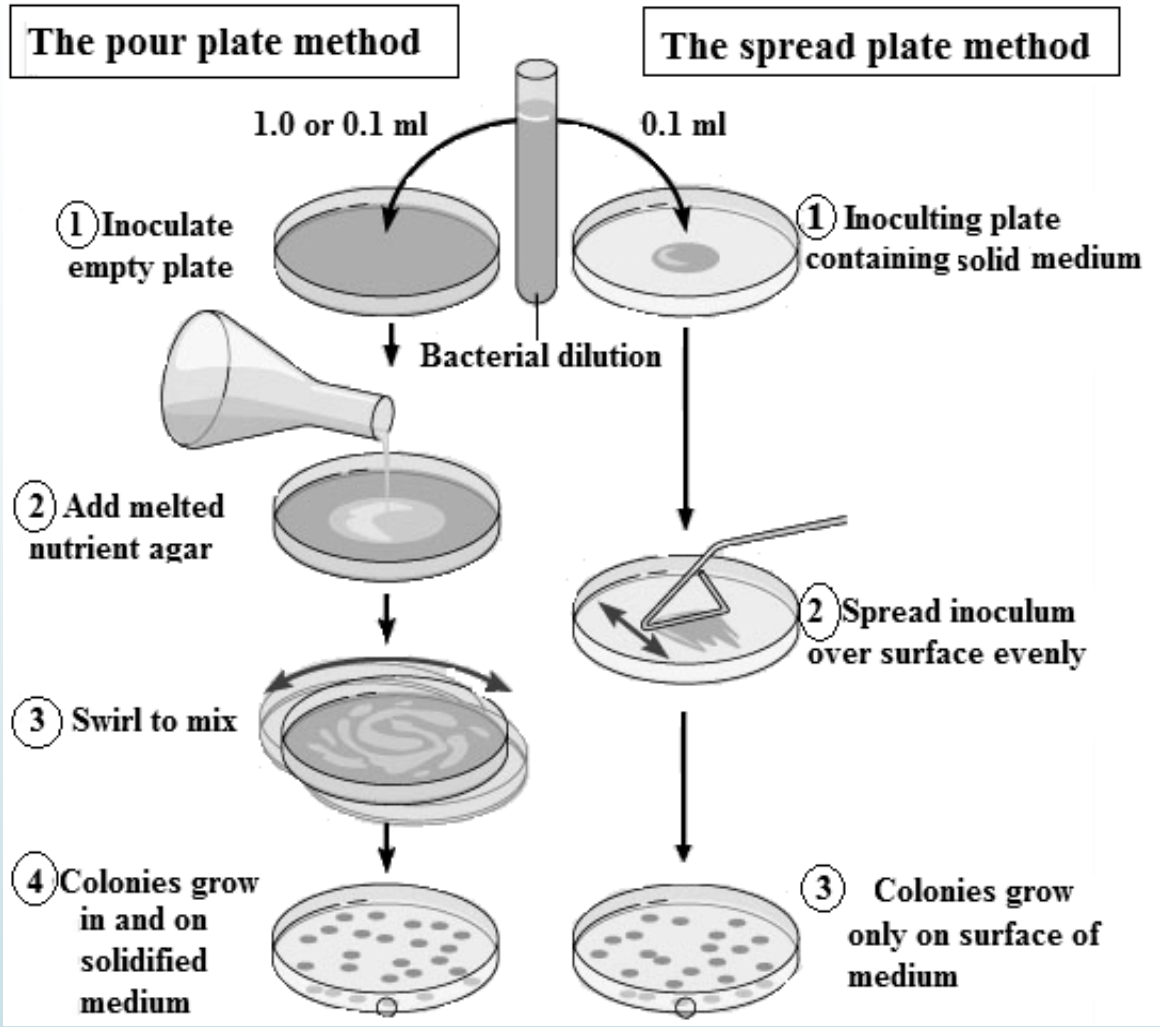
Results:

Goal: Single colonies in 4th quadrant

B







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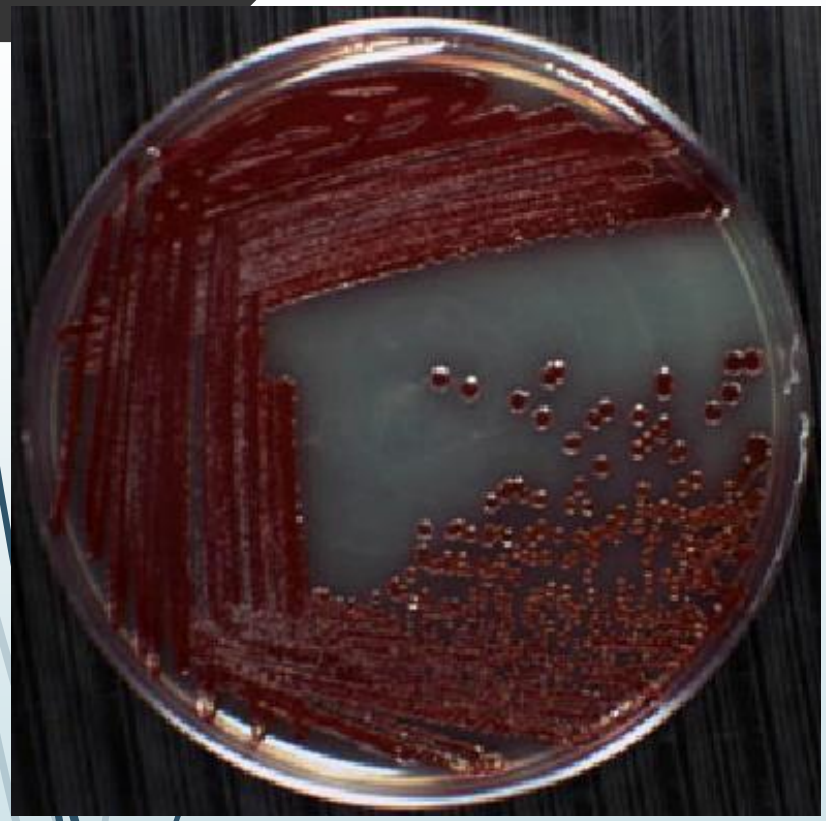
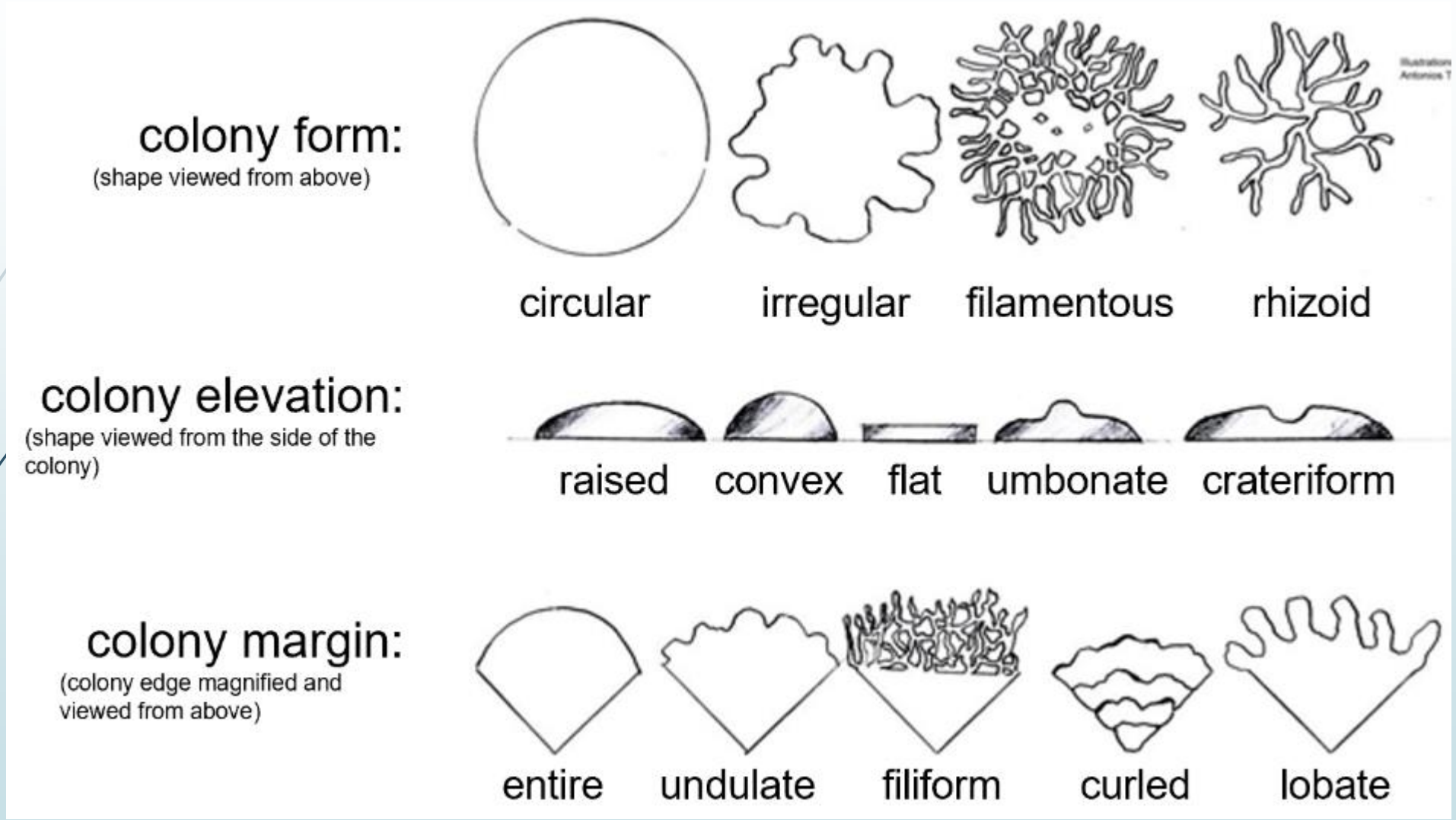


***Serratia marcescens*, a Gram negative, rod-shaped**

***Legionella*, a Gram negative, rod-shaped**

Bacteria growth on a petri plate streaked using the quadrant streak plate technique.

Characteristics of Colonies on a Petri Plates



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Preservation of Pure Culture

- **Periodic transfer to fresh media**
- **Refrigeration (stored at 0-4°C, 2-3 weeks for bacteria, 3-4 months for fungi; metabolic activity is slowed but not ceased)**
- **Paraffin method**
- **Cryopreservation**
- **Lyophilization(freeze drying)**

Periodic Transfer to Fresh Media

Strains can be maintained by periodically preparing a fresh culture from the previous stock culture. The culture medium, the storage temperature, and the time interval at which the transfers are made vary with the species and must be ascertained beforehand. The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible. Many of the more common heterotrophs remain viable for several weeks or months on a medium. The transfer method has the disadvantage of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

Refrigeration

Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold-rooms. This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped. Thus their growth continue slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

Paraffin Method/ preservation by overlaying cultures with mineral oil

This is a simple and most economical method of maintaining pure cultures of bacteria and fungi. In this method, sterile liquid paraffin is poured over the slant (slope) of culture and stored upright at room temperature. The layer of paraffin **ensures anaerobic conditions and prevents dehydration of the medium**. This condition helps microorganisms or pure culture to **remain in a dormant state** and, therefore, the culture can be preserved form months to years (varies with species).

The advantage of this method is that we can remove some of the growth under the oil with a transfer needle, inoculate a fresh medium, and still preserve the original culture. The simplicity of the method makes it attractive, but changes in the characteristics of a strain can still occur.

- Simple, most economical method.
- Agar slants are inoculated & incubated.
- Then, covered with sterile mineral oil to a depth of 1cm above the tip of slant surface.
- Transfers are made by removing a loop full of growth- touching the tip to the glass surface to drain off excess oil- inoculating a fresh medium- preserving the initial stock culture.
- Functions- providing an aerobic condition, prevents the dehydration of the medium and decreases the metabolic rate of the organisms.

Cryopreservation

Cryopreservation (i.e., freezing in liquid nitrogen at -196°C or in the gas phase above the liquid nitrogen at -150°C) helps survival of pure cultures for long storage times. In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents such as glycerol or Dimethyl Sulfoxide (DMSO) that prevent the cell damage due to formation of ice crystals and promote cell survival. This liquid nitrogen method has been successful with many species that cannot be preserved by lyophilization and most species can remain viable under these conditions for 10 to 30 years without undergoing change in their characteristics, however this method is expensive.

Lyophilization (Freeze-Drying)

Freeze-drying is a process where water and other solvents are removed from a frozen product **via sublimation.** Sublimation occurs when a frozen liquid goes directly to a gaseous state without entering a liquid phase. It is recommended using slow rates of cooling, as this will result in the formation of vertical ice crystal structures, thus allowing for more efficient water sublimation from the frozen product.

Freeze-dried products are hygroscopic and must be protected from moisture during storage. Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years. Lyophilized or freeze-dried pure cultures and then sealed and stored in the dark at 4°C in refrigerators.

Freeze-drying method is the most frequently used technique by culture collection centers. Many species of bacteria preserved by this method have remained viable and unchanged in their characteristics for more than 30 years.

Cultivation of Anaerobic Bacteria

Type 1. Special Anaerobic Culture Media (Prereduced Media):

During preparation, the liquid culture medium is boiled by holding in a boiling water both for 10 minutes to drive off most of the dissolved oxygen.

Liquid media soon become aerobic thus a reducing agent (e.g., cysteine 0.1%, ascorbic acid 0.1%, sodium thioglycollate 0.1%), is added to further lower the oxygen content.

Oxygen-free N_2 is bubbled through the medium to maintain anaerobic condition. The medium is then dispensed into tubes, which are stoppered tightly and sterilized by autoclaving. Such tubes can be stored for many months before being used. During inoculation, the tubes are continuously flushed with oxygen free CO_2 by means of gas cannula, re-stoppered, and incubated.

Examples - ‘Robertson’s bullock-heart medium’, thioglycollate broth, Brucella blood agar, Bacteroides bile aesculin agar, phenylethyl alcohol agar, kanamycin blood agar.

Type 2. Anaerobic Chamber:

Anaerobic chamber is an ideal anaerobic incubation system, which provides oxygen- free environment for inoculating media and incubating cultures. It refers to a plastic anaerobic glove box that contains an atmosphere of H_2, CO_2, and N_2. Glove ports and rubber gloves are used by the operator to perform manipulations within the chamber. There is an air-lock with inner and outer doors.

Culture media are placed within the air-lock with the inner door. Air of the chamber is removed by a vacuum pump connection and replaced with N_2 through outer doors.

The culture media are now transferred from air-lock to the main chamber, which contains an atmosphere of H_2, CO_2, and N_2. A circulator fitted in the main chamber circulates the gas atmosphere through pellets of palladium catalyst causing any residual O_2 present in the culture media to be used up by reaction with H_2.

When the culture media become completely anaerobic they are inoculated with bacterial culture and placed in an incubator fitted within the chamber. The function of CO_2 present in the chamber is that it is required by many anaerobic bacteria for their best growth.

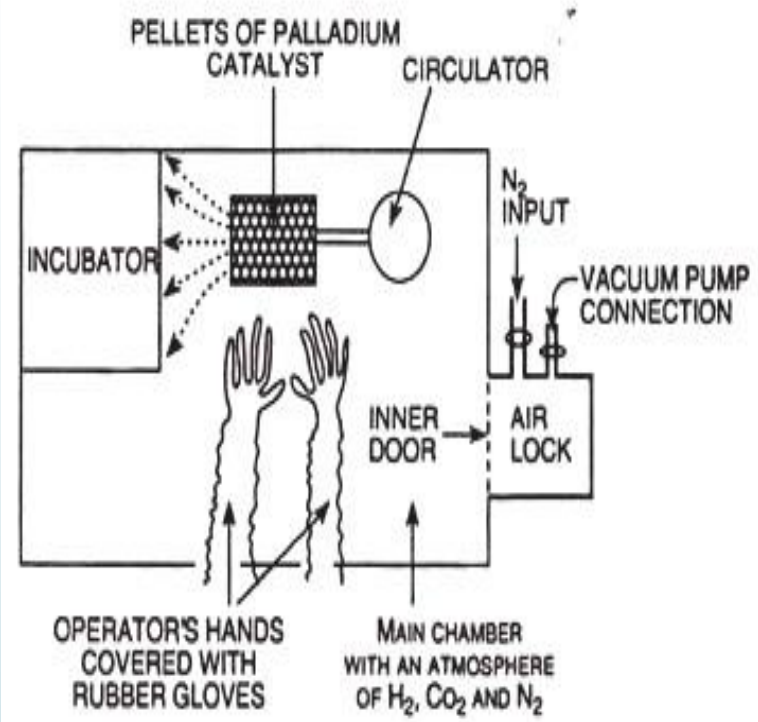

FIG. 16.19. Schematic representation of an anaerobic chamber showing its various parts.

Type 3. Anaerobic Bags or Pouches:

Anaerobic bags or pouches make convenient containers when only a few samples are to be incubated anaerobically. They are available commercially. **Bags or pouches have an oxygen removal system consisting of a catalyst and calcium carbonate to produce an anaerobic, CO_2-rich atmosphere.**

One or two inoculated plates are placed into the bag and the oxygen removal system is activated and the bag is sealed and incubated. Plates can be examined for growth without removing the plates from bag, thus without exposing the colonies to oxygen.

But as with anaerobic jar, plates must be removed from the bags in order to work with the colonies at the bench. These bags are also useful in transport of biopsy specimen for anaerobic cultures.

Type 4. Anaerobic Jars (or GasPak Anaerobic System):

When an oxygen-free or anaerobic atmosphere is required for obtaining surface growth of anaerobic bacteria, anaerobic jars are the best suited. The most reliable and widely used anaerobic jar is the Melntosh-Fildes' anaerobic jar. It is a cylindrical vessel made of glass or metal with a metal lid, which is held firmly in place by a clamp.

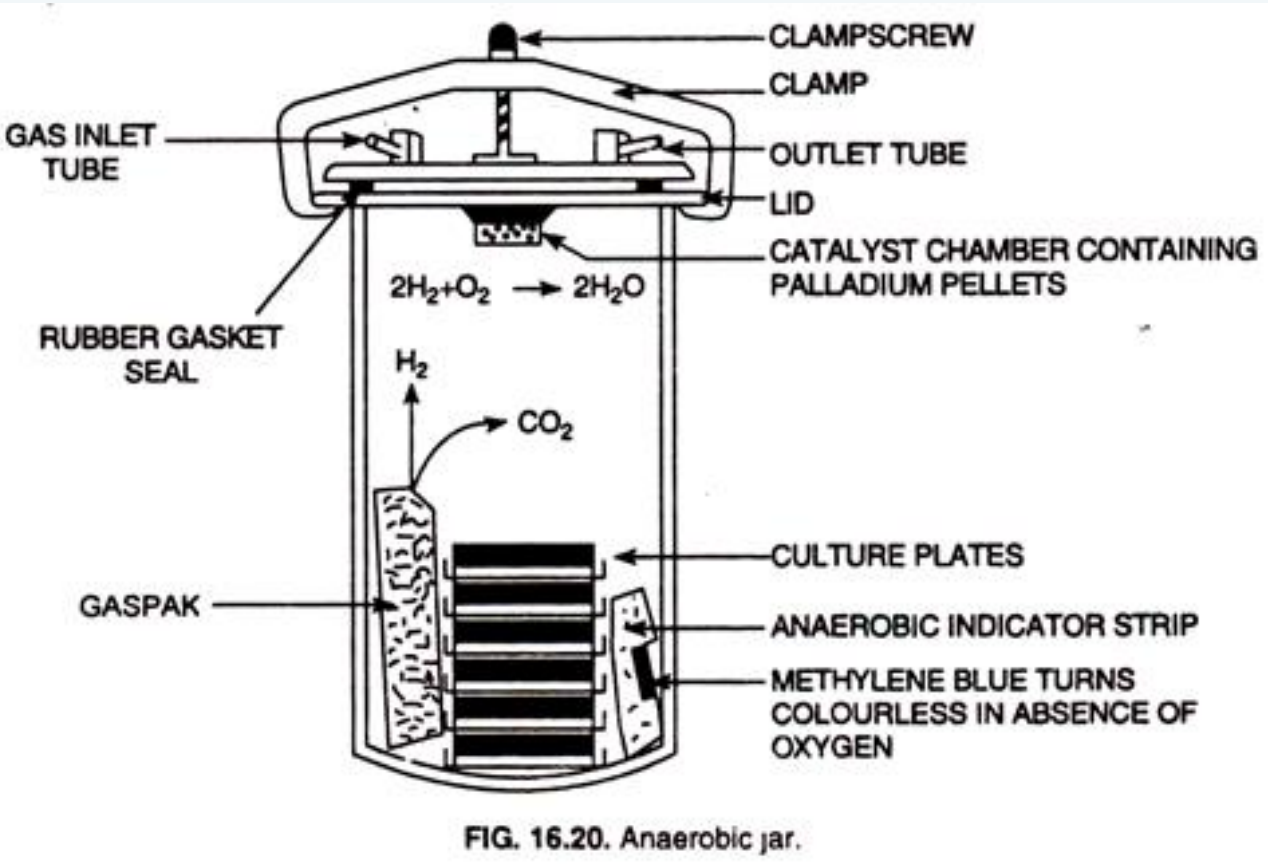

FIG. 16.20. Anaerobic jar.


Thank You