

2. Methods in Microbiology

Theory and Practice of Sterilization

- An important phase of microbiology is knowledge of methods of **killing, removing and inhibiting** (preventing growth of) microorganisms because of the undesirable consequences of the presence of microorganisms, or their products.
- Species of microorganisms vary in ease with which they may be destroyed, removed and inhibited,
- The situations in which they may occur

- The following are reasons for killing, removing or inhibiting microorganisms.
 - To prevent infections of human, animals and plants
 - To prevent spoilage of food and other commodities
 - To prevent interference of contaminating microorganisms in various industrial processes
 - To prevent contaminations of materials used in pure culture work in laboratories (diagnosis, research and industry)

Control of microorganisms can be achieved by a variety of **chemical** and **physical** methods.

Sterilization

- **Sterilization:** is the process by which an article, a surface or a medium is free of all microorganisms including virus, bacteria, their spores and fungi, both pathogenic and non-pathogenic.
- generally achieved by using physical means such as heat, radiation and filtration
- is the complete destruction or removal of all living microorganisms

- ❑ **Sterilization by heat:** is the most reliable, certain and rapid methods of sterilization .
- ✓ Unless the material to be sterilized is heat sensitive, this method should be preferred.
- ✓ The time required for sterilization by heat is inversely proportional to temperature of exposure, .i.e., higher the temperature shorter the time of exposure.

Dry heat

- **Red heat** – inoculating loops, forceps edge, searing spatulas.
- **Flaming** – mouth of culture tubes, culture bottles with medium, stoppers glass slides, cover slips, scalpel, needles.
- **Hot Air in Oven** – glass wares or metal instruments at higher temperature at 160 °C for 1hr, 170 °C for 30 min are required.
- **Incineration** – wound swab, dressings, clothing, animal autopsies.

Moist heat:

❖ At temperature less than 100°C

□ **Pasteurization of milk** at 72 °C for 15 seconds

✓ Beer, wine, etc

- it ensures the destruction of disease-causing organisms
- high temperatures can cause damage to the taste, texture and nutritional value of many food substances
- in such instances, it is sufficient to destroy vegetative cells by a process of pasteurization
 - ✓ Pasteur demonstrated that the microbial spoilage of wines could be prevented by short periods of heating.

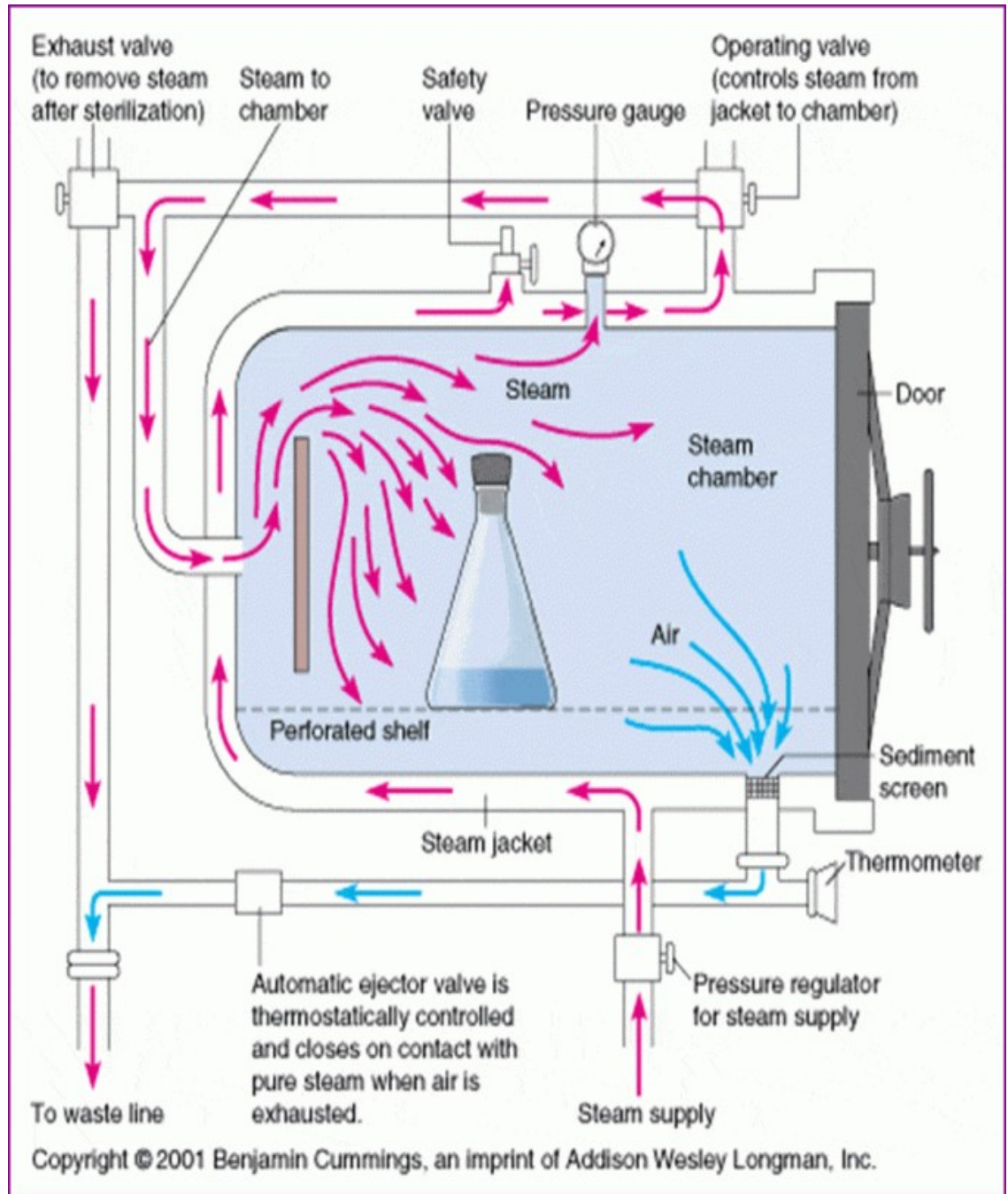
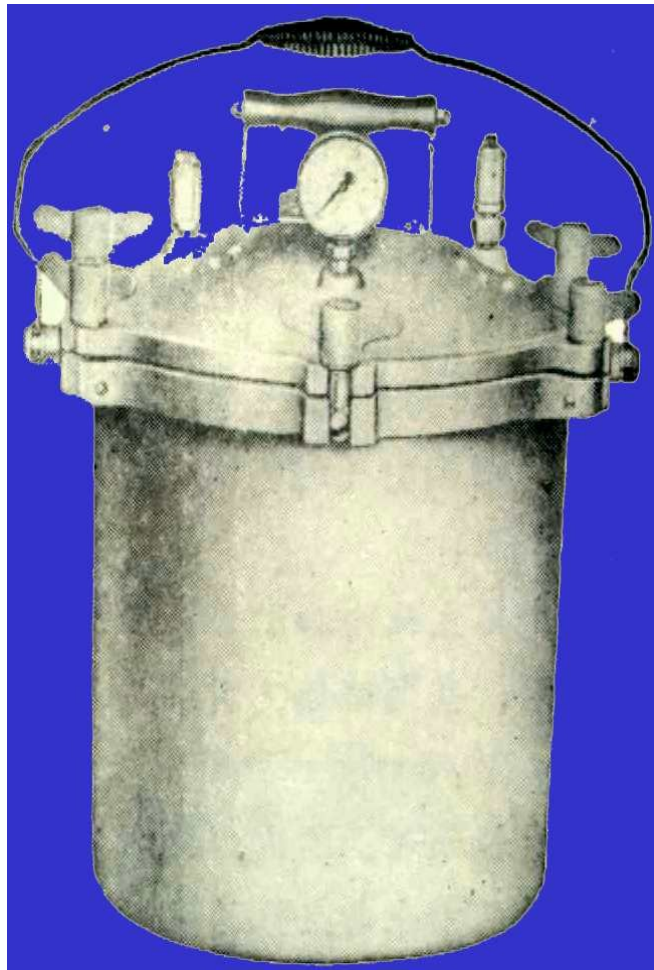
Moist heat cont'd:

❖ At temperature 100°C

- **Boiling**:- 100°C for 10 minutes in copper coated vessel.
- **Tyndallisation**:- Intermittent exposure at 100°C for 20-45 min on each three successive days kills vegetative cells.

❖ **At temperature greater than 100°C**

- Moist heat or steam under pressure of **103 kpa (15 psi)** in **autoclave** for 15 min raising the T⁰ of steam to 121 °C.
 - ✓ for sterilization of culture media and equipment and etc.



❑ **Sterilization by irradiation**

- Certain types of irradiation are used to control the growth of microorganisms. These include both ionizing and non-ionizing radiation.

❖ **Non-ionizing radiation**

- widely used form of non-ionizing radiation is ultraviolet (UV)
- **UV radiation** – at 250nm wavelength, biological safety cabinets, inoculation hood or chambers, work bench, operation theaters, tissue culture glass wares are sterilized

Non-ionizing radiation cont'd

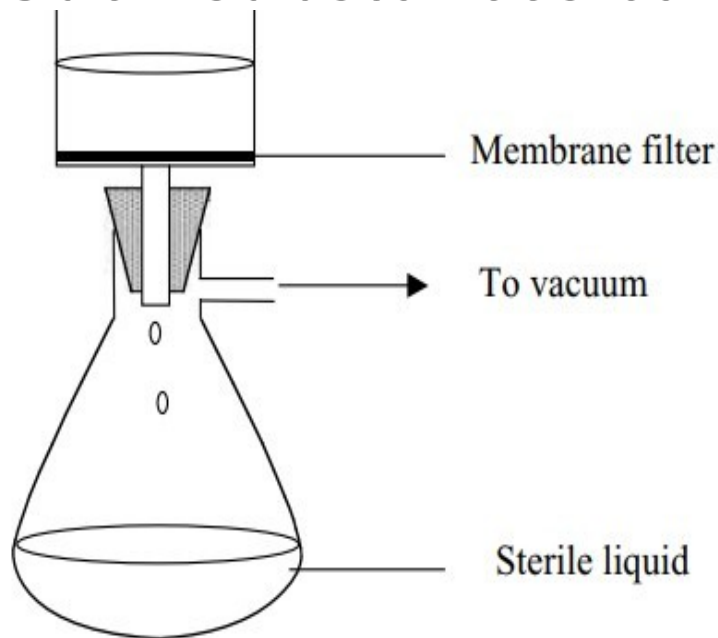
- ❖ B/c this wave length is absorbed by the purine and pyrimidine components of nucleic acids as well as certain aromatic amino acids in proteins.
- Rapture chemical bonds, so that normal cellular function impaired
- Causes thymine dimers which inhibit DNA replication

❖ Ionizing radiations

- have a shorter wavelength and much higher energy which give them greater penetrating powers
- It's effect is due to the production of highly reactive free radicals, which disrupt the structure of macromolecules such as DNA and proteins.
- Surgical supplies such as syringes, catheters and rubber gloves are commonly sterilized employing gamma (γ) rays from the isotope cobalt 60 (^{60}Co).
- Is used in situations where heat sterilization would be inappropriate, because of undesirable effects on the texture, taste or appearance of the product.

❑ Filtration

- Antibiotics solutions and certain components of culture media become chemically altered at high temperatures
- Such substances can be done for liquids and m through filters of an



B. Chemical Agents

- Chemical methods are used for **disinfection**

- ❖ Ethylene oxide

- ✓ for sterilizing large items of medical equipment, and materials such as plastics that would be damaged by heat treatment
- ✓ effective in sterilizing items such as dressings and mattresses, due to its great powers of penetration
- ✓ the materials to be treated are placed in a special chamber which is sealed and filled with the gas in a humid atmosphere at 40–50 °C for several hours.
- ✓ affect the structure of proteins and nucleic acids

Disinfection

- ❖ **Disinfection:** is the elimination or inhibition of pathogenic microorganisms in or on materials so that **they no longer pose a threat.**
- It does not necessarily kill all microorganisms, especially resistant bacterial endospores
- Less effective than sterilization
- The action of disinfectants is mainly due to their ability to react with microbial proteins, and therefore enzymes.

Disinfection cont'd

- E.g. **alcohols** (denatures proteins, act by dissolving lipids, and thus have a disruptive effect on membranes, and on the envelope of certain viruses)
- Halogens (Cl as gas and as component of chlorine releasing compounds such as hypochlorite and chloramines).
 - ✓ Sodium hypochlorite (household bleach) oxidizes sulfhydryl ($-SH$) and disulfide ($S-S$) bonds in proteins

- **Disinfectants:-** are chemical agents used to disinfect inanimate objects such as surface of materials and floors
- **Antiseptics:-** those chemicals which are used over living tissue.
 - ✓ is used to indicate a non-toxic disinfectant suitable for use on animal tissue

cont...

- **Antiseptics**: are **substances** which either kill microbes or inhibit their growth.
- **Asepsis**: a **technique** that is used in preventing infections from gaining access to uninfected tissue.
- **Bactericidal** - agents which destroy bacteria
- **Bacteriostasis** - agents Inhibits the growth of microorganisms
- **Cleaning**: is a removing process which may remove many Mos. It is a necessary **pre-requisite** before sterilization and disinfection.
- **Germicide**: chemical or physical agent that destroys most organisms but not spores
- **Sporocide**: chemicals that kills spores and vegetative cells as well
- **Fungicide**: chemicals that kill fungi
- **Fungistat**: chemicals that stop the growth of fungi

Factors affecting disinfection

- Time of exposure
- Temperature of application/ T^0 at which the agent is used
- Concentration of agents used
- Nature of the medium
- pH of the agents
- Types of bacteria and the number
- Effective permeability
- Presence of organic matter.

- The artificial culture of any organism requires a supply of the necessary nutrients, together with the provision of appropriate conditions such as temperature, pH and oxygen concentration.
- **Culture media** are artificially prepared media containing the required nutrients used for propagation of microorganisms or living tissue cells.
- **Culture:** is microbes that grow and multiply in or on a culture medium.

Cont'd

Uses of culture medium

- ✓ growth of microorganisms at *in-vitro* level
 - ✓ isolation and identification of microorganisms
 - ✓ performing antimicrobial sensitivity tests
-
- A synthetic growth medium may be **defined** (its exact chemical composition is known)
 - **Undefined or complex medium** (one whose precise chemical composition is not known.)
 - ✓ Undefined medium may have a variable composition due to the inclusion of a component such as blood, yeast extract or tap water

Types of culture medium

- ❑ Culture media can be classified by their **consistency (form)** as:

A. Solid media- solidified by agar

- ✓ used mainly in Petri dishes as plate cultures, in tubes as a **slant (for stab) or slope cultures**.
 - ✓ to isolate discrete colonies of each organism present in the specimen
 - ✓ which enable pure cultures to be produced for identification and sensitivity testing.
- **Gelatin had been used before agar found for solidifying agent**

Cont'd

- ❑ **Gelatin-** collagen sourced from **animal hides and bone marrow**
- ❑ **Agar-** a polymer made up of galactose, and is a component of the cell walls of **red algae**
- **Drawbacks of gelatin**
 - ✓ Melts below 37 °C and degraded by bacteria
- **Unique Properties of Agar:**
 - ✓ Melts above 95 °C. Once melted, does not solidify until it reaches 40 °C.
 - ✓ Cannot be degraded by most bacteria.

B. Semi solid media

- Are culture media prepared by adding small amount of agar (0.4 to 0.5% W/V) to a fluid medium.
- used mainly as transport media, and for motility and biochemical tests.

C. Liquid/fluid culture media

- used for biochemical test as an enrichment media and blood Culture. e.g. Peptone water.

Cont'd

 Based on chemical composition

1. Basic media/general purpose medium
2. Enrichment media
3. Selective media
4. Differential (indicator) media
5. Transport media

1. Basic media/general purpose media

- support the growth of microorganisms that do not need special nutritional requirements.

- ✓ **non-fastidious** microorganisms

Example: Nutrient agar, nutrient broth

Purposes of basic media

- used in preparation of enriched media.
- used to maintain **stock culture**.

2. Enriched media

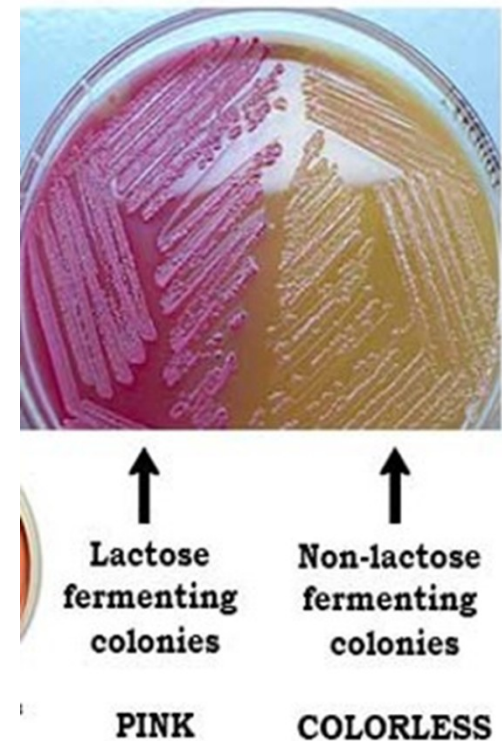
- Media required for growth of microorganisms with extra nutritional requirements such as *H. influenza*, *Neisseria spp.*, and some *streptococcus* species.
- An enriched medium increases the number of a pathogen by containing all the necessary ingredients to promote its growth.
- The media can be enriched with whole blood, lysed blood, serum, vitamins, and other growth factors.
- Example:- Blood Agar (contain whole blood)
Chocolate blood agar (contain lysed blood)

3. Selective media

- Contain substances that prevent or slow down growth of unwanted microbes and encourage the growth of desired microbes.
 - Designed for the isolation and identification of particular types
 - Is made selective by incorporation of certain substances like bile salt, crystal violet, antibiotics, etc.
 - Used when culturing a specimen from a site having normal microbial flora to prevent unwanted contaminants.
- **Example:** Thiosulphate citrate bile salt sucrose agar(TCBS) is alkaline medium and selective for **V.**

4. Differential (Indicator) media

- are media to which dyes or other substances are added to differentiate microorganisms.
- Many differential media distinguish between bacteria by incorporating an indicator which changes colour when acid is produced following fermentation of a specific carbohydrate.



Example: MacConkey agar- contain neutral red as an indicator and lactose as carbohydrate.

➤ The colony of lactose fermenting bacteria will become **pink/red** and other bacteria become

Differential (Indicator) media cont'd

- ❖ Many media act as both selective and differential
 - ✓ **MacConkey agar** contains **bile salts and the dye crystal violet**, both of which serve to inhibit the growth of **unwanted Gram-positive bacteria**.
 - ✓ **Mannitol salt agar** is also both selective and differential. The high (7.5%) salt content suppresses growth of most bacteria, whilst a combination of mannitol and an indicator permits the **detection of mannitol fermenters**

5. Transport media

- These are mostly semisolid media that contain ingredients to prevent the overgrowth of **commensals** and ensure the survival of aerobic and anaerobic pathogens when specimens cannot be cultured immediately after collection.
- Their use is particularly important when transporting microbiological specimens from **health centres to the public health laboratory and etc.**

E.g. Cary and Blair medium, Stuarts medium

Obtaining a Pure Culture

Techniques

Microorganisms in the natural world do not live in pure cultures; they exist as part of complex ecosystems comprising numerous other organisms.

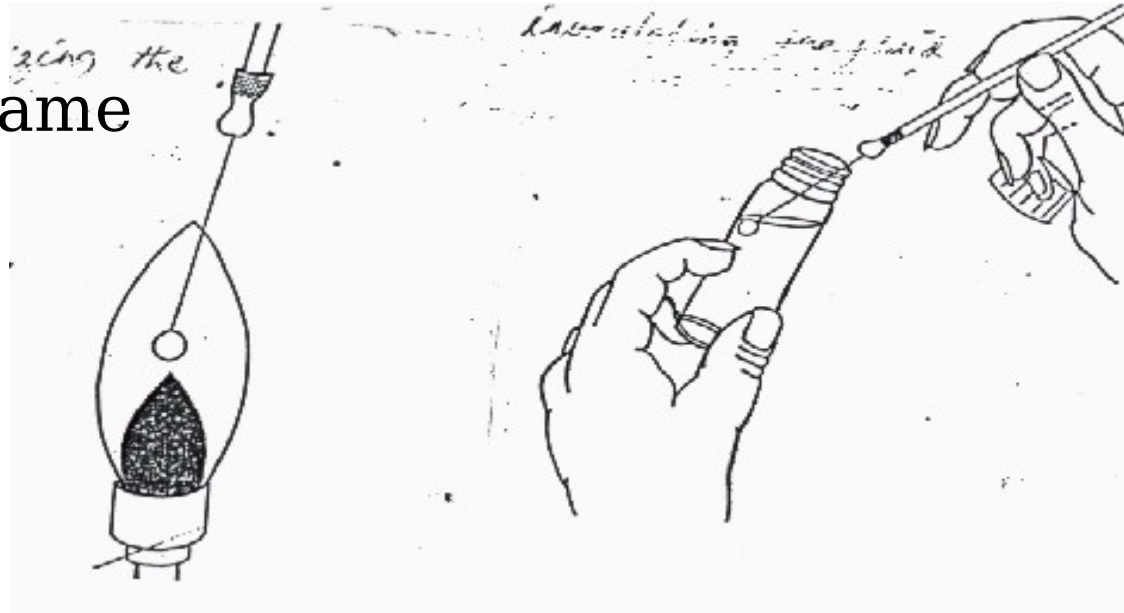
- **Pure culture (axenic culture):-** is a microbial culture containing a single species of organism.

Inoculation of culture media

- **Inoculation:-** is artificial seeding or introduction of microorganisms on/in to culture media or animal body.
- When inoculating culture media, an **aseptic technique** must be used to prevent **contamination of specimens** and **culture media**, and **laboratory worker** and the **environment**

- Before inoculating a culture medium, check the medium for visual contamination which may indicate **deterioration of the medium.**
- The aim of inoculating media (solid) is to get pure colony
- The technique used to inoculate media in Petridishes must provide single colonies for identification
- Before inoculating, the surface of the solid medium must be **dried**, other wise single colonies will not be formed.

Sterilizing
the
inoculating
loop
with flame



Inoculating
the fluid
media with
sterilized
loop

Cont'd

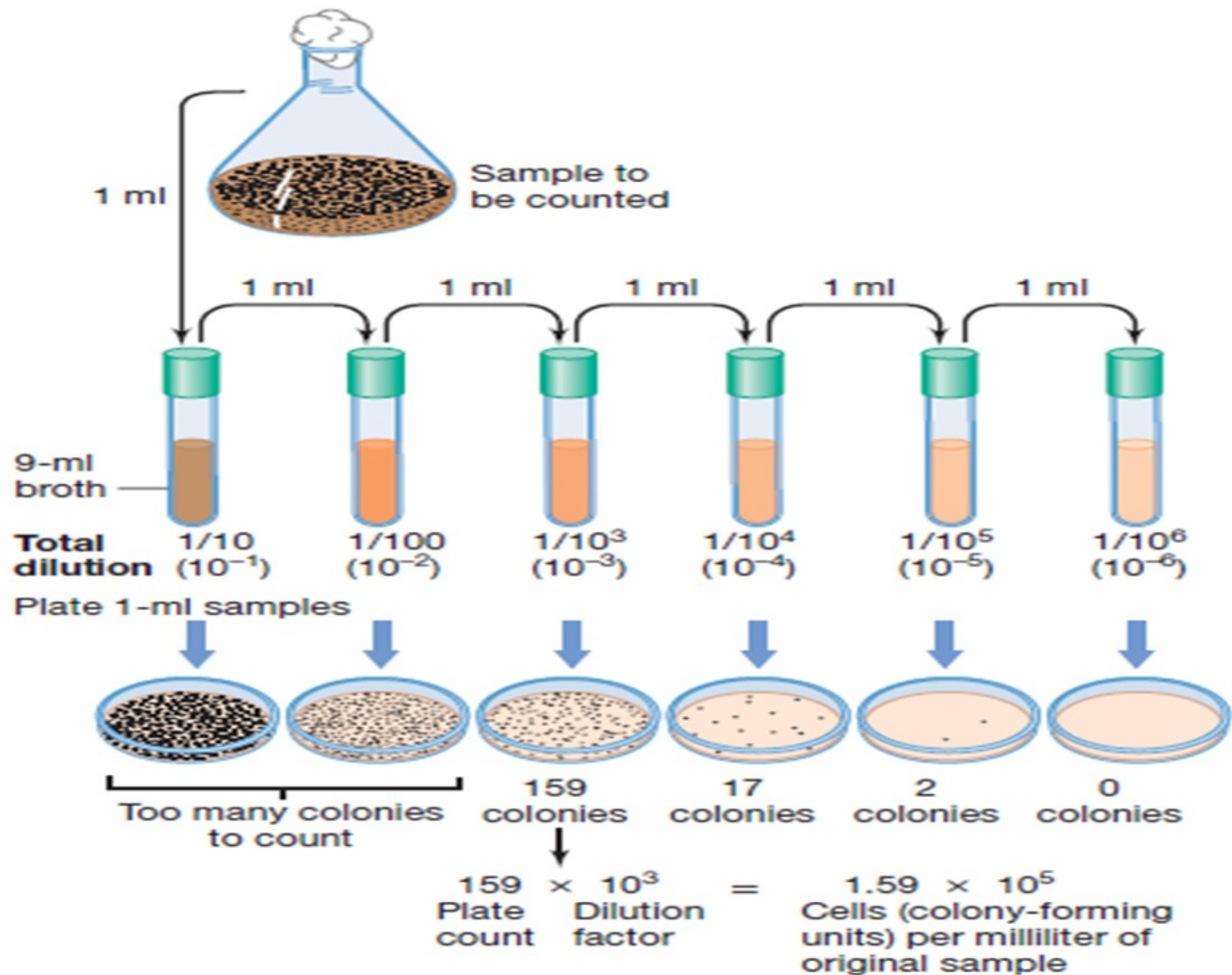
Obtaining Pure Culture of Microorganisms:

The methods are:

- Serial Dilution Method
- Spread Plate Method
- Pour Plate Method
- Streak Plate Method
- Use of specialized media.

Serial Dilution Method

- A sample containing a mixture of bacteria is serially diluted with a sterile broth in culture test tubes
- Used to isolate a microbe that is numerically dominant
- The technique of excluding the microbe that is numerically minor is called **extinction effect**



- involves plating of 0.1ml of diluted samples on solid medium and spreading it over the surface of the medium
- the mixed culture diluted in a series of tubes containing sterile liquid, usually, water or physiological saline.

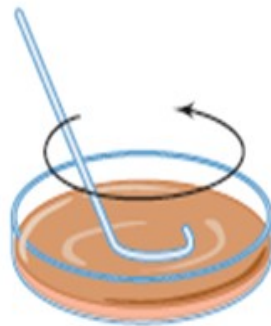
❑ **Pour Plate Method:**

- involves plating of diluted samples mixed with melted agar medium.
- The main principle is to dilute the inoculum in successive tubes containing liquefied agar medium so as to permit a thorough distribution of bacterial

Spread-plate method

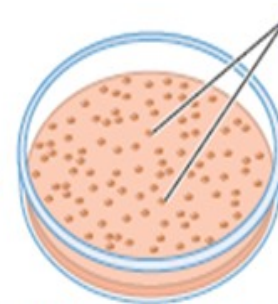


Sample is pipetted onto surface of agar plate (0.1 ml or less)



Sample is spread evenly over surface of agar using sterile glass spreader

Incubation



Typical spread-plate results

Surface colonies

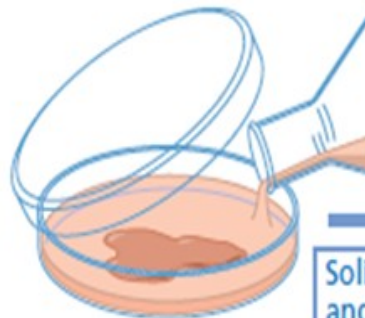


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Pour-plate method

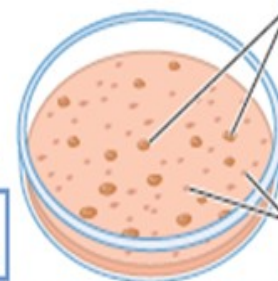


Sample is pipetted into sterile plate



Sterile medium is added and mixed well with inoculum

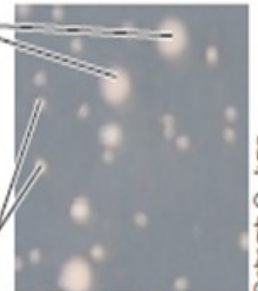
Solidification and incubation



Typical pour-plate results

Surface colonies

Subsurface colonies



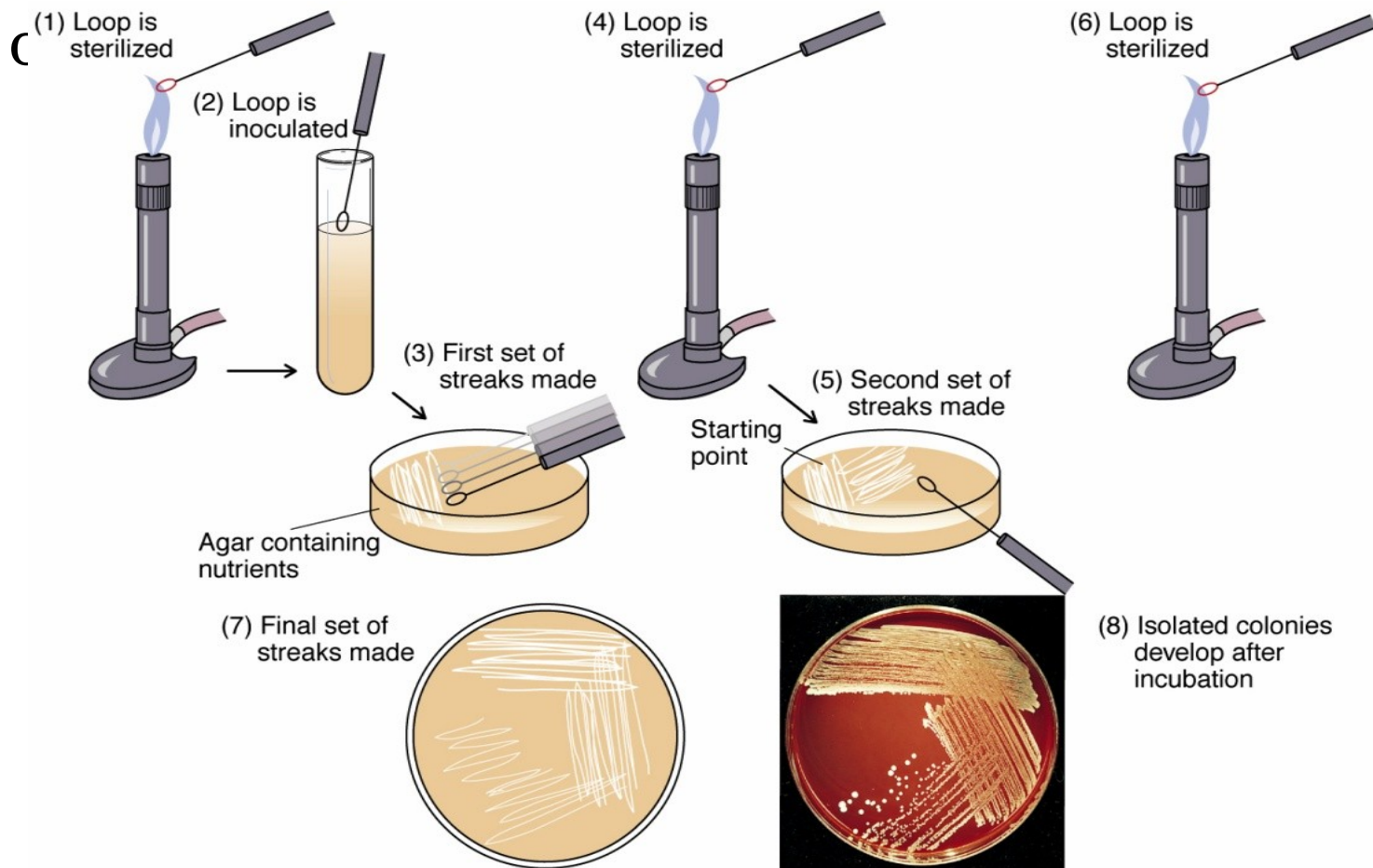
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Figure 5.15 Two methods for the viable count. In the pour-plate method, colonies form within the agar as well as on the agar surface. On the far right are photos of colonies of *Escherichia coli* formed from cells plated by the spread-plate method (top) or the pour-plate method (bottom).

follows:

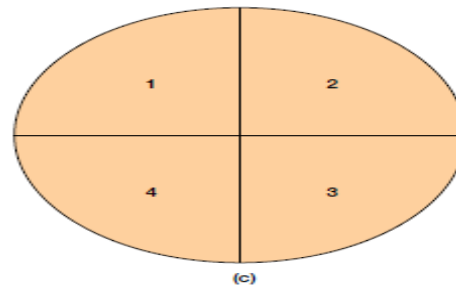
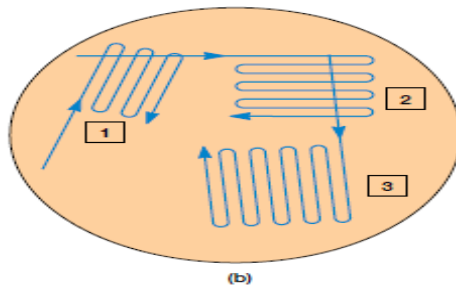
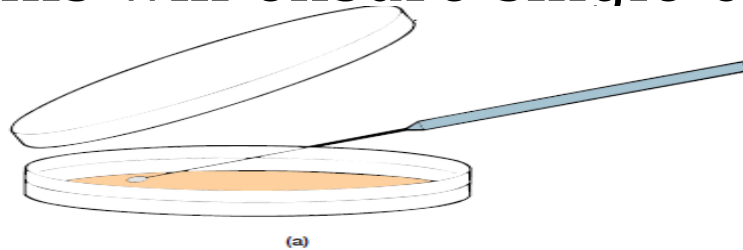
- (i) The picking up of subsurface colonies needs digging them out of the agar medium thus interfering with other colonies, and
 - (ii) The microbes being isolated must be able to withstand temporary exposure to the 42-45° temperature of the liquid agar medium
- ✓ therefore this technique proves unsuitable for the isolation of psychrophilic microorganisms.

- ❑ **Streak Plate Method:-** this method is used most commonly to **isolate pure cultures of bacteria**
- ❑ It's standard method of obtaining a pure bacterial



Inoculation Techniques

1. Using a sterile loop or swab of the specimen, apply the inoculation to a **small area of the plate.**
2. Flame to sterilize the loop, when cool or using a second sterile loop, spread the inoculation systematically. This will ensure single colony growth



Staining techniques

- **Stains (dyes)** are coloured chemical compounds that are used to selectively give colour to the colourless structures of **bacteria or other cells**.
- Bacterial staining is the process of **imparting colour** to the **colourless structures** (cell wall, **spore**, etc) of the bacteria in order to make it visible under the microscope.

Uses

1. To observe the morphology, size and arrangement of bacteria
 2. To differentiate one group of bacteria from the other group.
-
- Staining reactions are made possible because of the:
 - ✓ physical phenomena of capillary osmosis, solubility and absorption of stains by cells of microorganisms.
 - ✓ individual variation in the cell wall constituents among different groups of bacteria will consequently produce variations in colours during microscopic examination.

Why dyes colour microbial cells?

- Because dyes absorb radiation energy in visible region of electromagnetic spectrum i.e., “light” (wave length 400-650).
- And absorption of anything outside this range it is colourless.

E.g. Acid fuchsin absorbs blue green and transmit red.

Type of staining methods

1. Simple staining method
2. Differential staining method
3. Special staining method

❖ Simple staining method

- It is a type of staining method in w/c **only a single dye** is used.
- Used to determine the **size**, **shape** and **arrangement** of prokaryotic cells
- There are **two** kinds of simple staining methods
 - Positive staining and Negative staining

A. Positive staining:- the **bacteria or its parts** are **stained** by the dye.

e.g. Methylene blue stain, Crystal violet stain

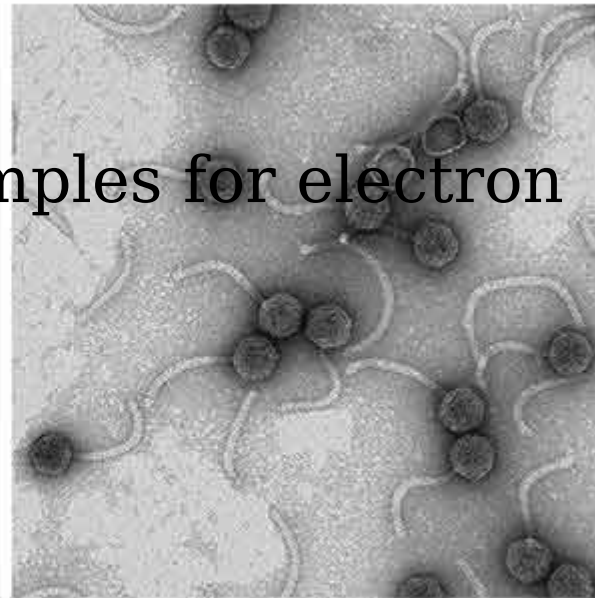
B. Negative staining:- the dye stains the **background** and the bacterial component remain unstained. e.g. Indian ink stain.

➤ Used to study the morphological shape, size and arrangement of the **bacteria cells that is difficult to stain** eg: *Spirilla*.

➤ to prepare biological samples for electron microscopy



Negatively Stained Cocci



Bacteriophage
(TEM Negative Stain)

❖ **Differential staining method**

- A method in which **multiple stains** are used to distinguish different group of bacteria. e.g. Gram's stain, Ziehl-Neelson stain.

Gram's stain

- ✓ This method was developed by the Danish bacteriologist Hans Christian Gram in 1884.

Basic concepts:

- Most bacteria are differentiated by their Gram reaction due to differences in their **cell wall structure**.

Gram staining technique

- Differentiate bacteria into **Gram Positive** and **Gram Negative Bacteria**
- Widely used differential stain in bacteriology.
- Gram-positive bacteria **retain** the crystal violet-iodine complex when washed with the decolorizer, **Ethanol**, whereas
- Gram-negative bacteria **lose** their crystal violet-iodine complex and become colorless.

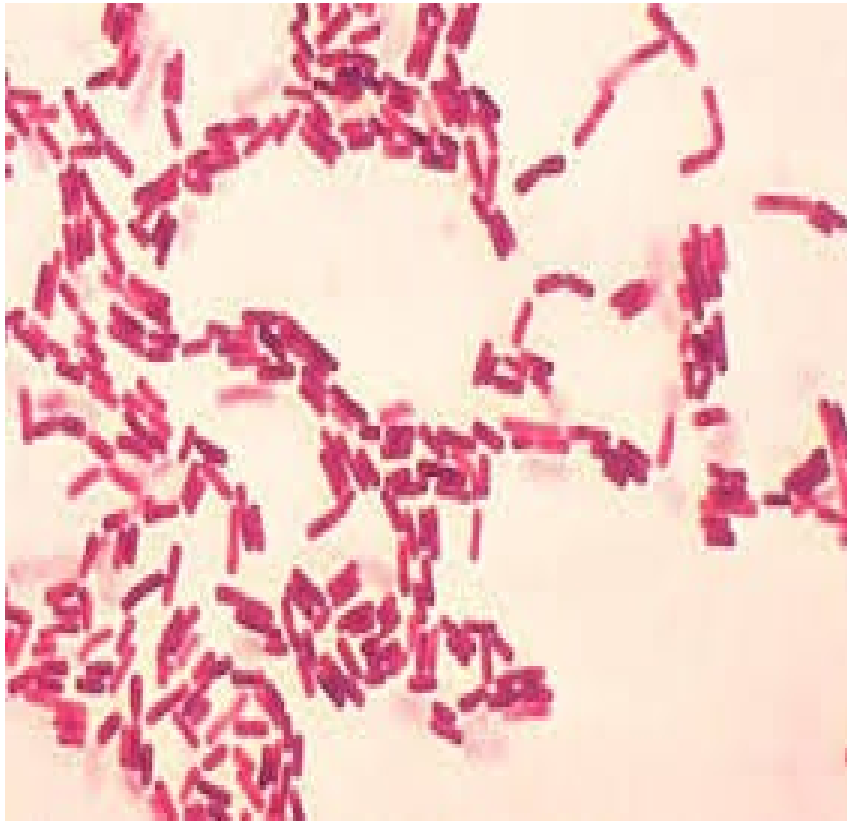
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- The cell walls of Gram positive bacteria have a thick layer of protein-sugar complexes called **peptidoglycan** and lipid content is low.
 - Decolorizing the cell **causes this thick cell wall to dehydrate and shrink**, which closes the pores in the cell wall and prevents the stain from exiting the cell.
 - So ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of Gram positive bacteria and

Cont'

- In case of Gram negative bacteria, cell wall also takes up the Crystal Violet-Iodine complex but due to the **thin layer of peptidoglycan** and thick outer layer which is formed of lipids, CV-Iodine complex gets washed off.
- ❖ When they are exposed to alcohol, decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells.



**Gram Negative
Bacteria**



**Gram Positive
Bacteria**

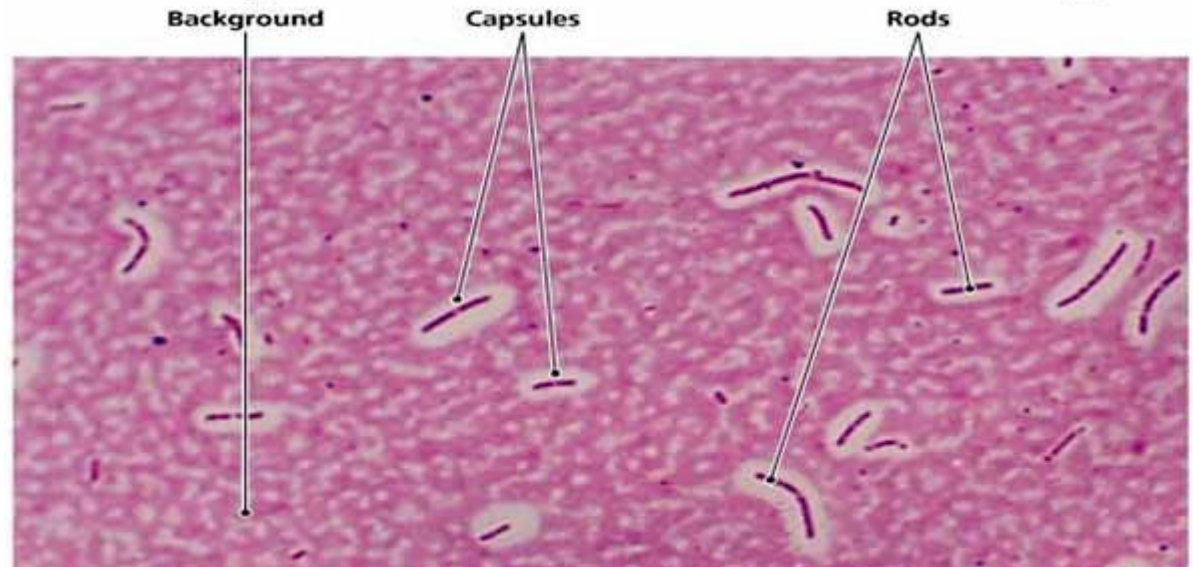
3. Special Staining method

- These are stains, which are used to stain capsules and spores.

A. Capsule staining method

- This technique is used for showing the presence of capsules and

Capsule Staining



B. Spore staining method

- ✓ The primary dye **malachite green** is a relatively weakly binding dye to the cell wall and spore wall.
- ✓ In fact, if washed well with water, the dye comes right out of the cell wall. That is why there does not need a decolourizer in this stain.
- ✓ It is based on the binding of the malachite green and the permeability of the spore vs. cell wall.
- ✓ The steaming helps the malachite green to permeate the spore wall.



Interpretation

- Spores- **light green**
- Vegetative cell walls will pick up the counter stain **safranin-Red/pink**