

ISOLATION & PRESERVATION METHODS FOR PURE CULTURE



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Pure Culture Technique



- **Culture** : Act of cultivating microorganisms or the microorganisms that are cultivated

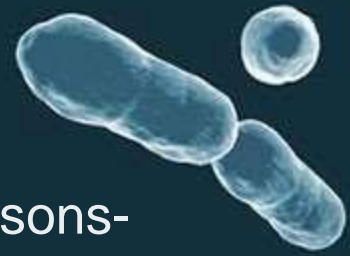
Mixed culture : more than one microorganism

Pure culture : containing a single species of organism.

- A pure culture is usually derived from a mixed culture (one containing many species) by transferring a small sample into new, sterile growth medium in such a manner as to disperse the individual cells across the medium surface or by thinning the sample many times before inoculating the new medium.



Why important ?



Pure cultures are important in microbiology for the following reasons-

1. Once purified, the isolated species can then be cultivated with the knowledge that only the desired microorganism is being grown.
2. A pure culture can be correctly identified for accurate studying and testing, and diagnosis in a clinical environment.
3. Testing/experimenting with a pure culture ensures that the same results can be achieved regardless of how many times the test is repeated.

- Pure culture spontaneous mutation rate is low
- Pure culture clone is 99.999% identical



ISOLATION TECHNIQUE OF PURE CULTURE



- Cultures composed of cells arising from a single progenitor
- Progenitor is termed a CFU
- Aseptic technique prevents contamination of sterile substances or objects
- Common isolation techniques
 - Streak plate method
 - Pour plate method
 - Spread plate method
 - Roll tube method





**Special methods
of isolation on of
pure culture**

**Single cell isolation
methods**

**Enrichment culture
method**

**Capillary pipette
method**

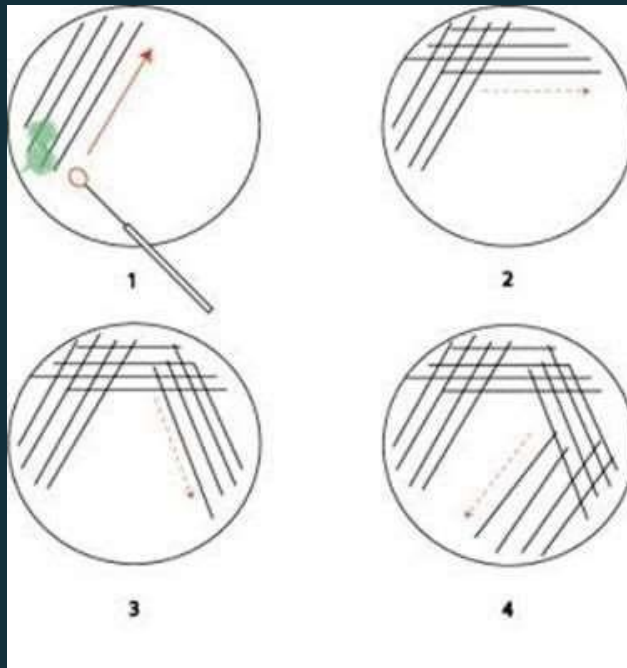
**Micromanipulator
method**

The enrichment culture strategy provides a specially designed cultural environment by incorporating a specific nutrient in the medium and by modifying the physical conditions of the incubation.

1. Streak plate method



- Streaking is the process of spreading the microbial culture with an inoculating needle on the surface of the media.
- Sterilize the inoculating needle by flame to make red hot and allow it to cool for 30 seconds.
- The sample is streaked in such a way to provide a series of dilution.
- purpose - thin out inoculum to get separate colonies.
- subculturing can be done by streaking well isolated colonies from streak plate to new plate.



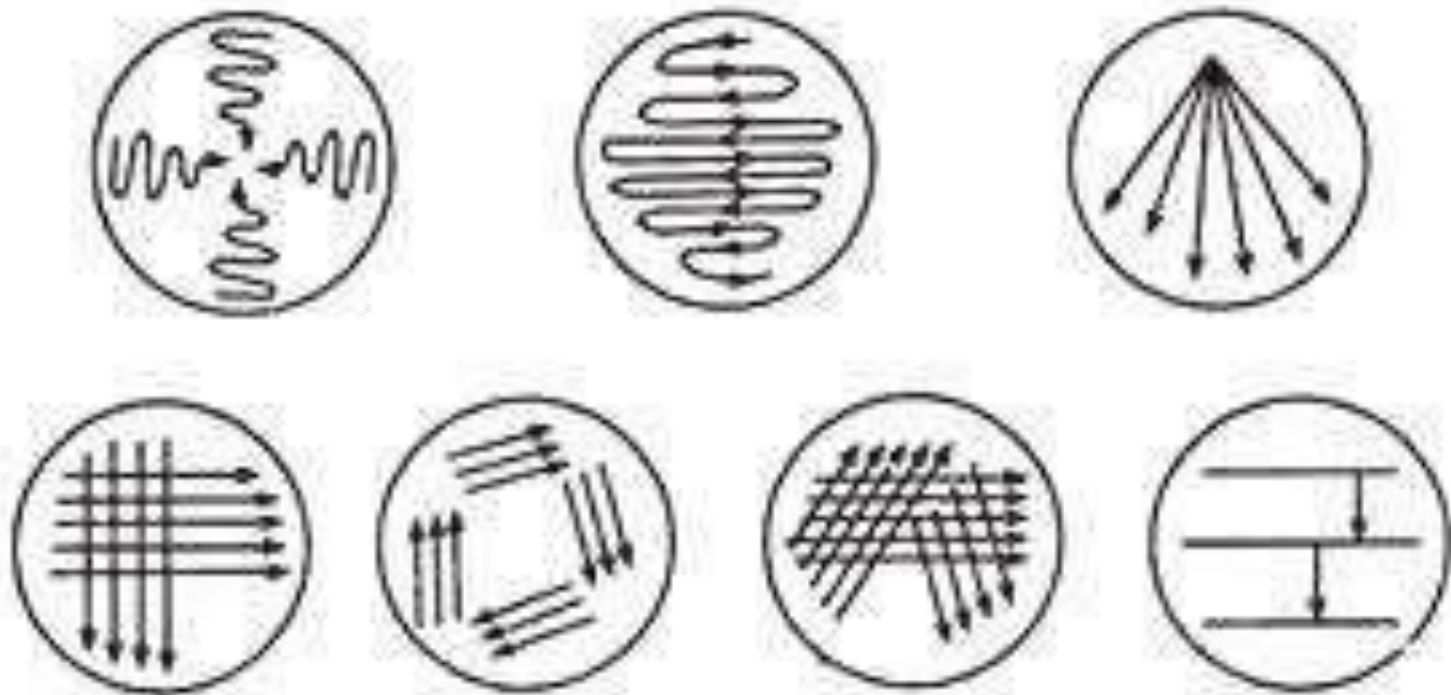


FIG. 16.13. Various methods of streaking.



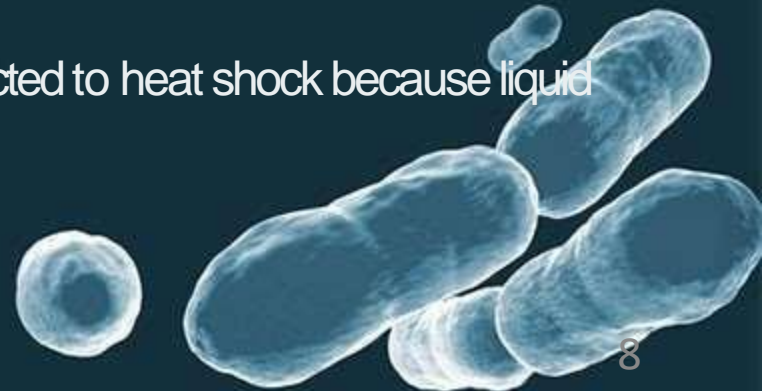
2. Pour plate method

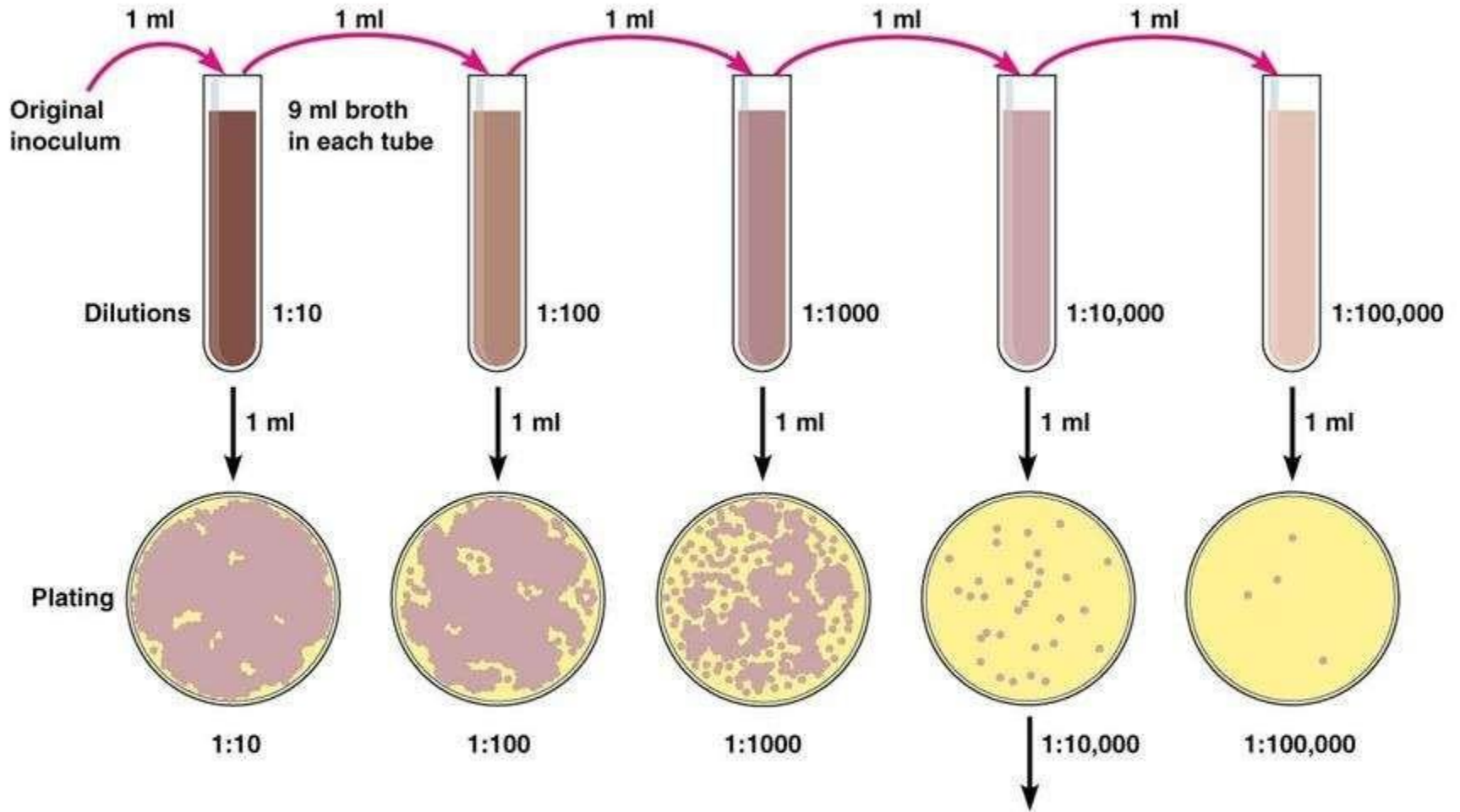


- The bacterial culture and liquid agar medium are mixed together.
- After mixing the medium, the medium containing the culture poured into sterilized petridishes (petriplates), allowed solidifying and then incubated.
- After incubation colonies appear on the surface.

DISADVANTAGES-

1. Microorganism trapped beneath the surface of medium hence surface as well as subsurface colonies are developed which makes the difficulties in counting the bacterial colony.
2. Tedious and time consuming method, microbes are subjected to heat shock because liquid medium maintained at 45°C.
3. Unsuitable-*Psychrophile*





Calculation: Number of colonies on plate \times reciprocal of dilution of sample = number of bacteria/ml
 (For example, if 32 colonies are on a plate of $1/10,000$ dilution, then the count is $32 \times 10,000 = 320,000$ bacteria/ml in sample.)

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3. Spread plate method

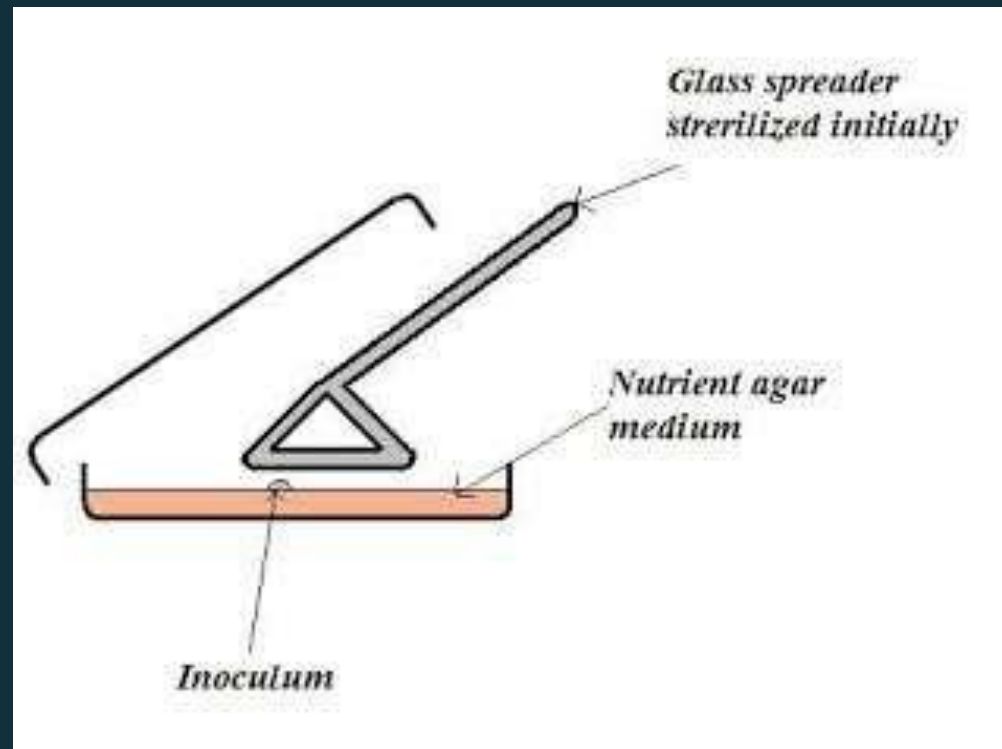


- This is the best method to isolate the pure colonies.
- In this technique, the culture is not mixed with the agar medium. Instead it is mixed with normal saline and serially diluted.
- 0.1 ml of sample taken from diluted mixture, which is placed on the surface of the agar plate and spread evenly over the surface by using L-shaped glass rod called spreader.
- Incubate the plates
- After incubation, colonies are observed on the agar surface.



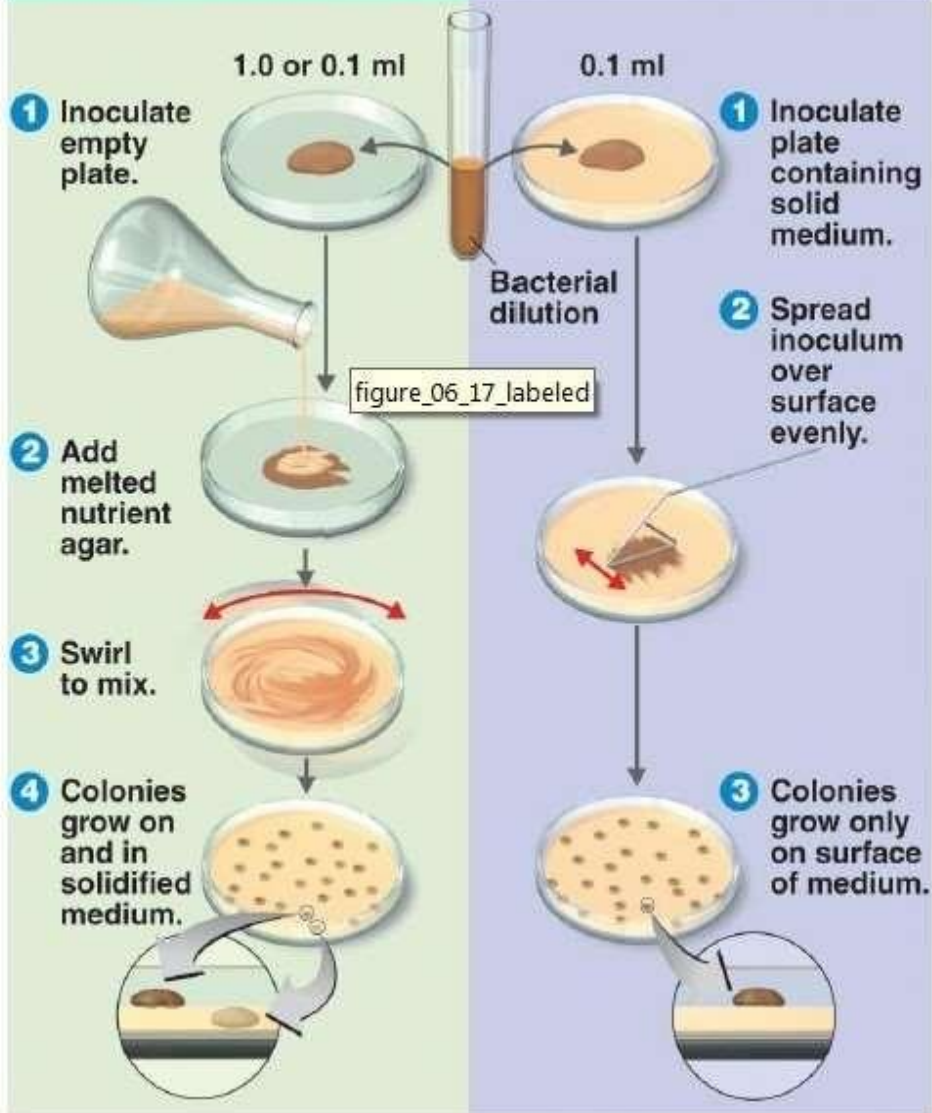
ADVANTAGES

1. It is a simple method.
2. In this method only surface colonies are formed.
3. Micro-organisms are not exposed to higher temperature.

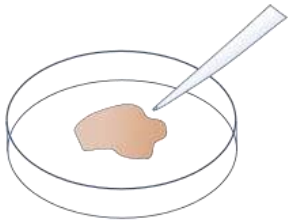


(a) The pour plate method

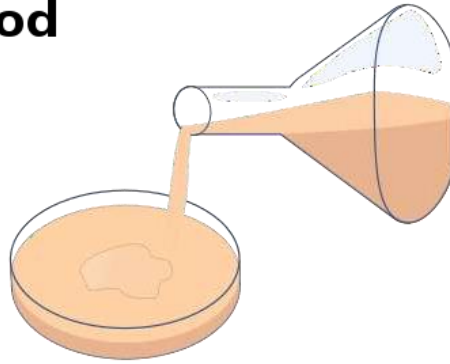
(b) The spread plate method



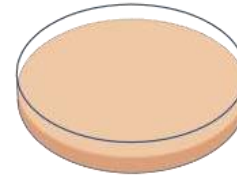
Pour plate method



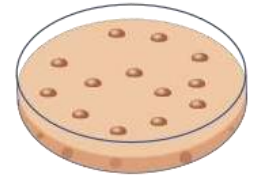
pipette inoculum onto sterile plate



add sterile medium



swirl to mix and incubate

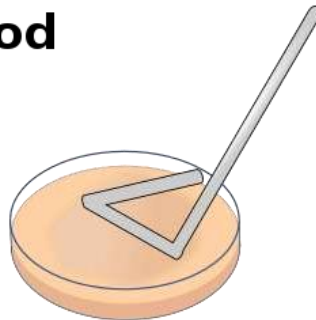


colonies grow in and on medium

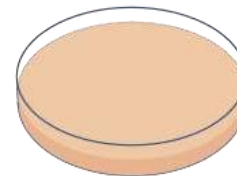
Spread plate method



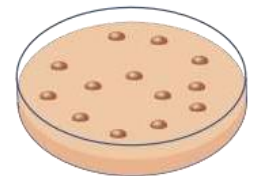
pipette inoculum onto the surface of agar plate



spread evenly over the agar surface

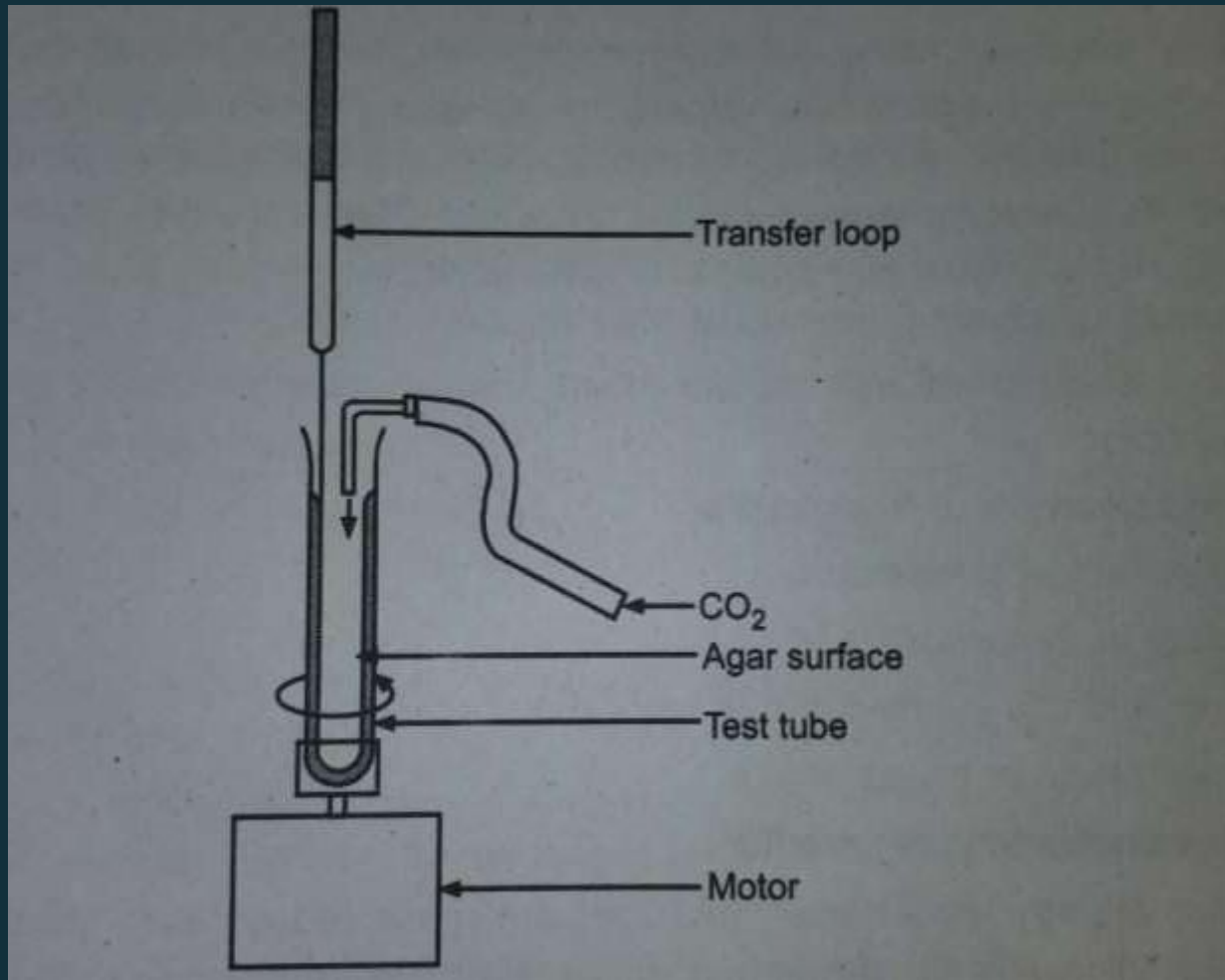


incubate



colonies grow only on the surface of medium

4. Roll tube method.



5. Micromanipulator method



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 single cell of microbe sucked into micropipette and transferred to large amount of sterile medium.

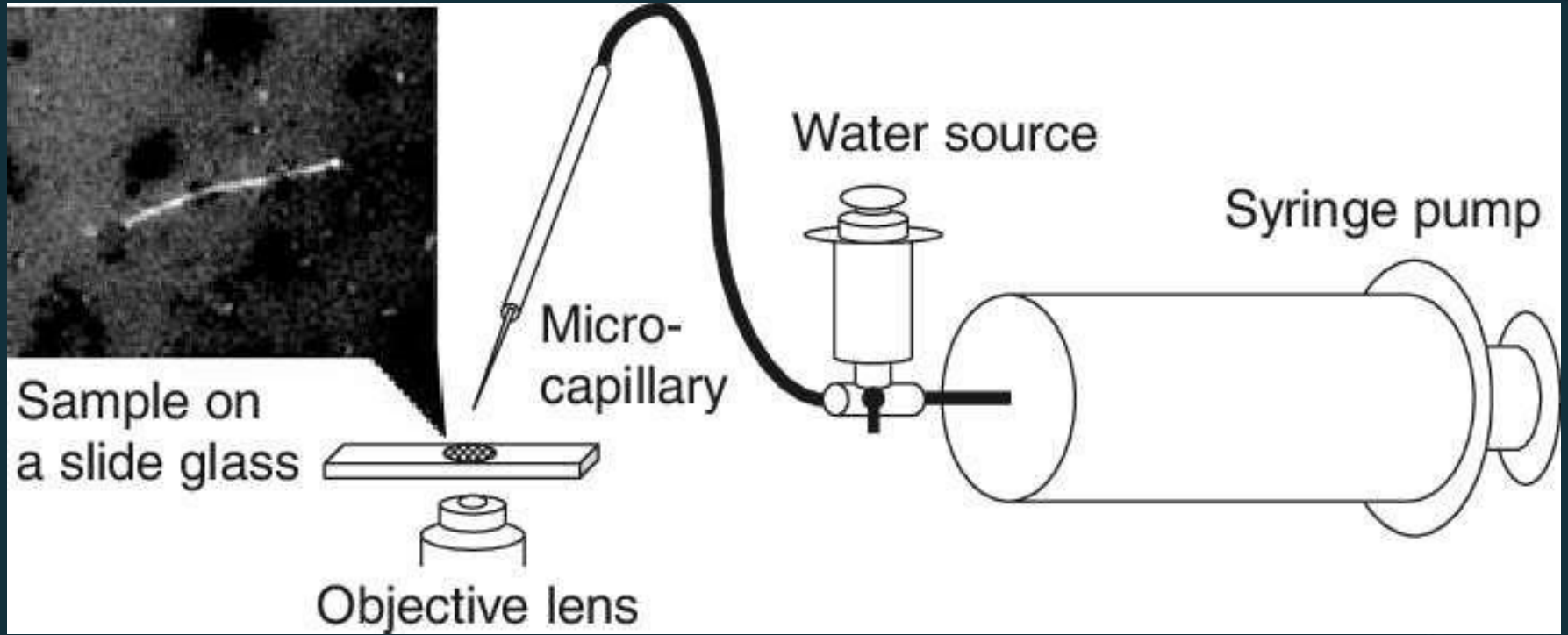
ADVANTAGES OF MICROMANIPULATOR METHOD-

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.

DISADVANTAGES-

The disadvantages are that the equipment is expensive, its manipulation is very tedious, and it requires a skilled person.





PRESERVATION OF PURE CULTURE



To maintain pure culture for extended periods in viable condition without any genetic change is referred as Preservation.

During preservation most important factor is to stop microbial growth or at least lower the growth rate.

Due to this toxic chemicals are not accumulated and hence viability of microorganism is not affected.



Objectives of preservation

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

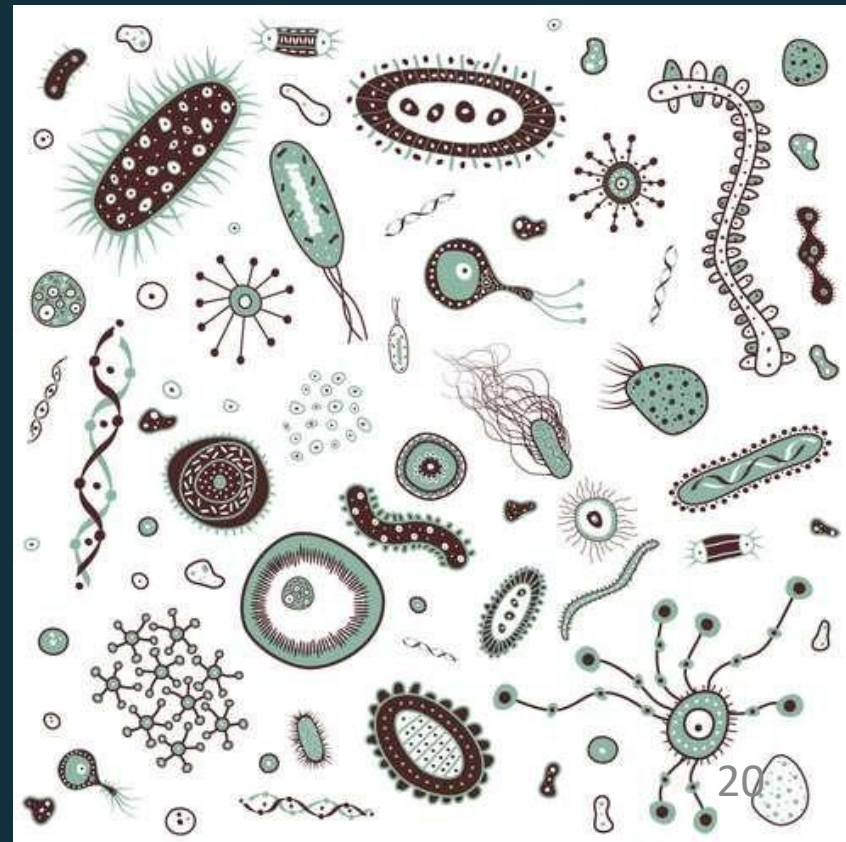


Why to Preserve Bacteria?

- ❖ In nature there are only 1% bacteria which is pathogenic and harmful to Animalia and Plantae.
- ❖ 99% of bacterial populations are of economic importance for human beings and plants.
- ❖ In soil for nutrient uptake in food industry, in sewage treatment, in medical industry.
- ❖ So the preservation of bacteria is one of the most profitable practice economically as well as environmentally.



1. Academic purpose
2. Reserch Purpose
3. Biotechnology field
4. Fermentation Industry



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



1. Periodic transfer to fresh medium

- 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.
- 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.



2. Storage at low temperature



1. REFRIGERATION

2. CRYOPRESERVATION



1. 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



2. 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.



3. 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.

2 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 spp which are also maintained in soi for several years.



4. 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).

ADVANTAGES

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



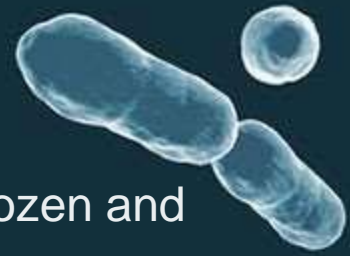
Paraffin Method/ Preservation by Overlaying Cultures with Mineral Oil

- Simple, most economical method.
- Agar slants are inoculated & incubated.
- Then, covered with sterile mineral oil to a depth of 1 cm 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.



5. Lyophilization or freeze drying

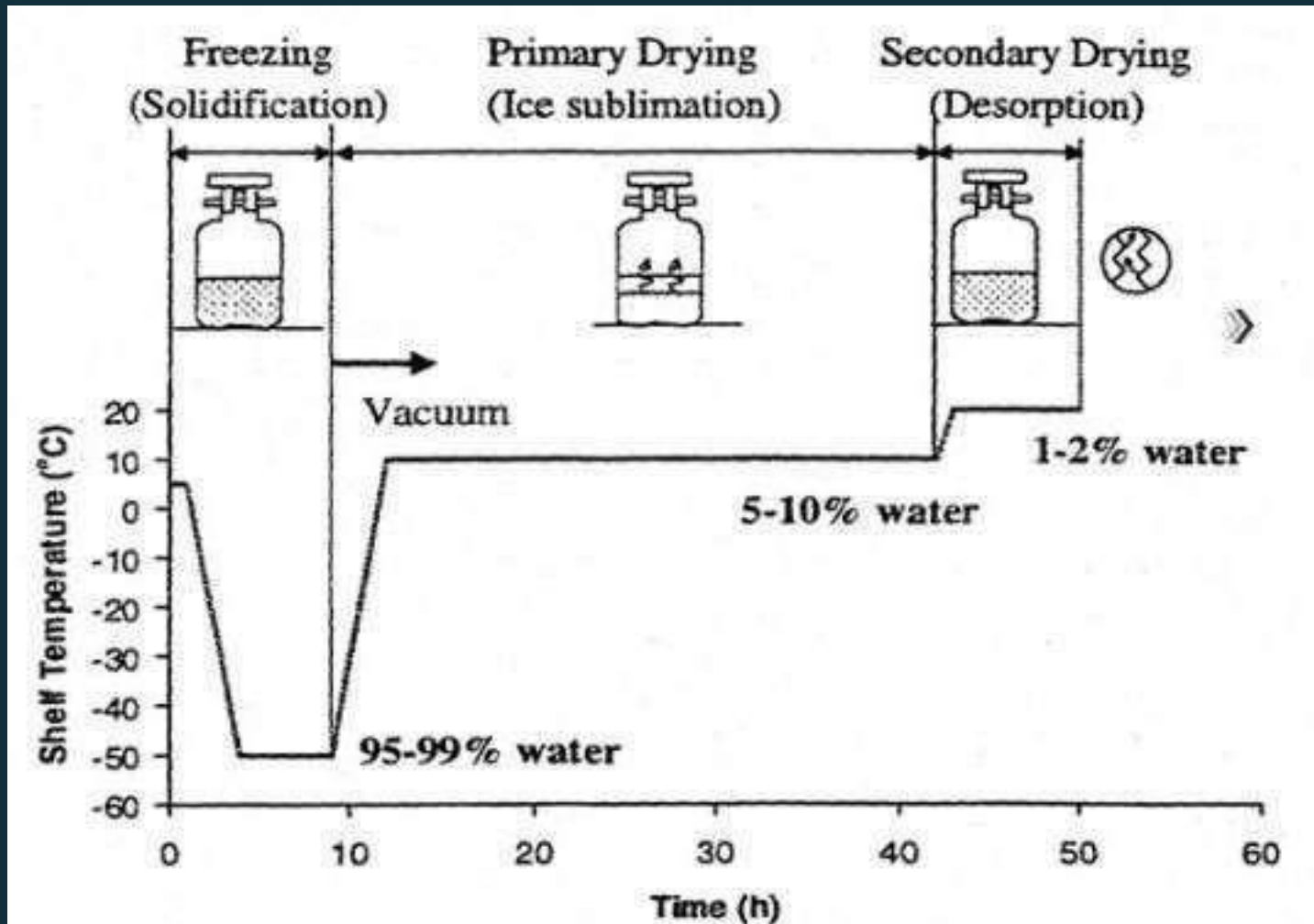
- Freeze drying is a stabilizing process in which a substance is first frozen and then the quantity of the solvent is reduced, first by sublimation (primary drying stage) and then desorption (secondary drying stage)
- Better preservation occurs with freeze-drying than with other methods because freeze-drying **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



Lyophilization Process



Storage Vials

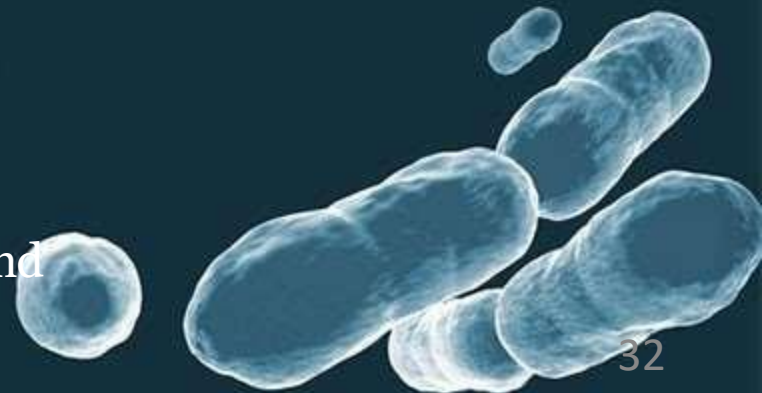


1. Glass vials are used for all freeze-dried specimens
2. When freeze-drying is performed in a chamber, double glass vials are used
3. (chamber method) : an outer soft glass vial is added for protection and preservation of the dehydrated specimen
4. Silica gel granules are placed in the bottom of the outer vial before the inner vial is inserted and cushioned with cotton
5. Manifold method- a single glass vial is used
6. storage vial must be sealed to maintain the vacuum and the dry atmospheric condition

Cryoprotective Agents

Two most commonly used agents are

- ❖ Skim milk for chamber lyophilization, and
- ❖ sucrose for manifold lyophilization



ADVANTAGES

- ❖ Removal of water at low temperature
- ❖ Thermolabile materials can be dried.
- ❖ Sterility can be maintained.
- ❖ Reconstitution is easy



DISADVANTAGES



- ❖ Many biological molecules are damaged by the stress associated with freezing, freeze-drying, or both.
 - ❖ E.g. the process of drying causes extensive damage to molds, protozoa, and most viruses
 - ❖ Hence, these microorganisms can not be stored by this method
- ❖ The product is prone to oxidation, due to high porosity and large surface area. Therefore the product should be packed in vacuum or using inert gas
- ❖ Cost may be an issue, depending on the product

