

UNIT-I

NUTRITIONAL REQUIREMENTS AND ISOLATION AND PRESERVATION OF BACTERIA



CENTURION
UNIVERSITY
*Shaping Lives...
Empowering Communities!*

RASMITA JENA
ASSISTANT PROFESSOR
SoPLS, CUTM, BBSR

Nutritional requirements, raw materials used for culture media

- Nutrition is substances used in **biosynthesis and energy production** and therefore are required for all living things. Bacteria, like all living cells, **require energy and nutrients to build proteins and structural membranes and drive biochemical processes.**
- Bacteria require sources of **carbon, nitrogen, phosphorus, iron** and a large number of other molecules. **Carbon, nitrogen, and water** are used in the highest quantities.
- The nutritional requirements for bacteria can be grouped according to the carbon source and the energy source.
- Some types of bacteria must consume preformed organic molecules to obtain energy, while other bacteria can generate their own energy from inorganic sources.

- **CULTURE** - the growth or crop of microorganisms obtained in a culture medium after its incubation period.
- **CULTURE MEDIUM** - any material where microorganisms may thrive for their nourishment and reproduction.
- **INCUBATION PERIOD** – is the time needed to let previously inoculated culture media to show a distinct colony or colonies within a desired temperature.
 - It is also the time needed for the microorganisms to adapt, grow and multiply in its new environment.
- **COLONY** - a group of microorganisms growing together characteristically in a culture medium.
- **INOCULUM** – the fished-out colony hanging on the wire loop, cotton swabs, etc. that is ready for transfer to another culture medium for cultivation and/or for further isolation

Bacteriological Media:

- Bacteriological media or culture media are an artificially prepared mixture of various nutrients for the growth, multiplication, differentiation and transportation of microorganisms.
- A culture media must supply suitable carbon, nitrogen, energy sources and other nutrients.
- A lot of culture media are available but there is no single media suitable for growth of all microorganisms.

Common ingredients used in bacteriological media :

Water:

- Microbes contain almost 70 to 80% water in their Protoplasm.
- Water is used for preparation of culture media by dissolving various organic and inorganic compounds.
- Copper distilled water should not be used for preparation of culture media as copper is known to inhibit bacterial growth.

Peptone:

- Peptone is a partially digested protein obtained from partial hydrolysis of lean meat.
- The important constituents of peptone are amino acids, proteases, certain inorganic salt and vitamins.
- Peptone acts as a nitrogen source and as a buffer.
- It must be stored in a tightly closed container as it is highly hygroscopic and becomes very sticky on contact with air.

Yeast Extract:

- It is prepared from Baker's Yeast i.e. **Saccharomyces**.
- It contains carbohydrates, amino acids, inorganic salts and vitamins from the B complex group.
- It is mainly used as a source of vitamins and can be substituted for meat extract.

Meat Extract:

- It is prepared by hot water extraction of fresh lean meat.
- Contents are gelatin peptones, proteases amino acids, mineral salt and certain vitamins.

Agar: It is long chain polysaccharide obtained from extraction of seaweeds called Agarophytes. Agar contains a mixture of two polysaccharides Agarose and Agaropectin along with certain inorganic salts, a protein like substance and many inorganic ions.

- Important properties of agar are as follows
 - Acts as a good solidifying agent (2%).
 - Has no nutritional value in the media.
 - Bacteriologically inert.
 - Resistant to the action of all microorganisms.
 - Stable at different temperatures used for incubation.
 - Melts @ 95 to 98 °C and remain liquid @ 40-45 °C.
 - Gets solidified below 40°C temp.
 - Economical and easily available.

Types of Culture Media

- They are classified in different way as follows;
- ***Depending on Physical State (Consistency):***
 - **Solid Media** (1.5 to 2.5 % Agar): e.g. Nutrient Agar.
 - **Semisolid Media** (0.2 to 0.5 % Agar): e.g. Nutrient broth containing 0.5% Agar.
 - **Liquid Media** (Absence of Agar): Fluid Thioglycollate broth.
- ***Depending on Oxygen Requirement:***
 - **Aerobic Media:** e.g. Mackonkey's Broth.
 - **Anaerobic Media:** e.g. Robertson's Cooked Meat medium.
- ***Depending on Chemical Composition:***
 - **Simple or Basal Media.**
 - **Synthetic or Defined Media.**
 - **Non-synthetic or Undefined or Complex Media.**

Culture Media used in Microbiology



- **Special Media:**
 - Enriched Media.
 - Enrichment Media.
 - Selective Media.
 - Indicator Media.
 - Differential Media.
 - Sugar Media.
 - Transport Media.
 - Assay Media.
 - Storage Media.

Simple or Basal Media: Basal media are basically simple media that supports most non-fastidious bacteria. (which don't require any special condition or substance for their growth)

- **Peptone-water, nutrient broth, and nutrient agar (NA)** are considered as basal medium.
- These media are generally used **for the primary isolation of microorganisms.**



Synthetic or Defined Media:

- These are **prepared from pure chemicals** and hence exact **chemical composition is known**.
- Used for research purposes and to study metabolic activities of bacteria.

Non-synthetic or Undefined or Complex Media:

- They **contain complex chemical materials from biological origin** whose exact **composition is unknown**.
- These biological products provide growth nutrients required by bacteria. These culture mediums **may contain added nutritional materials**.

Indicator Media:

- These media **contain an indicator that changes color** on growth of a specific microbe.
- e.g. **Wilson and Blair Media** which contains **sulphite**, in which *Salmonella typhi* reduces this **sulphite to sulphide** in presence of glucose and produces colonic having **black color with metallic shine**.



EMB Agar



MacConkey Agar



XLD Agar

Bacterial Culture Media



Blood Agar



Chocolate Agar



Nutrient Agar

Differential Media:

- A differential medium is used to **distinguish colonies of different bacteria.**
- e.g. **Macconkey's Agar** which **contains lactose, peptone, neutral red** and **differentiates lactose fermentation in red colored colonies from non lactose fermentation in pale or white colonies.**



Sugar Media:

- In this **1% sugar** is added to peptone water along with a suitable indicator.
- Contains a small tube called **Durham's tube** kept inverted for **detection of production of gas by bacteria.**

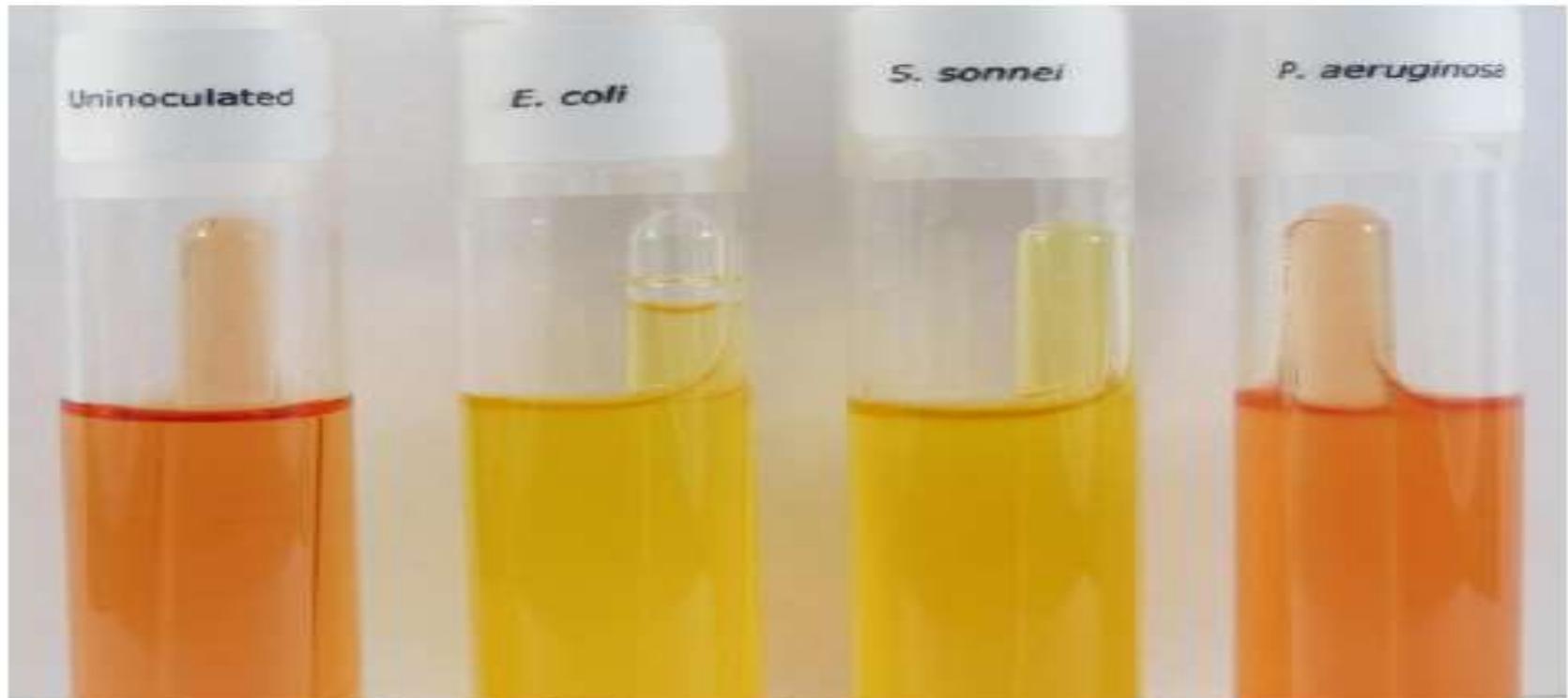


Figure 1. Peptone media with phenol red indicator. From left to right: Uninoculated tube; glucose fermenter with gas production (visible air bubble in the inverted Durham tube); glucose fermenter without gas production (no visible air bubble in the inverted Durham tube); non-fermenter.

Transport Media:

- Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms. This can be achieved by using transport media.
- Such media **prevent drying** (desiccation) of a specimen, maintain the pathogen viable, and **inhibit the overgrowth of unwanted bacteria**. Some of these media (Stuart's & Amie's) are semi-solid in consistency. **Addition of charcoal** serves to **neutralize inhibitory factors**.
- E.g. **Cary Blair transport medium** and **Venkatraman Ramakrishnan (VR) medium** are used to **transport feces from suspected cholera patients**.
e.g. **Pike's medium** is used to transport **streptococci** from **throat specimens**.

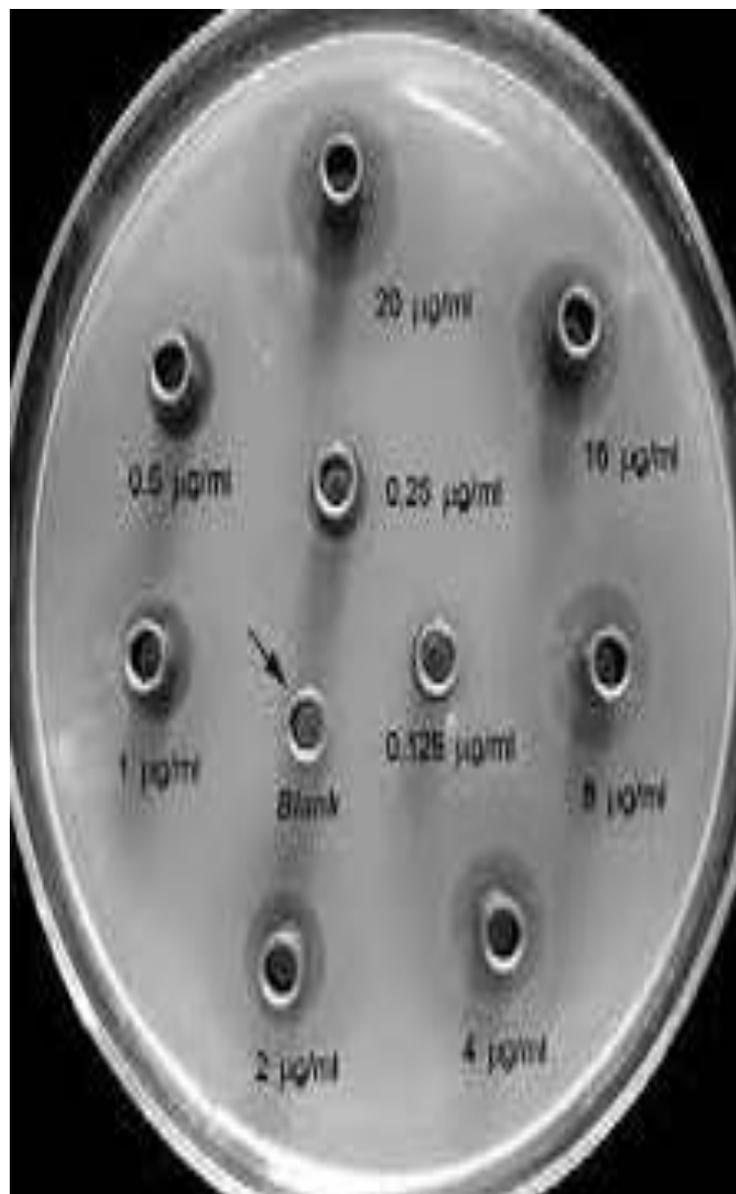
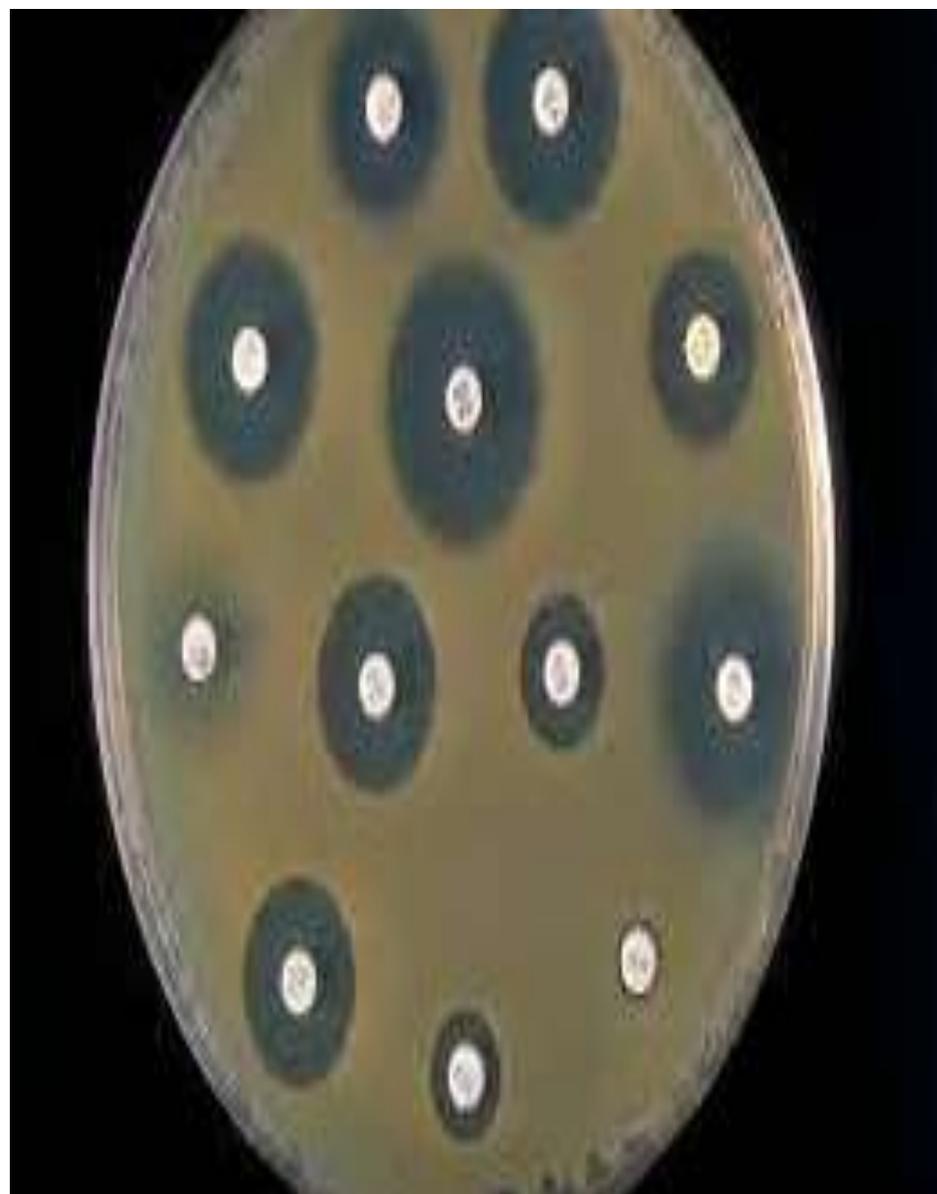


Assay Media:

- These media are used for the **assay of vitamins, amino acids, and antibiotics.**
- e.g. **antibiotic assay media** are used for determining **antibiotic potency by the microbiological assay technique.**

Storage Media:

- These help in **preservation and storage of bacteria for a longer duration.**
- Dorset's Egg Medium, Nutrient agar stabs, Robertsons cooked meat medium etc.



Physical parameters for growth, growth curve

Microbial Growth:

- Refers to an increase in **cell number, not in** cell size. Bacteria grow and divide by **binary fission**, a rapid and relatively simple process.
- Bacteria which derive energy from sunlight are called **phototrophs**.
- Those that obtain energy from chemical reactions are called **chemotrophs**.
- **Bacteria that can synthesise all their organic** compounds are called **autotrophs**.
- Some bacteria are unable to synthesize own metabolites. They depend on preformed organic compounds. They are called **heterotrophs**.

Requirements for Growth

Physical Requirements

1. **Temperature: Microbes are loosely** classified into several groups based on their preferred temperature ranges.
 - A. **Psychrophiles: “Cold-loving”.** Can grow at 0°C. Two groups:
 - **True Psychrophiles: Sensitive to temperatures over 20°C.** Optimum growth at 15oC or below. Found in very cold environments (North pole, ocean depths). Seldom cause disease or food spoilage.
 - **Psychrotrophs: Optimum growth at 20 to 30oC.** Responsible for most low temperature food spoilage.

B. Mesophiles: “Middle loving”. Most bacteria

- Include most pathogens and common spoilage organisms. Best growth between 25 to 40°C.
- Optimum temperature commonly 37°C.
- Many have adapted to live in the bodies of animals.

C. Thermophiles: “Heat loving”.

- Optimum growth between 50 to 60°C.
- Many cannot grow below 45°C.
- Adapted to live in sunlit soil, compost piles, and hot springs.
- Some thermophiles form extremely heat resistant endospores.
- **Extreme Thermophiles (Hyperthermophiles):**
 - Optimum growth at 80°C or higher. Archaeobacteria.
 - Most live in volcanic and ocean vents.

Physical Requirements

2. pH:

- Most **bacteria prefer neutral pH (6.5-7.5)**.
- **Molds and yeast grow in wider pH range, but prefer pH between 5 and 6.**
- **Acidity inhibits most microbial growth and is used frequently for food preservation (e.g.: pickling).**
- **Alkalinity inhibits microbial growth, but not commonly used for food preservation.**
- Acidic products of bacterial metabolism interfere with growth. Buffers can be used to stabilize pH.

2. pH: Organisms can be classified as:

A. Acidophiles: “Acid loving”. Grow at very low pH (0.1 to 5.4)

Lactobacillus produces lactic acid, tolerates mild acidity.

B. Neutrophiles:

- Grow at pH 5.4 to 8.5.
- Includes most human pathogens.

C. Alkaliphiles: “Alkali loving”.

- Grow at alkaline or high pH (7 to 12 or higher)
- *Vibrio cholerae* and *Alkaligenes faecalis* optimal pH 9.
- Soil bacterium *Agrobacterium* grows at pH 12.

3. Osmotic Pressure : Cells are 80 to 90% water.

A. Hypertonic solutions: High osmotic pressure removes water from cell, causing shrinkage of cell membrane (plasmolysis).

- Used to control spoilage and microbial growth. Ex: Sugar in jelly. Salt on meat.

B. Hypotonic solutions: Low osmotic pressure causes water to enter the cell. In most cases cell wall prevents excessive entry of water. Microbe may lyse or burst if cell wall is weak.

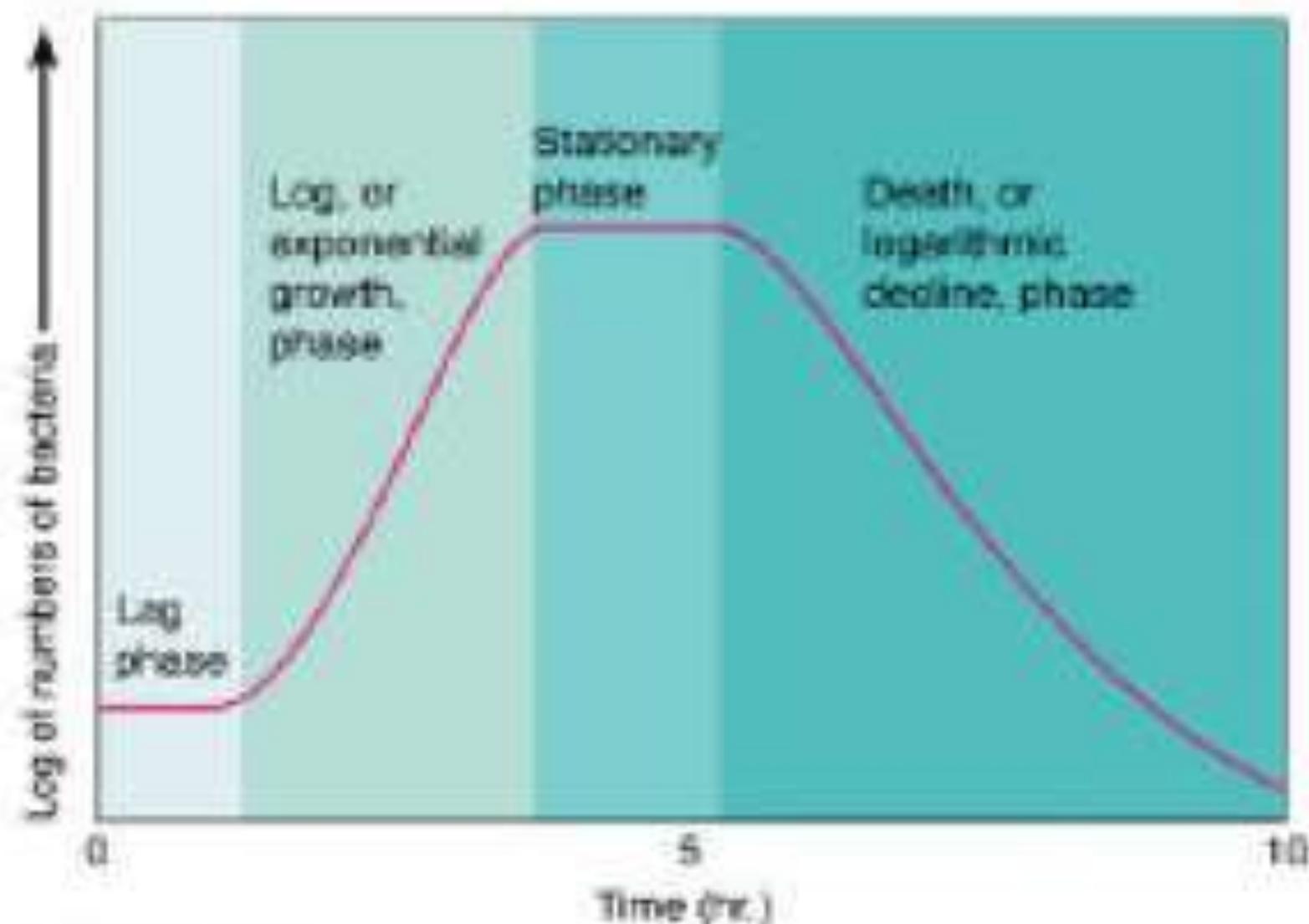
3. Osmotic Pressure :

- **Halophiles: Require moderate to large salt concentrations.** Ocean water contains 3.5% salt.
- Most bacteria in oceans.
- **Extreme or Obligate Halophiles: Require very high salt concentrations (20 to 30%).**
- Bacteria in Dead Sea, brine vats.
- **Facultative Halophiles: Do not require high salt concentrations for growth, but tolerate 2% salt or more.**

Bacterial growth curve

- When a bacterium is seeded into a suitable liquid medium and incubated, its growth follows a definite course. If bacterial counts are made at intervals after inoculation and plotted in relation to time, a growth curve is obtained. The curve shows the following phases:
 - **Lag phase**
 - **Log (logarithmic) or exponential phase**
 - **Stationary phase**
 - **Phase of decline**

Four Phases of Bacterial Growth Curve



1. **Lag Phase:** Immediately following the seeding of a culture medium, there is no appreciable increase in number, though there may be an increase in the size of the cells.
 - This initial period is the time required for adaptation to the new environment. The necessary enzymes and metabolic intermediates are built up in adequate quantities for multiplication to proceed.
 - The maximum cell size is obtained towards the end of lag phase.
 - The duration of the lag phase varies with the species, size of the inoculum, nature of the culture medium and environmental factors such as temperature.

- Period of adjustment to new conditions.
- Little or no cell division occurs, population size doesn't increase.
- Phase of intense metabolic activity, in which individual organisms grow in size.
- May last from one hour to several days.

2. Log Phase: Following the lag phase, the cells start dividing and their numbers increase exponentially or by geometric progression with time. If the logarithm of the viable count is plotted against time, a straight line will be obtained. In this phase, cells are smaller and stain uniformly.

- Cells begin to divide and generation time reaches a constant minimum.
- Period of most rapid growth.
- **Number of cells produced > Number of cells dying**
- Cells are at highest metabolic activity.
- Cells are **most susceptible to adverse** environmental factors at this stage. Ex: Radiation and antibiotics

3. Stationary Phase: After a varying period of exponential growth, cell division stops due to depletion of nutrients and accumulation of toxic products. The number of new cells formed is just enough to replace the number of cells that die. Equilibrium exists between the dying cells and the newly formed cells. So, the viable count remains stationary. In this phase, cells are frequently gram variable and show irregular staining. Sporulation occurs at this stage. Population size begins to stabilize. **Number of cells produced = Number of cells dying**

- Overall cell number does not increase. Cell division begins to slow down. Factors that slow down microbial growth:
 - Accumulation of toxic waste materials
 - Acidic pH of media
 - Limited nutrients
 - Insufficient oxygen supply

4. Death or Decline Phase: This is the phase when the population decreases due to cell death. Besides nutritional exhaustion and toxic accumulation, cell death may also be caused by autolytic enzymes.

- Population size begins to decrease.
- **Number of cells dying > Number of cells produced**
- Cell number decreases at a logarithmic rate.
- Cells lose their ability to divide.
- A few cells may remain alive for a long period of time.

Isolation and preservation methods for pure cultures

- In nature, microorganisms are never found in pure cultures, they are always mixed.
- Hence to study them, the pure cultures are obtained from various laboratories where they are prepared and preserved for longer durations.
- The notable laboratories are National Chemical Laboratory, India Ltd., American Type Culture Collection (ATCC) USA, National Collection of Type Cultures (NCTC) England etc.

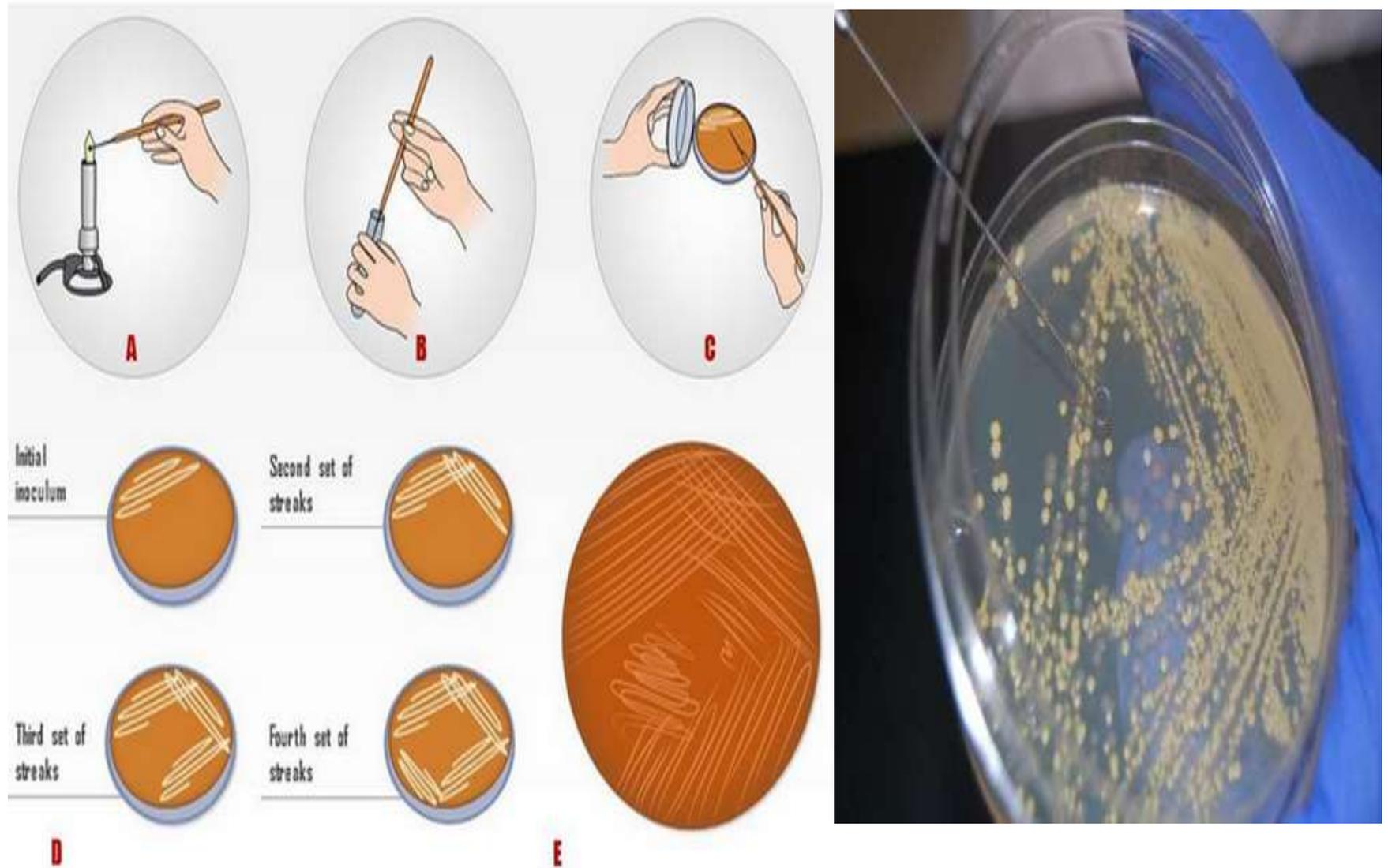
Pure culture techniques

- A pure culture is a population of only one specific species of a microorganism.
- The isolation of this only one kind of microorganism from a mixture is called “Pure Culture Techniques”. The important pure culture techniques are listed as follows,
 - 1. Streak Plate Method.
 - 2. Pour Plate Method.
 - a. Loop Dilution Technique.
 - b. Serial Dilution Technique.
 - 3. Spread Plate Method.
 - 4. Micromanipulator Method.
 - 5. Roll Tube Method.

Streak Plate Method:

- Most commonly used method to isolate pure cultures of bacteria.
- A small amount of mixed culture is placed on the tip of an inoculation loop/needle and is streaked across the surface of the agar medium.
- The successive streaks “thin out” the inoculum sufficiently and the micro-organisms are separated from each other. These plates are incubated to allow the growth of colonies.
- The key principle of this method is that, by streaking, a dilution gradient is established across the face of the Petri plate as bacterial cells are deposited on the agar surface.
- Because of this dilution gradient, confluent growth does not take place on that part of the medium where few bacterial cells are deposited.
- Presumably, each colony is the progeny of a single microbial cell thus representing a clone of pure culture.
- Such isolated colonies are picked up separately using sterile inoculating loops/needle and re-streaked onto fresh media to ensure purity.

The Streak Plate Isolation Method



Pour Plate Method:

- This method involves plating of diluted samples mixed with melted agar medium.
- Here, the mixed culture of bacteria is diluted directly in tubes containing melted agar medium maintained in the liquid state at a temperature of 42-45°C. The bacteria and the melted medium are mixed well.
- The contents of each tube are poured into separate Petri plates, allowed to solidify, and then incubated.
- When bacterial colonies develop, one finds that isolated colonies develop both within the agar medium (subsurface colonies) and on the medium (surface colonies). These isolated colonies are then picked up by an inoculation loop and streaked onto another Petri plate to insure purity

Pour Plate Method

1 Bacterial sample mixed with warm agar (45–50 °C)



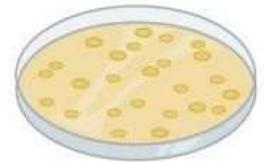
2 Sample poured onto sterile plate



3 Sample swirled to mix, allowed to solidify



4 Plate incubated until bacterial colonies grow



Disadvantages of Pour Plate Technique:

- Formation of subsurface colonies, which are difficult to separate and count.
- Use of heat.
- Not useful for isolating psychrophile bacteria.
- Time consuming and requires skilled persons.

(a) The pour plate method

(b) The spread plate method

1 Inoculate empty plate.

1.0 or 0.1 ml



2 Add melted nutrient agar.



3 Swirl to mix.



4 Colonies grow on and in solidified medium.



0.1 ml

Bacterial dilution

1 Inoculate plate containing solid medium.



2 Spread inoculum over surface evenly.

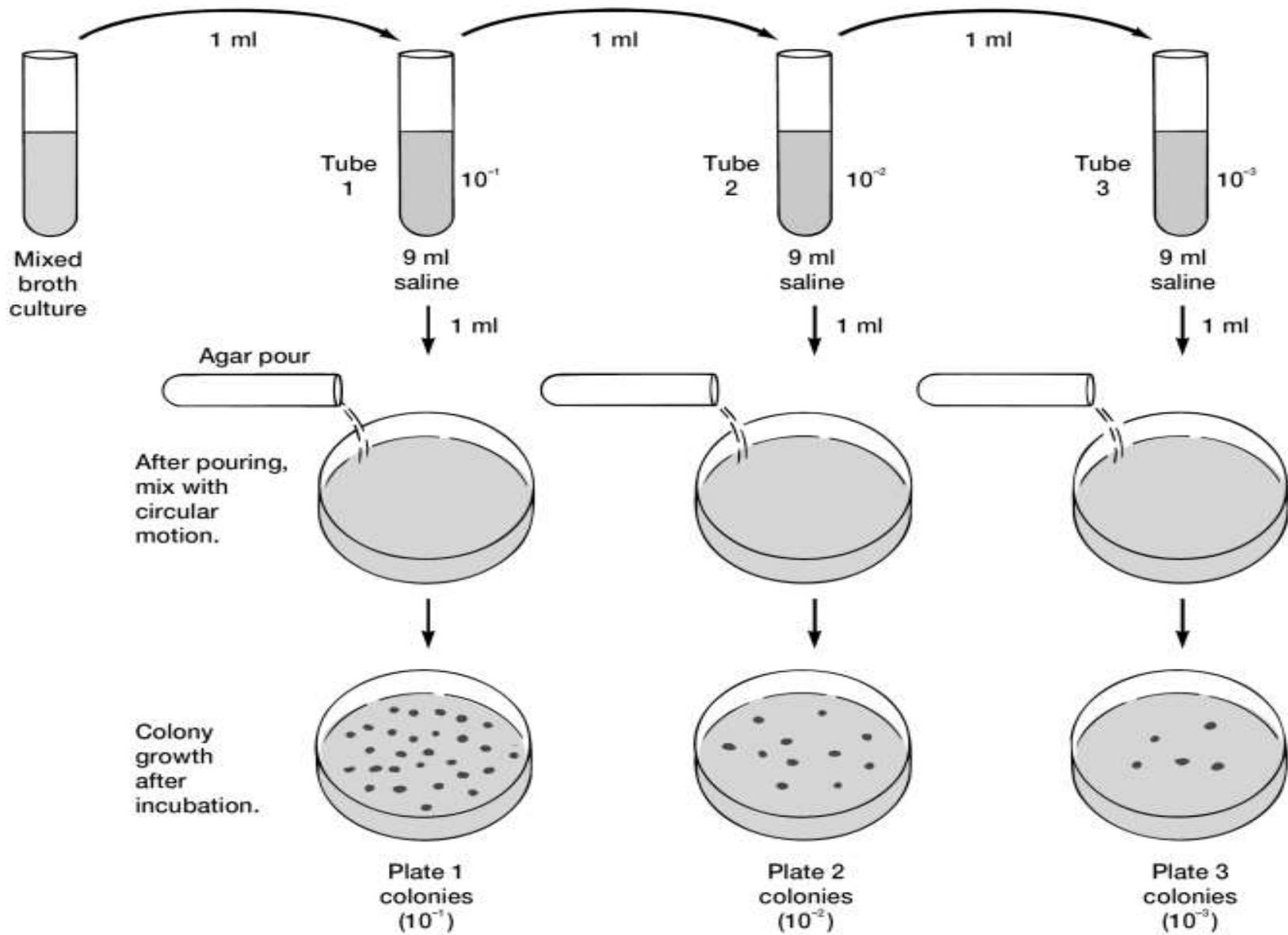


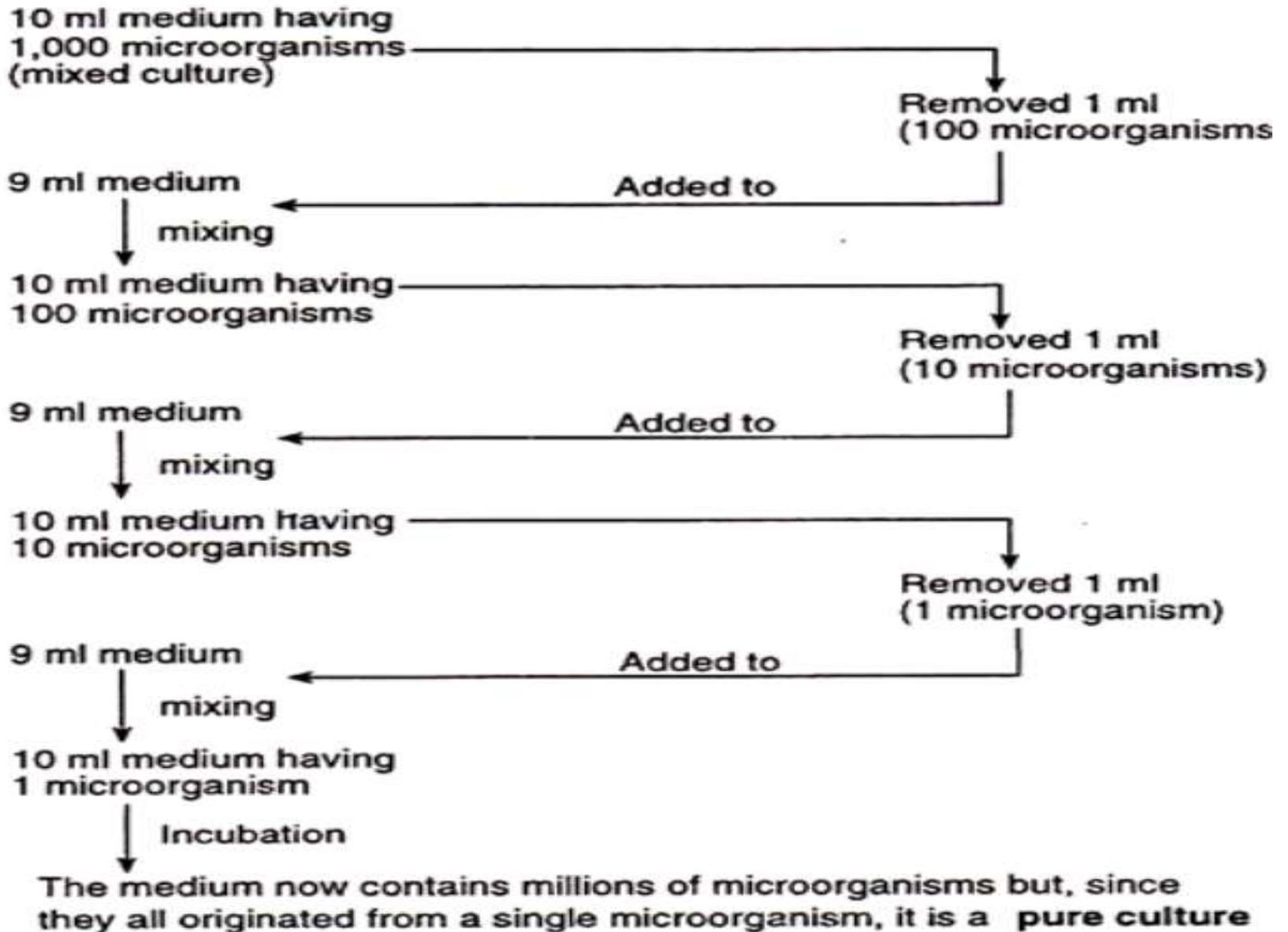
3 Colonies grow only on surface of medium.



Serial Dilution Technique:

- In this technique the original inoculum is diluted with sterile water or saline solution.
- The dilutions are then mixed with liquid nutrient agar @ 45°C and poured in sterile petridishes allowed to solidify and then incubated.

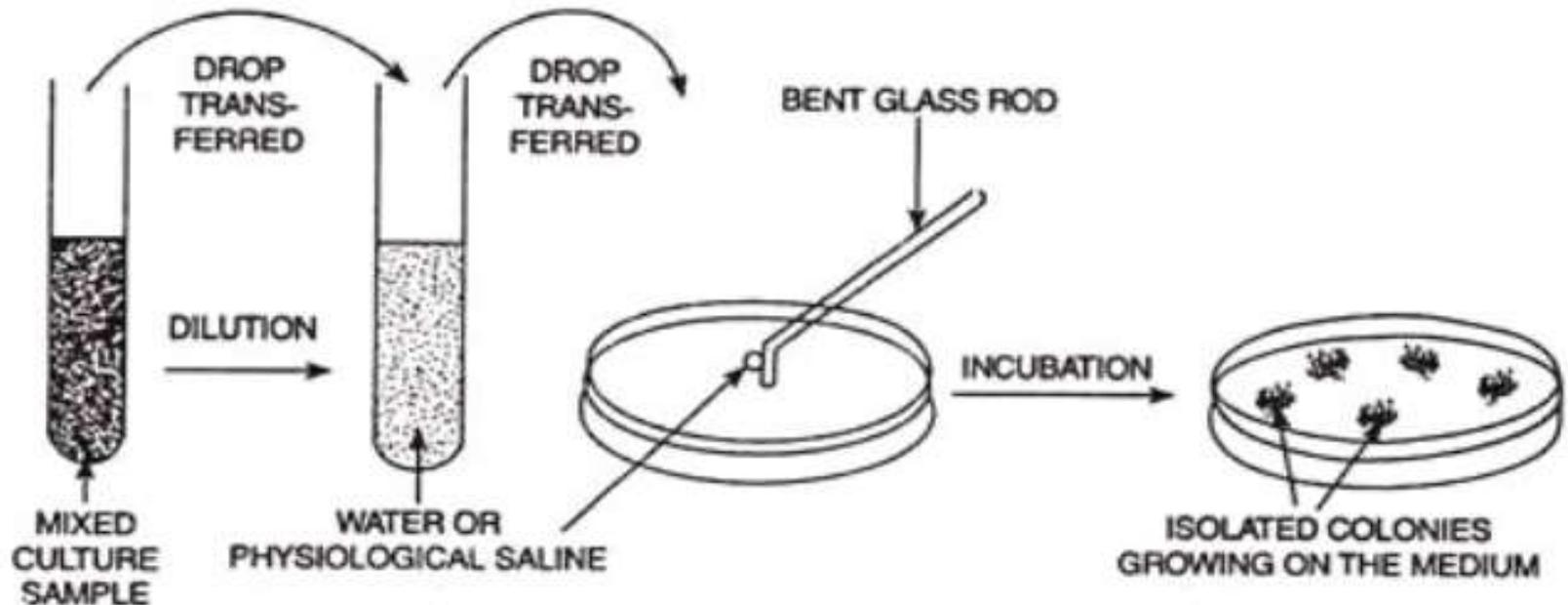




Spread Plate Method:

- In this method, the mixed culture or microorganisms is not diluted in the melted agar medium (unlike the pour plate method); it is rather diluted in a series of tubes containing sterile liquid, usually, water or physiological saline.
- A drop of so diluted liquid from each tube is placed on the center of an agar plate and spread evenly over the surface by means of a sterilized bent-glass-rod then medium is incubated.
- When the colonies develop on the agar medium plates, it is found that there are some plates in which well-isolated colonies grow.

- This happens as a result of separation of individual microorganisms by spreading over the drop of diluted liquid on the medium of the plate.
- The isolated colonies are picked up and transferred onto fresh medium to ensure purity.
- In contrast to the pour plate method, only surface colonies develop in this method and the microorganisms are not required to withstand the temperature of the melted agar medium.



Pour plate method



pipette inoculum onto sterile plate



add sterile medium



swirl to mix and incubate

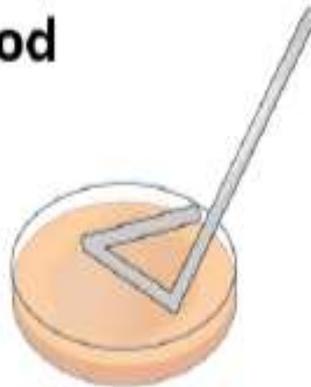


colonies grow in and on medium

Spread plate method



pipette inoculum onto the surface of agar plate



spread evenly over the agar surface



incubate



colonies grow only on the surface of medium

Micromanipulator Method:

- This is a single cell isolation technique.
- Micromanipulators have been built, which permit one to pick out a single cell from a mixed culture.
- This instrument is used in conjunction with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation.
- The micro-manipulator has micrometer adjustments by means of which its micropipette can be moved right and left, forward, and backward, and up and down.
- A series of hanging drops of a diluted culture are placed on a special sterile cover slip by a micropipette.
- Now a hanging drop is searched, which contains only a single microorganism cell.

- This cell is drawn into the micropipette by gentle suction and then transferred to a large drop of sterile medium on another sterile coverslip.
- When the number of cells increases in that drop as a result of multiplication, the drop is transferred to a culture tube having suitable medium.
- This yields a pure culture of the required microorganism.
- The advantages of this method are that one can be reasonably sure that the cultures come from a single cell and one can obtain strains within the species.
- The disadvantages are that the equipment is expensive, its manipulation is very tedious, and it requires a skilled operator.
- This is the reason why this method is reserved for use in highly specialized studies.

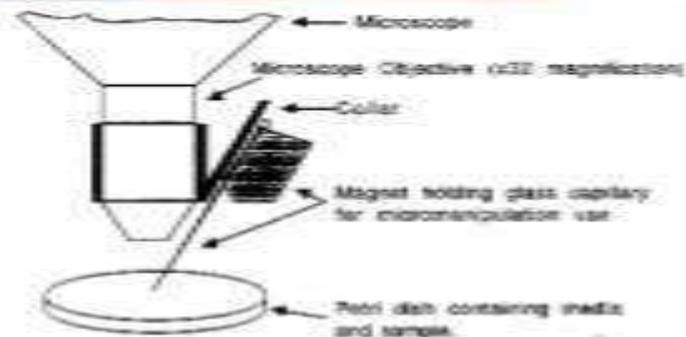
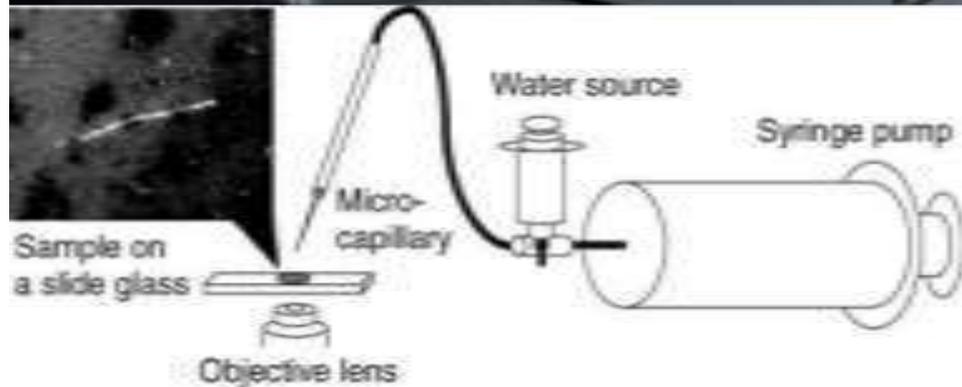


Figure 2. Diagram of Electrical Micromanipulator parts as set-up for cell manipulation

MAINTENANCE OF PURE CULTURE

- To maintain pure culture for extended periods in viable condition without any genetic change is referred as Preservation.
- Necessary to maintain the viability and purity of the microorganism by keeping the pure culture free from contamination.
- Transferred periodically onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms. Subject to aseptic conditions to avoid contamination.

- Microbial culture preservation aims at maintaining a microbial strain alive, uncontaminated, and without variation or mutation, as like original isolate.
- During preservation most important factor is to stop microbial growth or at least lower the growth rate.
- D u e to this toxic chemicals are not accumulated and hence viability of microorganism is not affected.

Objectives of preservation

- To maintain isolated pure culture for extended periods in a viable conditions.
- To avoid contamination
- To restrict Genetic Mutation

Preservation methods of Bacteria

1. Periodic transfer to fresh medium
2. Storage at low temperature
3. Storage in sterile soil
4. Preservation by overlaying culture with mineral oil
5. Lyophilization or freeze drying

Periodic transfer to fresh medium

- Strains can be maintained by periodically preparing a fresh interval at which the culture from the previous stock culture.
- The culture medium, the storage temperature, and the time transfers are made vary with the species .
- 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 like Nutrient Agar.
- 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.

Storage at low temperature

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 micro organism.

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.

Storage in sterile soil

- Storing organisms in soil fall into two groups; 1 sterile soil infested with small amount of inoculum, immediately dried and stored in refrigerator.
- Soil infested with the organism, than incubated allowing
- The organism to grow; thus the mycelium and propagative unit of second generation are preserved.
- The soil preservation method is useful for fungi, and by this method actinomycetes are maintained in soil for 4 to 5 years, and there are several bacterial species which are also maintained in soil for several years.

Preservation by overlaying culture with mineral oil

1. This is a simple and most economical method of maintaining pure cultures.
2. 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.
3. 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).

Lyophilization or freeze drying

- Freeze drying is a stabilizing process in which a substance is first frozen and then the quantity of the solvent is reduced, first by sublimation (primary drying stage) and then desorption (secondary drying stage).
- Better preservation occurs with freeze-drying than with other methods because freeze-drying reduces the risk of intracellular ice crystallization that compromises viability.
- Removal of water from the specimen effectively prevents this damage. Lyophilization is greatest with gram-positive bacteria (spore formers) and decrease with gram -negative bacteria but viability can be maintained as long as 30 years.

- Large numbers of vials of dried microorganisms can be stored with limited space, and organisms can be easily transported long distances at room temperature.
- The process combines freezing and dehydration- Organisms are initially frozen and then dried by lowering the atmospheric pressure with a vacuum apparatus.
- Specimens can be connected individually to the condenser (manifold method) or can be placed (in a chamber) where they are dehydrated in one larger airspace.

Novel Preservation Techniques for Microbial Cultures

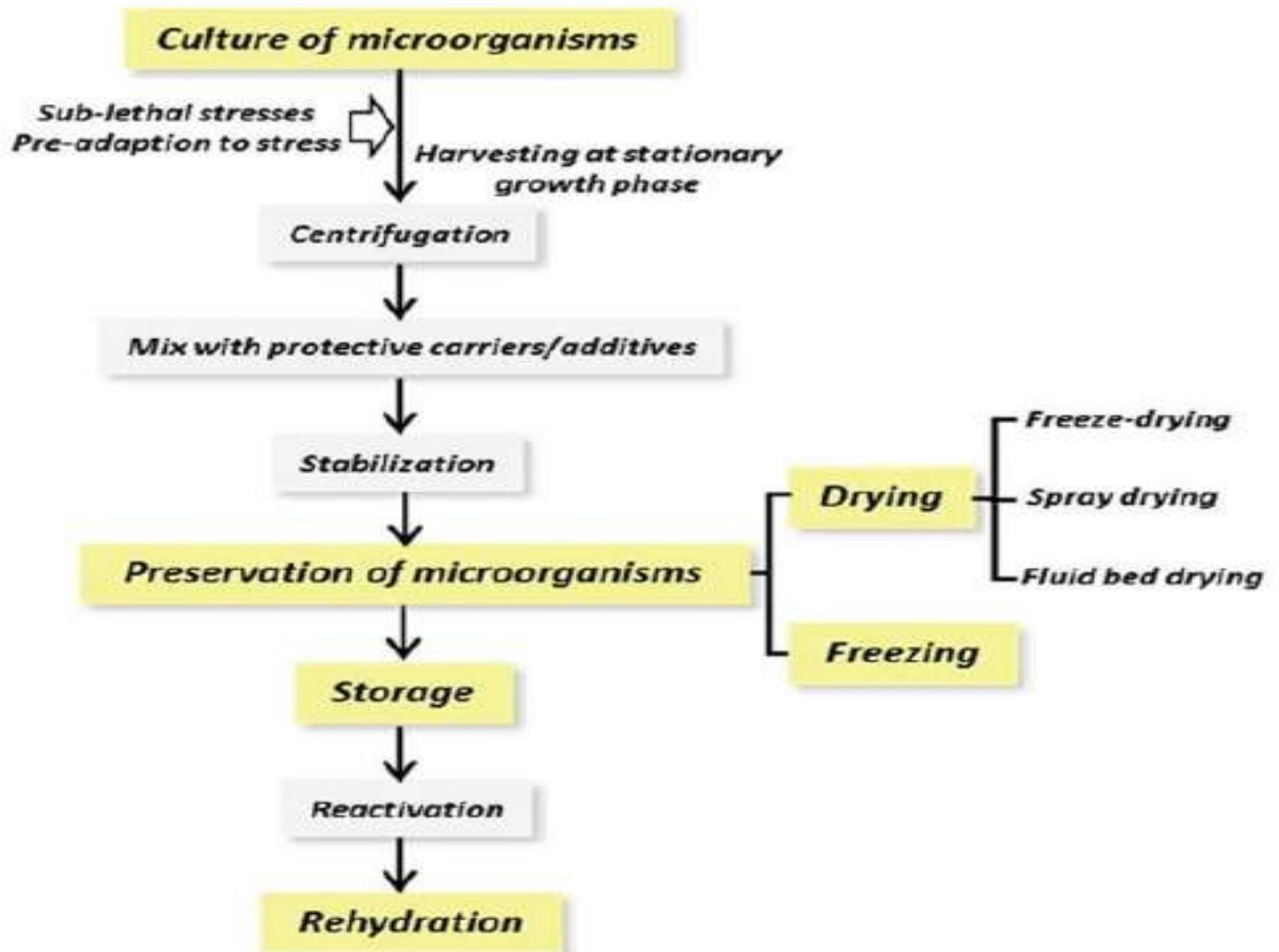


Table 2.1 Comparison of main advantages and disadvantages of the techniques used for cell preservation

Process	Advantages	Disadvantages
Cryopreservation	– Low cost protocol	– High energy consumption
	– High success rate	– Storage temperature dependent
	– High cell density	– Cryoprotectants required
Freeze-drying	– Easy handling	– High operating costs
	– Easy transportation	– Complex process
	– Long-term storage	– Cryoprotectants required
Spray drying	– Scalable operation	– Strain dependent
	– Continuous production	– Thermal stresses
	– Cost effective	– Rehydration dependent
Fluid bed drying	– Lower thermal stresses	– Limited strain applicability
	– Rapid heating	– Rehydration dependent
	– Scalable operation	
Microencapsulation	– High survival ratio	– Short-term storage
	– Easy resuscitation	– Storage temperature dependent

Cultivation of anaerobes

- Anaerobic microorganisms are widespread and very important.
 - Do not require oxygen for growth
 - Oxygen is often extremely toxic for them.

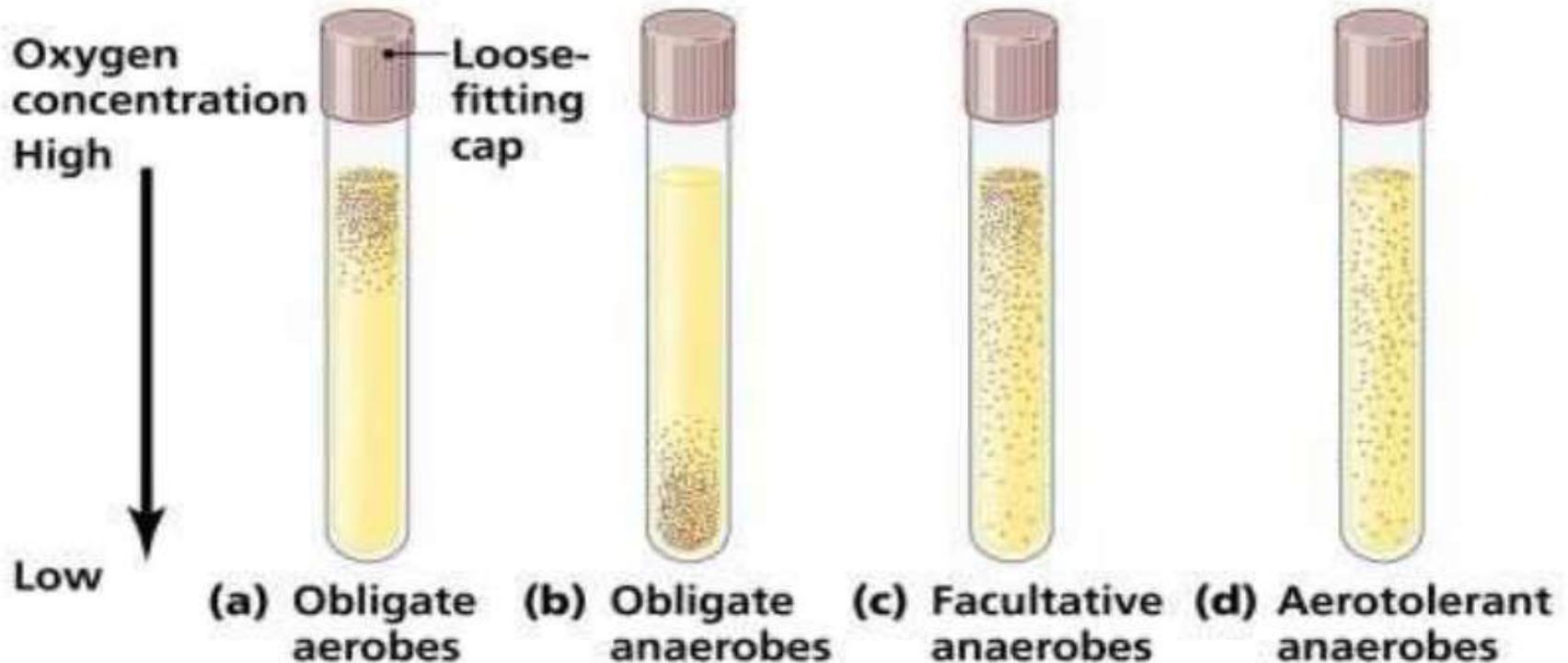


Fig. 1 Different categories of bacteria - on the basis of oxygen requirements.

- On the basis of oxygen requirements, bacteria can be divided into following different categories :

1. Aerobes: Grow in ambient air, which contains 21% oxygen and small amount of (0.03%) of carbon dioxide (*Bacillus cereus*).

2. Obligate aerobes: They have an absolute requirement for oxygen in order to grow. (*Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*).

3. **Obligate anaerobes:** These bacteria grow only under condition of high reducing intensity and for which oxygen is toxic. (*Clostridium perfringens*, *Clostridium botulinum*).
4. **Facultative anaerobes:** They are capable of growth under both aerobic and anaerobic conditions. (Enterobacteriaceae group, *Staphylococcus aureus*).
5. **Aero-tolerant anaerobes:** These are anaerobic bacteria that are not killed by exposure to oxygen.

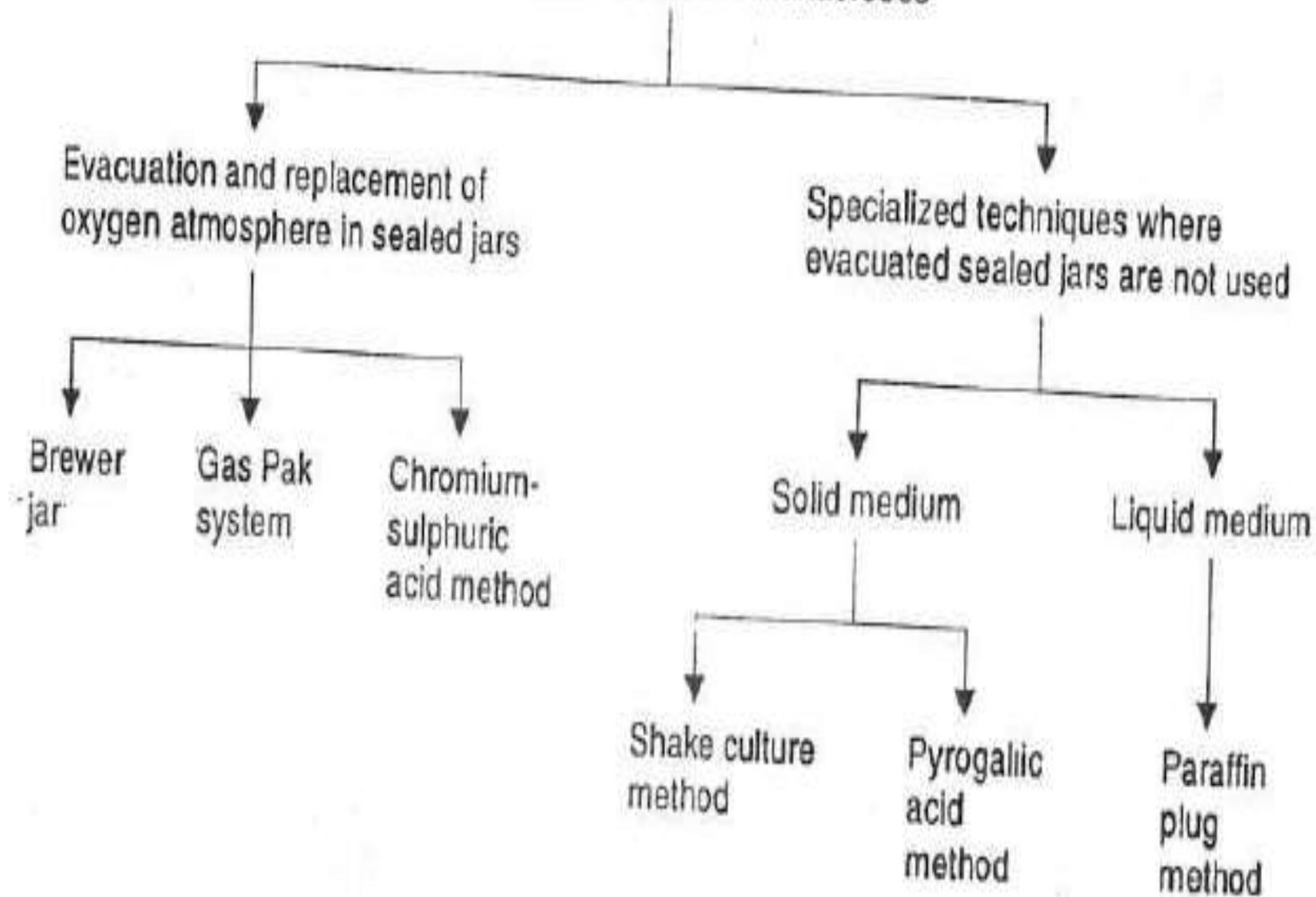
6. **Capnophiles:** Capnophilic bacteria require **increased concentration of carbon dioxide** (5% to 10%) and approximately 15% oxygen.

- This condition can be achieved by a candle jar (3% carbondioxide) or carbon dioxide incubator, jar or bags. (*Haemophilus influenzae*, *Neisseria gonorrhoeae*).

7. **Microaerophiles:** Microaerophiles are those groups of bacteria that **can grow under reduced oxygen** (5% to 10%) and increased carbon dioxide (8% to 10%).

- Higher oxygen tensions may be inhibitory to them. This environment can be obtained in specially designed jars or bags. (*Campylobacter jejuni*, *Helicobacter pylori*).

Methods for cultivation of anaerobes



ANAEROBIC BACTERIA - CULTIVATION

- An anaerobic bacteria culture is a method used to grow anaerobes from a clinical specimen. Obligate anaerobes are bacteria that can live only in the absence of oxygen. Obligate anaerobes are destroyed when exposed to the atmosphere for as briefly as 10 minutes.
- Some anaerobes are tolerant to small amounts of oxygen. Facultative anaerobes are those organisms that will grow with or without oxygen.
- The methods of obtaining specimens for anaerobic culture and the culturing procedure are performed to ensure that the organisms are protected from oxygen.
- It is crucial that the health care provider obtain the sample for culture via aseptic technique. Anaerobes are commonly found on mucous membranes and other sites such as the vagina and oral cavity.

- Therefore, specimens likely to be contaminated with these organisms should not be submitted for culture (throat or vaginal swab).
- Some types of specimens should always be cultured for anaerobes if an infection is suspected.
- These include abscesses, bites, blood, cerebrospinal fluid and exudative body fluids, deep wounds, and dead tissues.
- The specimen must be protected from oxygen during collection and transport and must be transported to the laboratory immediately.
- **Cultures should be placed in an environment that is free of oxygen, at 35°C for at least 48 hours before the plates are examined for growth.**

Anaerobic Growth Media

- Most strict anaerobes require not only the absence of oxygen to initiate growth, but also a redox potential below -300mV , which can be only achieved by the supplementation of media with reducing agents.
- **Thioglycolate broth is a multi-purpose, enriched differential medium used primarily to determine the oxygen requirements of microorganisms.**
- Sodium thioglycolate in the medium consumes oxygen and permits the growth of obligate anaerobes.
- This, combined with the diffusion of oxygen from the top of the broth produces a range of oxygen concentrations in the media along its depth.

- The oxygen concentration at a given level is indicated by a redox sensitive dye like resazurine that turns pink in the presence of oxygen.
- Reducing media chemically remove molecular oxygen (O₂) that might interfere with the growth of anaerobes.
- Thioglycolate combines with dissolved O₂ to deplete in media.
- The primary plating media for inoculating anaerobic specimen includes a nonselective blood agar and one or all of the following mentioned selective media.

Non selective media used in anaerobic bacteriology:

1. **Anaerobic blood agar:** It is a nonselective medium for isolation of anaerobes and facultative anaerobes.
2. **Egg-yolk agar (EYA):** Nonselective for determination of lecithinase and lipase production by clostridia and fusobacteria.
3. **Cooked meat broth:** Nonselective for cultivation of anaerobic organisms; with addition of glucose, can be used for gas-liquid chromatography.
4. **Peptone-yeast extract glucose broth (PYG):** Nonselective for cultivation of anaerobic bacteria for gas-liquid chromatography.

Selective and differential media used in anaerobic bacteriology

1. **Bacterioides bile esculin agar (BBE):** It is selective and differential for *Bacterioides fragilis* group and good for presumptive identification.
2. **Laked Kanamycin-vancomycin blood agar (LKV):** It is selective for isolation of *Prevotella* and *Bacterioides* spp.
3. **Anaerobic phenylethyl alcohol agar (PEA):** Selective for inhibition of gram negative rods and swarming by some clostridia.
4. **Cycloserine cefoxitin fructose agar (CCFA):** selective for clostridium difficile.
5. **Thioglycollate broth:** Non selective for cultivation of anaerobes; as well as facultative anaerobes and aerobes.

Special culture techniques for anaerobic bacteria

Candle jar

- A **micro-aerophile** is a microorganism that requires oxygen to survive, but requires environments containing lower levels of oxygen than atmospheric oxygen (20% concentration).
- Many microphiles are also **capnophiles**, as they require an elevated concentration of carbon dioxide. In the laboratory they can be easily cultivated in a candle jar.
- A candle jar is a container into which a lit candle is introduced before sealing the container's airtight lid .
- The candle's flame burns until extinguished by oxygen deprivation, which creates a carbon dioxide-rich, oxygen-poor atmosphere in the jar.

- Many labs also have access directly to carbon dioxide and can add the desired carbon dioxide levels directly to incubators where they want to grow microaerophiles.
- Candle jars are used to grow bacteria requiring an increased CO₂ concentration (capnophiles). Candle jars increase CO₂ concentrations and still leave some O₂ for aerobic capnophiles.

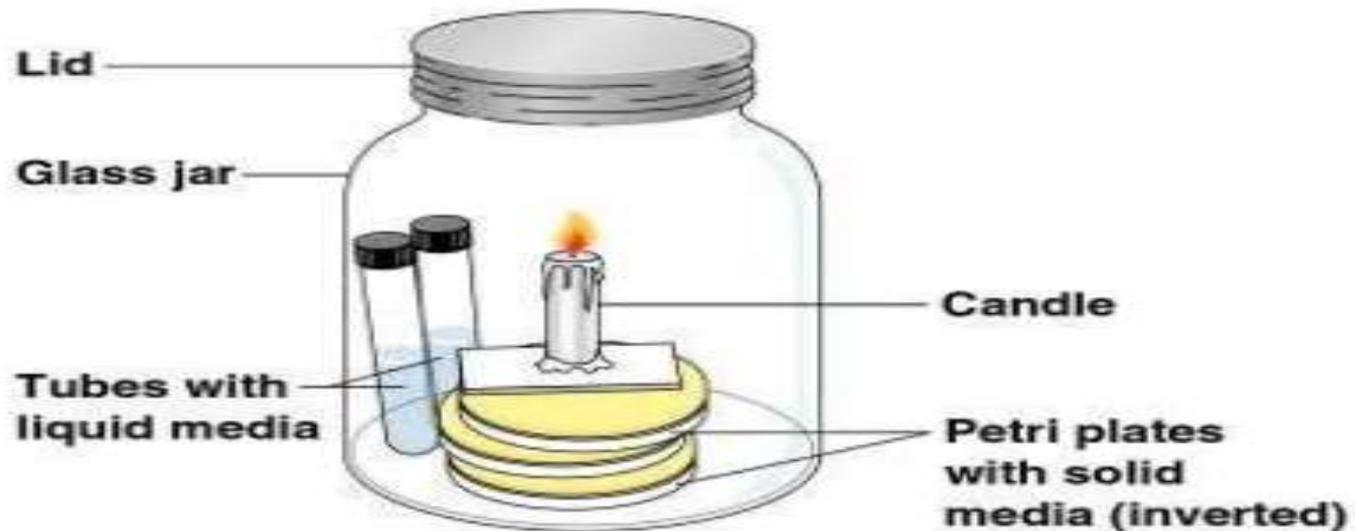


Fig. 15 Candle jar.

Gas pack

- Gas packs can generate CO₂ also and are generally used in place of candle jars.
- The packet consist of a bag containing a Petri plate and CO₂ gas generator.
- The gas generator is crushed to mix the chemicals it contains and start the reaction that produces CO₂.
- This gas reduces the oxygen concentration in the bag to about 5% and provides CO₂ concentration of about 10%.

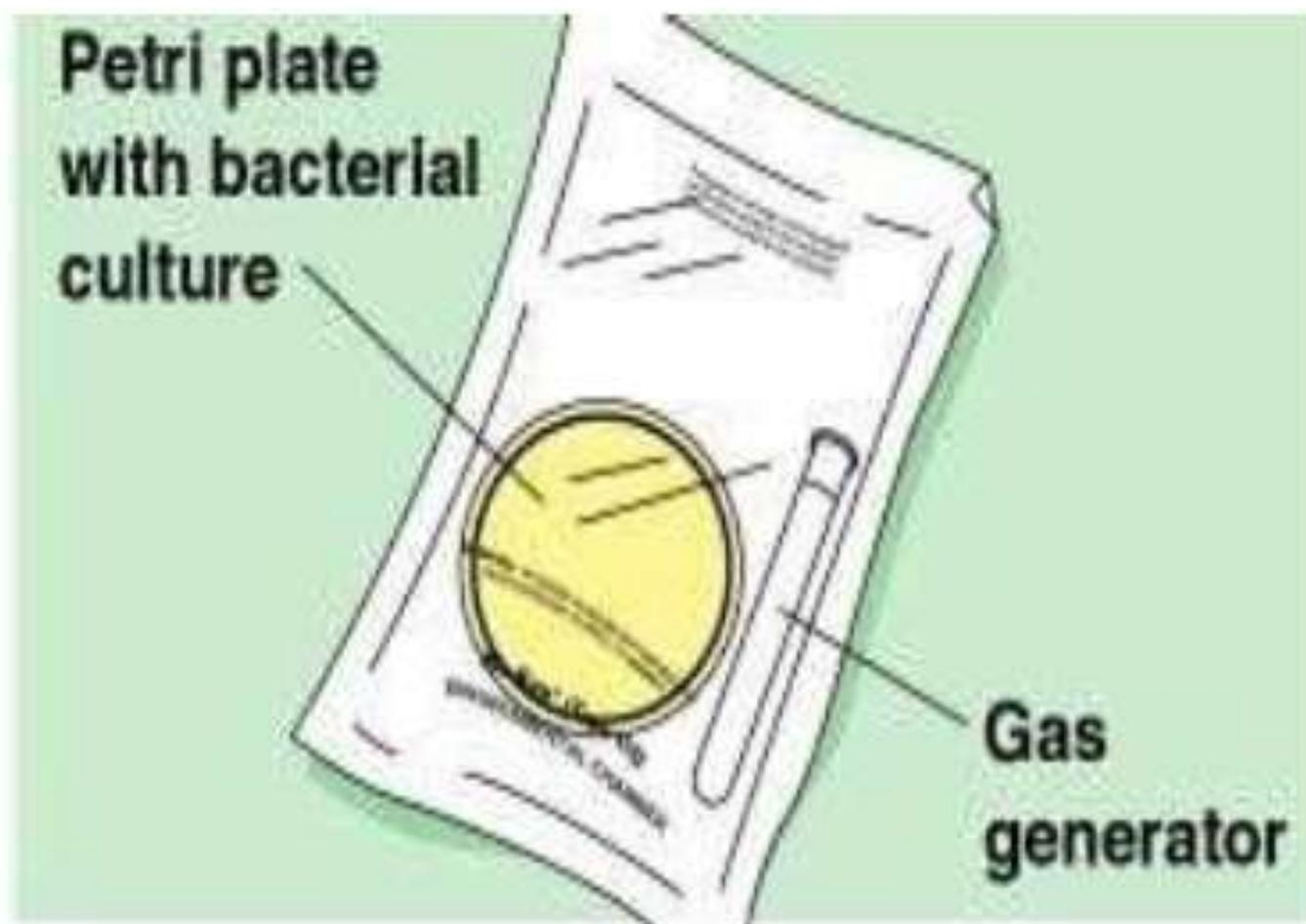


Fig. 16 Gas pack.

Anaerobic jar

- Petri plates can be incubated in an anaerobic jar or anaerobic chamber.
- **Sodium bicarbonate** and **sodium borohydride** are mixed with a small amount of **water** to produce **CO₂** and **H⁺**.
- A **palladium catalyst** in the jar **combines with the Oxygen and hydrogen to remove O₂**.

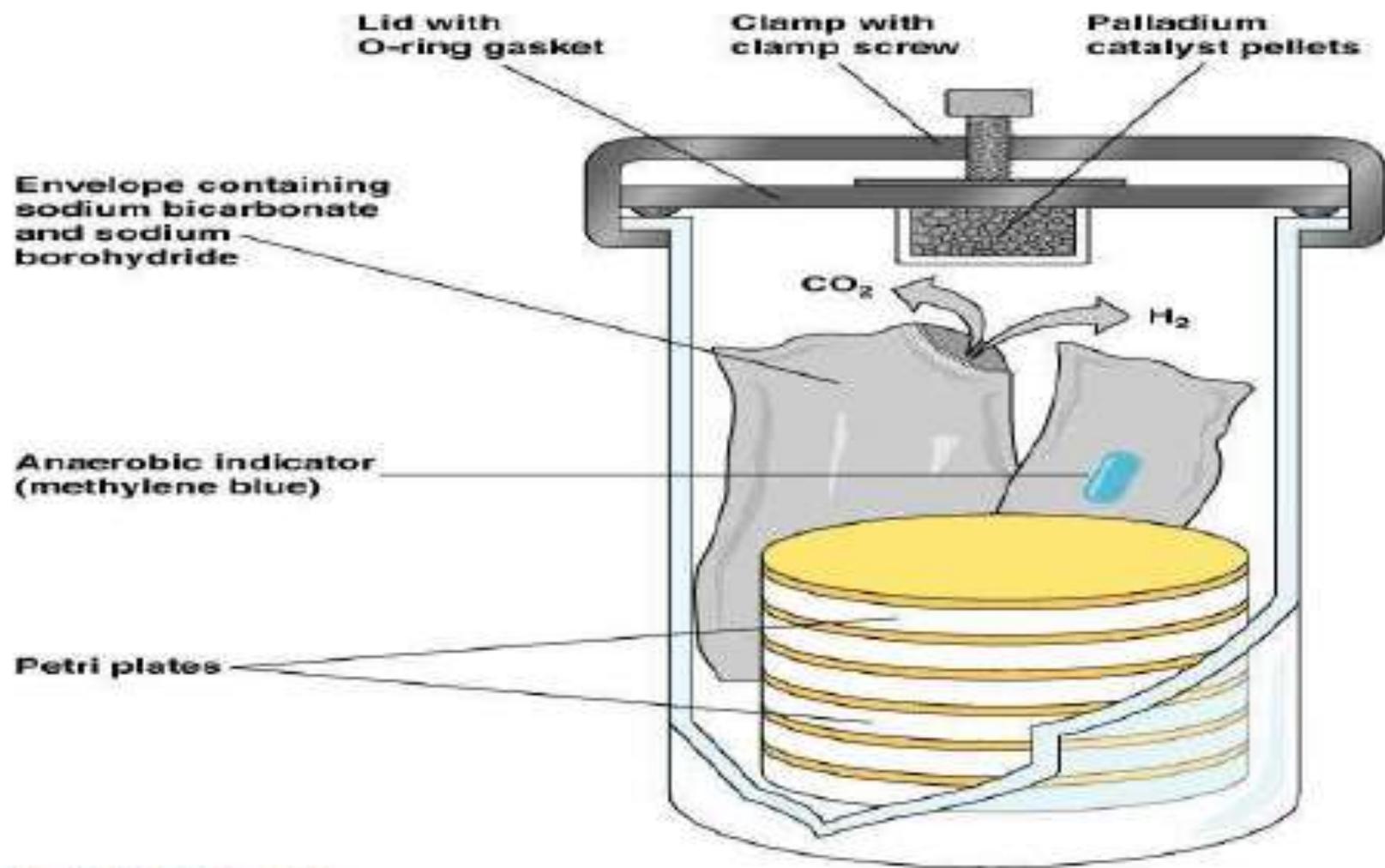


Fig. 17 Anaerobic jar.

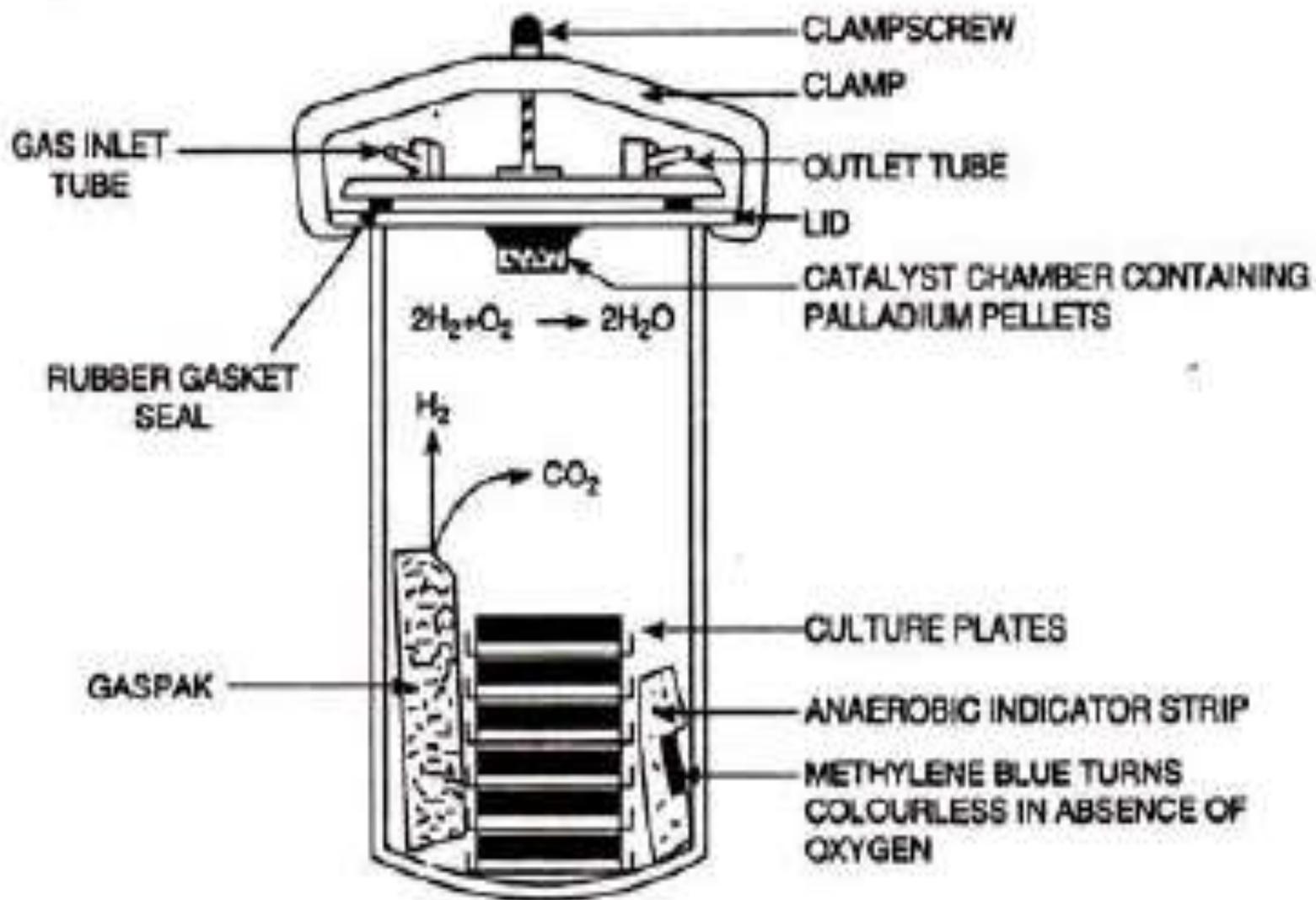


FIG. 16.20. Anaerobic jar.

Quantitative measurement of bacterial growth (Total Population Count)

Direct methods :

- An individual cell can be counted manually by observing each one under **phase contrast microscope** on a specialized counting chamber slide.
- A machine, such as the **Coulter Counter**, can also be used **to calculate the number of cells passing through a narrow passage**. Milliliter counts are the result of these methods.
- Despite phase contrast, these techniques have some challenges, such as the fact that bacteria are small and hard to see. Bacteria often cluster together or stick together. Often, the **actual number of bacteria is higher** than what is reported because clumped bacteria are considered one big bacterium.
- Counting all bacteria present in the sample, whether dead or alive, is also part of these methods. This process takes only a short time.

Quantitative measurement of bacterial growth(Total Count)

Indirect methods :

- **Turbidity may also be measured quantitatively in a spectrophotometer** or qualitatively in **McFarland's standard** against which a total population count can be determined.
- Here are the steps you need to follow in order to **use a spectrophotometer to measure bacterial** population.
- In order to standardize antimicrobial sensitivity testing, we will utilize the McFarland standards. As the **number of bacteria in a sample increase, the turbidity of the sample does as well.**
- **Turbidity can be used to estimate how many viable cells are in a culture that is growing exponentially (logarithmically).**
- In the **stationary phase**, however, both the **dead and live organisms in the culture will contribute to your total counts** exceeding the viable numbers.

Quantitative measurement of bacterial growth (viable count)

Viable Counts:

- The **number of live bacteria present in a sample is often needed to determine growth rates or disinfectant effectiveness.**
- Bacteria samples are **serially diluted and then plated** on appropriate growth media. Alternatively, you can place **the membrane on a pad soaked in growth media and filter your samples through that.**
- **Colonies are visible** when the plates have been **incubated for 18-24 hours.** As long as the colonies you see appear to have started from one viable bacterial unit, they are assumed to have originated from that unit.
- Generally, bacteria do not occur in individual form, so **the colony that you see may have started from one cell or from a group of cells.** CFUs (colony forming units) are used to report the results.

Measurement the growth of microbes

- Various methods of measuring microbial growth can be used to determine the growth rate and generation time of microbes.
- Growth can be determined by measuring either the mass or population, which both increase as a result of growth.
- A number of different measurements can be used to measure growth:
 - In this method, the growth is measured either **using an electronic particle counter or microscopy or indirectly through a colony count.**
 - In this growth, the **cell mass** can be measured directly using **an analytical weighing scale**, or indirectly by measuring the nitrogen concentration in the cells or by using a **spectrophotometer to determine turbidity.**
 - Analyzing the degree of biochemical activity in relation to the size of the population can measure the level of cell activity in this growth.