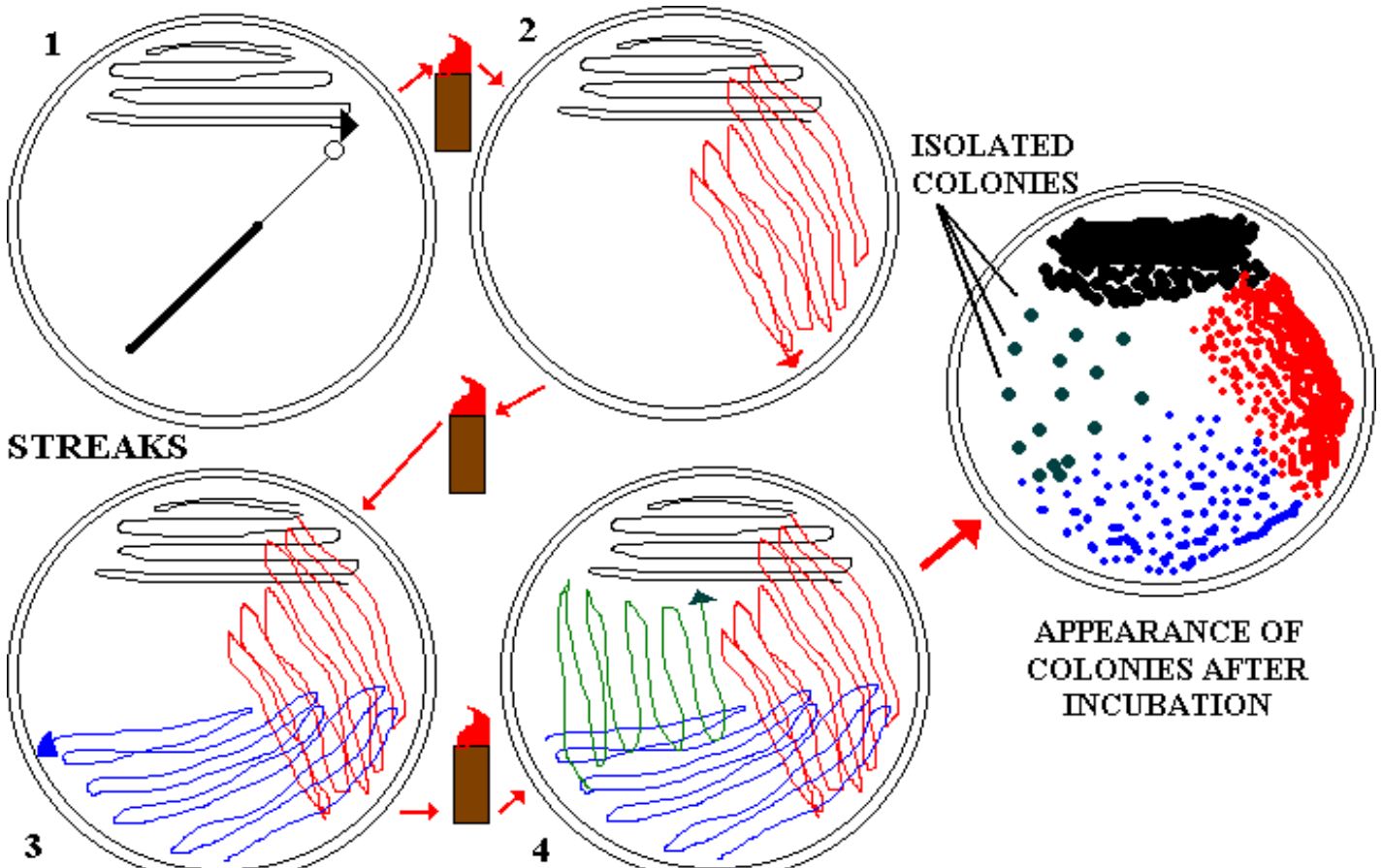
Isolation
and
Preservation of microorganism(bacteria)



ISOLATION OF MICROBIAL PURE CULTURE

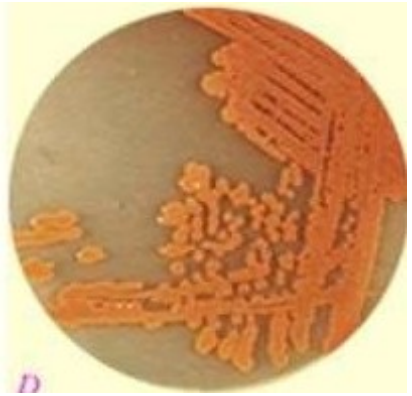
- **Microorganisms** are generally found in nature **(air, soil and water) as mixed populations.**
- Even the diseased parts of plants and animals contain a great number of microorganisms, which differ markedly from the microorganisms of other environments.
- To study the specific role played by a specific microorganism **in its environment, one must isolate the same in pure culture.**

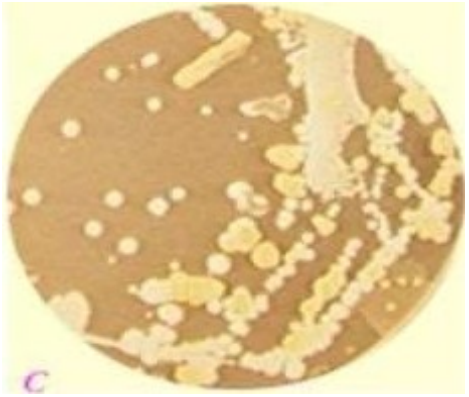
➢ **The two major steps of obtaining a pure culture are as follows :**

- Firstly, the culture has to be diluted until the various individual microorganisms are separated far apart on agar surface that after incubation they form visible colonies isolated from the colonies of other microorganisms.
- Secondly, an isolated colony has to be aseptically picked off the isolation plate

Pure vs mixed culture

- Pure: originate from 1 bacteria strain
  - All colonies look the same
- Mixed: originate from many bacteria strains
  - Colonies have different size/shape





http://smccd.net/accounts/case/biol240/streakpltae.html

- **Points to be taken into consideration during the inoculation are :**
- **The inoculation loop has to be sterilized before every inoculation of colonies from the agar plate**
- **Every time the loop is sterilized by heat it must be cooled before inoculating the next colony**
- There are several methods of isolating the pure culture of bacteria, like, **streak plate method, pour plate method, spread plate method and serial dilution.**

Why important ?

Pure cultures are important in microbiology for the following reasons

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

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

COMMON METHODS OF ISOLATION OF PURE CULTURE

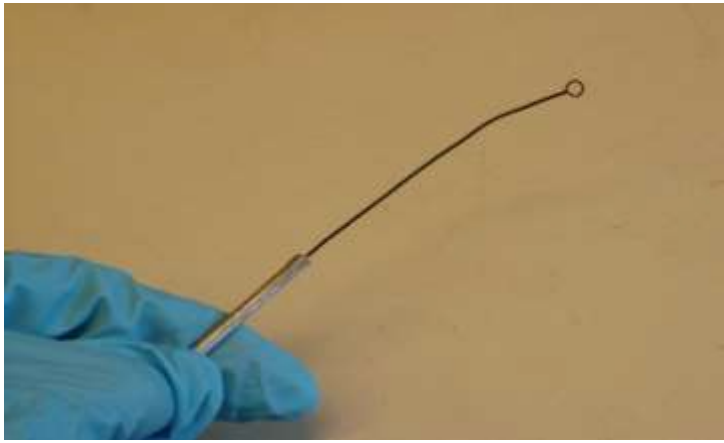
- The process of screening a pure culture by separating one type of microbes from a mixture is called Isolation.

Some common isolation methods are;

- I) Streak plate method
- II) Pour plate method-a) Loop dilution technique
  b) Serial Dilution technique
- III) Spread plate method
- IV) Micromanipulator method
- V) Roll tube method

I. STREAKING OR STREAK PLATE TECHNIQUE

- In This method the tip of a fine structure wire loop called Inoculation needle consist of a wooden or glass handle with a Nichrome wire the end of which is bend to form a loop is used to transfer microbes from culture.
- The straight wires are similar to wire loop except they do not have loop. These are used to transfer culture in colony formed on solid culture medium.
- In such cases, the colony from solid medium is streaked on the surface of nutrient agar medium in a sterile petri dish.



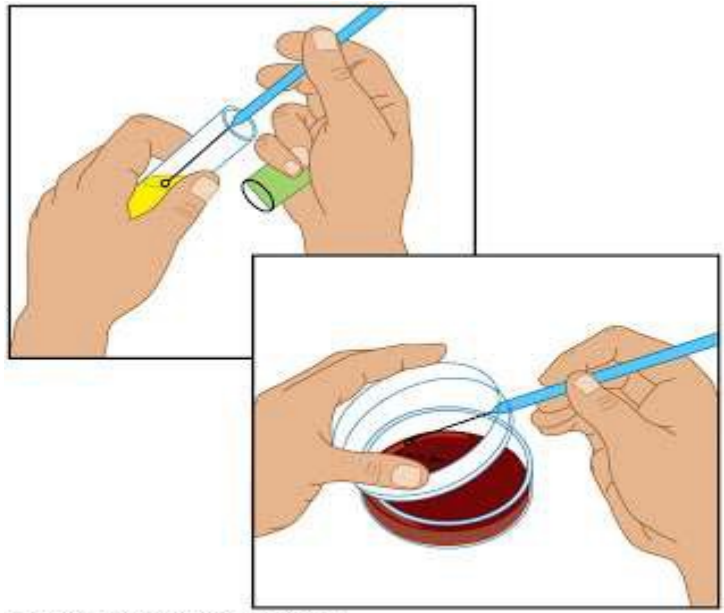
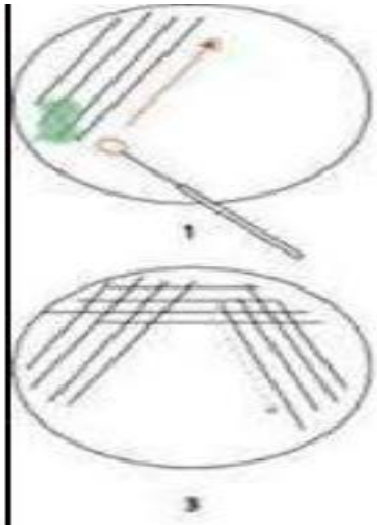
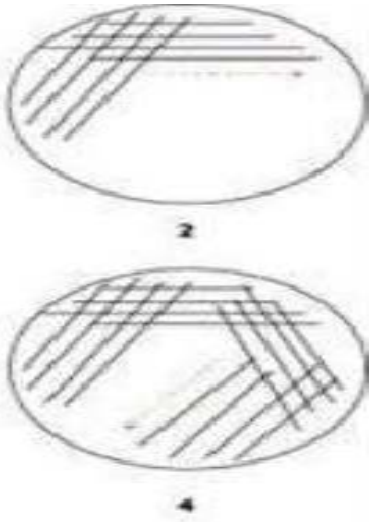


Figure 5-4ab Brock Biology of Microorganisms 11/e
© 2006 Pearson Prentice Hall, Inc.

This technique consist of the following steps-

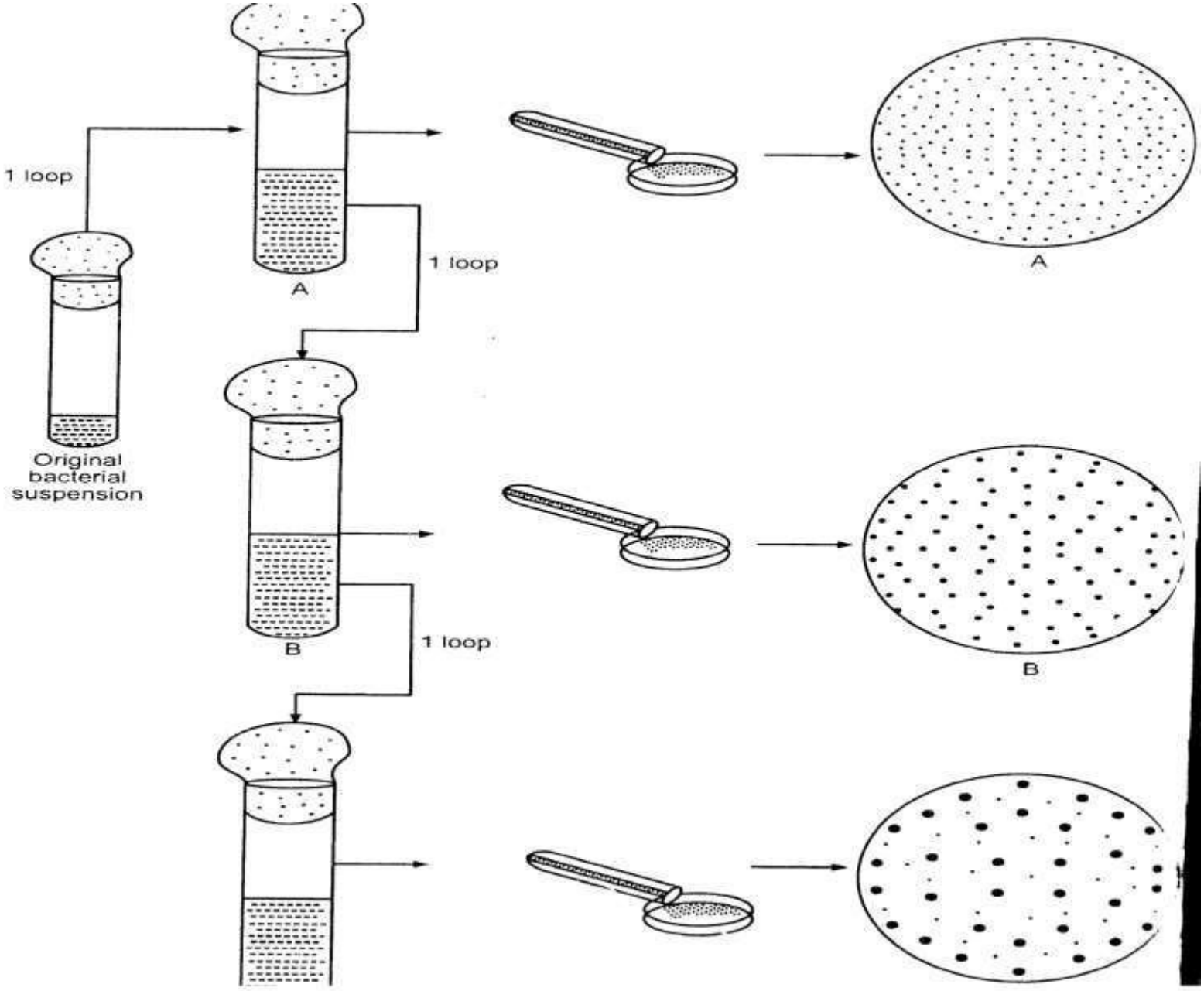
- A. Hold the broth culture containing tube in left hand and shake it.
- B. Sterilize the wire loop of the inoculation needle on burner flame .
- C. Remove the cotton plug of the broth culture tube by little finger of right hand.
- D. Flame the mouth of the test tube immediately.
- E. Insert the wire loop to form a thin film and replace the cotton plug.
- F. The thin film in the loop is streaked in either a zig-zag manner by removing the loop backwards and forwards firmly. Care should be taken that loop should not be firmly pressed against the agar surface.
- G. Incubate the petri dish in incubator at a required temperature.
- H. Growth of the bacteria will be visible (after an overnight incubation)on the streaked marks.





II) 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.
- Link: https://microbeonline.com/pour-plate-method-principle-procedure-uses-dis-advantages/


1 loop
Original bacterial suspension
A
1 loop
B
1 loop
A
B

Disadvantages of Pour plate method

1. The microorganisms are trapped beneath the surface of medium when it solidifies. Hence, surface as well as subsurface colonies are developed and it is very difficult to isolate and count the subsurface colonies.
2. This method is tedious, time consuming and requires skill.
3. The microorganisms are subjected to hot shock because liquid medium is maintained at 45°C temperature.
4. This method is unsuitable for isolation of psychrophile bacteria.

PROCEDURE FOR SPREAD AND POUR PALTE METHOD

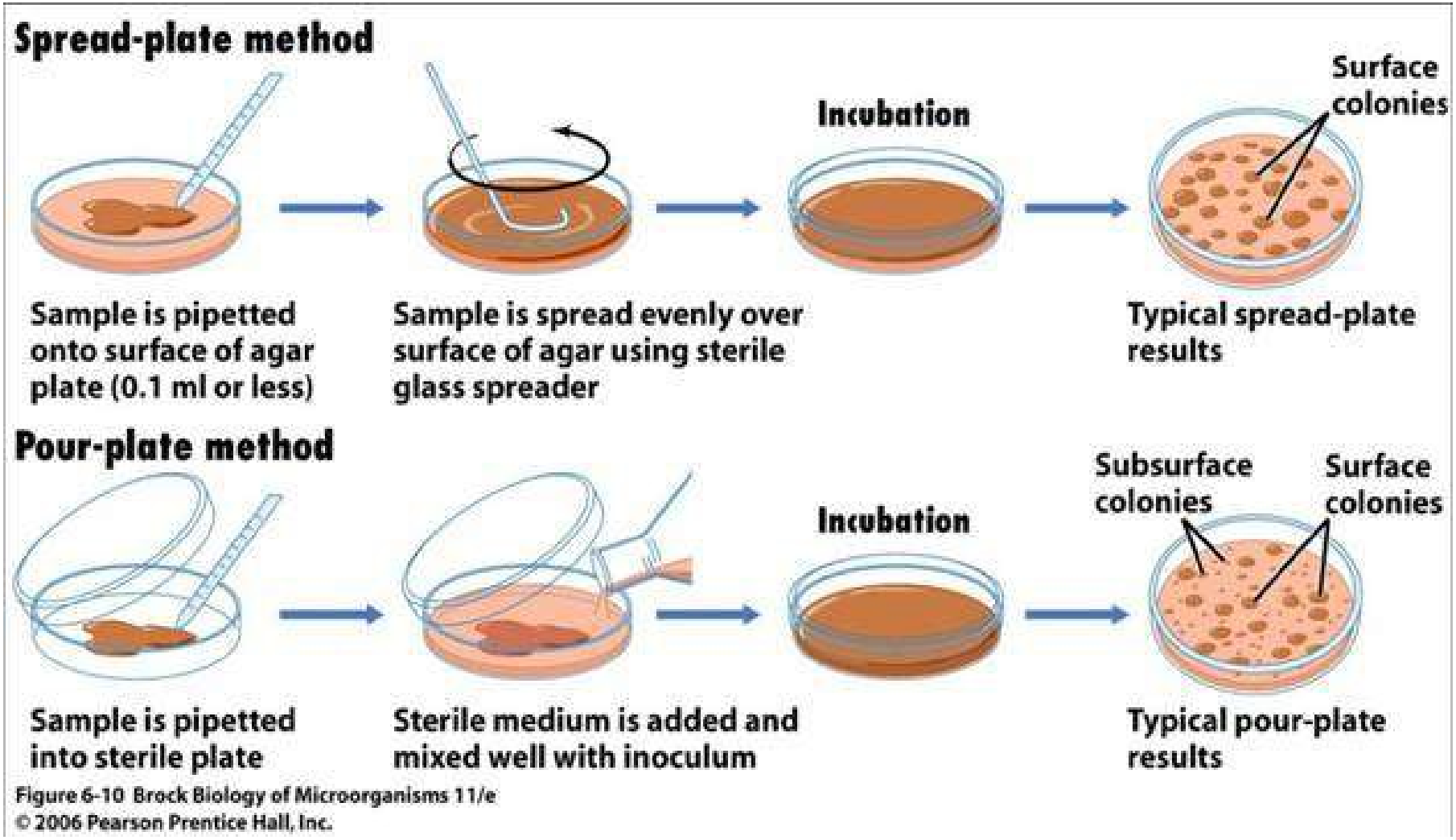
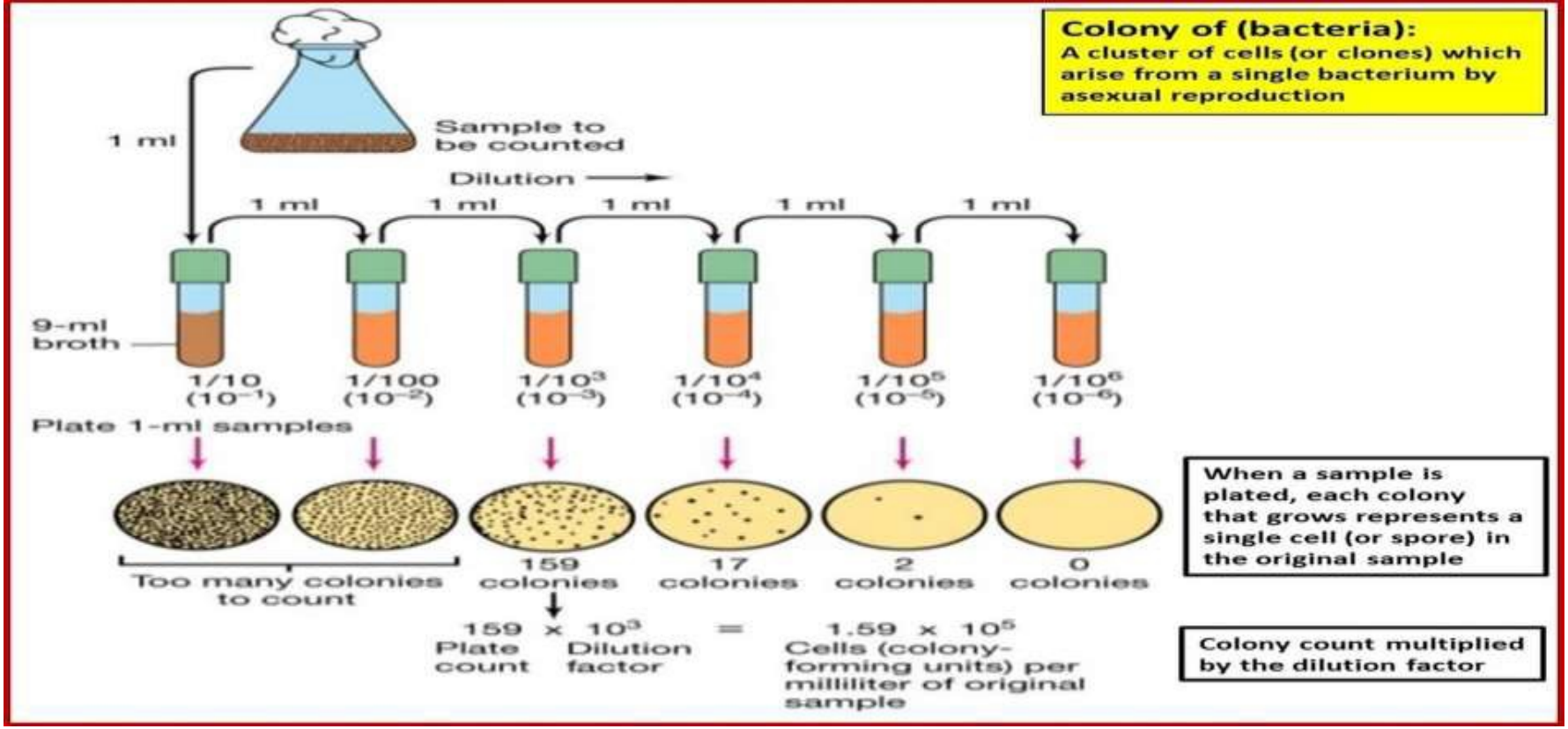


Figure 6-10 Brock Biology of Microorganisms 11/e

a) SERIAL DILUTION

- This method is commonly used to obtain pure cultures of those microorganisms that have not yet been successfully cultivated on solid media and grow only in liquid media.
- A microorganism that predominates in a mixed culture can be isolated in pure form by a series of dilutions.
- The inoculum is subjected to serial dilution in a sterile liquid medium, and a large number of tubes of sterile liquid medium are inoculated with aliquots of each successive dilution.

- Link: https://www.sciencedirect.com/topics/earth-and-planetary-sciences/dilution



- If we take out 1 ml of this medium and mix it with 9 ml of fresh sterile liquid medium, we would then have 100 microorganisms in 10 ml or 10 microorganisms/ ml.
- If we add 1 ml of this suspension to another 9 ml. of fresh sterile liquid medium, each ml would now contain a single microorganism.
- If this tube shows any microbial growth, there is a very high probability that this growth has resulted from the introduction of a single microorganism in the medium and represents the pure culture of that microorganism.

III) 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.
- Links for further study:https://vlab.amrita.edu/?sub=3&brch=73&sim=213&cnt=1
- https://jcm.asm.org/content/jcm/29/7/1462.full.pdf

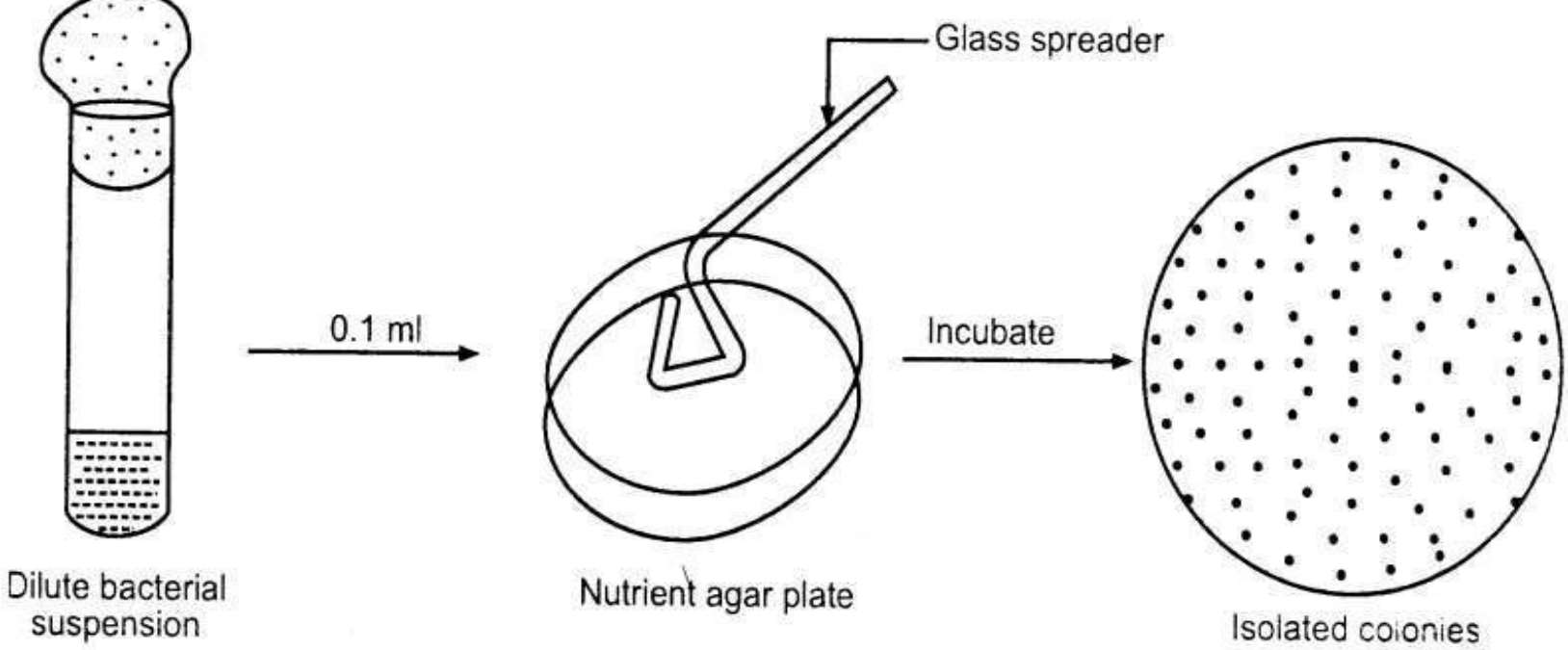


Fig. 6.3 : Spread plate technique

Advantages of spread plate method

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

IV-MICROMANIPULATOR METHOD
Micromanipulators

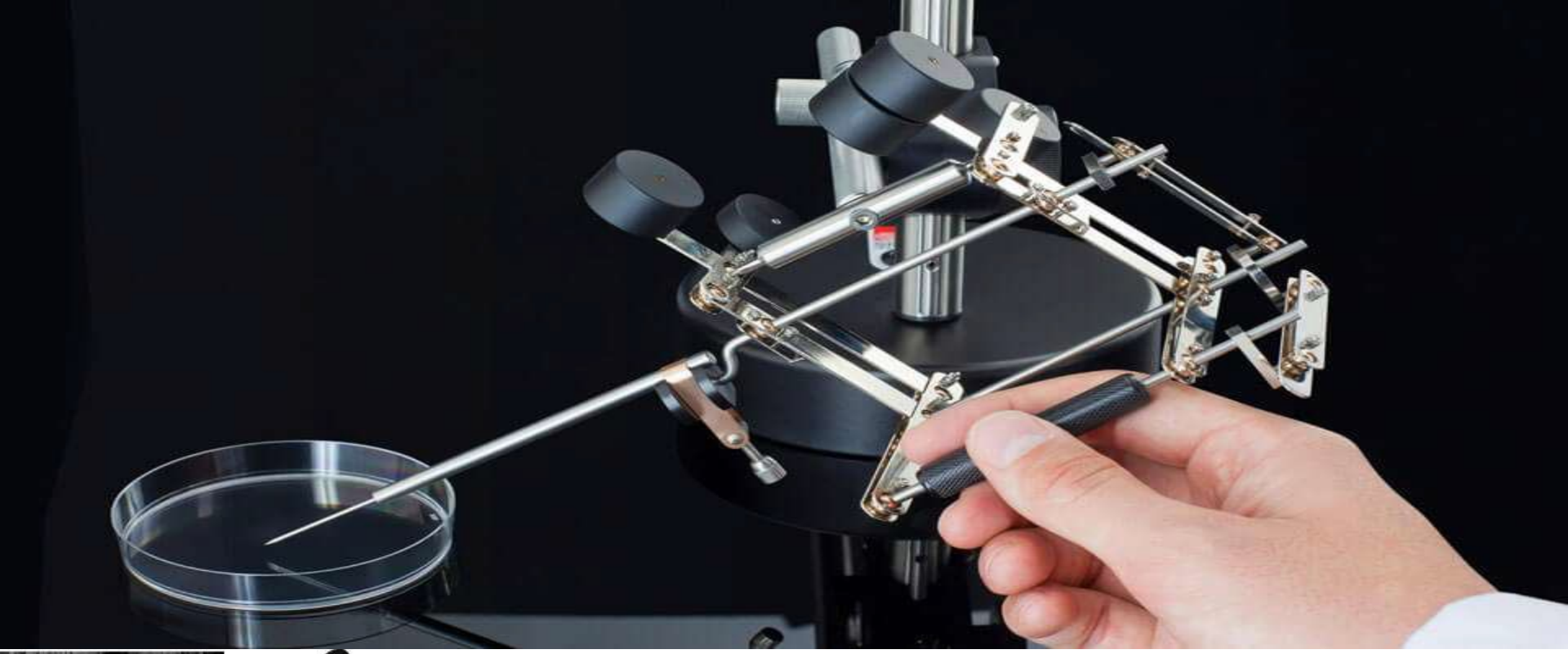
- Micromanipulators have been built, which permit one to pick out a single cell from a mixed culture. This instrument is used in conjunction with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation.

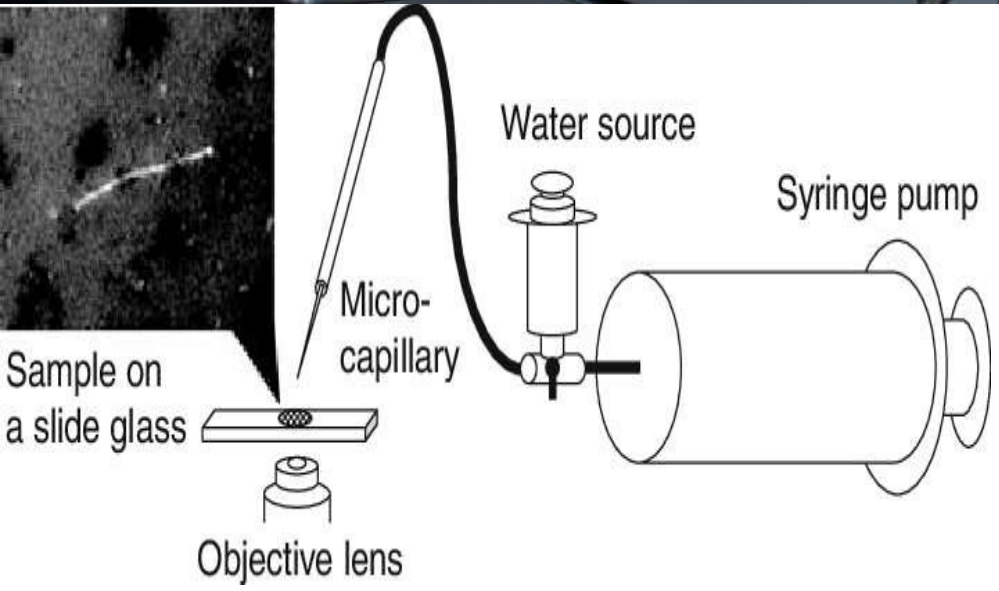
ADVANTAGES OF MICROMANIPULATOR METHOD

- 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 with in the species.

DISADVANTAGES

- Disadvantages are that the equipment is expensive,
- Its manipulation is very tedious, and it requires a skilled operator.





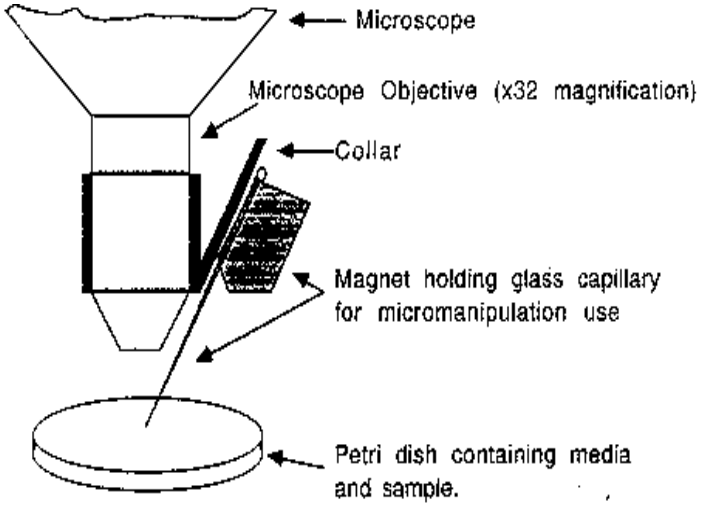


Figure 2. Diagram of Skerman Micromanipulator parts as set-up for cell manipulation

Maintenance and Preservation of Pure Cultures

- Once a microorganism has been isolated and grown in pure culture, it becomes **necessary to maintain the viability** and **purity** of the **microorganism by keeping the pure cultures free from contamination.**
- Normally in laboratories, the pure cultures are transferred periodically **onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms.**
- The transfer is always **subject to aseptic conditions to avoid contamination.**

Objectives of Preservation

- To maintain isolated pure cultures for extended periods (future use) in a viable conditions.
- To avoid the contamination
- To restrict genetic change(Mutation)

Application of Preservation

- **Academic Use:**
- **Research Purpose:**
- **Fermentation Industry:**
- **Biotechnological Field:**

- Once a microorganism has been isolated and grown in pure culture, it becomes necessary **to maintain the viability and purity of the microorganism** by keeping the pure culture free from contamination.
- Normally in laboratories, the pure cultures are transferred periodically onto or into a fresh medium (sub-culturing) to allow continuous growth and viability of microorganisms. The transfer is always subject to aseptic conditions to avoid contamination.
- **Since repeated sub-culturing is time consuming,**
- **It becomes difficult to maintain a large number of pure cultures successfully for a long time.**
- In addition, there is **a risk of genetic changes as well as contamination.**
- Therefore, it is now being replaced by some modern methods that do not need

Methods of Preservation

1. Periodic transfer to fresh media (Sub-culturing)
2. Storage in sterile soil
3. Storage at low temperature
4. Preservation by overlaying cultures with mineral oil
5. Freeze dying/Lyophilization

1. Periodic transfer to fresh media

- Strains can be maintained by periodically preparing a fresh culture from the previous stock culture.
- The culture medium, the storage temperature, and the time interval at which the transfers are made vary with the species and must be ascertained beforehand.
- The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible.
- Many of the more common heterotrophs remain viable for several weeks or months on a medium 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.

Advantages

1. It is a simple method, any special apparatus are not required.
2. Easy to recover the culture

Disadvantage

1. Risk of contamination is more
2. It may be possible to change in genetic and biochemical characteristics

2. Lyophilization (Freeze-Drying)

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

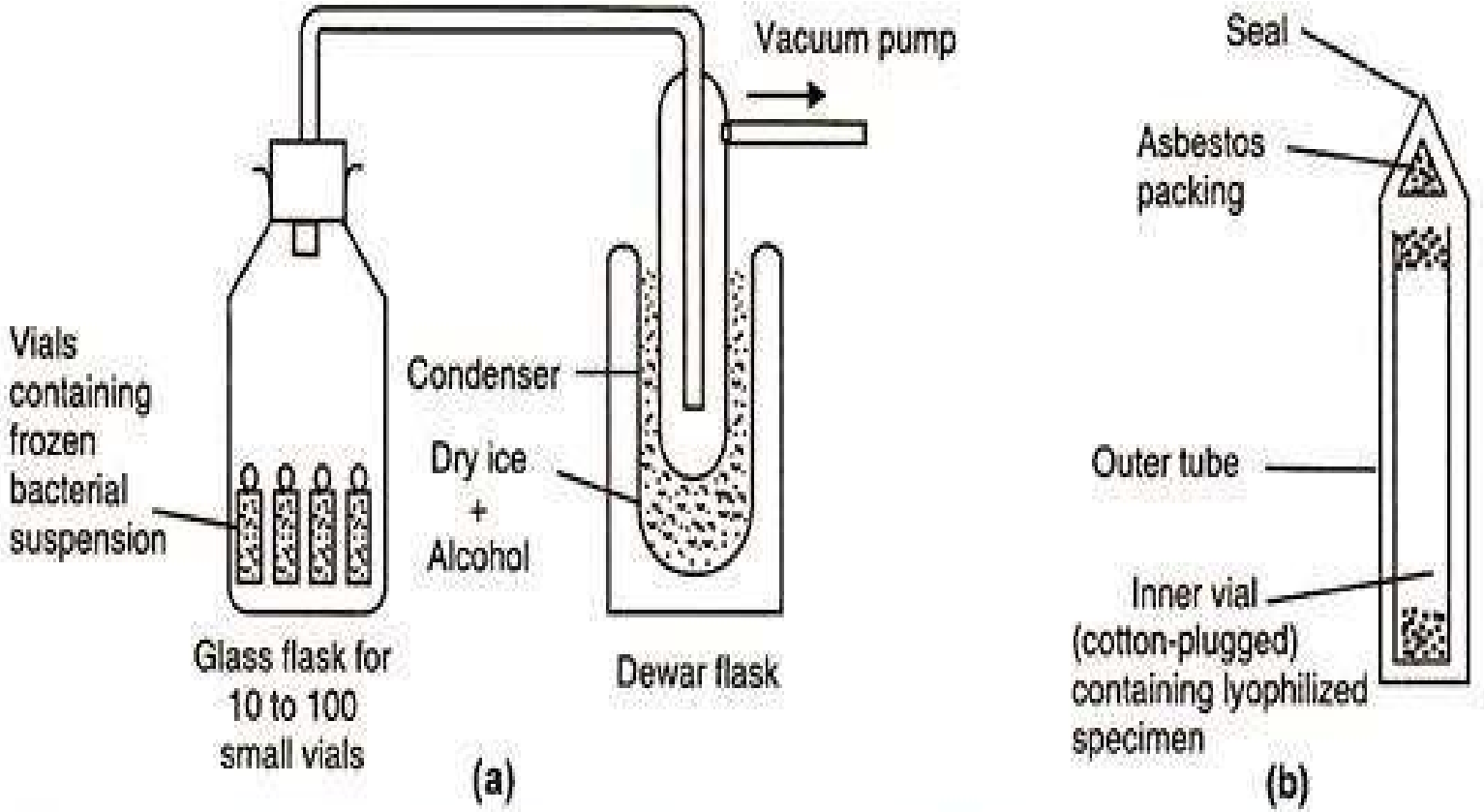


Fig. 2.16: (a) Small cotton-plugged vials containing frozen suspension of the microorganisms are placed in the glass-flask, which is attached to a condenser. The condensor is connected with a high-vacuum pump and this system brings about desiccaton of the cultures.

(b) After desiccation of the cultures as in (a) the vials are removed, placed individually in a large tube covered with asbestos packing and under vacuum.

Procedure

- In this process, a dense cell suspension is placed in small vials and frozen at -60 to -70°C.
- The vial are immediately connected to a high vacuum line.
- The ice present in the frozen suspension evaporates(sublime) under the vacuum.
- This results in dehydration of bacterial cell and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years.
- The vials are then sealed off under a vacuum and stored in the dark at 4°C in refrigerators.

Advantage of Lyophilization

- Only minimal storage space is required; hundreds of lyophilized cultures can be stored in a small area.
- Remained viable for more than 30 years.
- Frequent sub-culturing is not required
- Maintained without contamination
- Lyophilised strains remains genetically stable
- Small vials can be sent conveniently through the mail to other microbiology laboratories when packaged in a special sealed mailing containers.
- This method is employed for the preservaton of sera, toxin, enzymes and other biologicals.

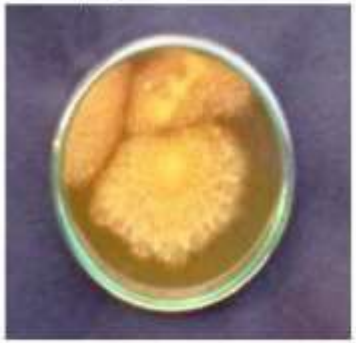
Storage in sterile soil

- It is mainly applied for the preservation of **sporulating microorganisms** (a single spore (endospore) within the cell).
- *Fusarium, Penicillium, Alternaria, Rhizopus, Bacillus, Aspergillus, Penicillium, etc. proved successful for store in* sterile soil.
- Soil storage involves inoculation of 1ml of spore suspension into soil (autoclaved twice) and incubating at room temperature for 5-10 days.
- The initial growth period allows the fungus to use the available moisture and gradually to become dormant.
- The bottles are then stored at refrigerator.
- Viability of organisms found around 70-80 years.


Aspergillus niger


Aspergillus melleus


Aspergillus ustus


Aspergillus terreus

Storage at low temperature

- Culture medium can be successfully stored in refrigerators or cold rooms, when the temperature is maintained at 4˚C.
- Another: **liquid nitrogen** can provide long term preservation of culture. In this method, dense suspension of microbes is prepared in a medium containing a protective agent(**Glycerol or dimethyl sulfoxide)** which prevent cell damage due to ice crystal formation. Suspension is sealed into small ampoules or vials and then frozen at -150°c
- At this temperature range the metabolic activities of microbes slows down greatly and only small quantity of nutrients will be utilized.
- This method cannot be used for a very long time

Preservation by overlaying culture with mineral oil or liquid paraffin storage

- In this method sterile liquid paraffin is poured over the slant culture of microbes and stored upright at room temperature.
- Where as cultures can also be maintained by covering agar slants by sterile mineral oil which is stored at room temperature or preferably at 0-5°C.
- It limit the oxygen access that reduces the microorganism's metabolism and growth, as well as to cell drying during preservation.
- The preservation period for bacteria from the genera *Azotobacter and Mycobacterium is from 7-10 years, for Bacillus it is 8-12 years.*

Adv:

- *Simple*
- *Mainly used anaerobic microorganism*
- *Cost effective method*
- *Can preserve 10-15 years*

Three Stories

Once, all villagers decided to pray for rain. On the day of prayer all people gathered but only one boy came with umbrella.

-That's Faith.

When you toss a one year old baby in the air, he laughs because he knows his father will catch him.

-That's Trust

Every night we go to bed, we have no assurance to wake up alive next morning, but still we set alarm for tomorrow

-That's Hope