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MICROSCOPIC RESEARCH METHODS IN PRACTICAL MICROBIOLOGY

Educational and Methodical Manual

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The manual describes the basic methods of microscopic examination of microorganisms. It provides information about safe handling of microorganisms in the teaching laboratory, preparation of specimens, their staining and observing with the help of light microscope. The manual is compiled in accordance with the program of practical classes of the discipline "Microbiology, Virology" for students studying in English in the specialties "Medical science" and "Dentistry". This manual is intended for students of higher educational institutions, graduate students, and teachers.

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CONTENTS

1. MICROBIOLOGY LAB PRACTICES AND SAFETY RULES

Microbiology laboratory work inherently involves exposure to potential hazards surpassing those encountered in many other educational settings. Personnel may handle infectious agents, alongside chemical substances, open flame heat sources, and electrical equipment. Epidemiological data document instances of laboratory-acquired infections (LAIs) among microbiology workers. Approximately 20% of these LAIs are traceable to specific, identifiable incidents, while the remaining 80% are attributed to non-specific laboratory practices. Individuals with compromised immune function, a recent history of prolonged illness, or those who are pregnant are advised to consult with the teacher prior to commencing laboratory activities. This consultation is crucial for assessing individual risk and implementing appropriate safety measures to mitigate potential harm.

A microbiology laboratory presents a distinct environment necessitating specialized practices and engineered containment strategies to ensure the safety of personnel working with microorganisms. Prioritizing safety is paramount. Effective containment of microorganisms relies on three fundamental pillars: (1) adherence to rigorous laboratory practices and aseptic techniques, (2) utilization of appropriate personal protective equipment (PPE) and biosafety equipment, and (3) implementation of a facility design that minimizes risk of exposure and contamination.

The microbiology laboratory can be a fascinating and rewarding learning environment. However, it is essential to recognize that working with microorganisms, even those considered non-pathogenic, requires strict adherence to safety protocols. These rules are designed to protect you, your classmates, and the integrity of the experiments. Your safety and the success of this laboratory depend on your cooperation and responsible behavior. *Failure to comply with these rules will result in being denied access to the laboratory. Missed classes are not caught up.*

1.1. Microbiology Laboratory Safety Rules and Procedures

1.1.1. General Safety Practices

Treat all microorganisms as potential pathogens. While many microorganisms are not typically harmful to humans, some can become opportunistic pathogens under certain conditions. Environmental samples may contain potentially hazardous microbes. Therefore, all cultures and samples should be handled with caution and appropriate Biosafety Level 1 (BSL-1) containment practices, as outlined below. Under no circumstances are cultures to be removed from the laboratory.

Personal Protective Equipment (PPE)

- **Lab Coat** (fig. 1): A long-sleeved cotton lab coat must be worn at all times in the laboratory. The lab coat must adequately cover your arms and torso. The lab coat should be buttoned or snapped closed and must be easily removable in case of an emergency. Leave protective clothing in the lab and do not wear it to other non-lab areas. Avoid loose fitting items of clothing.
- **Footwear:** A designated pair of closed-toe shoes must be worn in the laboratory. Outdoor shoes are not allowed to be worn in the laboratory.
- **Hair Covering** (fig. 2): Hair should be completely contained under a cotton headdress such as a medical cap, bandana, kerchief or headscarf. Shoe covers, disposable spunbond or outdoor scarf are not allowed. Long hair must be tied back to prevent contamination and interference with experiments.
- **Hand Protection:** Cover any cuts or abrasions on your hands with a sterile bandage. Gloves may be worn as extra protection. Your teacher will inform you when gloves are required for a specific procedure.

Workspace Management

- Maintain a clean and uncluttered workspace. Only essential materials (lab manual, notebook – a 12- or 18-sheet exercise book, a pen, red and blue pencils, matches, permanent marker with sharp tip, disposable gloves, and paper handkerchiefs) should be present at your bench.
- Personal belongings (coats, backpacks, bags, purses, cell phones) should be stored in the designated area (cloakroom or at feet), away from

aisles and work surfaces. Put on your laboratory coat before you enter the laboratory.

Fig. 1. Wear a cotton laboratory coat in the class. This garment must be long sleeved and cover your arms. The lab coat should be fully buttoned or snapped and suitable for quick removal without pulling it over your head

Fig. 2. Your hair should be hidden under the cotton headdress such as medical cap, bandana, kerchief or headscarf. Shoe covers, disposable spunbond or outdoor scarf are not allowed. Long hair should be tied at the back of the head

Hygiene

• Wash your hands thoroughly with disinfectant soap and water:

– Before entering the laboratory;

– After handling cultures or equipment;

– Before leaving the laboratory.

• Absolutely no food, drinks, chewing gum, or smoking is allowed in the laboratory. Do not place any objects (pens, pencils, labels, fingers) in your mouth. Do not store food or beverages in the laboratory.

1.1.2. Safe Handling of Microorganisms and Equipment

Aseptic technique: Practice strict aseptic technique at all times to prevent contamination of cultures and the environment.

Bunsen or alcohol burners: Turn off Bunsen or alcohol burners when not actively in use. Never leave an open flame unattended.

Pipetting: *Never pipette by mouth.* Use mechanical pipetting devices (pipette bulbs, pipettes) for all liquid transfers.

Labeling: Clearly and accurately label all cultures, chemicals, disinfectants, and media with the date, your initials, and the identity of the substance or organism.

Sterilization and disinfection

Sterilization is the complete elimination of all viable microorganisms.

• All materials, media, tubes, plates, loops, needles, pipettes, and other items used for handling of microorganisms should be sterilized. Otherwise, use commercially sterilized products.

• Inoculating loops and needles must be sterilized by flaming in a Bunsen or alcohol burner *before and after* each use. Ensure the loop has cooled before touching it to a culture.

• On being opened, the neck of a test tube or flask and cotton plug must be immediately flamed. This heats the air inside the tube, so the air moves out of the tube, preventing contaminants from entering the tube.

• The cotton plugs should not be placed on the table top; they should be held in your hand while inoculating.

• **Disinfection of work surfaces:** Laboratory equipment and work surfaces should be decontaminated with an appropriate disinfectant (for example, 70% ethanol or a freshly prepared 10% bleach solution) on a routine basis, and especially after spills, splashes, or other contamination. Be aware of the possible dangers of these disinfectants. 70% ethanol is flammable. Keep away from open flames and heat sources. 10% Bleach is corrosive and can cause skin and eye irritation. Avoid contact with skin and clothing. Bleach, if spilled, can ruin your clothing. It can release toxic fumes if mixed with acids. In the case of contact with skin or eyes, flush the affected area with plenty of water for at least 15 minutes and seek medical attention if necessary.

1.1.3. Emergency Procedures

Familiarize yourself with the location and proper use of the following safety equipment:

– Sink,
– Fire extinguisher,
– First aid kit,
– Emergency gas shut-off valve,
– Fire blanket.

Fire blankets are fire-resistant sheets of fabric that one uses to extinguish small fires by preventing the flames from being fed with oxygen. In the event of a clothing fire, use a fire blanket to smother the flames. Wrap the blanket tightly around the person and have them drop to the floor and roll. The fire blanket can also be used to cover the spilled burning ethanol on the bench. Do not throw the blanket, but lay it down gently.

Carbon dioxide fire extinguisher is applied for extinguishing solid, gaseous, liquid substances and electrical devices (up to 1000 V voltage). Never spray a person with a fire extinguisher.

If a cotton plug catches fire, it should not be thrown away; stop the flame by immediate plugging the tube not by blowing or soaking in water. If the burning plug falls out of your hands, you need to cover it with a glass or other non-flammable object.

• Report all spills, injuries, or accidents, no matter how minor they may seem, to the teacher or teacher' assistant immediately.

• Splashes on the skin should be treated as soon as possible; washing thoroughly with soap and hot water should be sufficient, but if necessary the skin can be disinfected.

• Clean up spills with care. Use lab cleanup and disposal supplies to keep the lab safe and clean. Cover any spills or broken culture tubes with a 70% ethanol or 10% bleach solution; then cover with paper towels. After allowing the spill to sit with the disinfectant for a short time, carefully clean up and place the materials in a biohazard autoclave bag to be autoclaved. Wash the area again with disinfectant.

• For broken glass, use a brush and dustpan to collect the fragments. Never pick up glass fragments with your fingers. Dispose of broken glass in the designated sharps container.

1.2. Biosafety Levels and Practices

To ensure the safety of personnel and the environment, all microbiological work must adhere to established Biosafety Levels (BSLs). The Center for Disease Control (CDC) and the National Institutes of Health (NIH) have defined four BSLs, each with specific requirements for microbiological practices, laboratory facilities, and safety equipment. These requirements are designed to provide appropriate protection against the hazards associated with different biological agents.

Biosafety Level 1 (BSL1)

Individual Risk: LOW
Community Risk: LOW

Examples of BSL1 Agents:
Bacillus subtilis,
Naegleria gruberi,
many *Escherichia coli*,
Saccharomyces cerevisiae,
Infectious Canine Hepatitis Virus

BSL1 containment is suitable for work involving well-characterized agents not known to cause disease in healthy adult humans, and of minimal potential hazard to laboratory personnel and the environment.

A BSL1 lab requires no special design features beyond those suitable for a well-designed and functional laboratory. Biological safety cabinets (BSCs) are not required.

Work may be done on an open bench top, and containment is achieved through the use of practices normally employed in a basic microbiology laboratory.

Biosafety Level 2 (BSL2)

Individual Risk: MODERATE
Community Risk: LOW

Examples of BSL2 Agents:
Bacillus anthracis,
Bordetella pertussis,
Brucella spp.,
Cryptococcus neoformans,
Clostridium botulinum,
Clostridium tetani,
Helicobacter pylori,
most *Salmonella* spp.,
Yersinia pestis,
Mycobacterium leprae,
Shigella spp.,
Human Immunodeficiency Virus,
Human blood

The primary exposure hazards associated with organisms requiring BSL2 are through the ingestion, inoculation and mucous membrane route. Agents requiring BSL2 facilities are not generally transmitted by airborne routes, but care must be taken to avoid the generation of aerosols (aerosols can settle on bench tops and become an ingestion hazard through contamination of the hands) or splashes. Primary containment devices such as BSCs and centrifuges with sealed rotors or safety cups are to be used as well as appropriate personal protective equipment (i.e., gloves, protective eyewear). As well, environmental contamination must be minimized by decontamination facilities (autoclaves).

Biosafety Level 3 (BSL3)

Individual Risk: HIGH
Community Risk: MODERATE

Laboratory personnel have specific training in handling these pathogenic and potentially lethal agents and are supervised by scientists who are experienced in working with these agents. These agents may be transmitted by the airborne route, often

Examples of BSL3 Agents:
Mycobacterium tuberculosis,
Salmonella typhi,
Vesicular Stomatitis Virus,
Yellow Fever Virus,
Francisella tularensis,
Coxiella burnetti,
SARS-CoV-2

have a low infectious dose and can cause serious or life-threatening disease. BSL3 emphasizes additional primary and secondary barriers to minimize the release of infectious organisms into the immediate laboratory and the environment. Additional features to prevent transmission of BSL3 organisms are appropriate respiratory protection, HEPA filtration of exhausted laboratory air and strictly controlled laboratory access.

Biosafety Level 4 (BSL4)

Individual Risk: HIGH
Community Risk: HIGH

<u>Examples of BSL4 Agents:</u>
smallpox virus,
Ebola virus,
hemorrhagic fever viruses.

This is the maximum containment available and is suitable for facilities manipulating agents that are dangerous/exotic, which pose a risk of life-threatening disease. These agents have the potential for aerosol transmission, often have a low infectious dose and produce very serious and often fatal disease; there is generally no treatment or vaccine available. This level of containment represents an isolated unit, functionally and, when necessary, structurally independent of other areas.

BSL4 emphasizes maximum containment of the infectious agent by complete sealing of the facility perimeter with confirmation by pressure decay testing; isolation of the researcher from the pathogen by his or her containment in a positive

pressure suit or containment of the pathogen in a Class III BSC line; and decontamination of air and other effluents produced in the facility.

2. ESSENTIAL LABORATORY EQUIPMENT

This chapter introduces the essential equipment used in a microbiology laboratory (table 1). Understanding the function and proper use of each item is crucial for successful experimentation and maintaining a safe working environment.

2.1. Culture Vessels

Microorganisms require a suitable environment to grow, and various vessels are used to culture them in the lab.

Petri dishes (fig. 3A), first introduced by Julius Richard Petri (1852-1921) who worked as an assistant for Robert Koch in Berlin at the Imperial Health office, are indispensable tools in the microbiology lab, serving as the primary vessels for cultivating microorganisms on solid media. These shallow, cylindrical, lidded dishes, provide a contained and sterile environment for bacterial, fungal, and viral growth. The lid, while not airtight, minimizes contamination from airborne microbes while still allowing for gas exchange necessary for microbial respiration. Petri dishes are often used for isolating pure cultures, performing antibiotic sensitivity testing, and observing colony morphology. Understanding proper handling techniques, including sterile pouring of nutrient agar media, labeling, and safe disposal, is crucial for successful microbiology experiments utilizing Petri dishes.

Petri dishes are made from glass or plastic. Usually, glass Petri dishes are made from borosilicate glass, and plastic Petri dish from polystyrene or polycarbonate. The glass Petri dishes are reusable, while the plastic Petri dishes are discarded after use. The commonly used in the laboratory plastic Petri dish has a 90 mm diameter. Petri dishes are available in different sizes like 35 mm, 50 mm, 60 mm, 90, 100 mm, 150 mm, etc., which vary according to the purpose of the laboratory work.

Always incubate the Petri dishes in the inverted position – upside down, not right side up. During incubation, water tends to condense in the lid of Petri dishes. If the plate is incubated in a right-side-up position, this condensation will "rain" onto the surface of the agar. Incubating the plate upside down keeps water condensation in the lid and away from your culture.

Always label the Petri dishes on the bottom of the plate, not on the lid to avoid the risk of misplacing the lids and confusing samples.

Test tubes (fig. 3B) are ubiquitous in the microbiology lab, serving as versatile and indispensable vessels for a multitude of procedures. These cylindrical, typically glass or plastic, containers are used for holding, mixing, and heating small volumes of liquids or solids. They come in various sizes, but the most common in microbiology labs are around 13 mm × 100 mm or 16 mm × 150 mm. In microbiology test tubes are utilized for growing microbial cultures in liquid media (broths), performing biochemical tests, preparing serial dilutions, and even conducting small-scale chemical reactions. Their rounded bottom allows for even heating and easy mixing, and they are typically closed with caps or cotton plugs to maintain sterility and prevent contamination. Proper handling, labeling, and sterilization of test tubes are essential to ensure accurate and reliable experimental results.

Erlenmeyer flasks (fig. 3C) are indispensable tools in the microbiology laboratory, serving a multitude of purposes. Their conical shape with a flat bottom makes them ideal for swirling liquids without spillage, crucial for aeration and mixing of cultures. The narrow neck minimizes evaporation and allows for easy capping with cotton plugs, foam stoppers, or sterile closures, maintaining sterility while allowing gas exchange. Erlenmeyer flasks are utilized for preparing and storing media, and culturing microorganisms in liquid broth. Their versatility and ease of sterilization make them a staple in any microbiology lab setting.

Cotton plugs are used to cover the neck of test tubes and flasks to prevent contamination from airborne microbes while still allowing oxygen for aerobic bacterial growth.

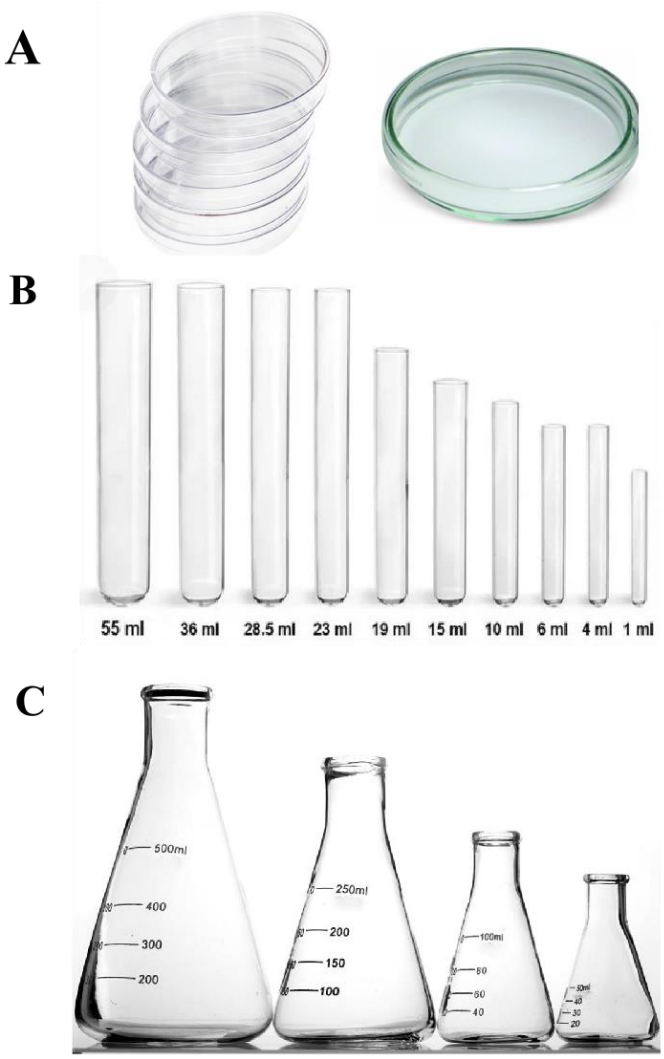


Fig. 3. Culture vessels: Petri dishes (A), test tubes (B), and Erlenmeyer flasks (C)

Flaming the Neck of Flasks and Test Tubes

1) Loosen the cotton plug so that it can be removed easily.
2) Lift the flask or test tube with the left hand (fig. 4).
3) Remove the cotton plug with the little finger of the right hand.
4) Do not put down the cotton plug, keep it in hand during the procedure.
5) Flame the neck of the flask or test tube and their cotton plug by passing them forwards and back through a hot flame.
6) After carrying out the procedure required, sterilize the neck and the plug again and replace the cotton plug using the little finger.

Fig. 4. Aseptic technique: flaming the neck of a test tube

2.2. Inoculation Tools

These tools are essential for transferring microorganisms from a source culture to a sterile medium. They must be sterile before and after each use to prevent contamination.

A wire loop (also called an inoculation loop, transfer loop, or a smear loop) is a simple tool used by microbiologists to pick up and transfer a small sample of microorganisms.

The tool consists of a thin handle with a loop about 5 mm wide or smaller at the end. It was originally made of twisted metal wire (such as platinum, tungsten or nichrome), but disposable molded plastic versions are now common (fig. 5). The size of the loop determines the volume of liquid the inoculation loop can transfer. Loops can transfer volumes ranging from 1 to 10 microliters, though pipettes have replaced inoculation loops as more reliable tools to deliver small volumes of liquid.

If a loop does not hold any liquid it has an incomplete circle. To correct the problem, first ensure that the loop has been sterilized and then reshape the loop with forceps. Do not use your fingers because of the possibility of puncturing the skin.

Wire loops are sterilized by flaming in the Bunsen or alcohol burners before and after use. They must be heated to red hot to make sure that any contaminating bacterial spores are destroyed. The handle of the wire loop

is held close to the top, as you would a pen, at an angle that is almost vertical. This leaves the little finger free to take hold of the cotton wool plug/screw cap of a test tube/bottle.

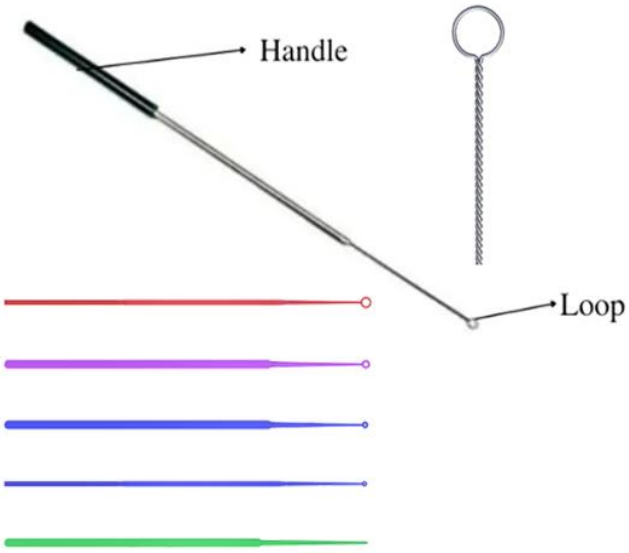


Fig. 5. Inoculation loops: metal (grey) and plastic (colored)

Flaming Procedure for a Wire Loop

The flaming procedure (fig. 6) is designed to heat the end of the loop gradually because after use it will contain culture, which may “splutter” on rapid heating with the possibility of releasing small particles of culture and aerosol formation.

Fig. 6. Flaming procedure for a wire loop

1) Hold the loop as you would a pen, close to the top of loop holder at an angle that is almost vertical.

2) Position the loop in the light blue cone of the flame. This is the cool area of the flame.

2) Draw the loop upwards slowly up into the hottest region of the flame (immediately above the light blue cone).

3) Hold there until it is red hot.

4) Ensure the full length of the wire receives adequate heating.

5) Allow to cool then use immediately.

6) Do not put the loop down or wave it around.
7) Re-sterilize the loop immediately after use.

An inoculation needle (needle), similar to the loop, has a handle with a thin wire, but the wire ends in a straight point instead of a loop. The needle is used for transferring microorganisms to a solid medium such as agar deeps for motility testing or in stabbing inoculation for biochemical tests especially in isolation of very defined regions of the cultures and the requirements of least disturbance between two closely crowded microbial colonies.

A spreader (L-shaped spreader, "hockey stick") is a L-shaped rod, usually made of glass, plastic or stainless steel (fig. 7). Spreaders are used to evenly distribute a liquid culture over the surface of an agar plate. This is especially useful for creating a bacterial lawn or for quantitative plating techniques. Sterile spreaders are available pre-packaged. Alternatively, glass or metal spreaders can be sterilized by dipping them in ethanol and then briefly passing them through a flame to burn off the ethanol (flaming). Allow the spreader to cool before use.

Fig. 7. Spreaders

2.3. Flame Sterilization Tools (Fire Sources)

A Bunsen burner (fig. 8A) is a gas burner that produces a single open flame. It is connected to a gas source (usually natural gas or propane).

The hottest part of the gas flame is known as the non-luminous zone. The non-luminous zone flame is blue (fig. 8B). In this zone, complete combustion of the fuel takes place because there is plenty of air around it.

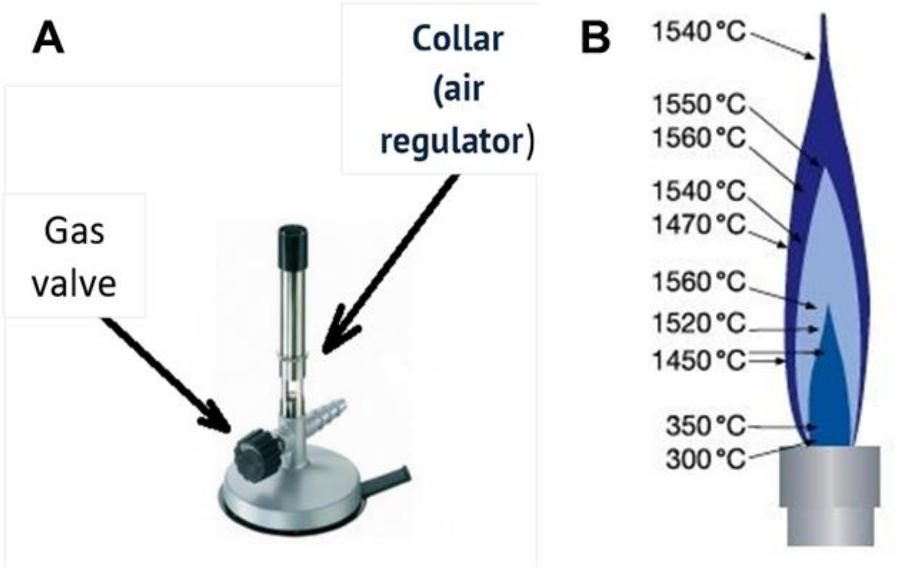


Fig. 8. A Bunsen burner (A)
and the temperature distribution in its flame (B)

Bunsen burners are used to sterilize inoculation loops and needles. Other items such as glass spreaders can also be sterilized by passing them through the flame.

A Bunsen burner keeps contaminants away from a sterilized work area by pushing contaminated air upwards and away from the flame (fig. 9). This creates a sterile area around the Bunsen burner which has a radius of about 10 cm (as the length of a pen). When working with microorganisms, it is important to work within this sterile area to prevent contamination.

Manual: Handling a Bunsen Burner

1) Remove all flammable materials such as paper from the work area.
2) Turn on the handle of the yellow valve on a yellow gas pipe so it is in line with the tubing connecting the burner to the gas.
3) Light up a match.
4) Open the black valve on the Bunsen burner.
5) Bring the lit match to the edge of the burner and light it.
6) Extinguish the match and throw it into metallic container.

7) Adjust the Bunsen burner to produce a roaring blue flame.
8) Turn off the valves at the Bunsen burner and the gas pipe when the work is finished.

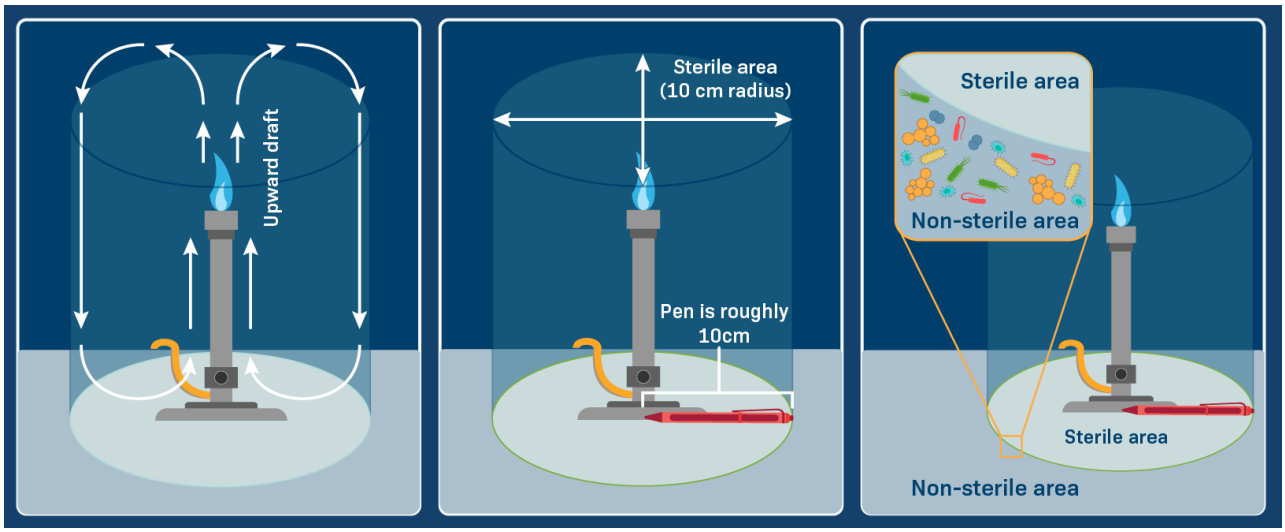


Fig. 9. Sterile area of the Bunsen burner (Image by LabXchange © The President and Fellows of Harvard College)

– Never leave the lit Bunsen burner unattended.
– Be careful around Bunsen burners. Flames cannot always been clearly seen.
– Gloves should never be worn, instead, wash hands thoroughly before and after working with microbiological samples or disinfect hands with hand sanitizer.
– Ethanol should only be used while the Bunsen burner is off.
– Place only necessary materials in the sterile work area and arrange them so that the upward current that creates the sterile field is not disturbed.
– Minimize movements that can disturb the sterile field around the Bunsen burner.
– Sterilize the necks of tubes and flasks as well as cotton plugs during their opening and closing by passing them through the flame.
– Hold any lids and closures in the hand in the sterile field rather than placing them on the work surface.

– Use either a new disposable item (such as pipette tips) for each action or sterilize reusable equipment (such as inoculation loops or glass spreaders) before and after each action by holding or passing them through the flame.

An alcohol burner (spirit lamp) (fig. 10) consists of a small container filled with ethanol and a wick. Ethanol burners provide a smaller, more portable flame than Bunsen burners.

Fig. 10. An alcohol burner

Handling an Alcohol Burner

1) Remove all flammable materials such as paper at a safe distance from the work area.

2) Before lighting-up the alcohol lamp, make sure that it has no cracks, the wick has been pulled out to the required height and is quite fluffy, the neck and wick holder are completely dry. If the wick holder and the neck of the spirit lamp are moistened with alcohol, an explosion of vapors inside will almost inevitably occur, resulting in a breakage, spreading of alcohol and fire. You should wait a while and let it dry. The wick should fit tightly into the guide tube of the holder, otherwise the possibility of a flash of vapor inside the spirit lamp is not excluded.

3) The burner can be light up only with matches or lighter and never with another burner.

4) Used matches should be thrown into metallic can on the bench.

5) It is prohibited to move burner with open flame.

6) Never leave the lit burner unattended.

7) Minimize movements that can disturb the sterile field around the Bunsen burner.

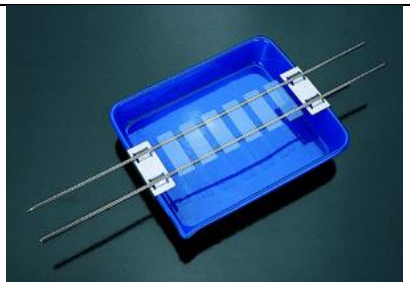
8) When the burner is not actively in use the flame should be extinguished.

9) There is only one way to put out the alcohol lamp – it is to cover the flame of the wick with a cap. The cap should always be at hand.

10) If spilling burning alcohol on the table, immediately cover it with a fire blanket, and if necessary, use a fire extinguisher.

Table 1

Other tools, devices and equipment of a microbiological laboratory

N.	Items	Function	Figure
1	Glass slides and coverslips (cover glasses)	Microscopic observations	
2	Pipettes	Transfer of measured volumes/drops of culture/sterile solutions	
3	Forceps	Transfer of materials (e.g. antibiotic discs)	
3	Rack or staining stand	Holding slides in upright position during staining	
4	Racks for test tubes	Holding tubes in upright position	
5	Glass and plastic vials and mini bottles with lids	Store dyes in the form of liquids	

2.4. Sterilization Equipment

Sterilization is absolutely critical in microbiology to prevent contamination and ensure accurate results.

An autoclave (fig. 11A) is a pressure chamber used to sterilize culture media, equipment, and some plastics by subjecting them to high-pressure saturated steam at 121 °C (2 atm) typically for 15-30 minutes or 111 °C (1.5 atm) for thermolabile substrates.

A dry heat sterilizer (hot air oven) (fig. 11B) uses high temperatures typically 160 °C for 120 minutes or 180 °C for 60 minutes to sterilize glassware (Petri dishes, test tubes, flasks, etc.), metal instruments, and other materials that can withstand high temperatures but may be damaged by steam. It is also used for sterilizing anhydrous materials like powders.

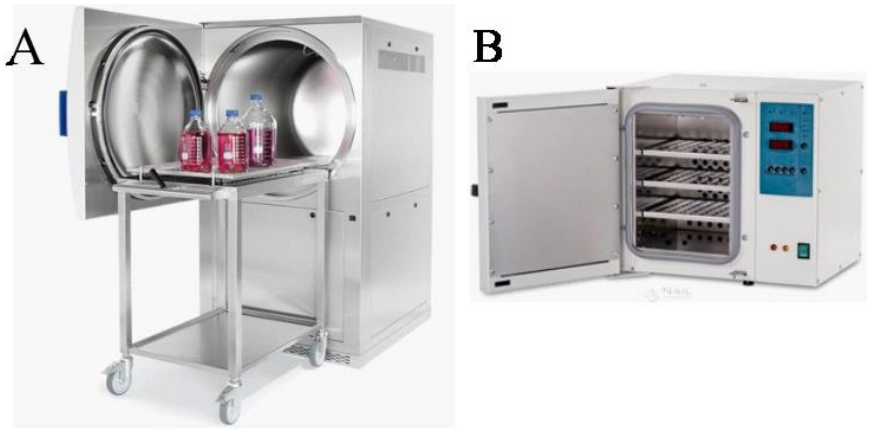


Fig. 11. Autoclave (A) and dry heat sterilizer (hot air oven) (B)

2.5. Incubation Equipment

An **incubator (thermostat)** is a specialized, insulated chamber designed to maintain a specific temperature, humidity, and sometimes other atmospheric conditions (like CO_2 concentration) optimal for microbial growth. Several types of incubators are available, each designed for specific applications.

Air Incubators (Standard Incubators) (fig. 12A) are the most basic and widely used type of incubators. They typically consist of an insulated cabinet with a heating element and a thermostat to maintain a constant temperature. Air is circulated within the chamber by convection or a fan to

ensure temperature uniformity. Generally, they can be set from slightly above ambient temperature (room temperature) to around 70 °C, although the most common temperature for bacterial culture is 37 °C (human body temperature).

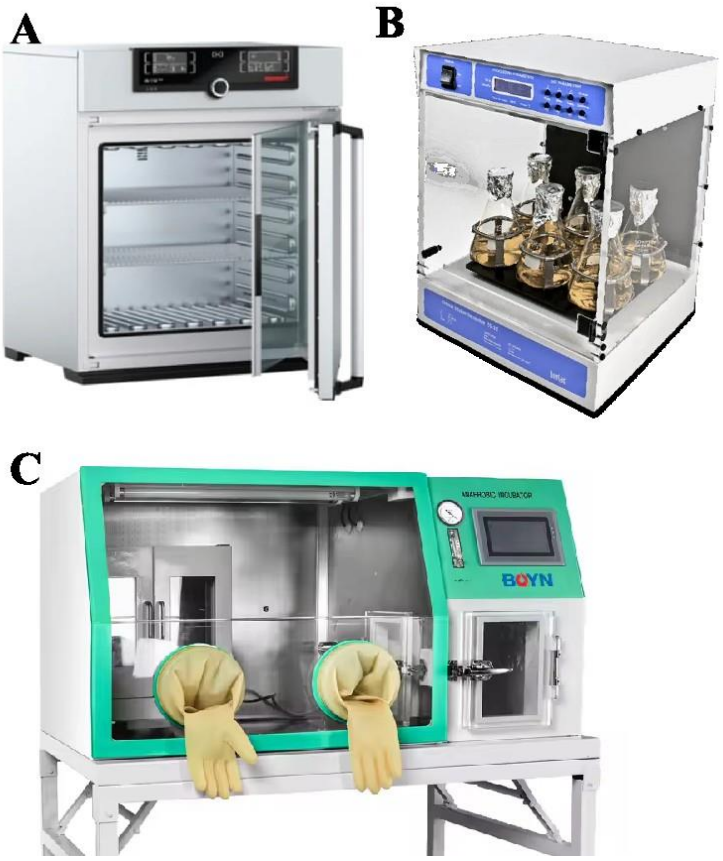


Fig. 12. Incubators: standard air incubator (A), shaking incubator (B), and anaerobic incubator (C)

Shaking incubators (fig. 12B) incorporate a shaking platform or orbital shaker to agitate the cultures. This is particularly useful for liquid cultures, as it increases aeration and nutrient mixing, promoting faster and more uniform growth.

Anaerobic incubators/chambers (fig. 12C) are specialized incubators which maintain a strictly anaerobic environment (lack of oxygen) for culturing anaerobic bacteria. They use various methods to remove oxygen, such as gas packs (chemical reactions that consume oxygen), gas flushing (replacing air with N_2, H_2, and CO_2), or catalytic converters.

3. MICROSCOPY IN MICROBIOLOGY

Microscopic research methods were an initial prerequisite for the emergence of microbiology as a science when a Dutch textile merchant, Antonie van Leeuwenhoek (1632-1723) observed bacteria for the first time using a single-lens microscope of his own design, and later continued to play an important role in the formation and development of this branch of life sciences. The use of light microscopy made it possible to gain insights into the diversity of microorganisms in the human body and natural ecosystems, as well as to identify among them the main variants with typical morphological and tinctorial characteristics.

Compound Light Microscope

The light microscope (fig. 13A) is an important tool in the study of microorganisms. The compound light microscope uses visible light to directly illuminate specimens in a two-lens system, resulting in the illuminated specimen appearing dark against a bright background (*a bright-field microscopy*). The two lenses present in a compound microscope are the ocular lens in the eyepiece and the objective lens located in the revolving nosepiece (fig. 13B).

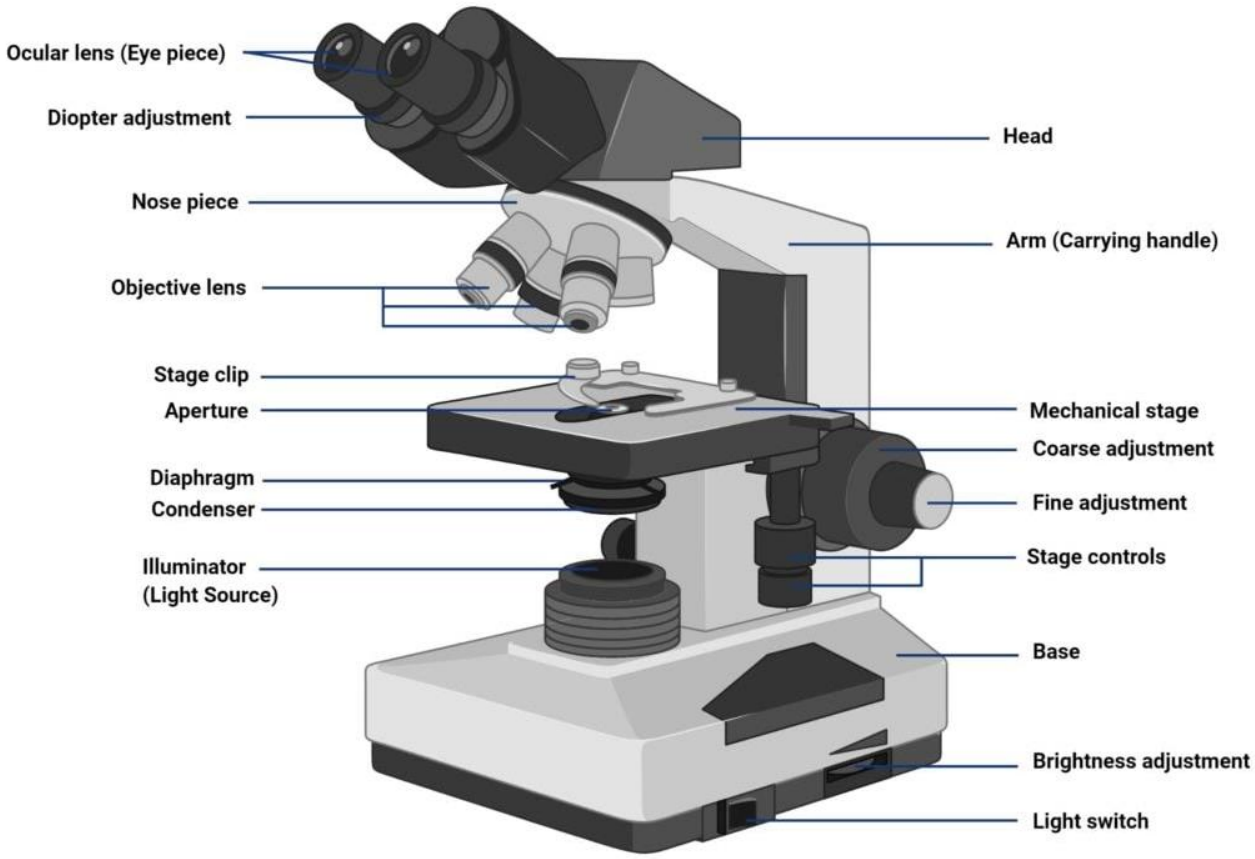
Resolution and Magnification

Magnification is the number of times that a real-life specimen has been enlarged to give a larger image. The magnification of 400× means that a specimen has been enlarged 400 times to give the image shown.

A light microscope has two types of lens which allow it to achieve different levels of magnification: (1) an eyepiece lens, which often has a magnification of 10×; (2) a series of objective lenses, each with a different magnification, e.g. 4×, 10×, 40× and 100×. To calculate the total magnification of a specimen being viewed, the magnification of the eyepiece lens and the objective lens are multiplied together:

$$\text{Total magnification} = \begin{matrix}\text{Eyepiece lens} \\ \text{magnification}\end{matrix} \times \begin{matrix}\text{Objective lens} \\ \text{magnification}\end{matrix}$$

A



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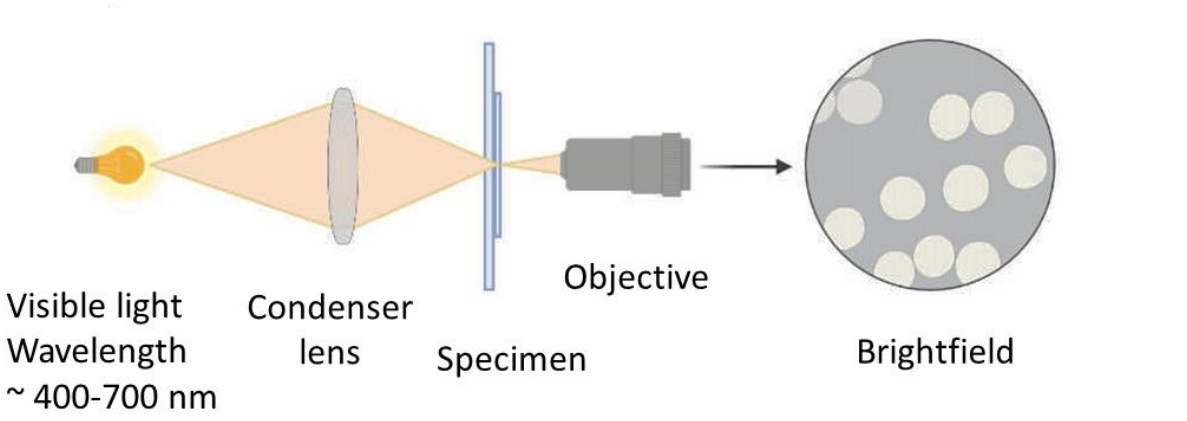


Fig. 13. Parts (A) and the principle (B)
of a compound light (bright-field) microscope

The resolution of a microscope is its ability to distinguish two separate points on an image as separate objects; this determines the ability of a microscope to show detail. If resolution is too low then two separate objects will be observed as one point, and an image will appear blurry, or an object will not be visible at all.

The resolution of a microscope limits the magnification that it can usefully achieve; there is no point in increasing the magnification to a higher level if the resolution is poor.

The resolution of a light microscope is limited by the wavelength of light. Visible light falls within a set range of light wavelengths (400–700 nm). The resolution of a light microscope cannot be smaller than half the wavelength of visible light. The shortest wavelength of visible light is 400 nm, so the maximum resolution of a light microscope is 200 nm.

For the light microscope, the magnification range extends from 10× to 1000×, with a resolving power of the order of 0.2 μm.

Manual: How to Use a Light Microscope

1) Place your microscope on a flat surface and connect its power cord into an outlet. Flip on the light switch, which is located on the bottom of the microscope. After flipping the switch, the light should come out of the illuminator, which is the light source.
2) Rotate the revolving nosepiece to the lowest power objective lens (10×) with a yellow band around it. Once you hear the lens click into place, stop rotating the nosepiece. Be gentle as you rotate the nosepiece to avoid breaking it or wearing it down.
3) Mount your specimen onto the stage using its metal clips.
4) Rotate the stage control knob until the objective lens hovers straight over the slide.
5) Start to adjust light and focus.

– Rotate the rim around the condenser that is underneath the stage until it focuses the maximum amount of light. Similarly, the diaphragm is the rotating disc located under the stage that has varying holes for different light intensities – rotate it until you achieve the maximum.

– Turning the coarse adjustment knob, adjust the objective lens until it is directly over the slide, with enough space in between to fit a piece of paper (raise the stage in the upper position). Never let the objective lens touch the slide.

– Then look at the oculars and adjust the focus. Slowly turn the coarse adjustment knob (the larger one) so that stage moves downward and away from the lens until the image comes into focus. Now, if necessary, use the fine adjustment knob (the smaller one) for additional clarity. Never rotate fine adjustment knob for more than one complete revolution.

– Rotate the stage control knob to move the slide so that the image is in the center of your vision.

6) Without turning the adjustment knobs switch to the next powerful objective lens. Rotate the revolving nosepiece to the objective lens that is next in terms of magnification intensity (40×, with blue band). After switching upward in intensity, adjust the fine focus knob to make any minor adjustments for clarity. At this point, your image should only need minimal focusing.

7) Examine your specimen. Always keep both eyes open. Even though you are only using one eye to look through the lens, closing the other eye can strain your eyes. And remember: everything is backward and upside down! Moving the slide to the right puts the image to the left and vice versa.

8) When you are done examining your specimen, turn the objective lens knob until it is at the highest point from the specimen. Turn the nosepiece back to the lowest power lens, carefully remove the slide, decrease the light intensity and then switch it off.

Microscopy with Immersion Oil

Immersion oil contributes to two characteristics of the image viewed through the microscope: finer resolution and brightness. These characteristics are most critical under high magnification; so, it is only the higher power, short focus objectives that are usually designed for oil immersion.

When light passes from a material of one refractive index to another (for example: from glass to air), it bends. In the space between the micro-

scope objective lens and the slide (where air is), light is refracted, the light scatters and it is lost. The refractive index of air is approximately 1.0, while the refractive index of glass is approximately 1.5. When light passes through both glass and air it is refracted. Light of different wavelengths bends at different angles, so as objects are magnified more, images become less distinct.

Basically, when using lower magnification microscope objective lenses (4×, 10×, 40×) the light refraction is not usually noticeable. However, once you use the 100x objective lens, the light refraction when using a dry lens is noticeable. If you can reduce the amount of light refraction, more light passing through the microscope slide will be directed through the very narrow diameter of a higher power objective lens. In microscopy, more light gives clear and crisp images. By placing a substance such as immersion oil with a refractive index equal to that of the glass slide in the space filled with air, more light is directed through the objective and a clearer image is observed.

Manual: How to Use Microscope Immersion Oil

1) Before using immersion oil, make sure that your 100× objective lens (with white band) is made for use with immersion oil.
2) Begin by focusing your sample using the 10× objective lens. Rotate the objective lens part way so you can reach the smear on your slide.
3) Place a drop of immersion oil on your smear (fig. 14).
4) Slowly rotate your 100× oil objective lens into place and adjust the fine focus until you get a crisp and clear image.
5) When finished viewing with your 100× oil immersion lens, carefully wipe the oil from all glass surfaces using a piece of lens cleaning paper.
6) Use a second piece of lens paper moistened with a small amount of alcohol (ethyl or isopropyl) or lens cleaning solution, wiping all glass surfaces again to remove any remaining oil. Failing to remove immersion oil from lenses will result in hardened oil on lenses that will affect future clarity.

Fig. 14. Microscopy with immersion oil

4. BASIC MICROSCOPIC TECHNIQUES FOR CHARACTERIZATION OF MICROORGANISMS

4.1. Wet Mount Microscope Slide Preparation

Wet mount slides, also known as temporary mounts, are easy to prepare with fresh specimens. The aqueous environment keeps the samples alive for examination over a short period before they eventually dry out.

Materials and Equipment

– Microscope slides and coverslips;
– Methylene blue (MB) dye solution;
– Filter paper;
– Dropper pipettes or inoculating loop;
– Bunsen or alcohol burner
– Light microscope;
– Specimens: 24-hour cultures of eukaryotic yeast cells *Saccharomyces cerevisiae* and prokaryotic cells *Bacillus megaterium* (*Priestia megaterium* after 2020).

Step-by-Step Procedure for Wet Mount Slide Preparation

1) Clean and degrease a microscopic slide using piece of cotton soaked in ethanol.
2) Apply a small drop of methylene blue on a clean microscope slide.
The drop should be slightly smaller than the size of the coverslip.

3) Transfer your specimen onto methylene blue and spread it evenly:
Flame the inoculating loop; cool it inside the Petri dish or tube with the culture; touch the culture with the loop and transfer it into the drop of methylene blue on the slide and spread evenly.
4) Gently lower a coverslip at an angle onto the sample to avoid trapping air bubbles. Apply slight pressure on the coverslip once it contacts the liquid to further eliminate any air pockets. Carefully blot away any excess liquid from under the coverslip using a filter paper.
5) Place the prepared slide on the microscope stage and secure it with stage clips. Start observing under the low-power objective (10×) first before switching to a higher magnification lens (high-dry objective 40×). The light should be reduced with the iris diaphragm and, if necessary, by lowering the condenser.
6) Record and draw your observations of the size, shape, cells arrangement.
7) Wash your slide and cover glass using soap and water.

Methylene Blue Stains Non-Viable Eukaryotic Cells

Methylene blue (methylthioninium chloride) is a thiazine dye with a pronounced affinity for acidic cellular components. It was first prepared in 1876, by Heinrich Caro. It is on the World Health Organization's List of Essential Medicines. While it has historically been used for various medicinal purposes, its primary application in the laboratory is as a staining agent that facilitates the visualization of microorganisms and cellular structures.

Methylene blue dye is oxidized to a colorless compound by a reaction which only takes place in living eukaryotic cells, so when added to a cell suspension, it stains all the dead cells and leaves only the living ones unstained (fig. 15).

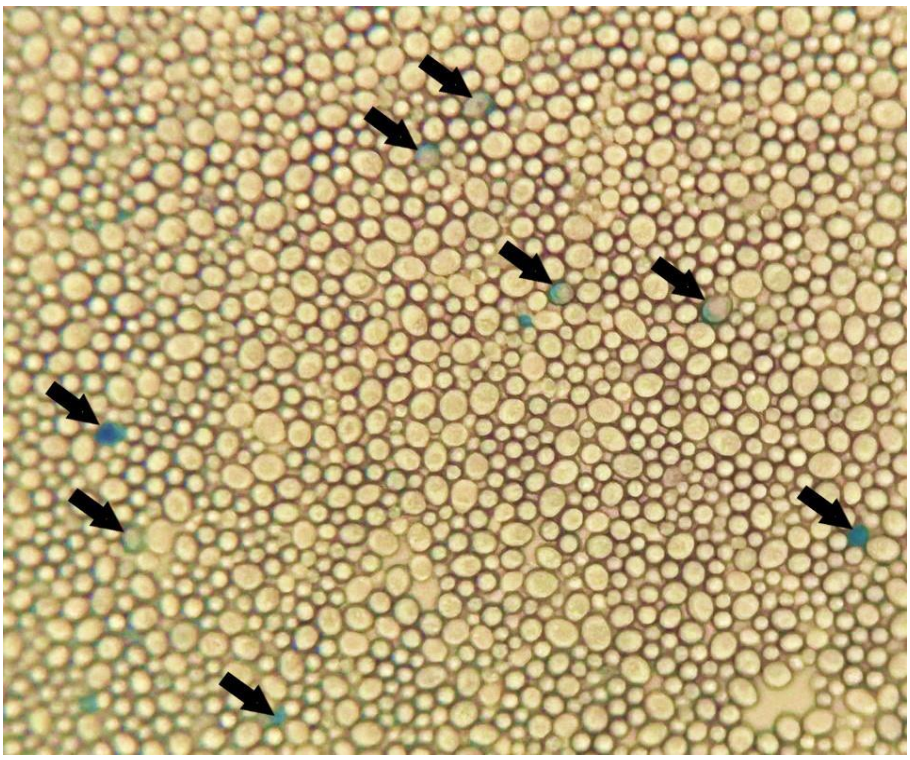


Fig. 15. *Saccharomyces cerevisiae* in methylene blue wet mount. Light microscopy, magnification 400×. Dead cells are labeled with arrows

4.2. Smear Preparation, Simple Staining

Fixation and simple staining is a fundamental technique in microbiology used to make microorganisms visible under a microscope. Most commonly, fixed and stained smears are used for the study of cell morphology, intracellular constituents and structures like spores.

Fixation, which may itself consist of several steps, is necessary to ensure that cells are not alive and safe; adhere to the slide; and more eagerly accept stains. Fixation always aims to preserve the shape of the bacteria as much as possible.

There are various fixation methods used in microbiology, each with its own advantages and disadvantages. The most common fixation methods include heat fixation and chemical fixation.

Heat fixation is a simple and rapid method that involves passing a slide with a microbial culture through a flame several times. This causes denaturation of proteins and coagulation of cell constituents, effectively killing and fixing the microorganisms to the slide. However, this method

may cause some distortion of the cell morphology and is not suitable for all types of microorganisms.

Chemical fixation involves the use of chemical agents such as formaldehyde, glutaraldehyde, or methanol to preserve the microbial cells. These agents cross-link proteins and other cell constituents, maintaining the cell structure and preventing decomposition. Chemical fixation provides better preservation of cell morphology compared to heat fixation and is suitable for a wide range of microorganisms. However, chemical fixation can be time-consuming and requires the use of hazardous chemicals.

Bacteria are transparent and colorless, so they would be visible if stained with certain dyes (table 2). **Simple staining** involves staining bacteria or other microscopic cells with a single dye, such as methylene blue or crystal violet. It is a quick and easy way to observe the overall shape, size and arrangement of microorganisms, as well as to identify certain structural features like spores.

Table 2

Types of Staining

Simple Staining	**Differential Staining**
Only one dye is used during the staining procedure.	More than one dye is used during the staining procedure.
Makes all of the organisms in a sample appear to be of the same color, even if the sample contains more than one type of organisms.	Distinguishes organisms (or different cell structures) based on their interactions with multiple stains.
	Examples: Gram staining, Ziehl-Neelsen staining (acid-fast staining), staining by Leifson (flagella staining), capsule staining, endospore staining.

The chemistry of simple staining is based on the principle that different charges attract, while similar charges repel each other. In an aqueous environment, at pH 7, the net electrical charge produced by most bacteria

is negative. Dyes applied for staining could be **acidic, basic** and **neutral** according to their chemical characteristics.

Each dye contains a cation (positive charge) and an anion (negative charge) and either one could be the chromophore (the part of the molecule that is coloured). Since **acidic dyes** carry a negative charge on their chromophore, the bacterial cells (also negatively charged) reject these dyes. Negative staining could also be conducted with dyes having a colloidal particle size that therefore cannot enter the cell (e.g., the black colored India Ink and Nigrosine). The chromophores of **basic dyes** have a positive charge and result the staining of bacterial cells (positive dyes), since they bind to proteins and nucleic acids (around neutral pH carrying a negative charge). Basic dyes include safranin (red), methylene blue (blue), crystal violet (violet), malachite green (green).

In case of differential staining, the first stain is called "primary stain" and the second one as "counterstain".

Materials and Equipment

- Microscope glass slides;
- Dropper pipettes;
- Inoculating loop;
- Saline solution / Tap water;
- Crystal violet (CV) dye solution;
- Filter paper;
- 70% ethanol;
- Bunsen or alcohol burner;
- Light microscope;
- Immersion oil;
- Specimens: 24-hour agar cultures of *Bacillus megaterium* (*Priestia megaterium* after 2020) and *Micrococcus luteus.*

  A hint: A culture on nutrient agar medium is preferable to a liquid culture for making a smear.

Step-by-Step Procedure for Bacterial Smear Preparation, Heat-Fixed and Simple Stained (fig. 16)

1) Degrease the surface of a glass slide with alcohol.
2) Label the backside of the slide.

3) Put the slide down on a rack/staining stand above the tray (dish) with the degreased surface upwards.
4) Put a small drop of saline (water) onto the slide.
5) Using inoculation loop transfer a small amount of bacterial culture in it, spread thoroughly to obtain a thin layer (smear) on a 2 cm^2 area and let it dry:
– Flame a wire loop to ensure that no culture accidentally remains from a previous operation.
– Allow to cool.
– Using aseptic technique, transfer a very small part of a single colony from a plate or slant agar medium into the drop of the tap water. If the amount of culture on the loop is easily visible you have taken too much!
– Spread the specimen thoroughly (fig. 17).
– Flame the loop.
– Air-dry the slide.
6) Fix your preparation with heat. To heat-fix the smear, hold the slide with tweezers (forceps) and pass the underside of the slide through the hottest part of the Bunsen or alcohol burner flame for 2 to 3 seconds. Repeat 3 or 4 times. Let it cool down before staining. A heat-fixed smear should be visible to the naked eye as a whitish area.
7) Drop basic dye onto the fixed smear and let it get stained for 2-5 minutes.
8) Wash the smear with tap water to remove excess dye solution (above the tub).
9) Dry the slide.
10) Examine under oil immersion. During microscopy, first use 10×, then switch to 100× objective lenses. In the latter case, use immersion oil.
11) Make a drawing of the observed microscopic field.
12) After finishing microscopic observation, clean all used objective lenses. Carefully wipe the oil from all glass surfaces using a piece of lens cleaning paper. Use a second piece of lens paper moistened with a small amount of alcohol (ethyl or isopropyl) or lens cleaning solution, wiping all glass sur-

faces again to remove any remaining oil. Clean your slide using piece of cotton wool soaked in ethanol. Wash your slide using soap and water.

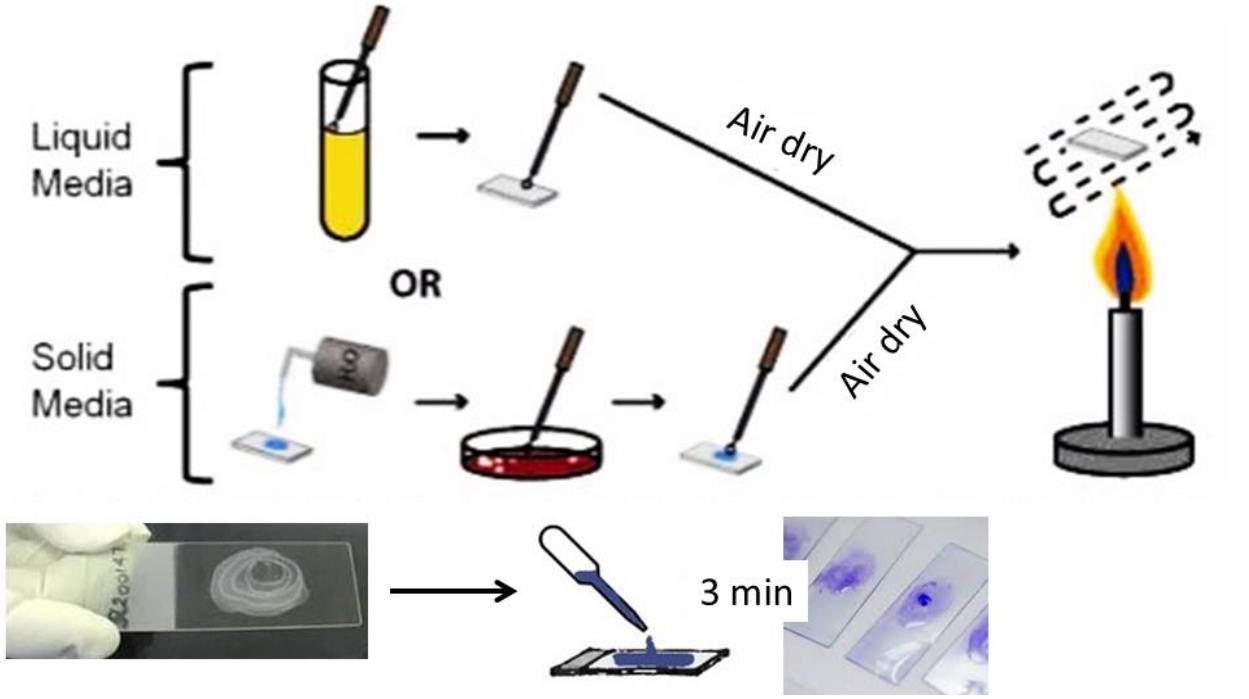


Fig. 16. Steps of heat-fixed and simple stained smear preparation

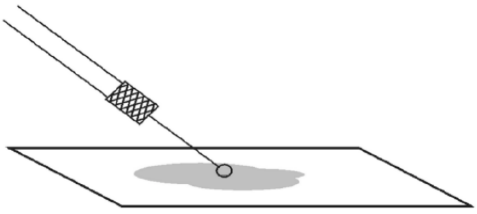


Fig. 17. Making a smear. Transfer a very small part of a single colony from a plate or an agar slant into the drop of water on a degreased slide. If the amount of culture on the loop is easily visible you have taken too much. Spread the resulting suspension evenly over an oval area of up to 2 cm length. A smear that is thin and even enables the shape and arrangement of cells to be clearly seen and ensures that the staining procedure is applied uniformly

4.3 Gram Staining

This important bacteriological staining procedure was discovered in 1884 by a Danish scientist, Christian Gram.

The staining is based on the cell wall structure of bacteria (fig. 18). When bacteria are stained with crystal violet, the cells of most Gram-

negative bacteria can be easily decolorized with organic solvents such as ethanol or acetone, while cells of most Gram-positive bacteria restrict decolorization. The ability of bacteria to either retain or lose the stain generally reflects fundamental differences in the cell wall and is an important taxonomic feature.

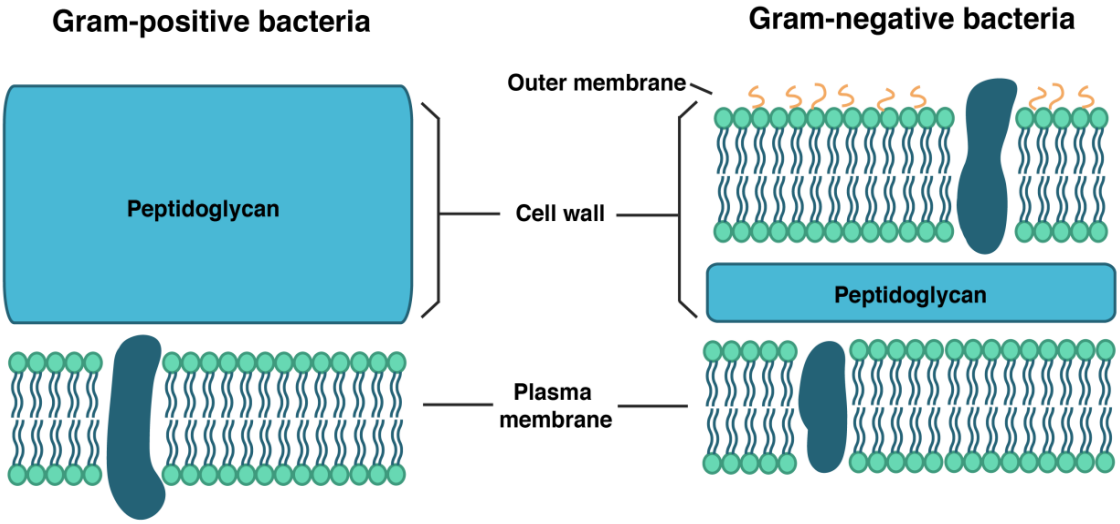


Fig. 18. Cell walls of Gram-positive and Gram-negative bacteria

Gram staining is therefore used as an initial step in the identification of bacteria. The cells of some bacteria are strongly Gram-positive when young, but tend to become Gram-negative in ageing cultures (e.g. *Bacillus* sp., *Clostridium* sp.), which may reflect degenerative changes in the cell wall. Some bacteria show a Gram-variable reaction: they are sometimes Gram-positive, sometimes Gram-negative; this could reflect minor variation in the staining technique or changes in cell wall thickness, etc. Yeast cells can be stained by Gram's method, but it is of no value in their identification.

Generally the steps of Gram staining are as follows:

1) Prepare a fixed smear of the bacterial culture on a microscope slide.
2) Stain with **crystal violet** solution.
3) Treat with **iodine** solution.
4) Decolorize with **96% ethanol**.
5) Counterstain with **safranin** solution.

Gram-positive bacteria appear violet/purple, while Gram-negative bacteria appear red/pink (fig. 19).

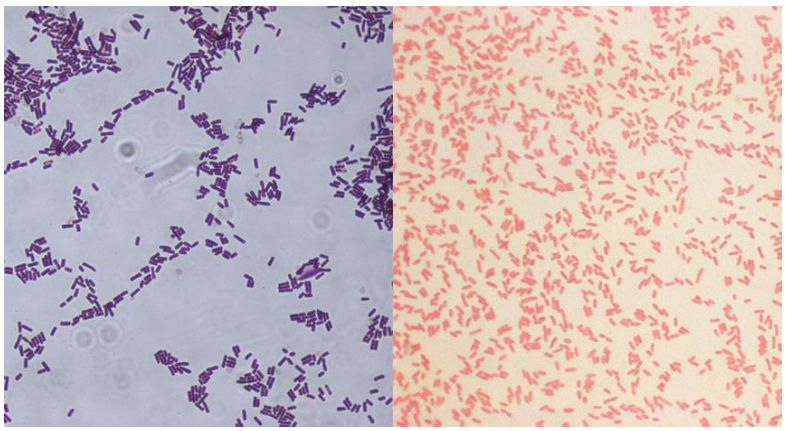


Fig. 19. After Gram staining, Gram-positive bacteria appear violet/purple, while Gram-negative bacteria appear red/pink

Materials and equipment

– Microscope glass slides
– Dropper pipettes
– Inoculating (transfer) loop
– Tap water/saline solution
– Crystal violet dye solution
– Iodine solution (Lugol's iodine)
– 96% ethanol
– Safranin dye solution
– Filter paper
– 70% ethanol
– Bunsen or alcohol burner
– Light microscope
– Immersion oil
– Specimens: 24-hour cultures of Gram-positive and Gram-negative bacteria (table 3) in Petri dishes

Table 3

Examples of Gram-positive and Gram-negative bacteria

Gram-positive bacteria	Gram-negative bacteria
Bacillus megaterium (*Priestia megaterium* after 2020)	*Escherichia coli*
Bacillus subtilis	*Serratia marcescens*
Clostridium sp.	*Salmonella* sp.
Lactobacillus sp.	*Pseudomonas* sp.
Staphylococcus sp.	
Streptococcus sp.	
Micrococcus luteus	

Step-by-Step Gram Staining Procedure

1) Prepare heat-fixed smears of two bacterial cultures on a microscope slide.

– Degrease the surface of a glass slide with alcohol.

– Label the backside of the slide adequately.

– Put the slide down on a rack/staining stand with the degreased surface upwards.

– Put a small drop of water onto the slide.

– Using aseptic technique, transfer a very small part of a single colony from a plate or slope of nutrient agar medium with a Gram-positive culture into the drop of water. If the amount of culture on the loop is easily visible you have taken too much!

– Flame a wire loop to ensure that no culture accidentally remains from a previous operation. Then transfer and mix a small amount of Gram-negative bacterial culture in the drop of water. A thin suspension will be formed this way.

– Apply a thin layer (smear) to a 2 cm^2 area with the inoculating loop and let it dry.

– Fix your preparation with heat. To heat-fix the smear, pass the underside of the slide through the hottest part of the Bunsen or alcohol burner flame for 2 to 3 seconds. Repeat 3 or 4 times. Let it cool down before staining. A heat-fixed smear should be visible to the naked eye as a whitish area.

2) Flood slide with **crystal violet**. Allow to stand for **2 minutes**, then shake off the dye.

3) Flood with **iodine** solution. Leave for **1 minute**, then shake off the iodine.

4) Decolorize with **96% ethanol** for **30 seconds**.

5) Rinse with tap water.

6) Apply **carbol fuchsin** or safranin (the counterstain) for **2 minutes**.

7) Rinse with tap water.

8) Drain and blot-dry the smear with filter/fibre-free blotting paper using firm pressure, but not sideways movements that might remove the smear.

Air dry the slide thoroughly before you examine the preparation under the microscope.

9) Examine the slide with immersion oil under the oil-immersion 100× objective.

10) Record and draw your observations (fig. 20–22).

11) After finishing microscopic observation, clean all used objective lenses. Carefully wipe the oil from all glass surfaces using a piece of lens cleaning paper. Use a second piece of lens paper moistened with a small amount of alcohol (ethyl or isopropyl) or lens cleaning solution, wiping all glass surfaces again to remove any remaining oil. Clean your slide using piece of cotton wool soaked in ethanol. Wash your slide using soap and water.

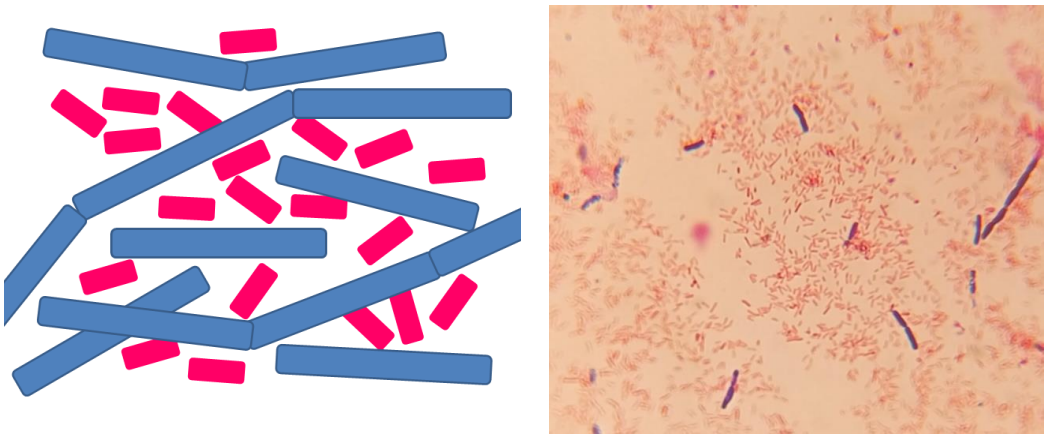


Fig. 20. Bright-field (Light) microscopy of the mixture of *Pseudomonas putida* and *Bacillus megaterium.* Gram staining. Magnification 1,600×

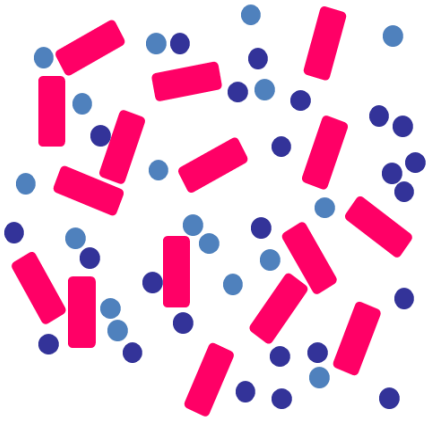


Fig. 21. Bright-field (Light) microscopy of the mixture of *Pseudomonas* sp. and *Micrococcus* sp. Magnification 1,600×

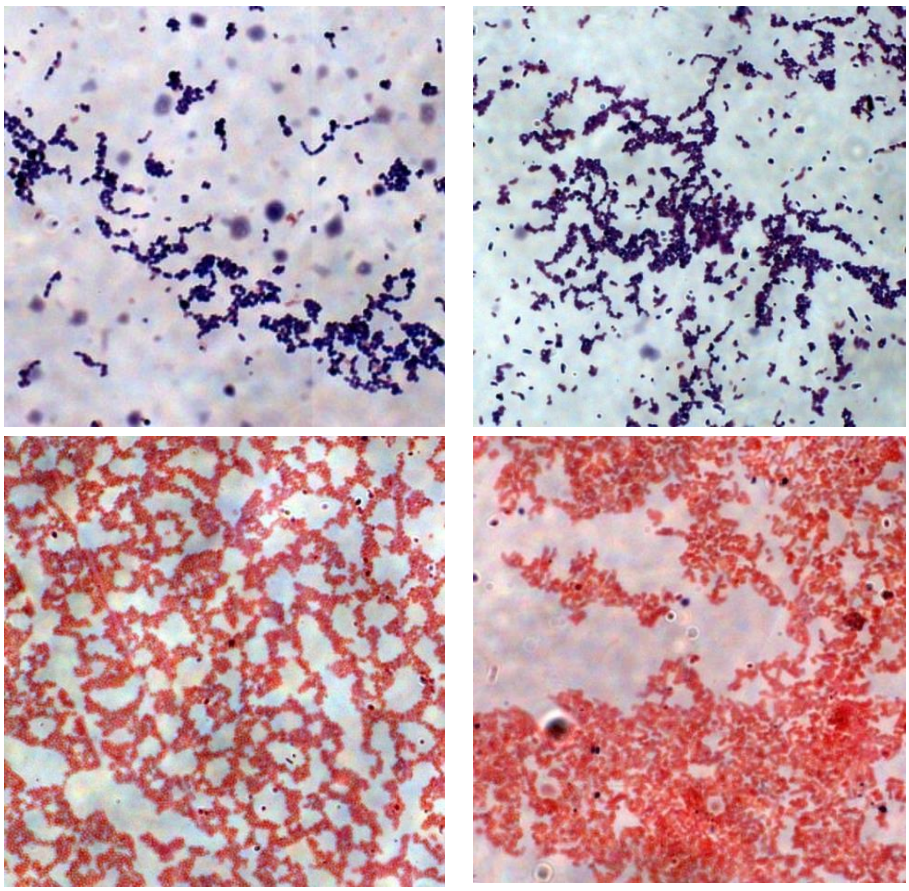
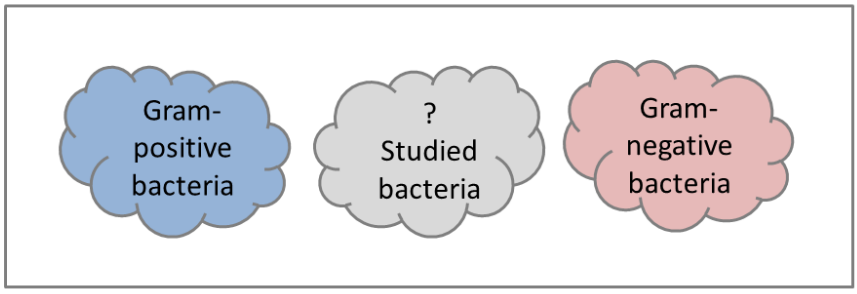


Fig. 22. Examples of typical Gram stain results.

Hints

1) To determine the Gram reaction of the culture, three cultures are prepared on a slide: control Gram-positive, control Gram-negative, and studied culture in the center.



2) Always use a fresh culture because older cultures of Gram-positive bacteria tend to lose the ability to retain the crystal violet-iodine complex and appear to be Gram-negative; but some bacteria are naturally only weakly Gram-positive.

3) The amount of alcohol treatment (the differential stage) must be judged carefully because over-treatment washes the crystal violet-iodine complex from Gram-positive bacteria and they will appear to be Gram-negative.
4) Make an even smear otherwise alcohol will continue to wash the violet/purple color from thick parts of the smear while thin parts are being over-decolorized.
5) At the end of the procedure, check that the labelling has not been washed off by the alcohol.
6) Do not despair if the stained smear is not visible to the naked eye; this may happen with a Gram-negative reaction.

5. CHARACTERISTICS OF MICROORGANISMS

5.1 Bacterial Cell Morphology

Cell morphology is a key feature in traditional bacterial classification. However, morphology alone is not enough for identification, as bacteria fall into a limited number of morphological categories (table 4, fig. 22). Cell size, typically measured in micrometers, is an important part of describing a bacterium's morphology. Cell size and shape are usually assessed after staining, but these characteristics can be influenced by factors like culture conditions, the age of the culture, and the bacteria's overall health.

Bacteria are generally classified by shape as rods, spheres (cocci), or spirals. While a typical rod-shaped bacterium measures 2-5 μm in length and 0.5-0.8 μm in diameter, and a typical sphere-shaped is about 0.8 μm across, there's considerable variation. Some bacteria, like certain spirochetes, are incredibly thin (around 0.2 μm), while others, such as *Thiomargarita namibiensis* and *Epulopiscium fishelsoni*, are giants, reaching sizes of 100-300 x 750 μm and 50 x 600 μm, respectively.

Table 4

Morphology of bacterial cells

Coccus (sphere)		
Micrococci	Following cell division, cells separate (singles)	*Micrococcus luteus*
Diplococci	Following cell division, cells remain in pairs	*Neisseria gonorrhoeae*
Streptococci	Chain of cocci	*Streptococcus lactis*
Staphylococci	Grape-like cluster of cocci	*Staphylococcus aureus*
Tetracocci	Cell division on 2 planes, cocci in tetrads	*Planococcus*
Sarcina	Cell division on 3 planes, cocci in aggregates (packets) of eight	*Sarcina* sp.
Rod (bacillus)		
	Shape and size very variable: long-short, wide-thin, coccoid, irregular	*Bacillus megaterium, Pseudomonas* sp., *Haemophilus influenzae, Corynebacterium* sp.
Curved rod (spiral shape)		
Vibrio	Cell with quarter or half a turn	*Vibrio cholerae*
Spirillum	Rigid cell wall, motility with flagella, cell with one or more turns	*Spirillum volutans*
Spirochaeta	Flexible cell wall, endoflagella, cell with one or more turns	*Treponema pallidum*
Filamentous		
	Actinomyces have branching cells, forming bacterial hyphae and their network (mycelium)	*Streptomyces* sp., *Nocardia* sp.
Variable		
	Intermediate forms (e.g. rod-coccus life cycle)	*Rhodococcus* sp., *Arthrobacter* sp.,

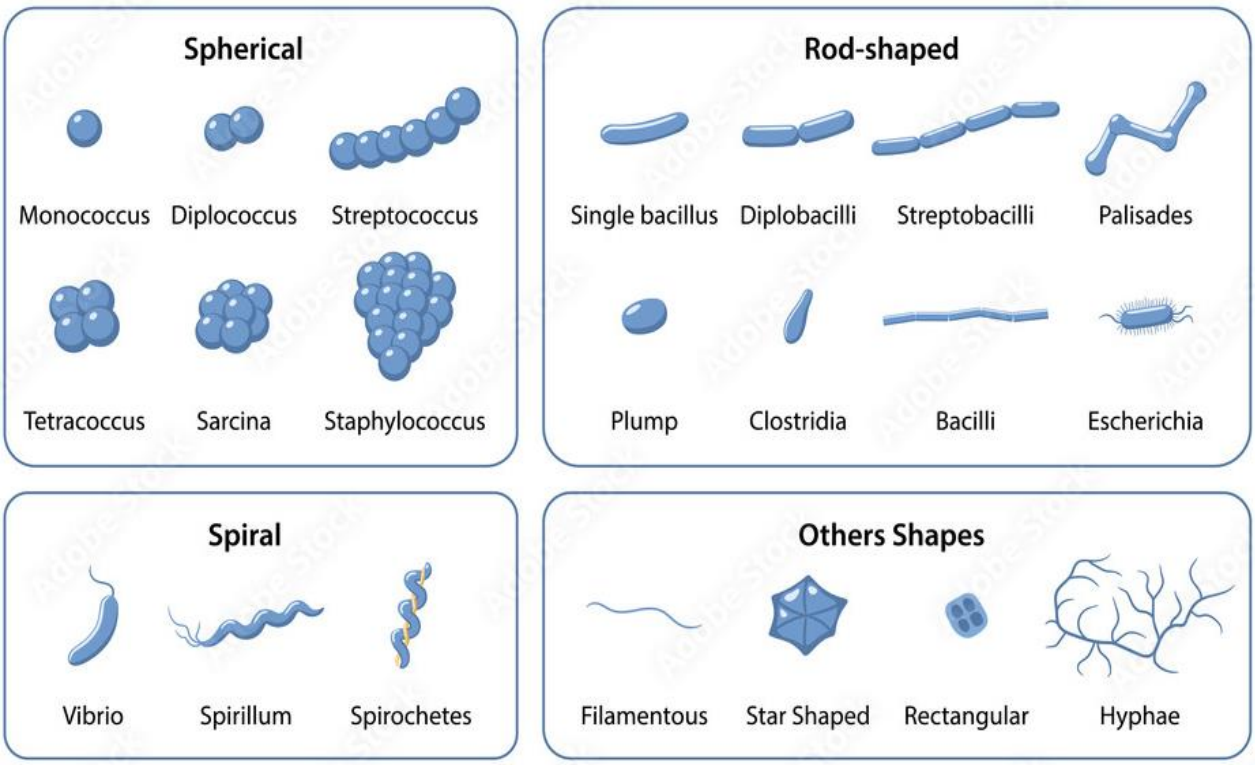


Fig. 23. Basic shapes of bacterial cells

5.2. Model Microorganisms

5.2.1. *Bacillus megaterium* (After 2020 – *Priestia megaterium*)

Systematics: Domain *Bacteria*; Kingdom *Bacillati*; Phylum *Bacillota*; Class *Bacilli*; Order *Bacillales*; Family *Bacillaceae*; Genus *Priestia.*

B. megaterium is a rod-like, Gram-positive, mainly aerobic spore forming bacterium found in widely diverse habitats. It frequently occurs in chains (fig. 24).

In 1884 de Bary named the Gram-positive bacterium *Bacillus megaterium* the "big beast" due to its large size. It is the largest known *Bacillus* species. *B. megaterium*, is about 1.5 μm across by 4 μm long. Compared to the model organism for Gram-negative bacteria, *Escherichia coli*, it has an up to 100-times higher volume (fig. 25).

Due to the dimensions of the vegetative form and spores, *B. megaterium* is an ideal model system for morphological research. It is well suited for the investigation of cell-wall biosynthesis, cytoplasmic membrane formation and the sporulation process including spore structure and organization. In the 1960s, prior to the development of *Bacillus subtilis*,

B. megaterium was the model organism of choice for intensive studies on biochemistry, sporulation and bacteriophages of Gram-positive bacteria. During the last years, it became more and more popular in the field of biotechnology for its recombinant protein production capacity.

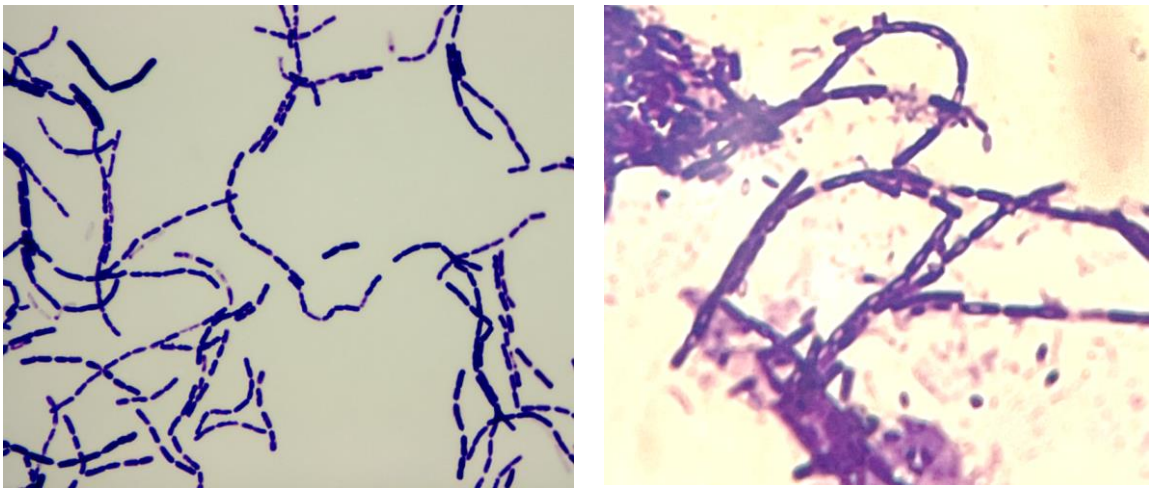


Fig. 24. *Bacillus megaterium*. Light microscopy, crystal violet staining, magnification 1,600×, immersion oil

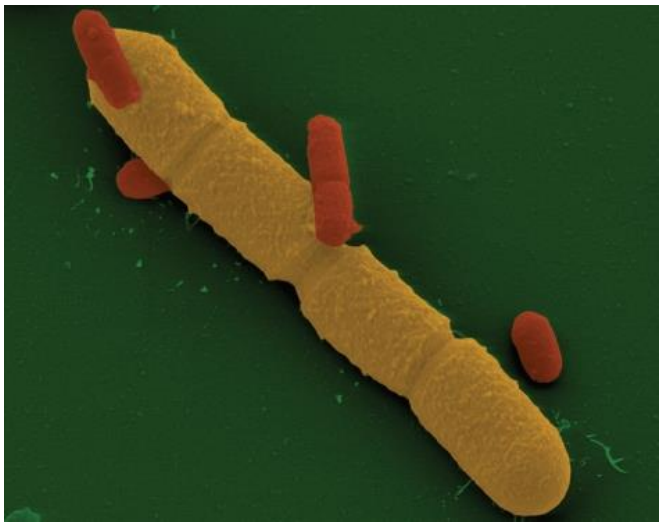


Fig. 25. Electron microscope image of *Bacillus megaterium* (yellow) and *Escherichia coli* (red) vegetative cells. Magnification 15,000×. Compared to the *E. coli* volume of 0.5 μm^3 (0.5×0.5×2), *B. megaterium* has at least up to 100-times higher volume[1]

[1] *Bunk B., Schulz A., Stammen S. et al.* A short story about a big magic bug // Bioengineered Bugs. 2010. V. 1. N. 2. P. 85–91.

5.2.2. *Micrococcus luteus*

Systematics: Domain *Bacteria*; Kingdom *Bacillati*; Phylum *Actinomycetota*; Class *Actinomycetes*; Order *Micrococcales*; Family *Micrococcaceae*; Genus *Micrococcus.*

M. luteus is a Gram-positive to Gram-variable, non-motile, coccus, saprotrophic bacterium (fig. 26). This bacterium is often arranged in circular tetrads or irregular clusters but not in chains. It forms bright yellow colonies on nutrient agar. *M. luteus* is found in soil, dust, water, and in human skin flora. *M. luteus* causes odours in humans when breaking down the components of sweat. It has also been isolated from foods such as milk and goat's cheese. This bacterium can withstand massive doses of UV radiation and also has the ability to degrade pollutants such as petrol.

Its name stands for: microscopic (micro), of spherical shape (coccus), and yellow (luteus). *M. luteus* was first known as *M. lysodeikticus* and was discovered by Alexander Fleming in 1928. *M. luteus* played an important part in Fleming's discovery of Lysozyme ('the body's natural antibiotic').

M. luteus has the ability to show dormancy without forming spores. Unlike other actinobacteria, *M. luteus* expresses only one resuscitation-promoting factor required for emergence form dormancy, and has few other dormancy-related proteins.

Infection: *M. luteus* is considered an opportunistic pathogen that can be responsible for nosocomial infections. *M. luteus* can cause skin infections and is sometimes clinically mistaken for *Staphylococcus aureus.* This bacterium can be transmitted due to poor hand-washing practices. *M. luteus* can cause septic shock in immunocompromised people.

M. luteus is an atmospheric microorganism commonly found on environmental monitoring plates and it is one of the most common contaminants of lab cultures. It is often observed on agar plates from bioburden testing of pre-sterilisation medical devices.

The distinct bright yellow organism can be fully identified using identification techniques such as MALDI-ToF (Matrix Assisted Laser Desorption Ionization-Time of Flight). It can commonly be mis-identified by

eye as *S. aureus* due to the golden / yellow colour so identification beyond colony morphology is required. Mannitol Salt Agar can be used to culture *Micrococcus* spp. as it is selective for certain Gram-positive microorganisms. However, it will also allow growth of *Staphylococcus* so further identification work must be conducted to from a strong identification of *Micrococcus* presence.

Other distinguishing identification features are that *M. luteus* is urease and catalase positive but coagulase negative.

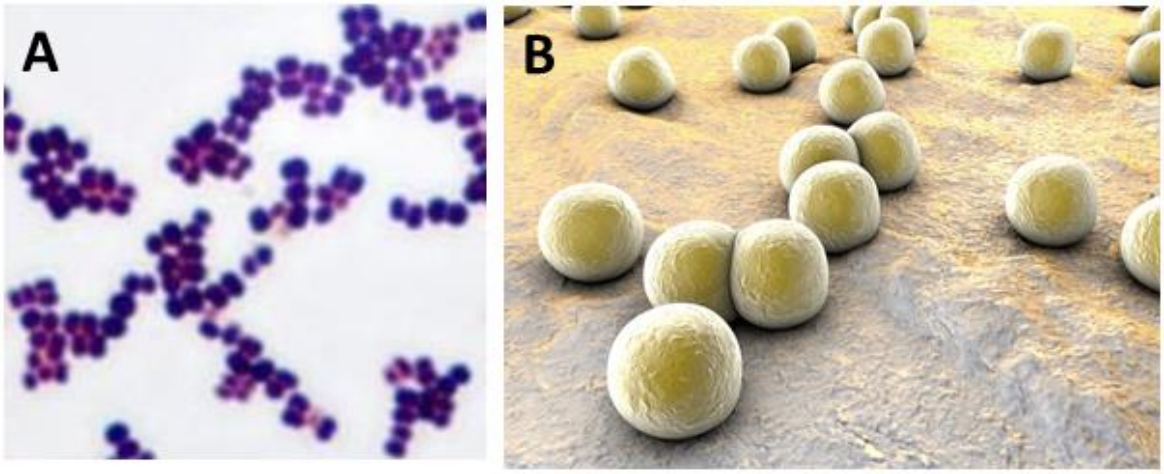


Fig. 26. *Micrococcus luteus.* Light immersion oil microscopy, crystal violet staining, magnification 1000× (A). Electron microscope image (B)

5.2.3. *Pseudomonas putida*

Systematics. Domain *Bacteria*; Kingdom *Pseudomonadati*; Phylum *Pseudomonadota*; Class *Gammaproteobacteria*; Order *Pseudomonadales*; Family *Pseudomonadaceae*; Genus *Pseudomonas.*

P. putida is a Gram-negative, rod-shaped, 0.5 to 1.0 μm in length, flagellated, spore-non-forming motile bacterium found in soil, water, and plant rhizospheres (fig. 27). It is a versatile saprophyte, meaning it obtains nutrients from decaying organic matter. *P. putida* is known for its remarkable metabolic capabilities, including the ability to degrade a wide range of organic compounds, making it a valuable tool in bioremediation. Colonies typically appear smooth and creamy on nutrient agar.

The genus name ‘putida’ is derived from the Latin term ‘putida’, meaning stinking or fetid, indicating the occurrence of the bacteria in

spoiled food items and the aromatic odor on solid media. The species was discovered by Trevisan in 1889. *P. putida* is considered an evolutionary group that includes other species like *P. fulva, P. parafulva, P. alkylphenolia* and *P. monteilii*.

P. putida is not considered a primary human pathogen, However, in rare cases, particularly in immunocompromised individuals, it can act as an opportunistic pathogen, potentially causing infections such as pneumonia or bacteremia. *P. putida* is often exploited for biotechnological applications. It is able to colonize a wide variety of environments, and is useful in the degradation of toxins. Genetically engineered strains are also used to produce useful chemicals.

P. putida's ability to metabolize a diverse array of compounds makes it a common contaminant in laboratory settings. Its presence in cultures can sometimes interfere with experiments or complicate the identification of other microorganisms. *P. putida* is the first patented microorganism in the world because of its ability to decompose hydrocarbons.

Identification of *P. putida* can be achieved through various methods, including biochemical testing, 16S rRNA gene sequencing, and MALDI-ToF MS. *P. putida* is typically oxidase-positive and catalase-positive, which can aid in differentiating it from other bacteria. Its ability to grow on minimal media with specific organic compounds as the sole carbon source is another key characteristic used for identification.

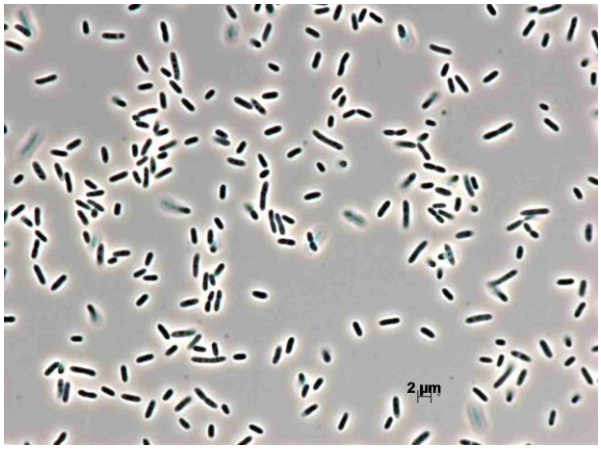


Fig. 27. Phase contrast microscopy of *Pseudomonas putida*

5.2.4. *Saccharomyces cerevisiae*

Systematics: Domain *Eukaryota*; Kingdom *Fungi*; Subkingdom *Dikarya*; Phylum *Ascomycota*; Subphylum *Saccharomycotina*; Class *Saccharomycetes*; Order *Saccharomycetales*; Family *Saccharomycetaceae*; Genus *Saccharomyces*.

S. cerevisiae is a species of yeast (single-celled fungal microorganisms). It has been instrumental in winemaking, baking, and brewing since ancient times. It causes many common types of fermentation. It is one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like *Escherichia coli* as the model bacterium. *S. cerevisiae* cells are round to ovoid, 5–10 μm in diameter (fig. 28, 29). It reproduces by budding – a bud is formed; it grows and may later leave its mother cell.

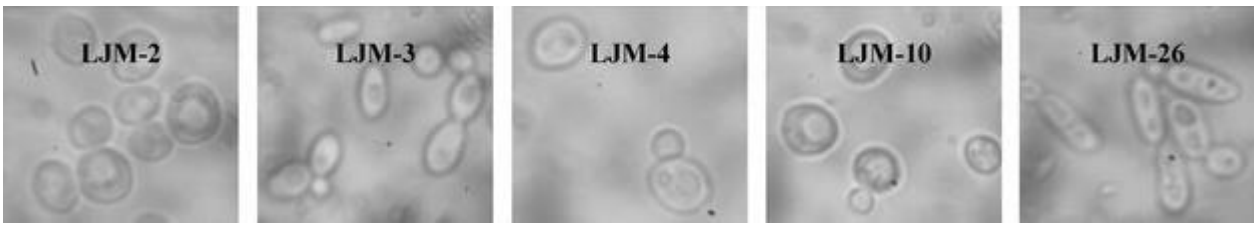


Fig. 28. Photomicrograph of a yeast cell type[2]

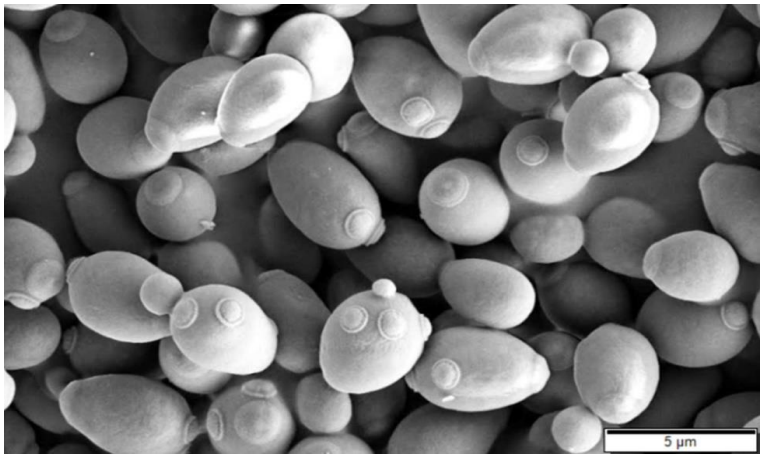


Fig. 29. Electron microscope image of *Saccharomyces cerevisiae*

[2] *Long J., Cai J., Gao X. et al.* Investigation on screening, identification, and fermentation characteristics of Yunnan olive in the fermented liquid utilizing five strains of *Saccharomyces cerevisiae* // Arch. Microbiol. 2024. V. 206. P. 164.

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Educational publication

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Educational and Methodical Manual