UNIT II (10 Periods)

Cultivation and Maintenance of microorganisms: Nutritional categories of micro-organisms, methods of isolation, Purification and preservation.

Nutrients – are substances used in biosynthesis and energy production.

Nutrient Requirements:

Microbial cell composition shows that 95% of cell dry weight is made up of a few major elements: Carbon, oxygen, hydrogen, nitrogen, sulfur, phosphorous, potassium, calcium, magnesium and iron.

Macronutrients or macro elements:

These are required by microorganisms in relatively large amounts. Carbon, oxygen, hydrogen nitrogen, sulfurs and phosphorous are components of carbohydrates, lipids, proteins and nucleic acids. The remaining four macro elements (K, Ca, Mg and Fe) exist in the cell as cations.

K^+ - is required for the activity by a number of enzymes, including those involved in protein synthesis.

Ca^{2+} - contributes to the heat resistance of bacterial endospores. 15% of spore contains dipicolinic acid and calcium.

Mg^{2+} - serves as a cofactor for many enzymes, complexes with ATP and stabilizes ribosomes and cell membranes.

Fe^{2+} and Fe^{2+} - part of cytochromes and a cofactor for enzymes and electron-carrying proteins.

Micronutrients or Trace elements:

These are manganese, zinc, cobalt, molybdenum, nickel and copper. These are normally part of enzymes and cofactors, and they aid in the catalysis of reactions and maintenance of protein structure.

Zn^{2+} - is present at the active site of some enzymes but is also involved in the association of regulatory and catalytic subunits in *E.coli* aspartate carbomoyl transferase.

Mn^{2+} - aids many enzymes catalyzing the transfer of phosphate groups.

Mo^{2+} - required for nitrogen fixation.

Co^{2+} - is a component of Vitamin B12.

Besides macro and micro nutrients, some microorganisms may have particular requirements that reflect the special nature of their morphology or environment. Diatoms need silicic acid to construct their beautiful cell walls of silica. Bacteria growing in saline lakes and oceans depend on the presence of high concentrations of sodium ion. Microorganisms require a balanced mixture of all the above nutrients for proper growth.

Growth factors:

Many microorganisms have the enzymes and pathways necessary to synthesize all cell components. Many lack one or more enzymes and hence require organic compounds because they are essential cell components or precursors of such components and cannot be synthesized by the organisms are called – growth factors. **There are three major classes of growth factors:**

Amino acids – needed for protein synthesis.

Purines and Pyramidines – for nucleic acid synthesis

Vitamins – small organic molecules that usually make up all or part of enzyme cofactors, and only very small amounts sustain growth.

Bacteria that can synthesize all they require from the basic ingredients are called **Prototrophs** – and most microorganisms that survive in the outside environment can do this. Microbes that have become adapted to life in a situation rich with nutrients such as human body may require other growth factors like vitamins, amino acids or nitrogenous bases to be provided. These are called **auxotrophs.**

MEDIA

The media that are used in microbiology laboratories to culture bacteria are referred to as artificial media or synthetic media, because they do not occur naturally; rather they are prepared in the laboratory. There are number of ways of categorizing the media, one way is to classify based on whether the exact contents of the media are known or not.

Chemically defined media : Is one in which all the ingredients are known; and was prepared in the laboratory by adding a certain number of grams of each of the components (Carbohydrates, amino acids, salts etc). Particularly photolithoautotrophs such as Cyanobacteria and eukaryotic algae can be grown on relatively simple media containing CO_2 as a carbon source (often added as sodium carbonate or bicarbonate), nitrate or ammonia as a nitrogen source, sulfate, phosphate and a variety of minerals. Chemoorganoheterotrophs can be grown in a defined media with glucose as a carbon source and an ammonium salt as a nitrogen source.

Complex media : contains undefined ingredients and the exact contents are not known. Complex medium may be sufficiently rich and complete to meet the nutritional requirements of many different microorganisms. They contain undefined components like peptones, meat extract and yeast extract.

Requirements for carbon, hydrogen and oxygen:

Carbon is needed for the skeleton or backbone of all organic molecules and molecules serving as carbon sources normally also contribute both oxygen and hydrogen atoms. One important carbon source that does not supply hydrogen or energy is CO_2 .

Autotrophs – can use CO_2 as their sole or principal source of carbon. Many microorganisms are autotrophic, and most of these carry out photosynthesis and use light as their energy source. Some autotrophs oxidize inorganic molecules and derive energy from electron transfer.

Heterotrophs – are organisms that use reduced pre-formed organic molecules as carbon sources. Ex. Glycolytic pathway produces carbon skeleton for use in biosynthesis and also releases energy as ATP and NADH.

Actinomycetes will degrade amyl alcohol, paraffin and even rubber.

Burkholderia cepacia can use over 100 different carbon compounds.

Some microorganisms can metabolize even relatively indigestible human-made substances such as pesticides. Indigestible molecules can be oxidized and degraded in the presence of a growth promoting nutrient that is metabolized at the same time, a process called Co-metabolism.

The products of this breakdown can then be used as nutrients by other microorganisms.

Nutritional types of microorganisms:

In addition to Carbon, hydrogen and oxygen all organisms require sources of energy and electrons for growth.

Carbon sources:

Autotrophs - CO_2 sole or principal biosynthetic carbon source

Heterotrophs – reduced, preformed organic molecules from other organisms.

Energy sources:

Phototrophs – use light as their energy source.

Chemotrophs – obtain energy from the oxidation of chemical compounds (either organic or in organic)

Electron sources:

Lithotrophs – use reduced inorganic substances as their electron source.

Organotrophs – extract electrons from organic compounds.

Four major nutritional classes based on their primary sources of carbon, energy and electrons is known.

1. Phtotolithotrophic autotrophs or photoautotrophs or photolithoautotrophs:

Source of energy – light energy

Source of electrons – Inorganic hydrogen/ electron

Carbon source - CO_2

Example: Algae, purple and green sulfur bacteria and cyanobacteria.

2. Photoorganotrophic heterotrophy or photoorganoheterotrophy:

Source of energy – light energy

Source of electrons – organic hydrogen/ electron

Carbon source – organic carbon sources (CO_2 may also be used)

Example: Purple and green nonsulfur bacteria (common inhabitants of lakes and streams)

3. Chemolithotrophic autotrophs or chemolithoautotrophy:

Source of energy – Chemical energy source (inorganic)

Source of electrons – Inorganic hydrogen/ electron donor

Carbon source - CO_2

Example: Sulfur-oxidizing bacteria, hydrogen bacteria, nitrifying bacteria, iron-oxidizing bacteria.

4. Chemoorganotrophic heterotrophs or chemoorganoheterotrophy:

Source of energy – Chemical energy source (organic)

Source of electrons – Inorganic hydrogen/ electron donor

Carbon source – organic carbon source

Example: Protozoan, fungi, most non-photosynthetic bacteria (including most pathogens)

The most common nutritional types are photolithoautotrophs and chemoorganoheterotrophs.

Mixotrophic Bacteria *Beggiatoa* rely on inorganic energy sources and organic (or sometimes CO_2) carbon sources. These microbes are called Mixotrophic because they combine chemolithoautotrophic and heterotrophic metabolic processes.

Media A **growth medium** or **culture medium** is a solid, liquid, or semi-solid designed to support the growth of a population of microorganisms or cells via the process of cell proliferation

Types of Media:

Selective Media: Are designed to suppress the growth of unwanted bacteria and encourage the growth of the desired microbes.

Examples: **MacConkey agar** inhibits growth of gram positive bacteria and thus is selective for gram-negative bacteria. Phenylethyl alcohol (PEA) agar and Colistinalidixic acid (CAN) agar are selective for gram positive bacteria.

Manitol salt agar contains a concentration of 7.5% NaCl that is quite inhibitory to most human pathogens. **Bile salts,** a component of fecus, inhibit most gram positive bacteria while permitting many gram negative rods to grow. This media is used for selecting intestinal pathogens which contain bile salts. **Dyes such as methylene blue and crystal violet** also inhibit certain gram positive bacteria (Staphylococcus can produce acid from mannitol and turn the phenol red dye to bright yellow.

A medium containing acetate as a carbon source would be selective for organisms that grow on acetate.

Sabaraud's dextrose agar is used to isolate fungi and cellulose for cellulose digesting bacteria.

Differential media :

To distinguish colonies of the desired organism from other colonies growing on the same plate.

MacConkey agar is also a differential and selective medium to distinguish between lactose fermenting organisms (e.g, *E.coli*) and other non-fermenters (e.g, *Shigella* sp.).

Lactose in the medium is fermented by *E.coli* producing acid which causes an indicator dye to change color to red and colonies that do not ferment lactose are white.

Dyes can be used as differential agents because many of them are pH indicators that change color in response to the production of an acid or base. MacConkey agar contains **neutral red,** a dye that is yellow when neutral and pink or red when acidic.

Mannitol salt agar is used to screen for *Staphylococcus aureus,* and it turns the originally pink medium to yellow due to its ability to ferment mannitol.

Blood agar is also differential because it is used to determine the hemolytic and non-hemolytic bacteria.

Blood agar is both differential and an enriched one.

It distinguishes between hemolytic and non-hemolytic bacteria. Hemolytic bacteria (eg., many *Streptococci* and *Staphylococci*) produce clear zones around their colonies because of red blood cell destruction.

Minimal medium

A defined medium that has just enough ingredients to support growth is called a "minimal medium". Minimal media are those that contain the minimum nutrients possible for colony growth, generally without the presence of amino acids, and are often used to grow "wild-type" microorganisms.

Enriched media

Enriched media contain the nutrients required to support the growth of a wide variety of organisms, including some of the more fastidious ones. They are commonly used to harvest as many different types of microbes as are present in the specimen.

Blood agar is an enriched medium in which nutritionally rich whole blood supplements the basic nutrients. Chocolate agar is enriched with heat-treated blood (40–45 °C or 104–113 °F), which turns brown and gives the medium the color for which it is named.

Transport media Temporary storage of specimens being transported to the laboratory for cultivation Maintain the viability of all organisms in the specimen without altering their concentration Contain only buffers and salt Lack of carbon, nitrogen, and organic growth factors so as to prevent microbial multiplication

Isolation of pure cultures:

In natural habitats, microorganisms usually grow in complex, mixed populations containing several species. One needs a pure culture, a population of cells arising from a single cell, to characterize an individual species. Robert Koch introduced pure culture techniques.

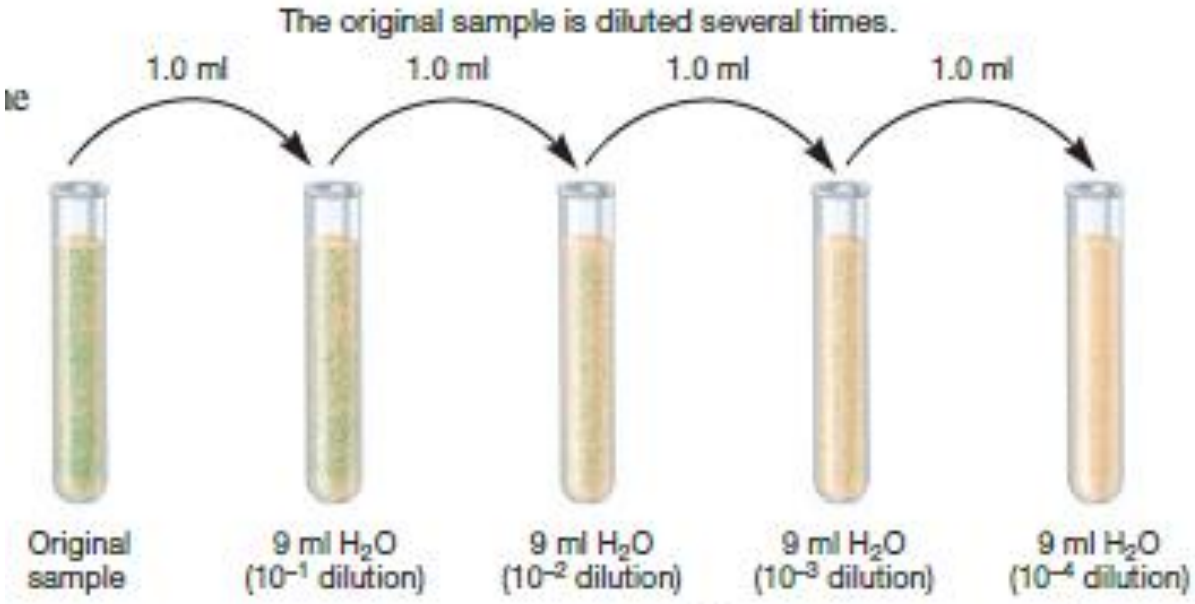
Spread plate and streak plate:

Spread plate - Mixture of cells is spread out on an agar surface so that every cell grows into a completely separate colony, each colony representing a pure culture. A small volume of around 30 to 300 cells (mixed) is transferred to the center of the plate and spread evenly over the surface with a sterile bent-glass rod.

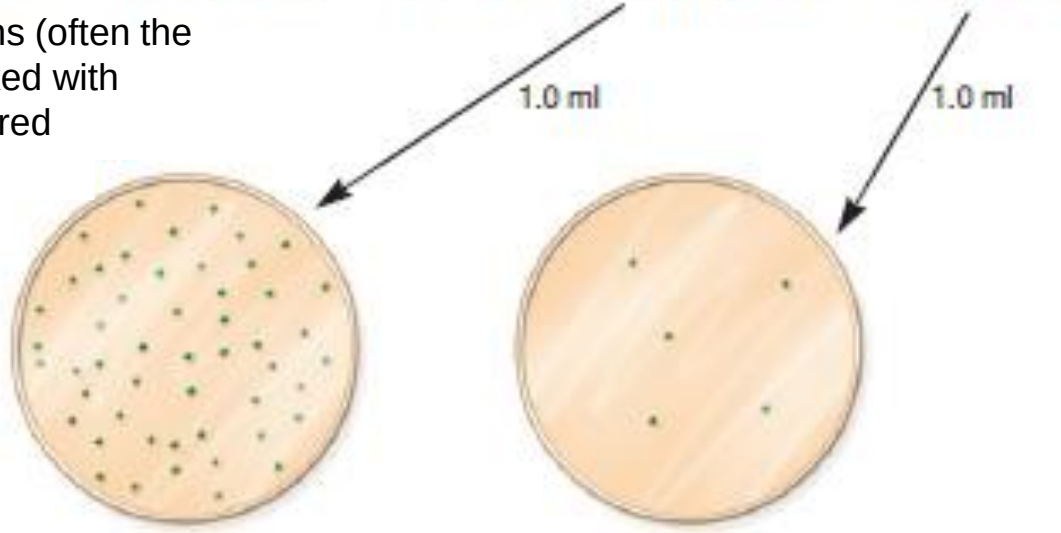
Streak plate – the microbial mixture is transferred to the edge of an agar plate with an inoculating loop or swab and then streaked out over the surface in one of several patterns. In both these techniques, successful isolation depends on spatial separation of single cells.

The pour plate : Extensively used with bacteria and fungi. The original sample is diluted several times to reduce the microbial population sufficiently to obtain separate colonies when plating. The microbes are mixed with molten agar which has been cooled at 45° C and then poured into perti dishes.

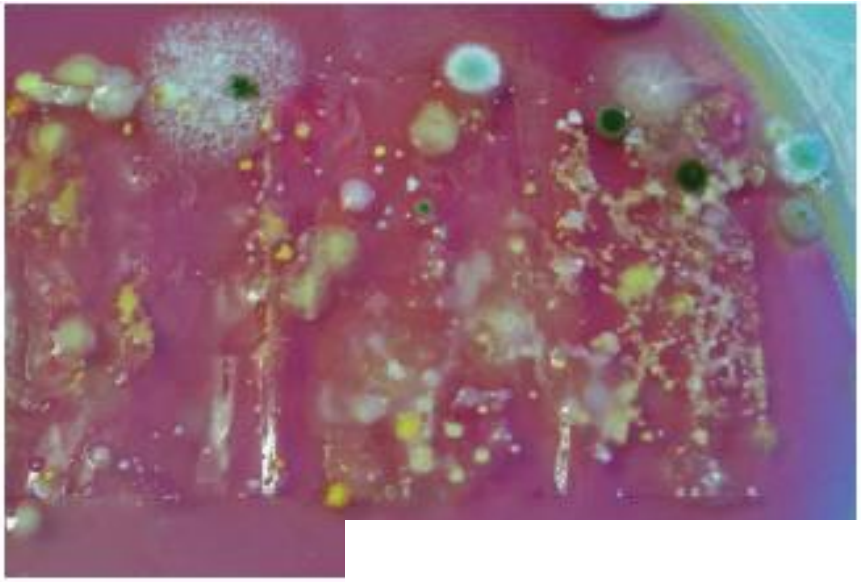
All these techniques require petri dishes (special culture dishes) after their inventor Julius Richard Petri (1887). Each bacterium replicates to form a colony that is visible to the naked eye. A colony contains up to 10^9 copies of the original bacterium.

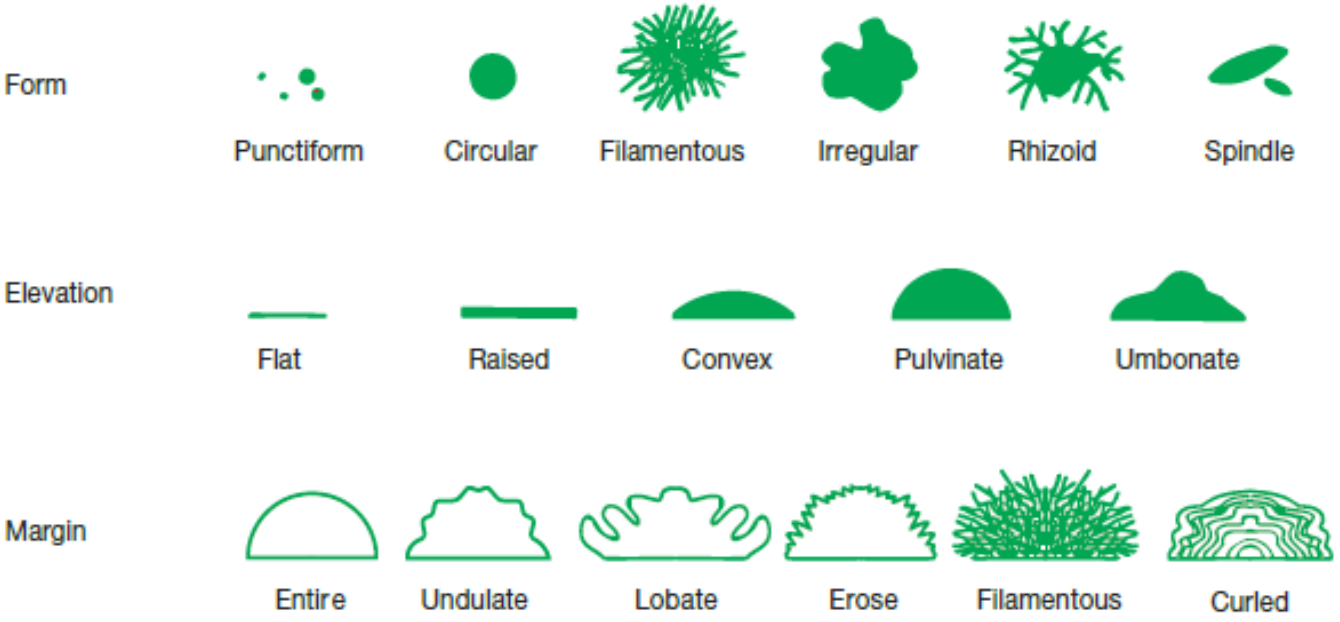


Some of the dilutions (often the most dilute) are mixed with warm agar and poured onto the plates.



Isolated cells grow into colonies on the surface (appear round) and within the medium (appear lens-shaped). The isolated colonies can be counted or used to establish pure cultures.




Form
Punctiform
Circular
Filamentous
Irregular
Rhizoid
Spindle
Elevation
Flat
Raised
Convex
Pulvinate
Umbonate
Margin
Entire
Undulate
Lobate
Erose
Filamentous
Curled

If replicate dilutions are prepared and the number of tubes yielding growth determined, then the number of viable cells in the sample can be estimated by the **most probable number method (MPN)**

Bacterial and Fungal Preservation Methods

Table of Contents

Maintenance Methods •

•**Pure culture-** Stock cultures- organisms of interest maintained in laboratory as reference

• • Necessary to prevent contamination, retain viability and remain true to type

•Two methods of maintenance of cultures 1. Agar Slants 2. Agar Stabs

Agar Slants Agar slants are a form of solid media generated from the addition of a gelling agent, such as agar, to a broth culture • Inoculated and incubated until reasonable growth – placed in a refrigerator after well sealing done to prevent drying • Hardy organisms (Spore formers, GPCs, Yeasts and Fungi)- remain viable for weeks to months • Regular transfer to fresh slants necessary

Agar Stabs An inoculation technique used when inoculating semi-solid medium for the analysis of motility or oxygen usage, or when inoculating certain types of solid medium • Inoculation is done with a straight needle having a culture – deep all the way to the bottom • Culture grows as a thin zone along the inoculation line • Acid produced as waste is diluted along the agar • Preserve upto 2 weeks

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 continues slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

Paraffin Method

•This is a simple and most economical method of maintaining pure cultures of bacteria and fungi.

•In this method, sterile liquid paraffin in 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 is preserved for several years.

Saline Suspension

•Sodium chloride in high concentration is frequently an inhibitor of bacterial growth.

•Bacteria are suspended in 1% salt solution (sublethal concentration in screw cap tubes to prevent evaporation).

•The tubes are stored at room temperature. Whenever needed the transfer is made on agar slant.

Lyophilisation (Freeze-Drying)

•In this method, the culture is rapidly frozen at a very low temperature (around -70°C) and then dehydrated by vacuum.

•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 centres.

The Lyophilisation Process

•In this process the microbial suspension is placed in small vials.

•A thin film is frozen over the inside surface of the vial by rotating it in mixture of dry ice (solid carbon dioxide) and alcohol, or acetone at a temperature of −78^{o}C .

•The vials are immediately connected to a high vacuum line. This dries the organism while still frozen.

•Finally, the ampules are sealed off in a vacuum with small flame.

•These cultures can be stored for several years at 40°C.

•To revive microbial cultures it is merely necessary to break open the vial aseptically, add a suitable stale medium, and after incubation make further transfers.

•The process permits the maintenance of longer number of culture without variation in characteristics of the culture and greatly reduces the danger of contamination.

Preservation at Very Low Temperature

- The organisms are suspended in nutrient broth containing 15% glycerol.
- The suspension is frozen and stored at -15°C to -30°C.

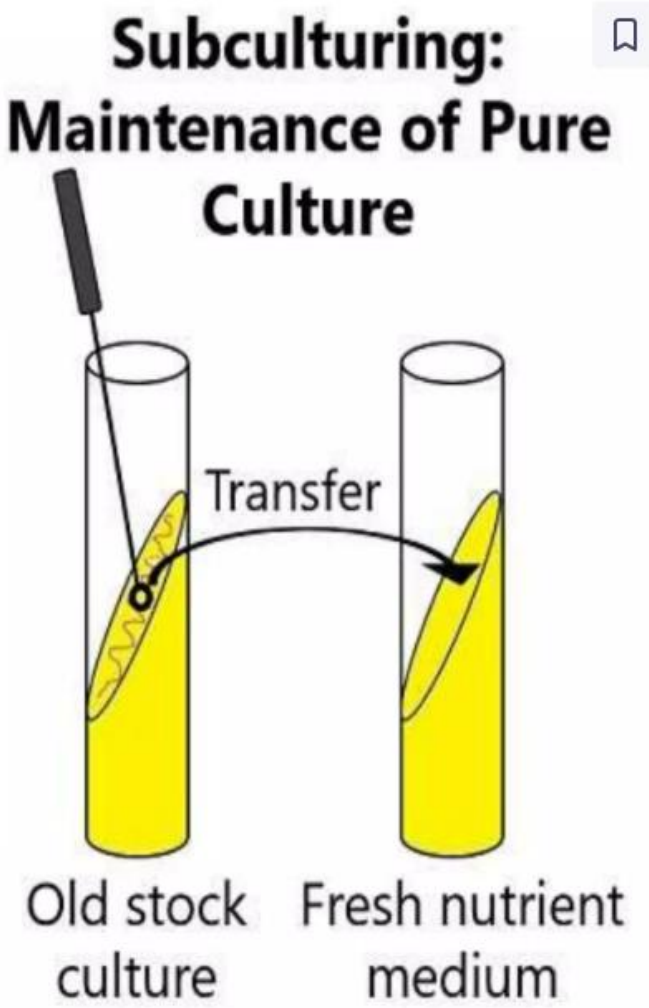
Preservation by Drying in Vacuum

- The organisms are dried over calcium chloride in the vacuum and are stored in the refrigerator.

• **PRESERVATION OF BACTERIA**

Bacterial species vary greatly in the ability of their cultures to remain alive after the completion of growth(e.g. after 24 hr at 37°C) • Problem maintaining them for various periods in a viable condition • Necessary to eliminate genetic instability, protection against contaminants and retain their original characters • Most laboratories maintain a large collection of pure cultures, frequently referred to as the stock culture collection • Some species such as Neisseria are poorly viable and their cultures usually die out within few days in routine conditions, must be subcultured regularly(2-4 days)

Culture collections of microorganisms are valuable resources for and scientific research in microbial diversity and evolution, patient care management, epidemiological investigations, and educational purposes • Preserved individual strains of microorganisms serve as permanent records of microorganisms' unique phenotypic profiles and provide the material for further genotypic characterizations • Effective storage is defined by the ability to maintain an organism in a viable state free of contamination and without changes in its genotypic or phenotypic characteristics


Subculturing:
Maintenance of Pure Culture
Transfer
Old stock culture
Fresh nutrient medium

Short term methods of culture preservation include:

- Periodic transfer to fresh media.
- Preservation using saline suspension.
- Preservation by drying method.
- Preservation by refrigeration.