I- Introduction

Handling microbial cultures or products containing microorganisms requires techniques which avoid contamination by the handler or the environment. The destruction of microorganisms or sterilization is essential when preparing equipment and culture media.

Microorganisms can only be identified once they have been isolated in their **pure state**. Both steps require the use of sterile culture media and suitable conditions for microorganism growth. As for identification techniques, they are numerous and vary according to the nature of the germ under study: bacterium, yeast, mold. They include **phenotypic** (or **phenetic**) and **genotypic** characteristics.

II- Sterilization

II-1- General information

Sterilization is an operation designed to kill the microorganisms contained in a preparation. The agents used for sterilization are either physical (temperature, radiation) or chemical.

II-2- Chemical agents

- **disinfectants**: chemical agents capable of destroying pathogenic germs in environments external to man: water, air, soil, objects and a wide variety of materials.
- **antiseptics**: an environment is said to be septic when pathogenic microorganisms can be present or develop in it. Otherwise, it is said to be aseptic.

Antiseptics are chemical substances capable of destroying microorganisms or stopping their growth. The term antiseptic is reserved for agents that exert a local action on living organisms, such as wounds or mucous membranes.

Disinfection and antisepsis are temporary operations designed to eliminate or kill microorganisms and/or inactivate viruses.

- **Antibiotics**: chemical substances that have a destructive effect on microorganisms, but are non-toxic to human or animal cells. These substances are known as chemotherapeutics. Antibiotics are produced by living microorganisms (fungi and bacteria). These substances can have a **bacteriostatic** or **fungistatic** effect when they prevent multiplication, and a **bactericidal** or **fungicidal effect** when they totally destroy bacteria and fungi.

II-3- Physical agents

The most commonly used physical agents are high temperatures and radiation.

II-3-1- Temperature: its action depends on the environment, the physico-chemical state of the cells and their number.

In aqueous solutions, vegetative forms of bacteria are generally killed at 100°C, whereas they are more resistant in dehydrated media. This is also the case with oily solutions, where thermostable lipid suspensions protect germs.

Practical sterilization processes use **moist** or **dry heat**. The **autoclave** is a device that delivers moist heat. Sterilization will achieve its goal when a temperature of 120°C is maintained for 15 to 20 minutes (e.g. liquid and solid culture media). However, many culture media and substances cannot withstand high sterilization temperatures (e.g. concentrated carbohydrate solutions, hydrolyzable substances), and are therefore subject to **tyndalinization**. This technique, described by Tyndall, involves heating the culture medium to 60°C or 70°C for 30 minutes or 1 hour, three consecutive times, with a 24-hour interval between each heating. At this temperature, all vegetative forms are destroyed. Thermoresistant spores may germinate between each interval, transforming into vegetative forms which are then eliminated.

Sterilization by boiling at a temperature close to 100°C is used for the preparation of certain culture media which cannot withstand autoclaving (milk, carbohydrate solutions, etc.).

Pasteurization is not a true sterilization method. It is applied to certain natural products to preserve them temporarily without altering them (e.g. milk, wine, etc.). This method inactivates pathogenic microorganisms.

For materials that cannot be sterilized by moist heat, dry heat is used. Objects are held at 180°C for 1 hour, or at 160°C for 2 hours.

Objects to be sterilized can also be held over a heated flame (Bunsen burner), as in the case of turntable handles.

II-3-2- Radiation

Radiation can have bactericidal effects. The main types are electromagnetic and electronic radiation.

II-3-3- Filtration

Filtration is used to sterilize solutions containing **thermolabile** substances (proteins, serum, carbohydrate solutions, etc.). It consists of passing a liquid through a porous wall or membrane, which retains solids and microorganisms.

III- Culture media

All culture media used in bacteriology can be classified according to several criteria:

1. Consistency: liquid or solid
2. Sterilization method: autoclaving, filtration or tyndallisation
3. Use: isolation, maintenance, identification or storage
4. Method of preparation and composition: synthetic, semi-synthetic and empirical media.

III-1- Synthetic and semi-synthetic media

III-1-1- Synthetic or defined media

These contain only chemically-defined substances dissolved in distilled water in the proportions required to form a buffered solution. They must contain a carbon source (carbohydrate, alcohol, organic acid, an amino acid and sometimes a carbonate), mineral compounds including trace elements, nitrate or ammonia as a nitrogen source, and growth factors (vitamins, purine and pyrimidine bases or amino acids) for demanding bacteria.

The composition of synthetic media varies widely depending on the germ to be grown.

For example:

Medium 1: Minimum mineral medium : N_4HCl, 1g ; KH_2PO_4, 1,2g ; $Na_2HPO_4.2H_2O$, 8,6 g ; $MgSO_4.H_2O$; 200 mg ; $FeSO_4.7H_2O$; 1 mg ; $CaCl_2$; 10 mg ; Traces de Mn, Mo, Cu, Zn, 0,02-0,5 mg ; distilled water ; 1000 mL.

Only autotrophic bacteria can grow on this medium, assimilating atmospheric CO_2 and drawing energy from the oxidation of ammonium chloride: nitrifying bacteria.

Medium 2: Same mineral solution + 5 g glucose. Suitable for the growth of many heterotrophic bacteria that do not require growth factors, e.g. *E. coli*.

III-1-2- Semi-synthetic media

Some germs require a lot of growth factors, making it difficult to use synthetic media. These are therefore supplied in the form of a complex organic extract (yeast extract, very rich in vitamins).

Example. Minimum mineral medium supplemented with 5 g glucose and 5 g yeast extract.

III-1-3- Complex empirical media

These media contain ingredients of indeterminate chemical composition. They contain undefined components such as peptones, meat extracts and yeast extracts. Peptones are protein hydrolysates prepared by partial proteolytic digestion of meat, casein, soy, gelatin

and other proteins. They serve as sources of carbon, energy and nitrogen. Beef extract contains amino acids, peptides, nucleotides, organic acids, vitamins and minerals. Yeast extract is an excellent source of B vitamins, nitrogen and carbon compounds.

Solid media are obtained by adding a gelling agent to a liquid medium. The most widely used gelling agent is **agar-agar**: this is a sulfated polygalactoside with the property of forming a solid gel with water at a temperature below around 60°C, while remaining liquefiable by boiling, in which case it remains in supercooling (liquid) until around 45°C.

IV- Main isolation and purification techniques

IV-1- Aim of isolation

Isolation is used to obtain the pure cultures needed for all bacteriological studies and identification. It separates and purifies the bacteria present in a product into distinct colonies.

It is essential to define the following terms:

- Inoculation: the process of depositing bacteria on or in a culture medium.

- Transplantation: transplantation of a pure culture onto a new medium.

- Isolation: inoculation carried out for separation purposes, resulting in clearly distinct colonies in pure culture.

IV-2- Main plating procedures

- Inoculation into the mass

This type of plating requires the use of solid media in supercooling (liquefied agar and 45-50°C). In the case of agar plates for bacterial counts, the liquid to be inoculated is pipetted under a given volume of supercooled agar. After homogenization, the medium is poured into a Petri dish and, after cooling, the dish is turned upside down and placed in an oven.

- Transverse streak plating

Streak plating on Petri dishes is often used for isolation purposes. The inoculum is deposited on a peripheral point of the medium and disseminated by the platinum loop or button pipette.

- Spreading or blanket plating

- Pipette rake: inoculum is spread using a pipette rake, which is slid over the surface of the medium while rotating the dish.

- By dabs: rich inoculum is inoculated by circular dabs (1 to 2 cm in diameter) or by small thick streaks (approx. 2 cm long). This technique allows several culture trials to be carried out on the same Petri dish.

- Inoculation by pricking

In pellet: the puncture is made in the center of the pellet from a culture in liquid or solid medium. It is performed either with a perfectly straight platinum loop, or with a resealed Pasteur pipette without bead formation.

- Marking of boxes and tubes

Marking includes the day and sometimes the time of inoculation, identification of the study (e.g. patient's name or registration number), nature of the product studied (bacterial strain, pathological product, etc.) and nature of the medium used.

- Checking the cap

Screw caps, which plug most of the tubes used, should be tightened all the way, or simply put on, or engaged by a quarter-turn; this variation in aeration must be respected.

- Incubation conditions

Temperatures, incubation times and any special atmospheric requirements (CO_2, anaerobiosis, etc.) must be respected. The seeded Petri dish must always be kept with the lid down, whether in the incubator or on the bench. Condensed water will fall to the surface of the medium, transforming the isolation into an unusable slick.

IV-3- Purification

- Medium-sized colonies: use a loop or tapered pipette to remove all or part of a well-isolated colony and suspend in a few drops of broth. Isolate on 2 or 3 slant agar tubes, after dilution.
- Small colonies: purified without prior dilution.

Colony purity must be checked before identification; one colony may conceal another, smaller colony corresponding to another species: mixed colony.

V- Microorganism identification techniques

Microorganisms must be identified on strains in pure culture. It comprises the following stages:

- Determination of cultural characteristics;
- Morphological characterization;
- Biochemical, physiological and immunological characterization and determination of pathogenicity and phage typing;
- Genetic characterization.

V-1- Determination of cell morphology

Cell morphology and association are determined by microscopy:

- Fresh examination between slide and coverslip of living bacteria.
- Examination after staining, generally on dried and fixed smears. Stains are classified into:
  * Standard stains: simple stains and Gram-type differential stains.
  * Special stains for bacterial structure: flagella, capsules, spores, cytoplasmic inclusions, etc.

V-1-1- Fresh observation

Fresh examination is the observation of living bacteria without fixation or staining. It enables us to observe bacterial morphology, grouping and mobility.

Example. Examination of cultures in liquid medium

A drop of culture is taken with a Pasteur pipette filled by capillary action, or with a platinum loop, and placed in the middle of a clean slide. The volume of the drop must be proportionate to the dimensions of the slide, so that the product does not overflow the slide, while avoiding the creation of air bubbles. Observation is made at × 10 and × 40.

V-1-2- Observation after staining

Microorganisms often need to be fixed and stained to increase visibility and accentuate specific morphological features.

Technique:

- *Smear preparation*

A small drop of liquid culture or bacterial suspension is spread in a thin film on a clean slide. After the slide has dried, the smear is fixed with a Bunsen burner flame. This process kills the bacteria without altering their structure.

- *Staining*

These are essential for studying bacterial structure.

- Simple staining

Use of dyes such as phenic methylene blue and toluidine blue.

Technique: Apply the chosen dye to the dried, fixed smear. Leave to act for 1 to 2 minutes, then wash and gently dry with filter paper. Examine by immersion.

- Gram staining

This technique is one of the foundations of bacterial classification. It was discovered by Hans Christian Gram in 1884, then applied by Roux to the identification of bacterial species.

* Technique: After fixation and cooling, wash the slide with water to remove impurities and artifacts.

- Cover the slide with filtered gentian violet and leave for 1 minute;
- Discard the violet by washing it away with the lugol solution. Never leave the slide uncovered. This etching time should be equal to or longer than that of the violet;
- Alcohol staining using absolute or 90° alcohol, poured drop by drop onto the tilted slide until the alcohol no longer carries any stain;
- Wash thoroughly with water;
- Cover the slide with water and add a few drops of filtered Ziehl's fuchsin at each end, rather than in the center, which would stain with pure fuchsin. Slides are examined by immersion.

* Results: This differential staining allows us to divide bacteria into two groups: Gram-positive bacteria retain their violet stain after alcohol decoloration, while Gram-negative bacteria are decolored by alcohol and stained pink or red by fuchsin.

This distinction is based on differences in parietal structure. The positivity or negativity of Gram positivity or negativity also depends, for the same species, on the age of the culture.

V-2- Determination of culture characteristics

The characteristic aspects of bacterial cultures on the usual media are orientation clues:

- Cultures in liquid media, we note:

1- Presence of significant or no turbidity in the medium

2- Presence of deposits, which may be mucous, granular, etc.

3- Presence of haze, observed with strict or facultative aerobic bacteria.

- Culture on solid media, we note:

1- Abundance of culture and time to appearance;

2- Colony appearance: observed with the naked eye or with a binocular magnifying glass, which can be used to examine colonies under different lighting conditions (transparency, reflection, etc.).

The characteristics of a bacterium are as follows:

* Size: diameter varies according to bacterial species (small colonies, diameter 1 mm; medium colonies, diameter 1.5 to 3 mm; large colonies, diameter 3 mm).

* General shape: contours (regular, irregular, jagged), relief (domed, flat, raised or umbilicated center).

* Transparency: some colonies are perfectly transparent, others translucent, lactescent or opaque.) Transparency should be assessed in both natural and artificial light.

* Surface appearance: smooth, sometimes shiny colonies, rough colonies with a matt surface, mucous colonies with a honeycomb appearance.

Consistency at the time of sampling: mucous colonies (stringy), fat colonies, creamy colonies, dry colonies, etc.

V-3- Physiological and biochemical characterization

V-3-1- Influence of physico-chemical parameters

The influence of temperature, pH and salt concentration (NaCl) on growth is determined by varying one of the parameters while keeping the other two constant.

V-3-2- Study of respiratory type and identification of enzymes

The aerobic-anaerobic character is determined using meat-liver agar (VF), seeded in depth and distributed in pellets. The agar is regenerated and, after cooling, the pipette containing the culture is introduced to the bottom of the tube, then brought to the surface in a spiral.

After cooling, the tubes are incubated at optimum T°. Depending on their O_2 behaviour, germs can be classified as strict aerobes, strict anaerobes, aero-anaerobes and microaerophiles.

- Detection of catalase

Catalase prevents the accumulation of H_2O_2 (product of substrate oxidation). The presence of this enzyme is demonstrated by dissociating a sufficient quantity of the culture on a glass slide containing a drop of 10-volume hydrogen peroxide, using the effilure of a Pasteur pipette. The presence of catalase is reflected, within a few seconds, by the formation of oxygen bubbles.

- Testing for cytochrome oxidase

This test is carried out using "Ox" discs, the reaction zone of which is made up of filter paper impregnated with N,N-dimethyl-1,4- phenylene diaminedichloride. A portion of the colony is removed using a Pasteur pipette and dissociated on filter paper soaked in distilled water. The presence of cytochrome oxidase results in the appearance of a red coloration, rapidly turning to a very dark violet, within 20 to 60 seconds.

V-3-3- Study of oxidative or fermentative metabolism

Hugh et Leifson culture medium is used to study glucose metabolism, which can be either oxidative or fermentative.

A sterile sugar solution at a final concentration of 1% is added to the previous sterile supercooled media. Homogenization and regeneration of the culture medium. Inoculation (2 tubes per germ) by central pricking with a platinum wire loaded with pure culture. Add a 1cm layer of sterile kerosene oil to one of the tubes and incubate at optimum T°.

Acid production is indicated by the color indicator (bromothymol blue) turning yellow in the case of Hugh and Leifson medium. If acidification takes place in both tubes, this is fermentation (fermentative bacteria). If acidification takes place only in the tube not covered with kerosene oil, it implies oxidation (oxidative bacteria). If neither tube turns yellow, the germ is said to be inert or inactive.

V-3-4- Study of carbohydrate metabolism

This is used to monitor carbohydrate attack. Several liquid (broth) or solid culture media can be used.

- Vogues Proskauer (VP) and Methyl Red reactions

Inoculation with Clark and Lubs medium and, after incubation, reading by adding 0.5 ml KOH 40% (w/v) (VP1 reagent) and 0.5 ml alpha-naphthol (v/p) (VP2 reagent) for the presence of acetoin (VP-positive strain), indicated by pink coloration.

Mixed acid production is tested on the same medium. A positive reaction is indicated by the broth turning red.

- Using the mannitol-mobility medium

Bacterial mobility and mannitol fermentation were studied by inoculating the semi-solid mannitol-mobility medium with a central prick using a straight wire. The presence of mobility is revealed by a greater or lesser invasion of the medium from the inoculation puncture, while the use of mannitol is reflected by a color change from red to yellow.

- Use of sugars on Triple Sugar Iron (TSI) medium

Triple Sugar Iron agar can be used to demonstrate glucose fermentation (with or without CO_2 production), lactose and/or sucrose oxidation and hydrogen sulfide production.

The use of one of the sugars contained in the medium results in acidification, yellowing of the pellet in the case of glucose, and of the slope in the case of lactose and/or sucrose. The production of hydrogen sulfide from thiosulfate is highlighted by the formation of a black coloration, and the release of CO_2 is revealed by the appearance of air bubbles in the pellet or the detachment of the agar.

- Testing for β-galactosidase

Dense culture suspensions are prepared in optimal saline solutions corresponding to each strain, then a disk impregnated with Ortho-Nitro-Phenyl- β-Galactoside (ONPG) is added to each suspension. After incubation for 18 to 24 hours, the appearance of a yellow coloration indicates the hydrolysis of ONPG, and consequently the presence of this enzyme.

V-3-5- Study of protein metabolism

- Research into gelatinase

Gelatin is a protein found in animal support tissues. Several methods are used to detect it. A gelatin medium (nutrient gelatin, etc.) is poured into a pellet and seeded by central pricking. The tube is observed after 48 hours to 2 weeks. Liquefaction of the gelatin indicates the presence of gelatinase.

Inoculation is by touch on base medium supplemented with 1% (w/v) gelatin. Hydrolysis is revealed by adding 1 to 2mL of the following reagent: $HgCl_2$ 15g, concentrated HCl: 20 ml, made up to 100 mL. A clear zone indicates gelatinase production.

- Caseinase assay

The base medium is modified by adding 1% (w/v) casein. After inoculation, Petri dishes are incubated. The presence of this activity is detected by a clear halo around the colonies, indicating casein hydrolysis.

- Detection of decarboxylases (lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase)

These enzymes, whose action is favored in acidic environments, form alkaline substances from amino acids.

The media used contain a single amino acid, lysine, ornithine or arginine respectively. Four tubes, one of which is a control, are inoculated with a bacterial suspension.

After incubation, a negative reaction results in a yellow coloration (acidification of the medium) whereas the appearance of a violet coloration (alkalinization of the medium) reveals a positive reaction.

- Indole production

Some bacteria deaminate and hydrolyze tryptophan to produce an indole molecule. The indole reacts with the aldehyde function of paradimethyl amino benzaldehyde to give a red-colored compound.

Strains are seeded on liquid culture medium supplemented with 0.5% (w/v) yeast extract. After incubation, indole production is detected by adding a few drops of Kovacs reagent. A positive reaction is indicated by the appearance of a red ring on the surface.

- Testing for urease

The urea-hydrolyzing enzyme is tested on Christensen's synthetic urea medium. Strains are plated on slant agar and incubated.

A positive reaction is indicated by a purplish-red or dark-orange coloration, whereas a yellow tint in the medium indicates a negative reaction.

V-3-6- Study of lipid metabolism

This activity is tested on a base medium containing 1% (v/v) Tween 80 or Tween 20, or 2.5% (v/v) olive oil. Strains are inoculated by touch. After incubation, the development of a precipitate around the streaks indicates the presence of a lipase.

V-4- Chemotaxonomy

Study of molecules resulting from a series of enzymatic reactions, and therefore from the expression of several genes. Determination of wall fatty acid and amino acid composition; membrane polar lipids and electrophoretic profiles of all proteins.

V-5- Genetic identification

Use of approaches enabling a complete or partial study of the genome:

- Study of the complete genome:
- G+C % DNA
- DNA/total DNA hybridization
- restriction polymorphism of total DNA
- Genome sequencing
- Study of a portion of the genome
- DNA portion restriction polymorphism
- Gene sequencing...