Methods of Isolation & Preservation Methods

Dr. Ekta Khare
Department of Microbiology,
School of Life sciences and Biotechnology
Chhatrapati Shahu Ji Maharaj University, Kanpur

Culture Media

- A culture medium is a solid or liquid preparation used to grow, transport, and store microorganisms.
- Although all microorganisms need sources of energy, carbon, nitrogen, phosphorus, sulfur, and various minerals, the precise composition of a satisfactory medium will depend on the species one is trying to cultivate because nutritional re quirements vary so greatly.
- Knowledge of a microorganism's normal habitat often is useful in selecting an appropriate culture medium because its nutrient requirements reflect its natural surroundings.
- Frequently a medium is used to select and grow specific microorganisms or to help identify a particular species. In such cases the function of the medium also will determine its composition.

Synthetic or Defined Media

- Some microorganisms, particularly photolithotrophic autotrophs such as cyanobacteria and eucaryotic algae, can be grown on rel atively 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.
- Such a medium in which all components are known is a **defined medium or synthetic medium**.
- Many chemoorganotrophic heterotrophs also can be grown in defined media with glucose as a carbon source and an ammonium salt as a nitrogen source.
- De fined media are used widely in research, as it is often desirable to know what the experimental microorganism is metabolizing.

Complex Media

- Media that contain some ingredients of unknown chemical com position are complex media.
- Such media are very useful, as a single complex medium may be sufficiently rich and complete to meet the nutritional requirements of many different microorganisms.
- In addition, complex media often are needed because the nutritional requirements of a particular microorganism are un known, and thus a defined medium cannot be constructed.
- Complex media contain undefined components like peptones, meat extract, and yeast extract.
- Peptones are protein hydrolysates prepared by partial proteolytic digestion of meat, casein, soya meal, gelatin, and other protein sources.
- They serve as sources of carbon, energy, and nitrogen. Beef extract and yeast extract are aqueous extracts of lean beef and brewer’s yeast, respectively.
- Beef extract contains amino acids, peptides, nucleotides, organic acids, vitamins, and minerals.
- Yeast extract is an excellent source of B vitamins as well as nitrogen and carbon compounds.
- Three commonly used complex media are (1) nutrient broth, (2) tryptic soy broth, and (3) MacConkey agar

Agar

- If a solid medium is needed for surface cultivation of microor ganisms, liquid media can be solidified with the addition of 1.0 to 2.0% agar; most commonly 1.5% is used.
- Agar is a sulfated polymer composed mainly of D-galactose, 3,6-anhydro-L-galactose, and D-glucuronic acid.
- It usually is extracted from red algae.
- Agar is well suited as a solidifying agent be cause after it has been melted in boiling water, it can be cooled to about 40 to 42°C before hardening and will not melt again until the temperature rises to about 80 to 90°C.
- Agar is also an excellent hardening agent because most microorganisms cannot degrade it.

Types of Media

- **General purpose media** support the growth of many microorganisms.
- **Enriched media:** Blood and other special nutrients may be added to general purpose media to encourage the growth of fastidious heterotrophs.
- These specially fortified media (e.g., blood agar) are called enriched media.
- **Selective media** favor the growth of particular microorganisms.
- Bile salts or dyes like basic fuchsin and crystal violet favor the growth of gram-negative bacteria by inhibiting the growth of gram-positive bacteria without affecting gram-negative organisms.
- Endo agar, eosin methylene blue agar, and MacConkey agar, three media widely used for the detection of *E. coli* and related bacteria in water supplies and elsewhere, contain dyes that suppress gram-positive bacterial growth.
- MacConkey agar also contains bile salts.
- Bacteria also may be selected by incuba tion with nutrients that they specifically can use.
- A medium containing only cellulose as a carbon and energy source is quite effective in the isolation of cellulose-digesting bacteria.

…Types of Media

- **Differential media** are media that distinguish between different groups of bacteria and even permit tentative identification of microorganisms based on their biological characteristics.
- Blood agar is both a differential medium and an enriched one.
- It distinguishes between hemolytic and nonhemolytic bacteria. Hemolytic bacteria (e.g., many streptococci and staphylococci isolated from throats) produce clear zones around their colonies be cause of red blood cell destruction.
- MacConkey agar is both differential and selective.
- Since it contains lactose and neutral red dye, lactose-fermenting colonies appear pink to red in color and are easily distinguished from colonies of nonfermenters.

Isolation of Pure Cultures

- In natural habitats microorganisms usually grow in complex, mixed populations containing several species.
- This presents a problem for the microbiologist because a single type of microorganism cannot be studied adequately in a mixed culture.
- **Pure culture**, a population of cells arising from a single cell, to characterize an individual species.
- Pure cultures are so important that the development of pure culture techniques by the German bacteriologist Robert Koch transformed microbiology.

Spread Plate Method

- If a mixture of cells is spread out on an agar surface so that every cell grows into a completely separate colony, a macroscopically visible growth or cluster of microorganisms on a solid medium, each colony represents a pure culture.
- The spread plate is an easy, direct way of achieving this result. A small volume of dilute microbial mixture containing around 30 to 300 cells is transferred to the center of an agar plate and spread evenly over the surface with a sterile bent-glass rod.
- The dispersed cells develop into isolated colonies.
- Because the number of colonies should equal the number of viable organisms in the sample, spread plates can be used to count the microbial population.

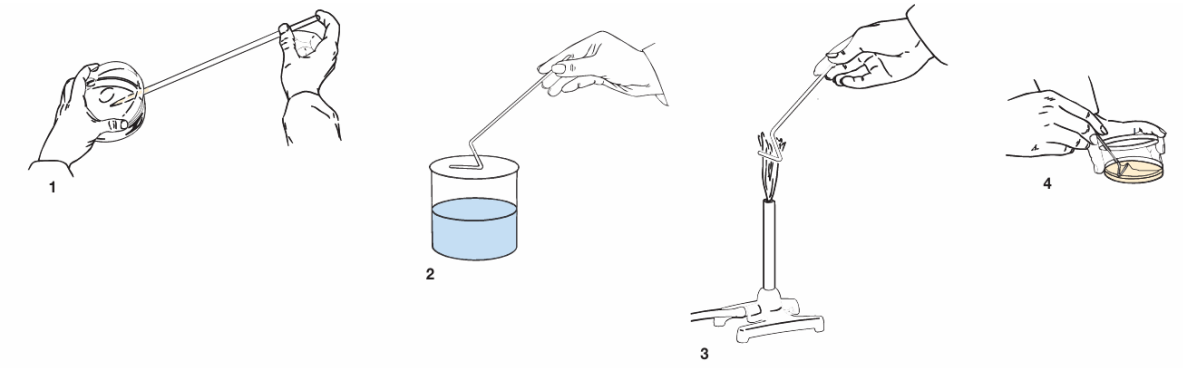


Figure 5.7 Spread-Plate Technique. The preparation of a spread plate. (*1*) Pipette a small sample onto the center of an agar medium plate. (*2*) Dip a glass spreader into a beaker of ethanol. (3) Briefly flame the ethanol-soaked spreader and allow it to cool. (*4*) Spread the sample evenly over the agar surface with the sterilized spreader. Incubate.

Streak-Plate Technique

- Pure colonies also can be obtained from streak plates.
- 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 (figure 5.8).
- While streaking in successive areas of the plate, the inoculum is diluted to the point where there is only one bacterial cell deposited every few millimeters on the surface of the agar plate.
- When these lone bacterial cells divide and give rise to thousands and thousands of new bacterial cells, an isolated colony is formed.
- Pure cultures can be obtained by picking well isolated colonies and re-streaking these on fresh agar plates.
- In both spread-plate and streak-plate techniques, successful isolation depends on spatial separation of single cells.

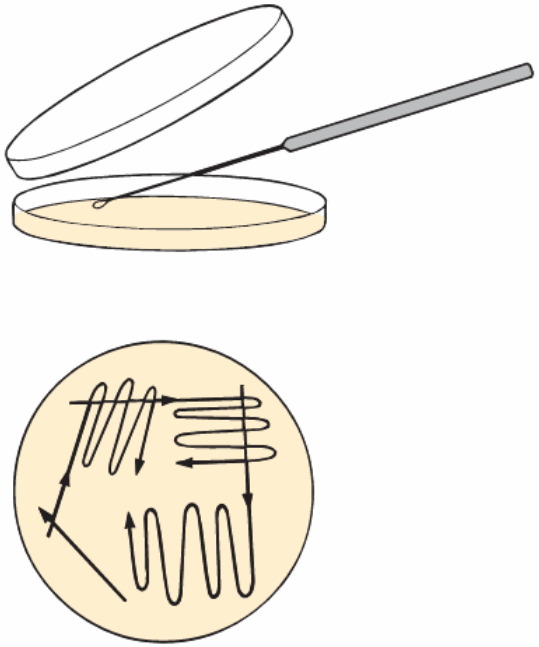
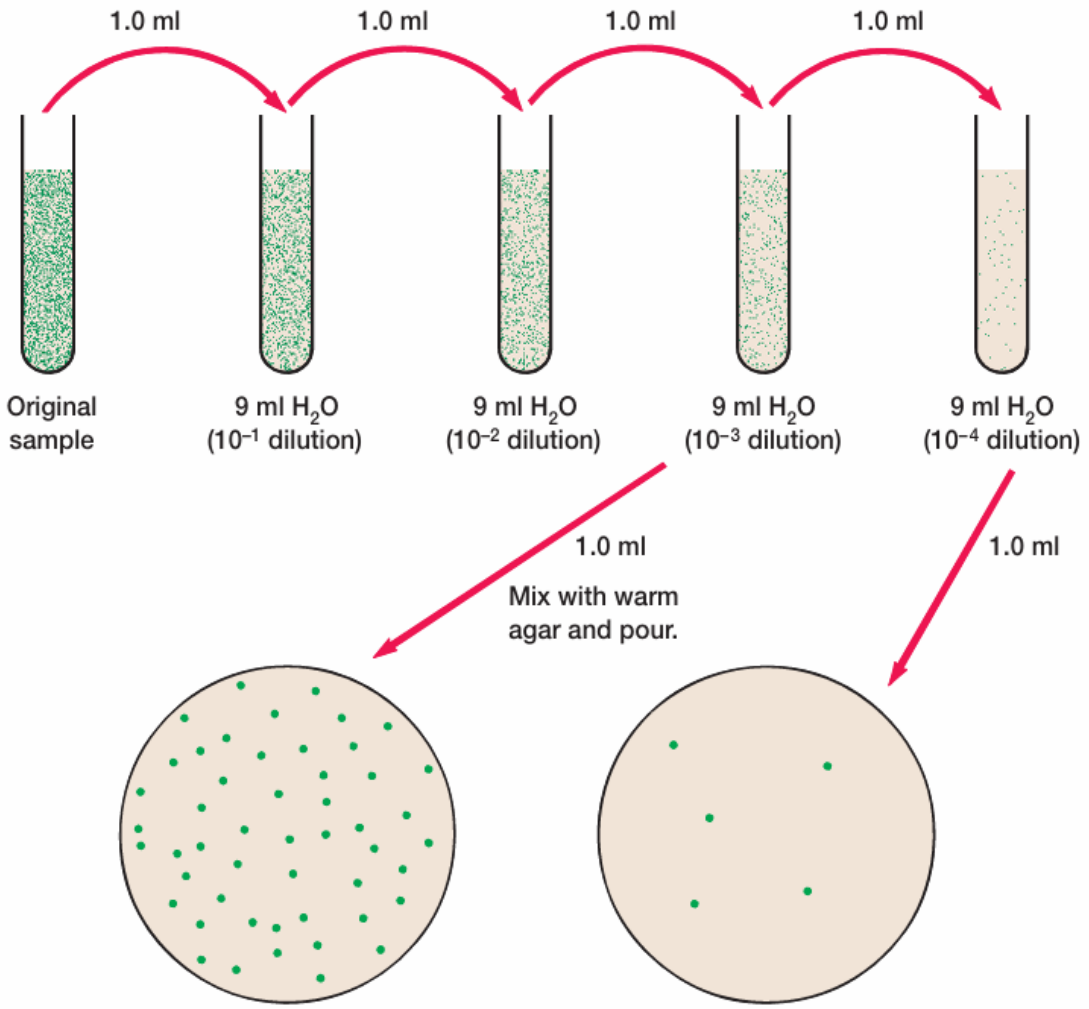


Figure 5.8 Streak-Plate Technique. Preparation of streak plates. The upper illustration shows a petri dish of agar being streaked with an inoculating loop. A commonly used streaking pattern is pictured at the bottom.

Pour Plate Technique

- Extensively used with bacteria and fungi, a pour plate also can yield isolated colonies.
- The original sample is diluted several times to reduce the microbial population sufficiently to obtain separate colonies when plating.
- Then small volumes of several diluted samples are mixed with liquid agar that has been cooled to about 45°C, and the mixtures are poured immediately into sterile culture dishes.
- Most bacteria and fungi are not killed by a brief exposure to the warm agar.
- After the agar has hardened, each cell is fixed in place and forms an individual colony.
- Plates containing between 30 and 300 colonies are counted.
- The total number of colonies equals the number of viable microorganisms in the diluted sample.
- Colonies growing on the surface also can be used to inoculate fresh medium and prepare pure cultures.
- The preceding techniques require the use of special culture dishes named petri dishes or plates after their inventor Julius Richard Petri, a member of Robert Koch's laboratory; Petri developed these dishes around 1887 and they immediately replaced agar-coated glass plates.
- They consist of two round halves, the top half overlapping the bottom.
- Petri dishes are very easy to use, may be stacked on each other to save space, and are one of the most common items in microbiology laboratories.

The Pour-Plate Technique.
The original sample is diluted several times to thin out the population sufficiently. The most diluted samples are then mixed with warm agar and poured into petri dishes. Isolated cells grow into colonies and can be used to establish pure cultures. The surface colonies are circular; subsurface colonies would be lenticular or lens shaped.



Preservation Methods

- Biopreservation is the process of preserving the integrity and functionality of cells.
- Most bacteriological laboratories maintain stock cultures of microorganisms for educational, research, bioassay, industrial, or other purposes.
- A wide variety of techniques are available for the preservation of bacteria and it may be difficult to choose a method for a particular strain, which not only assures survival, but which also makes certain that the genotype and hence the unique characteristics do not change.
- The primary aim of culture preservation is to maintain the organism alive, uncontaminated, and without variation or mutation, that is, to preserve the culture in a condition that is as close as possible to the original isolate.
- There are two types of microbial culture preservation;
    1. Short term culture preservation.
    2. Long-term culture preservation.

Short-term Culture Preservation.

- 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.
- Microbial cultures remain viable for several weeks or months on a medium like Nutrient agar.
- This is a simple method and any special apparatus are not required, however it is easy to recover the culture.
- The transfer has the disadvantage of failing to prevent changes in the characteristics of a strain due to development of variants and mutants and risk of contamination is also more in this process.

Short term methods of culture preservation include:

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

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.

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.

Storage By Drying

- Spores of some microbes which are sensitive to freeze- drying, can be preserved by drying from the liquid state rather than the frozen state.
  - Different procedures of drying methods are as follows:

1. **Paper disc:** A thick suspension of bacteria is placed on sterile discs of thick absorbent paper, which are then dried over phosphorus pentoxide in a desiccation under vacuum.
2. **Gelatin disc:** Drops of bacterial suspension in gelatin are placed on sterile plastic petri-plates and then dried off over P_2O_5 under vacuum.
3. **L-drying:** Bacteria in small ampoules are dried from the liquid state using a vacuum pump and desiccant and a water bath to control the temperature. In this suspension of the organisms are dried under vacuum from the liquid state without freezing taking place.

- Apart from the mentioned methods the organisms are also dried over **Calcium Chloride in vacuum** and are stored in the refrigerator.

Agar Slant Culture and Storage at Refrigerator

- All microbiology laboratories preserve micro-organisms on agar slant.
- The agar slants are inoculated and incubated until good growth appears.
- They are then covered with sterile mineral oil to a depth of 1 cm above the tip of slant surface.
- The slants are incubated for 24hr or more and are then stored in a refrigerator.
- These cultures are periodically transferred to fresh media.
- Transfers are made by removing a loop full of the growth, touching the loop to the glass surface to drain off excess oil, inoculating a fresh medium and then preserving the initial stock culture.
- Time intervals at which the transfers are made which varies with the origin and condition of growth.
- This is a simple and most economical method of preserving bacteria and fungi where they remain viable for several years at room temperature.
- The inoculated and preserved agar slants are stored in a refrigerator to slow down the metabolic activities of the microorganisms, further extending their viability.
- Despite this preservation method, periodic transfer of cultures to fresh media is essential to prevent the loss of viability and maintain the culture's genetic stability.
- The time intervals between transfers vary based on the origin and condition of the microorganism, but typically, transfers are made every six months.

Long Term Preservation Methods

- Long term microbial culture preservation methods include:
  - ➢Preservation by using liquid Paraffin/ Mineral oil
  - ➢Preservation by using of Glycerol
  - ➢Immersion in distilled water
  - ➢Preservation using sterile soil
  - ➢Lyophilization – Freeze drying
  - ➢Cryopreservation – Liquid Nitrogen

Paraffin Method

- This is a simple and most economical method of maintaining pure cultures of bacteria and fungi.
- 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.
- 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).
- The advantage of this method is that we can remove some of the growth under the oil with a transfer needle, inoculate a fresh medium, and still preserve the original culture.

Glycerol Method

- Bacteria can be frozen using 15% glycerol. The glycerol is diluted to 30% and an equal amount of glycerol and culture broth are mixed, dispensed into tubes, and then frozen at -10° C.
- When liquid bacteria cultures are added to a 50% glycerol solution, the glycerol infuses into the bacterial cells, making them structurally stable and allowing them to be stored safely.
- After mixing your samples, freeze them at −80 °C to ensure that they remain viable for as long as possible.
- They can be stored at -20 °C and transferred after 12–18 months.

Sterile Soil Method

- It is mainly applied for the preservation of sporulating microorganisms such as Fusarium, Penicillium, Alternaria, Rhizopus etc.
- 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 in refrigerator and the organisms can found viable after 70-80 years.

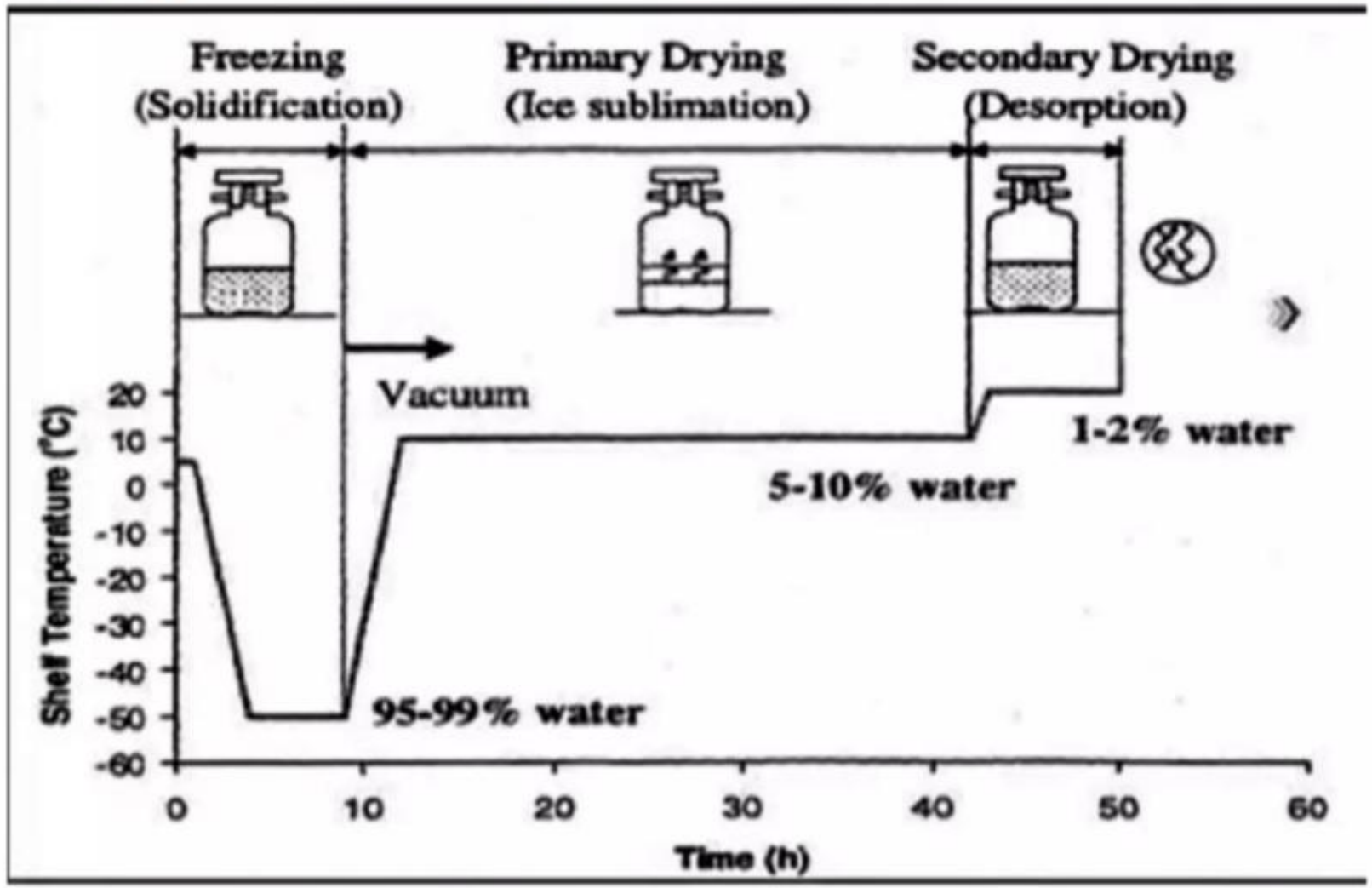
Lyophilisation (Freeze-Drying)

- Lyophilisation, commonly known as freeze-drying, is a preservation method used to maintain microbial cultures and other biological materials over long periods. This technique is widely employed in culture collection centers and other research facilities due to its effectiveness in preserving the viability of microorganisms.
- **Lyophilisation Process:**
  - **Rapid Freezing:** The initial step in lyophilisation involves rapidly freezing the microbial culture at an extremely low temperature, typically around -70°C. This rapid freezing is crucial for preventing the formation of large ice crystals, which can damage cellular structures.
  - **Dehydration by Vacuum:** Following freezing, the culture is subjected to a high vacuum environment. Under this vacuum, sublimation occurs, where ice transitions directly from a solid to a gas, bypassing the liquid phase. This process effectively removes the water content from the microbial cells.
- **Microbial Dormancy and Viability:**
  - **Cellular Dehydration:** The vacuum conditions ensure that the microbial cells are dehydrated while still in their frozen state. This dehydration halts all metabolic activities, placing the cells in a dormant condition.
  - **Long-Term Viability:** As a result of this dormancy, the microorganisms can retain their viability for several years. The lack of moisture prevents microbial growth and metabolic reactions, thereby preserving the integrity of the cultures.

…Lyophilisation

- **Storage and Preservation:**
  - **Sealing and Storage:** After dehydration, the vials containing the lyophilized cultures are sealed under vacuum using a small flame. This sealing process protects the cultures from moisture and contamination.
  - **Storage Conditions:** The sealed vials are stored in the dark at 4°C in refrigerators. This low-temperature storage further ensures the long-term preservation of the cultures.
- **Application and Benefits:**
  - **Culture Collection Centers:** Lyophilisation is frequently used by culture collection centers due to its effectiveness in maintaining a large number of cultures with minimal variation in characteristics.
  - **Versatility:** Besides microbial cultures, this method is also employed for preserving toxins, sera, enzymes, and other biological materials.
  - **Reduced Contamination Risk:** The freeze-drying process significantly reduces the risk of contamination and allows for the maintenance of culture characteristics over extended periods.
- **Revival of Cultures:**
  - **Thawing and Rehydration:** To revive a lyophilized culture, the vial is aseptically opened, and a suitable growth medium is added. The culture is then incubated to allow recovery and growth.
  - **Further Transfers:** Once revived, the microorganisms can be further transferred and used for research or applications.

Lyophilization process



Cryopreservation

- Cryopreservation is a highly effective method for the long-term storage of pure microbial cultures, utilizing extremely low temperatures to maintain cell viability over extended periods. This technique involves freezing cultures in liquid nitrogen at -196°C, providing a robust solution for preserving microorganisms for years.
- **Cryopreservation Process:**
  - **Freezing in Liquid Nitrogen:** The core of cryopreservation involves rapidly cooling microbial cultures to temperatures of -196°C using liquid nitrogen.
  - **Temperature Control:** This ultra-low temperature halts all metabolic and enzymatic activities within the cells, effectively putting them in a state of suspended animation.
- **Role of Stabilizing Agents:**
  - **Glycerol Addition:** To prevent cellular damage during the freezing process, stabilizing agents such as glycerol are added to the microbial suspension.
  - **Ice Crystal Prevention:** Glycerol functions as a cryoprotectant by lowering the freezing point of the solution and inhibiting the formation of ice crystals within the cells.
  - **Cell Survival:** By preventing ice crystal formation, glycerol helps maintain cell membrane integrity, thereby promoting higher survival rates upon thawing.
- **Advantages of Cryopreservation:**
  - **Long-Term Storage:** Cryopreservation allows for the preservation of microbial cultures for several years without significant loss of viability.
  - **Preservation of Genetic Material:** This method maintains the genetic and phenotypic characteristics of the cultures, ensuring that they remain representative of the original strain.
  - **Minimal Maintenance:** Once cultures are cryopreserved, they require minimal maintenance, making this method highly efficient for long-term storage.
- **Application and Procedure:**
  - **Preparation:** Before freezing, microbial cultures are mixed with a cryoprotectant solution, typically containing glycerol or other suitable agents.
  - **Freezing:** The culture is then rapidly frozen by immersing it in liquid nitrogen. This rapid cooling prevents the formation of ice crystals that could damage the cells.
  - **Storage:** The frozen cultures are stored in cryogenic containers at -196°C until needed.
- **Thawing and Reviving Cultures:**
  - **Thawing Procedure:** When required, the cryopreserved cultures are quickly thawed in a warm water bath or at room temperature.
  - **Revival:** Post-thawing, the cultures are inoculated into suitable growth media to recover and resume normal metabolic activities.

Storage in Distilled Water

- Many organisms die in distilled water because of water absorption by osmosis.
- However some have been known to survive for long periods in sterile distilled water.
- Usually such storage is accompanied by refrigeration; some organisms are however, harmed by refrigeration.
- Among organisms which have been stored for long periods with this method are *Pseudomonas solanaceanum, Saccharomyces cerevisiae*, and *Sarcina lutea.*
- *The* attractiveness of this method is its simplicity and inexpensiveness; since so few organisms seem to be storable in this manner, it should not, for fear of losing the organism, be adopted as the sole method for storing a newly acquired or isolated organism until it has been shown to be suitable.