SOUTH VALLEY UNIVERSITY
FACULTY OF SCIENCE
DEPARTMENT OF BOTANY

PRACTICAL MICROBIOLOGY

for

DIPLOMA of APPLIED MICROBIOLOGY

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PART (1). Safety & LABORATORY PRINCIPLES

SAFETY IN THE LABORATORY

To be safe in the laboratory, it will be necessary for you to learn how to handle cultures in such a way that they are not contaminated or inadvertently dispersed throughout the classroom. This involves learning aseptic techniques and practicing preventive safety measures. The procedures outlined here address these two aspects. It is of paramount importance that you know all the regulations that are laid down here as Laboratory Protocol.

LABORATORY PROTOCOL

Scheduling Before attending laboratory each day, check the schedule to see what experiment or experiments will be performed and prepare yourself so that you understand what will be done.

Each laboratory session will begin with a short discussion to brief you on the availability of materials and procedures. Since the preliminary instructions start promptly at the beginning of the period, it is **extremely important that you are not late to class.**

Personal Items When you first enter the lab, place all personal items such as jackets, bags, and books in some out of the way place for storage. Don't stack them on your desktop. Desk space is minimal and must be reserved for essential equipment and your laboratory manual.

A lab coat or apron must be worn at all times in the laboratory. It will protect your clothing from accidental contamination and stains in the lab. When leaving the laboratory, remove the coat or apron. In addition, long hair must be secured in a ponytail to prevent injury from Bunsen burners and contamination of culture material.

TERMINOLOGY

Various terms such as sterilization, disinfection, germicides, sepsis, and aseptic techniques will be used here. To be sure that you understand exactly what they mean, the following definitions are provided.

Sterilization is a process in which all living microorganisms, including viruses, are destroyed. The organisms may be killed with steam, dry heat, or incineration. If we say an article is sterile, we understand that it is completely free of all living microorganisms.

Generally speaking, when we refer to sterilization as it pertains here to laboratory safety, we think, primarily, in terms of steam sterilization with the autoclave. The ultimate method of sterilization is to burn up the infectious agents or incinerate them. All biological wastes must ultimately be incinerated for disposal.

Disinfection is a process in which vegetative, non spore- forming microorganisms are destroyed. Agents that cause disinfection are called disinfectants or germicides**.** Such agents are used only on inanimate objects because they are toxic to human and animal tissues.

Sepsis is defined as the growth (multiplication) of microorganisms in tissues of the body. The term **asepsis** refers to any procedure that prevents the entrance of infectious agents into sterile tissues, thus preventing infection.

Aseptic techniques refer to those practices that are used by microbiologists to exclude all organisms from contaminating media or contacting living tissues. **Antiseptics** are chemical agents (often dilute disinfectants) that can be safely applied externally to human tissues to destroy or inhibit vegetative bacteria.

GOOD LABORATORY PRACTICES

All activities that are performed by the laboratories can be grouped under a common term **good laboratory practices (GLP).** It indicates performance of all

the activities of the laboratory in the best possible way so that the results obtained are of the highest possible accuracy.

THE CONTROL OF MICROBIAL GROWTH

In the 19th century, surgery was risky and dangerous and patients, undergoing even the most routine operations, were at very high risk of infection. This was so because surgery was not performed under aseptic conditions. The operating room, the surgeon's hands, and the surgical instruments were laden with microbes, which caused high levels of infection and mortality.

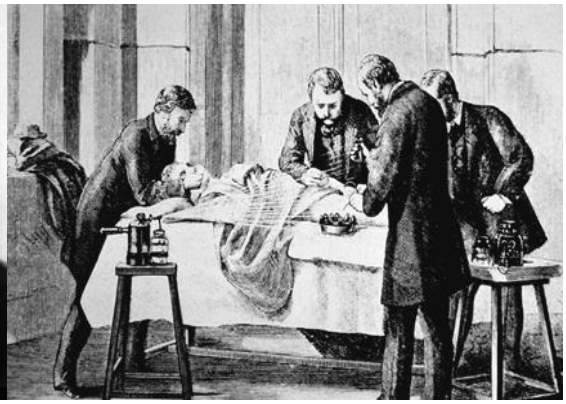
Surgeons in the mid-1800s often operated wearing their street clothes, without washing their hands. They frequently used ordinary sewing thread to suture wounds, and stuck the needles in the lapels of their frock coats in between patients. Surgical dressings were often made up of surplus cotton or jute from the floors of cotton mills. It was against this background that French scientist Louis Pasteur demonstrated that invisible microbes caused disease.

Louis Pasteur

Pasteur's work influenced the English surgeon Joseph Lister, who applied Pasteur's germ theory of disease to surgery, thus founding modern antiseptic surgery. To

disinfect, Lister used a solution of carbolic acid (phenol), which was sprayed around the operating room by a handheld sprayer.

Joseph Lister



19th Century surgery using Lister's carbolic acid sprayer.

It was clear that Lister's techniques were effective in increasing the rates of surviving surgery, but his theories were controversial because many 19th century surgeons were unwilling to accept something they could not see. Also, perhaps another reason that surgeons were slow to pick up on Lister's methods was the fact that during surgery they were required to breathe an irritating aerosol of phenol.

The control of microbial growth is necessary in many practical situations, and significant advances in agriculture, medicine, and food science have been made through study of this area of microbiology. "Control of microbial growth", means to inhibit or prevent growth of microorganisms. This control is affected in two basic ways: (1) by killing microorganisms or (2) by inhibiting the growth of microorganisms. Control of growth usually involves the use of physical or chemical agents that either kill or prevent the growth of microorganisms. Agents that kill cells are called **cidal** agents; agents which inhibit the growth of cells (without killing them) are referred to as **static** agents. Thus, the term **bactericidal** refers to killing bacteria, and **bacteriostatic** refers to inhibiting the growth of bacterial cells. A **bactericide** kills bacteria a **fungicide** kills fungi, and so on.

In microbiology, **sterilization** refers to the complete destruction or elimination of all viable organisms in or on a substance being sterilized. Sterilization procedures involve the use of heat, radiation or chemicals, or physical removal of cells.

Methods of Sterilization

Heat: most important and widely used. For sterilization one must consider the type of heat, and most importantly, the **time of application** and **temperature** to ensure destruction of all microorganisms. Endospores of bacteria are considered the most thermoduric of all cells so their destruction guarantees sterility.

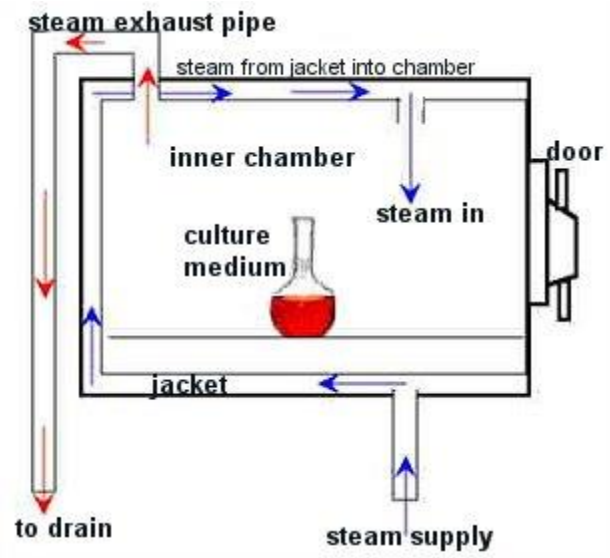
Incineration: burns organisms and physically destroys them. Used for needles, inoculating wires, glassware, etc. and objects not destroyed in the incineration process.

Boiling: 100^{o} for 30 minutes. Kills everything except some endospores. To kill endospores, and therefore **sterilize** a solution, very long (> 6 hours) boiling, or **intermittent boiling** is required (See the Table below).

Autoclaving (steam under pressure or pressure cooker)

Autoclaving is the most effective and most efficient means of sterilization. All autoclaves operate on a time/ temperature relationship. These two variables are extremely important. Higher temperatures ensure more rapid killing. The usual standard temperature/ pressure employed is 121ºC/ 15 psi for 15 minutes. Longer times are needed for larger loads, large volumes of liquid, and more dense materials. Autoclaving is ideal for sterilizing biohazardous waste, surgical dressings, glassware, many types of microbiologic media, liquids, and many other things. However, certain items, such as plastics, biological fluids (e.g. vitamins, milk, etc.) and certain medical instruments (e.g. fiber- optic endoscopes), cannot withstand autoclaving and should be sterilized with chemical or gas sterilants. When proper conditions and time are employed, no living organisms will survive a trip through an autoclave.

Why is an autoclave such an effective sterilizer? The autoclave is a large pressure cooker; it operates by using steam under pressure as the sterilizing agent. High pressures enable steam to reach high temperatures, thus increasing its heat content and killing power. Most of the heating power of steam comes from its latent heat of vaporization. This is the amount of heat required to convert boiling water to steam. This amount of heat is large compared to that required to make water hot. For example, it takes 80 calories to make 1 liter of water boil, but 540 calories to convert that boiling water to steam. Therefore, steam at 100º C has almost seven times more heat than boiling water.



Schematic diagram of a laboratory autoclave in use to sterilize microbiological culture medium. Sterilization of microbiological culture media is often carried out with the autoclave. The sterilization process is a 100% kill, and guarantees sterility.

Moist heat is thought to kill microorganisms by causing denaturation of essential proteins. Death rate is directly proportional to the concentration of microorganisms at any given time. The time required to kill a known population of microorganisms in a specific suspension at a particular temperature is referred to as **thermal death time (TDT)**. Increasing the temperature decreases TDT, and lowering the temperature increases TDT. Processes conducted at high

temperatures for short periods of time are preferred over lower temperatures for longer times.

Environmental conditions also influence TDT. Increased heat causes increased toxicity of metabolic products and toxins. TDT decreases with pronounced acidic or basic pH's. However, fats and oils slow heat penetration and increase TDT. It must be remembered that thermal death times are not precise values; they measure the effectiveness and rapidity of a sterilization process. Autoclaving at 121ºC/ 15 psi for 15 minutes exceeds the thermal death time for most organisms except some spore formers.

Dry heat (hot air oven): basically the cooking oven. The rules of relating time and temperature apply, but dry heat is not as effective as moist heat (i.e., higher temperatures are needed for longer periods of time). For example 160^{o}/ 2 hours or 170^{o}/ 1 hour is necessary for sterilization. The dry heat oven is used for glassware, metal, and objects that would not melt.

Irradiation: usually destroys or distorts nucleic acids. Ultraviolet light is commonly used to sterilize the surfaces of objects, although X- rays, gamma radiation and electron beam radiation are also used.

Ultraviolet lamps are used to sterilize workspaces and tools used in microbiology laboratories and health care facilities. UV light at germicidal wavelengths (two peaks, 185 nm and 265 nm) causes adjacent thymine molecules on DNA to dimerize, thereby inhibiting DNA replication (even though the organism may not be killed, it will not be able to reproduce). However, since microorganisms can be shielded from ultraviolet light in fissures, cracks and shaded areas, UV lamps should only be used for surfaces and as a supplement to other sterilization techniques.

An ultraviolet sterilization cabinet.

Gamma radiation and electron beam radiation are forms of ionizing radiation used primarily in the health care industry. Gamma rays, emitted from cobalt- 60, are similar in many ways to microwaves and X- rays. Gamma rays delivered during sterilization break chemical bonds by interacting with the electrons of atomic constituents. Gamma rays are highly effective in killing microorganisms and do not leave residues or have sufficient energy to impart radioactivity.

Electron beam (e-beam) radiation, a form of ionizing energy, generally characterized by low penetration and high- dose rates. E- beam irradiation is similar to gamma radiation in that it alters various chemical and molecular bonds on contact. Beams produced for e- beam sterilization are concentrated, highly-charged streams of electrons generated by the acceleration and conversion of electricity. E- beam and gamma radiation are for sterilization of items ranging from syringes to cardiothoracic devices.

Filtration involves the physical removal (exclusion) of all cells in a liquid or gas. It is especially important for sterilization of solutions that would be denatured by heat (e.g. antibiotics, injectable drugs, amino acids, vitamins, etc.). Portable units can be used in the field for water purification and industrial units can be used to "pasteurize" beverages. Essentially, solutions or gases are passed through a filter of sufficient pore diameter (generally 0.22 micron) to remove the smallest known bacterial cells.

Filter sterilization apparatus for heat-labile objects.

A typical set- up in a microbiology laboratory for filtration sterilization of medium components that would be denatured or changed by heat sterilization. The filter is placed (aseptically) on the glass platform, then the funnel is clamped and the fluid is drawn by vacuum into a previously sterilized flask. The recommended pore size of filter that will exclude the smallest bacterial cells is 0.22 micron. Other pore sizes are available for other purposes such as 0.45 μm.

Chemical and gas

Chemicals used for sterilization include the gases ethylene oxide and formaldehyde, and liquids such as glutaraldehyde. Ozone, hydrogen peroxide and peracetic acid are also examples of chemical sterilization techniques based on oxidative capabilities of the chemical.

Ethylene oxide (ETO) is the most commonly used form of chemical sterilization. Due to its low boiling point of 10.4°C at atmospheric pressure, ETO behaves as a gas at room temperature. ETO chemically reacts with amino acids, proteins, and DNA to prevent microbial reproduction. The sterilization process is carried out in a specialized gas chamber. After sterilization, products are transferred to an aeration cell, where they remain until the gas disperses and the product is safe to handle.

ETO is used for cellulose and plastics irradiation, usually in hermetically sealed packages. Ethylene oxide can be used with a wide range of plastics (e.g. Petri

dishes, pipettes, syringes, medical devices, etc.) and other materials without affecting their integrity.

An ethylene oxide sterilization gas chamber.

Ozone sterilization has been recently approved for use in the U.S. It uses oxygen that is subjected to an intense electrical field that separates oxygen molecules into atomic oxygen, which then combines with other oxygen molecules to form ozone. Ozone is used as a disinfectant for water and food. It is used in both gas and liquid forms as an antimicrobial agent in the treatment, storage and processing of foods, including meat, poultry and eggs. Many municipalities use ozone technology to purify their water and sewage. Los Angeles has one of the largest municipal ozone water treatment plants in the world. Ozone is used to disinfect swimming pools, and some companies selling bottled water use ozonated water to sterilize containers.

An ozone fogger for sterilization of egg surfaces. The system reacts ozone with water vapors to create powerful oxidizing radicals. This system is totally chemical free and is effective against bacteria, viruses and hazardous microorganisms which are deposited on egg shells.

An ozone sterilizer for use in the hospital or other medical environment.

Low Temperature Gas Plasma (LTGP) is used as an alternative to ethylene oxide. It uses a small amount of liquid hydrogen peroxide (H_2O_2), which is energized with radio frequency waves into gas plasma. This leads to the generation of free radicals and other chemical species, which destroy organisms.

A sterilizer that pumps vaporized H_2O_2 into the chamber.

Non sterilizing methods to control microbial growth

Many physical and chemical technologies are employed by our civilization to control the growth of (certain) microbes, although sterility may not the desired end- point. Rather, preventing spoilage of food or curing infectious disease might be the desired outcome.

Applications of Heat

The lethal temperature varies in microorganisms. The time required to kill depends on the number of organisms, species, nature of the product being heated, pH, and temperature. Autoclaving, which kills all microorganisms with heat, is commonly employed in canning, bottling, and other sterile packaging procedures. This is an ultimate form of preservation against microbes. But, there are some other uses of heat to control growth of microbes although it may not kill all organisms present.

Boiling: 100^{o}C for 30 minutes (more time at high altitude) kills everything except some endospores. It also inactivates viruses. For the purposes of purifying drinking water, 100^{o} for five minutes is a "standard" though there have been some reports that *Giardia* cysts can survive this process.

Pasteurization is the use of mild heat to reduce the number of microorganisms in a product or food. In the case of pasteurization of milk, the time and temperature depend on killing potential pathogens that are transmitted in milk, i.e., staphylococci, streptococci, *Brucella abortus* and *Mycobacterium tuberculosis*. But pasteurization kills many spoilage organisms, as well, and therefore increases the shelf life of milk especially at refrigeration temperatures (2°C). Milk is usually pasteurized by heating, typically at 63°C for 30 minutes (batch method) or at 71°C for 15 seconds (flash method), to kill bacteria and extend the milk's usable life. The process kills pathogens but leaves relatively benign microorganisms that can sour improperly stored milk. During the process of **ultrapasteurization**, also known as ultra high- temperature (UHT) pasteurization, milk is heated to temperatures of 140 °C. In the direct method, the milk is brought into contact with steam at 140°C for one or two seconds. A thin film of milk falls through a chamber of high- pressure steam, heating the milk instantaneously. The milk is flash cooled by application of a slight vacuum, which serves the dual purpose of removing excess water in the milk from condensing steam. In the indirect method of ultrapasteurization, milk is heated in a plate heat exchanger. It takes several

seconds for the temperature of the milk to reach 140°C, and it is during this time that the milk is scalded, invariably leading to a burned taste. If ultrapasteurization is coupled with aseptic packaging, the result is a long shelf life and a product that does not need refrigeration.

A review of protocols and recommendations for the use of heat to control microbial growth is given in Table 1.

Low temperature (refrigeration and freezing): Most organisms grow very little or not at all at 0^oC. Perishable foods are stored at low temperatures to slow rate of growth and consequent spoilage (e.g. milk). Low temperatures are not bactericidal. Psychrotrophs, rather than true psychrophiles, are the usual cause of food spoilage in refrigerated foods. Although a few microbes will grow in super cooled solutions as low as minus 20^oC, most foods are preserved against microbial growth in the household freezer.

Table 1. Recommended use of heat to control bacterial growth

Treatment	Temperature	Effectiveness
Incineration	$>500^o$	Vaporizes organic material on nonflammable surfaces but may destroy many substances in the process
Boiling	100^o	30 minutes of boiling kills microbial pathogens and vegetative forms of bacteria but may not kill bacterial endospores
Intermittent boiling	100^o	Three 30-minute intervals of boiling, followed by periods of cooling kills bacterial endospores
Autoclave and pressure cooker (steam under pressure)	121^o/15 minutes at 15# pressure	kills all forms of life including bacterial endospores. The substance being sterilized must be maintained at the effective T for the full time
Dry heat (hot air oven)	160^o/2 hours	For materials that must remain dry and which are not destroyed at T between 121^o and 170^o Good for glassware, metal, not plastic or rubber items

Dry heat (hot air oven)	170^{o}/1 hour	Same as above. Note increasing T by 10 degrees shortens the sterilizing time by 50 percent
Pasteurization (batch method)	63^{o}/30 minutes	kills most vegetative bacterial cells including pathogens such as streptococci, staphylococci and *Mycobacterium tuberculosis*
Pasteurization (flash method)	72^{o}/15 seconds	Effect on bacterial cells similar to batch method; for milk, this method is more conducive to industry and has fewer undesirable effects on quality or taste
Ultrapasteurization (direct method)	140^{o}/2 seconds	Effect on most bacterial cells is lethal. For milk, this method creates a product with relatively long shelf life at refrigeration temperatures.

Drying (removal of H_2O): Most microorganisms cannot grow at reduced water activity (A_w < 0.90). Drying is often used to preserve foods (e.g. fruits, grains, etc.). Methods involve removal of water from product by heat, evaporation, freeze-drying, and addition of salt or sugar.

Irradiation (UV, x-ray, gamma radiation): destroys microorganisms as described under "sterilization". Many spoilage organisms are readily killed by irradiation.

In some parts of Europe, fruits and vegetables are irradiated to increase their shelf life up to 500 percent. The practice has not been accepted in the U.S. UV light can be used to pasteurize fruit juices by flowing the juice over a high intensity ultraviolet light source. UV systems for water treatment are available for personal, residential and commercial applications and may be used to control bacteria, viruses and protozoan cysts.

The FDA has approved irradiation of poultry and pork to control pathogens, as well as foods such as fruits, vegetables, and grains to control insects, and spices, seasonings, and dry enzymes used in food processing to control microorganisms. Food products are treated by subjecting them to radiation from radioactive

sources, which kills significant numbers of insects, pathogenic bacteria and parasites.

According to the FDA, irradiation does not make food radioactive, nor does it noticeably change taste, texture, or appearance. Irradiation of food products to control food-borne disease in humans has been generally endorsed by the United Nation's World Health Organization and the American Medical Association. Two important Disease-causing bacteria that can be controlled by irradiation include *Escherichia coli* 0157:H7 and *Salmonella* species.

Control of microbial growth by chemical agents

Antimicrobial agents are chemicals that kill or inhibit the growth microorganisms. Antimicrobial agents include chemical preservatives and antiseptics, as well as drugs used in the treatment of infectious diseases of plants and animals. Antimicrobial agents may be of natural or synthetic origin, and they may have a static or cidal effect on microorganisms.

Types of antimicrobial agents

Antiseptics: microbicidal agents harmless enough to be applied to the skin and mucous membrane; should not be taken internally. Examples include alcohols, mercurials, silver nitrate, iodine solution, alcohols, detergents.

Disinfectants: agents that kill microorganisms, but not necessarily their spores, but are not safe for application to living tissues; they are used on inanimate objects such as tables, floors, utensils, etc. Examples include hypochlorites, chlorine compounds, copper sulfate, quaternary ammonium compounds, formaldehyde and phenolic compounds.

Common antiseptics and disinfectants and their uses are summarized in Table 2. Note: disinfectants and antiseptics are distinguished on the basis of whether they are safe for application to mucous membranes. Often, safety depends on the concentration of the compound.

Preservatives: static agents used to inhibit the growth of microorganisms, most often in foods. If eaten they should be nontoxic. Examples are calcium propionate, sodium benzoate, formaldehyde, nitrate and sulfur dioxide. Table 3a and 3b are lists of common preservative and their uses.

Table 2. Common antiseptics and disinfectants

Chemical	**Action**	**Uses**
Ethanol (50-70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Isopropanol (50-70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Formaldehyde (8%)	Reacts with NH_2, SH and COOH groups	Disinfectant, kills endospores
Tincture of Iodine (2% I2 in 70% alcohol)	Inactivates proteins	Antiseptic used on skin Disinfection of drinking water
Chlorine (Cl_2) gas	Forms hypochlorous acid (HClO), a strong oxidizing agent	Disinfect drinking water; general disinfectant
Silver nitrate ($AgNO_3$)	Precipitates proteins	General antiseptic
Mercuric chloride	Inactivates proteins by reacting with sulfide gro	Disinfectant, although occasiona used as an antiseptic on skin
Detergents (e.g. quaternary ammoni compounds)	Disrupts cell membranes	Skin antiseptics and disinfectants
Phenolic compounds (e.g. carbolic a lysol, hexylresorcinol, hexachlorophene)	Denature proteins and dis cell membranes	Antiseptics at low concentration disinfectants at high concentratio
Ethylene oxide gas	Alkylating agent	Disinfectant used to sterilize hea sensitive objects such as rubber a plastics
Ozone	Generates lethal oxygen radicals	Purification of water, sewage

Table 3a. Some common preservatives added to processed foods

Salt - retards bacterial growth. Not good for blood pressure.

Nitrates - can be found in some cheeses, adds flavor, maintains pink color in cured meats and prevents botulism in canned foods. Can cause adverse reactions in children, and potentially carcinogenic.

Sulfur Dioxide and Sulfites - are used as preservatives and to prevent browning in alcoholic beverages, fruit juices, soft drinks, dried fruits and vegetables. Sulfites prevent yeast growth and also retard bacterial growth in wine.

Sulfites may cause asthma and hyperactivity. They also destroy vitamins.

Benzoic Acid and Sodium Benzoate - are used to preserve oyster sauce, fish sauce, ketchup, non-alcoholic beverages, fruit juices, margarine, salads, confections, baked goods, cheeses, jams and pickled products. They have also been found to cause hyperactivity.

Propionic Acid and Propionates - used in bread, chocolate products, and cheese for lasting freshness.

Sorbic Acid and Sorbates - prevent mold formation in cheese and flour confectioneries

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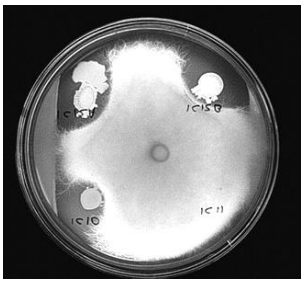
Table 3b. Common food preservatives and their uses

Preservative	Effective Concentration	Uses
Propionic acid and propionates	0.32%	Antifungal agent in breads, cake, Swiss cheeses
Sorbic acid and sorb	0.2%	Antifungal agent in cheeses, jellies, syrups, cakes
Benzoic acid and benzoates	0.1%	Antifungal agent in margarine, cider, relishes, soft d
Sodium diacetate	0.32%	Antifungal agent in breads
Lactic acid	unknown	Antimicrobial agent in cheeses, buttermilk, yogurt a pickled foods
Sulfur dioxide, sulfi	200-300 ppm	Antimicrobial agent in dried fruits, grapes, molasses
Sodium nitrite	200 ppm	Antibacterial agent in cured meats, fish
Sodium chloride	unknown	Prevents microbial spoilage of meats, fish, etc.
Sugar	unknown	Prevents microbial spoilage of preserves, jams, syru jellies, etc.
Wood smoke	unknown	Prevents microbial spoilage of meats, fish, etc.

Chemotherapeutic agents (synthetic antibiotics): antimicrobial agents of synthetic origin useful in the treatment of microbial or viral disease. Examples are sulfonilamides, isoniazid, ethambutol, AZT, nalidixic acid and chloramphenicol. Note that the microbiologist's definition of a chemotherapeutic agent requires that the agent be used for antimicrobial purpose and excludes synthetic agents used for therapy against diseases that are not of microbial origin. Hence, pharmacology distinguishes the microbiologist's chemotherapeutic agent as a "synthetic antibiotic".

Antibiotics: antimicrobial agents produced by microorganisms that kill or inhibit other microorganisms. This is the microbiologist's definition. A more broadened definition of an antibiotic includes any chemical of natural origin (from any type of cell) which has the effect to kill or inhibit the growth of other types cells



Three bacterial colonies growing on this plate secrete antibiotics that diffuse into the medium and inhibit the growth of a mold.

Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. Most of these microorganisms form some type of a spore or other dormant cell, and there is thought to be some relationship between antibiotic production and the processes of sporulation. Among the molds, the notable antibiotic producers are *Penicillium* and *Cephalosporium*, which are the main source of the beta-lactam antibiotics (penicillin and its relatives). In the Bacteria, the Actinomycetes, notably *Streptomyces* species, produce a variety of types of antibiotics including the aminoglycosides (e.g. streptomycin), macrolides (e.g. erythromycin), and the

tetracyclines. Endospore- forming *Bacillus* species produce polypeptide antibiotics such as polymyxin and bacitracin. The table below (Table 4) is a summary of the classes of antibiotics and their properties including their biological sources. **Semi-synthetic antibiotics** are molecules produced by a microbe that are subsequently modified by an organic chemist to enhance their antimicrobial properties.

Antimicrobial agents for the treatment of infectious disease

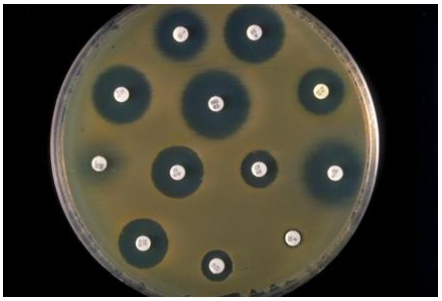
The modern era of antimicrobial chemotherapy began after Fleming's discovery in 1929 of the powerful bactericidal substance penicillin, and Domagk's discovery in 1935 of synthetic chemicals (sulfonamides) with broad antimicrobial activity. In the early 1940's, spurred partially by the need for antibacterial agents in WW II, penicillin was isolated, purified and injected into experimental animals, where it was found to not only cure infections but also to possess incredibly low toxicity for the animals. This fact encouraged an intense search for similar antimicrobial agents of low toxicity to animals that might prove useful in the treatment of infectious disease. The rapid isolation of streptomycin, chloroamphenicol and tetracycline soon followed, and by the 1950's, these and several other antibiotics were in clinical usage. The most important property of a clinically-useful antimicrobial agent, especially from the patient's point of view, is its **selective toxicity**, i.e., the agent acts in some way that inhibits or kills bacterial pathogens but has little or no toxic effect on the animal taking the drug. This implies that the biochemical processes in the bacteria are in some way different from those in the animal cells, and that the advantage of this difference can be taken in chemotherapy. Antibiotics may have a cidal (killing) effect or a static (inhibitory) effect on a range of microbes. The range of bacteria or other microorganisms that are affected by a certain antibiotic is expressed as its **spectrum of action**. Antibiotics effective against prokaryotes which kill or inhibit a wide range of Gram- positive and Gram- negative bacteria are said to be **broad spectrum** . If effective mainly against Gram- positive or Gram- negative bacteria, they are

narrow spectrum. If effective against a single organism or disease, they are **limited spectrum**.

Table 4. Some classes of antibiotics and their properties

Chemical class	Examples	Biological source	Spectrum (effective against)	Mode of action
Beta-lactams (penicillins and cephalosporins)	Penicillin G, Cephalothin	*P. notatum* and *Cephalosporium* species	Gram-positive bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Semisynthetic penicillin	Ampicillin, Amoxycillin		Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Clavulanic Acid	Clavamox is clavulanic acid plus amoxycillin	*Streptomyces clavuligerus*	Gram-positive and Gram-negative bacteria	Suicide inhibitor of beta-lactamases
Aminoglycosides	Streptomycin	*Streptomyces griseus*	Gram-positive and Gram-negative bacteria	Inhibit translation (protein synthesis)
Glycopeptides	Vancomycin	*Streptomyces orientales*	Gram-positive bacteria, esp. *Staph. aureus*	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Lincomycins	Clindamycin	*Streptomyces lincolnensis*	Gram-positive and Gram-negative bacteria esp. anaerobic Bacteroides	Inhibits translation (protein synthesis)
Macrolides	Erythromycin	*Streptomyces erythreus*	Gram-positive bacteria, Gram-negative bacteria not enterics, *Neisseria*, *Legionella*, Mycoplasma	Inhibits translation (protein synthesis)
Polypeptides	Polymyxin	*Bacillus polymyxa*	Gram-negative bacteria	Damages cytoplasmic membranes
	Bacitracin	*Bacillus subtilis*	Gram-positive bacteria	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Polyenes	Amphotericin	*Streptomyces nodosus*	Fungi	Inactivate membranes containing sterols
	Nystatin	*Streptomyces noursei*	Fungi (*Candida*)	Inactivate membranes containing sterols
Rifamycins	Rifampicin	*Streptomyces mediterranei*	Gram-positive and Gram-negative bacteria, *M. tuberculosis*	Inhibits transcription (eubacterial RNA polymerase)
Tetracyclines	Tetracycline	*Streptomyces species*	Gram-positive and Gram-negative bacteria, Rickettsias	Inhibit translation (protein synthesis)

The basis of bacterial resistance to antibiotics



An antibiotic sensitivity test performed on an agar plate. The discs are seeded with antibiotics planted on the agar surface. Interpretation of the size of the bacterial "zones of inhibition" relates to the possible use of the antibiotic in a clinical setting.

Bacterial resistance to an antimicrobial agent may be due to some innate property of the organism or due to acquisition of some genetic trait as described below.

Inherent (Natural) Resistance - Bacteria may be inherently resistant to an antibiotic. For example, a streptomycete may have some natural gene that is responsible for resistance to its own antibiotic; or a Gram-negative bacterium has an outer membrane that establishes a permeability barrier against the antibiotic; or an organism lacks a transport system for the antibiotic; or it lacks the target or reaction that is hit by the antibiotic.

Acquired Resistance - Bacteria can develop resistance to antibiotics, e.g. bacterial populations previously-sensitive to antibiotics become resistant. This type of resistance results from changes in the bacterial genome. Acquired resistance is driven by two genetic processes in bacteria: (1) mutation and selection (sometimes referred to as vertical evolution); (2) exchange of genes between strains and species (sometimes called horizontal evolution or horizontal gene transmission).

Alternatives to Antibiotics

Phage therapy is the therapeutic use of lytic bacteriophages to treat pathogenic bacterial infections. Phage therapy is an alternative to antibiotics being developed for clinical use by research groups in Eastern Europe and the U.S. After having

been extensively used and developed mainly in former Soviet Union countries for about 90 years, phage therapies for a variety of bacterial and poly microbial infections are now becoming available on an experimental basis in other countries, including the U.S. The principles of phage therapy have potential applications not only in human medicine, but also in dentistry, veterinary science, food science and agriculture.

An important benefit of phage therapy is derived from the observation that bacteriophages are much more specific than most antibiotics that are in clinical use. Theoretically, phage therapy is harmless to the eukaryotic host undergoing therapy, and it should not affect the beneficial normal flora of the host. Phage therapy also has few, if any, side effects, as opposed to drugs, and does not stress the liver. Since phages are self-replicating in their target bacterial cell, a single, small dose is theoretically efficacious. On the other hand, this specificity may also be disadvantageous because a specific phage will only kill a bacterium if it is a match to the specific subspecies. Thus, phage mixtures may be applied to improve the chances of success, or clinical samples can be taken and an appropriate phage identified and grown.

Phages are currently being used therapeutically to treat bacterial infections that do not respond to conventional antibiotics, particularly in the country of Georgia. They are reported to be especially successful where bacteria have constructed a biofilm composed of a polysaccharide matrix that antibiotics cannot penetrate.

MICROSCOPY

Microscopes in a laboratory represent a considerable investment and require special care to prevent damage to the lenses and mechanicals. The fact that a laboratory microscope may be used by several different individuals during the day, and moved around from one place to another, results in a much greater chance for damage to occur than if the instrument was used by only one individual. The complexity of some of the more expensive microscopes also requires that certain adjustments be made periodically. Knowing how to make these adjustments to get the equipment to perform properly is very important.

BRIGHTFIELD MICROSCOPY

A microscope that allows light rays to pass directly through to the eye without being deflected by an intervening opaque plate in the condenser is called a brightfield microscope**.** All brightfield microscopes have certain things in common, yet they differ somewhat in mechanical operation.

CARE OF THE INSTRUMENT

Microscopes can be damaged rather easily if certain precautions are not observed. The following suggestions cover most hazards.

Transport When carrying your microscope from one part of the room to another, use both hands when holding the instrument. And, incidentally, under no circumstances should one attempt to carry two microscopes at one time.

Clutter Keep your workstation uncluttered while doing microscopy. Keep unnecessary books, lunches, and other unneeded objects away from your work area. A clear work area promotes efficiency and results in fewer accidents.

Electric Cord Don't let the light cord on your microscope dangle in such a way as to hazard foot entanglement.

Lens Care At the beginning of each work period, check the lenses to make sure they are clean. At the end of each period, be sure to wipe any immersion oil off the immersion lens if it has been used.

Dust Protection In most laboratories dustcovers are used to protect the instruments during storage. If one is available, place it over the microscope at the end of the period.

COMPONENTS

Framework. The basic frame structure includes the **arm** and **base.** To this framework all other parts are attached. On many of the older microscopes the base is not rigidly attached to the arm; instead, a pivot point is present that enables one to tilt the arm backward to adjust the eyepoint height.

Stage The horizontal platform that supports the microscope slide is called the stage. Note that it has a clamping device, which is used for holding and moving the slide around on the stage. Note, also, the location of the mechanical stage control (see the following figure).

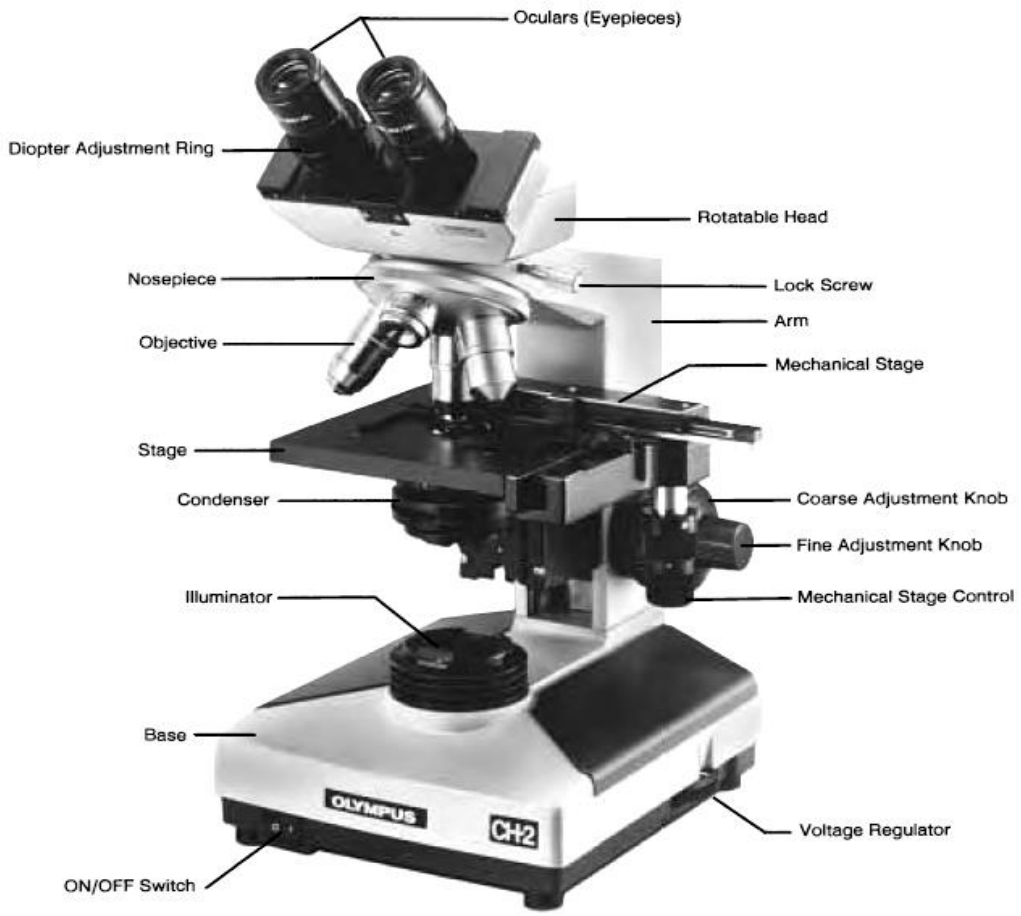
Light Source In the base of most microscopes is positioned some kind of light source. Ideally, the lamp should have a voltage control to vary the intensity of light. Most microscopes have some provision for reducing light intensity with a neutral density filter**.** On some microscopes a filter is built into the base.

Lens Systems All microscopes have three lens systems: the oculars, the objectives, and the condenser.

The ocular**,** or eyepiece, is a complex piece, located at the top of the instrument, which consists of two or more internal lenses and usually has a magnification of 10X. Although the microscope in the above figure has two oculars (binocular), a microscope often has only one. Three or more objectives are usually present.

Note that they are attached to a rotatable nosepiece, which makes it possible to move them into position over a slide. Objectives on most laboratory microscopes have magnifications of 10X, 45X, and 100X, designated as low power, high-dry, and oil immersion, respectively. Some microscopes will have a fourth objective for rapid scanning of microscopic fields that is only 4X.

The third lens system is the condenser, which is located under the stage. It collects and directs the light from the lamp to the slide being studied. The condenser can be moved up and down by a knob under the stage.



A diaphragm within the condenser regulates the amount of light that reaches the slide. Microscopes that lack a voltage control on the light source rely entirely on the diaphragm for controlling light intensity. On the Olympus microscope in the figure above, the diaphragm is controlled by turning a knurled ring. On some microscopes a diaphragm lever is present.

Focusing Knobs The concentrically arranged coarse adjustment and fine adjustment knobs on the side of the microscope are used for bringing objects into focus when studying an object on a slide. On some microscopes these knobs are not positioned concentrically as shown here.

Ocular Adjustments On binocular microscopes, one must be able to change the distance between the oculars and to make diopter changes for eye differences. On most microscopes the interocular distance is changed by simply pulling apart or pushing together the oculars.

RESOLUTION

The resolution limit or resolving power**,** of a microscope lens system, is a function of its numerical aperture, the wavelength of light, and the design of the condenser. The optimum resolution of the best microscopes with oil immersion lenses is around 0.2 µm. This means that two small objects that are 0.2 µm apart will be seen as separate entities; objects closer than that will be seen as a single object.

To get the maximum amount of resolution from a lens system, the following factors must be taken into consideration:

• A **blue filter** should be in place over the light source because the short wavelength of blue light provides maximum resolution.

• The **condenser** should be kept at its highest position where it allows a maximum amount of light to enter the objective.

• The **diaphragm** should not be stopped down too much. Although stopping down improves contrast, it reduces the numerical aperture.

• **Immersion oil** should be used between the slide and the 100X objective. Of significance is the fact that, as magnification is increased, the resolution is not increased.

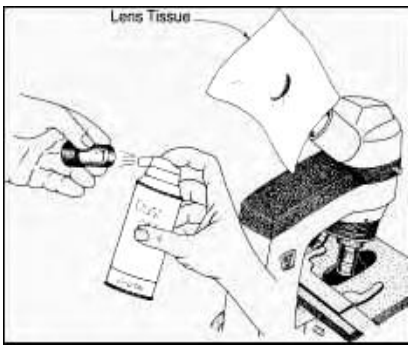
LENS CARE

Keeping the lenses of your microscope clean is a constant concern. Unless all lenses are kept free of dust, oil, and other contaminants, they are unable to achieve

the degree of resolution that is intended. Consider the following suggestions for cleaning the various lens components:

Cleaning Tissues Only lint-free, optically safe tissues should be used to clean lenses. Tissues free of abrasive grit fall in this category. Booklets of lens tissue are most widely used for this purpose. Although several types of boxed tissues are also safe, use only the type of tissue that is recommended by your instructor.

Solvents Various liquids can be used for cleaning microscope lenses. Green soap with warm water works very well. Xylene is universally acceptable. Alcohol and acetone are also recommended, but often with some reservations. Acetone is a powerful solvent that could possibly dissolve the lens mounting cement in some objective lenses if it were used too liberally. When it is used it should be used sparingly.



When oculars are removed for cleaning, cover the ocular opening with lens tissue. A blast from an air syringe or gas cannister removes dust and lint.

Oculars The best way to determine if your eyepiece is clean is to rotate it between the thumb and forefinger as you look through the microscope. A rotating pattern will be evidence of dirt. If cleaning the top lens of the ocular with lens tissue fails to remove the debris, one should try cleaning the lower lens with lens tissue and blowing off any excess lint with an air syringe or gas canister.

Whenever the ocular is removed from the microscope, it is imperative that a piece of lens tissue be placed over the open end of the microscope as illustrated in the figure below.

Objectives Objective lenses often become soiled by materials from slides or fingers. A piece of lens tissue moistened with green soap and water, or one of the acceptable solvents mentioned above, will usually remove whatever is on the lens. Sometimes a cotton swab with a solvent will work better than lens tissue. At any time that the image on the slide is unclear or cloudy, assume at once that the objective you are using is soiled.

Condenser Dust often accumulates on the top surface of the condenser; thus, wiping it off occasionally with lens tissue is desirable.

PROCEDURES

If your microscope has three objectives you have three magnification options: (1) low-power, or 100X total magnification, (2) high-dry magnification, which is 450X total with a 45X objective, and (3) 1000X total magnification with a 100X oil immersion objective.

Note that the total magnification seen through an objective is calculated by simply multiplying the power of the ocular by the power of the objective. Whether you use the low-power objective or the oil immersion objective will depend on how much magnification is necessary. However, it is best to start with the low-power objective and progress to the higher magnifications as your study progresses. Consider the following suggestions for setting up your microscope and making microscopic observations.

Viewing Setup If your microscope has a rotatable head, there are two ways that you can use the instrument. Note that you have to position the arm of the microscope near you, with the advantage of the stage easier to observe and when focusing the instrument you will be able to rest your arm on the table. If the

microscope head is not rotatable, it will be necessary to use the other position (the microscope arm is away from you).

Low-Power Examination The main reason for starting with the low-power objective is to enable you to explore the slide to look for the object you are planning to study. Once you have found what you are looking for, you can proceed to higher magnifications. Use the following steps when exploring a slide with the low-power objective:

1. Position the slide on the stage with the slide held in place by the mechanical stage retainer lever.
2. Turn on the light source, using a minimum amount of voltage. If necessary, reposition the slide so that the stained material on the slide is in the exact center of the light source.
3. Check the condenser to see that it has been raised to its highest point.
4. If the low-power objective is not directly over the center of the stage, rotate it into position. Be sure that as you rotate the objective into position it clicks into its locked position.
5. Turn the coarse adjustment knob to lower the objective until it stops before touching the slide. A built-in stop in modern microscopes will prevent the objective from touching the slide.
6. While looking down through the ocular (or oculars), bring the object into focus by turning the fine adjustment focusing knob. Don't readjust the coarse adjustment knob. If you are using a binocular microscope it will also be necessary to adjust the interocular distance to match your eyes.
7. Manipulate the diaphragm lever to reduce or increase the light intensity to produce the clearest, sharpest image. Note that as you close down the diaphragm to reduce the light intensity, the contrast improves and the depth of field increases. Stopping down the diaphragm when using the low-power objective does not decrease resolution.

8. Once an image is visible, move the slide about to search out what you are looking for. The slide is moved by turning the knobs that move the mechanical stage.

9. Check the cleanliness of the ocular, using the procedure outlined earlier.

10. Once you have identified the structures to be studied and wish to increase the magnification, you may proceed to either high-dry or oil immersion magnification. However, before changing objectives, be sure to center the object you wish to observe.

High-Dry Examination To proceed from low power to high-dry magnification, all that is necessary is to rotate the high-dry objective into position and open up the diaphragm somewhat. It may be necessary to make a minor adjustment with the fine adjustment knob to sharpen up the image, but the coarse adjustment knob should not be touched. If a microscope is of good quality, only minor focusing adjustments are needed when changing from low power to high-dry.

High-dry objectives should be used only on slides that have cover glasses; without them, images are usually unclear. When increasing the lighting, be sure to open up the diaphragm first instead of increasing the voltage on your lamp to extend the microscope lamp life. If the field is not bright enough after opening the diaphragm, feel free to increase the voltage and Keep the condenser at its highest point.

Oil Immersion Techniques The oil immersion lens derives its name from the fact that a special mineral oil is interposed between the lens and the microscope slide. The oil is used because it has the same refractive index as glass, which prevents the loss of light due to the bending of light rays as they pass through air. The use of oil in this way enhances the resolving power of the microscope.

Once the microscope has been brought into focus at one magnification, the oil immersion lens can be rotated into position without fear of striking the slide. Before rotating the oil immersion lens into position, a drop of immersion oil must

be placed on the slide. An oil immersion lens should never be used without oil. Incidentally, if the oil appears cloudy it should be discarded.

When using the oil immersion lens it is best to open the diaphragm as much as possible. Stopping down the diaphragm tends to limit the resolving power of the optics. In addition, the condenser must be kept at its highest point. If different coloured filters are available for the lamp housing, it is best to use blue or greenish filters to enhance the resolving power.

Since the oil immersion lens will be used extensively in all bacteriological studies, it is important that you learn how to use this lens properly. Using this lens takes a little practice due to the difficulties usually encountered in manipulating the lighting. At the end of the laboratory period remove all immersion oil from the lens tip with lens tissue.

PUTTING IT AWAY

The next person to use the instrument after you have used it will expect that it clean and working properly. A few moments of care at the end of the period will ensure these conditions. Check over this list of items at the end of each period before you return the microscope to its place or leave it for another period of work.

1. Remove the slide from the stage.
2. If immersion oil has been used, wipe it off the lens and stage with lens tissue. (Do not wipe oil off slides you wish to keep. Simply put them into a slide box and let the oil drain off.
3. Rotate the low-power objective into position.
4. If the microscope has been inclined, return it to an erect position.
5. If the microscope has a built-in movable lamp, raise the lamp to its highest position.
6. If the microscope has a long attached electric cord, wrap it around the base.
7. Adjust the mechanical stage so that it does not project too far on either side.

8. Replace the dustcover.

9. If the microscope has a separate transformer, return it to its designated place.

10. If moved, return the microscope to its correct place.

PART (2). BASIC BACTERIOLOGY

STAINING OF BACTERIA

1- NEGATIVE STAIN

Materials

24- to 48-hour nutrient broth cultures of *Bacillus subtilis*, *Micrococcus luteus*, and *Pseudomonas aeruginosa* (or any other available strains.

Nigrosine solution, India ink, or eosin blue ,clean microscope slides, inoculating loop, immersion oil, microscope lens, paper and lens cleaner, Bunsen burner.

Learning Objectives

Each student should be able to:

1. Understand the reason for the negative staining procedure

2. Stain three different bacteria using the negative staining procedure

Procedure

1. Use an inoculating loop to apply a small amount of bacteria to one end of a clean microscope slide.

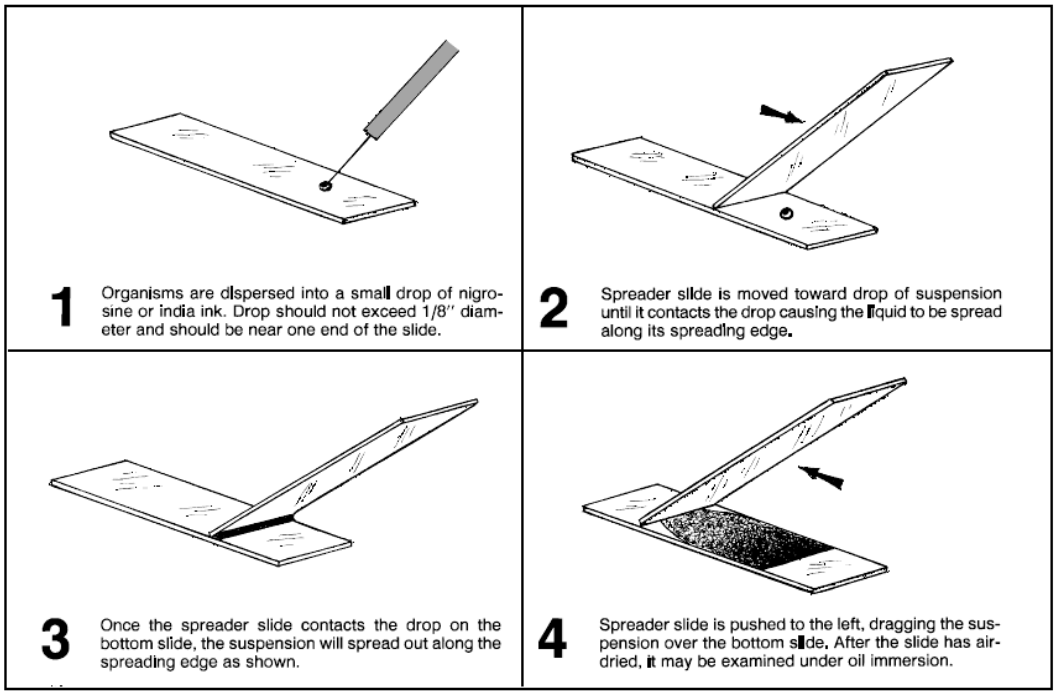
2. Add 1 to 2 loops of nigrosin, India ink, or eosin solution to the bacteria and mix thoroughly.

3. Spread the mixture over the slide using a second slide. The second slide should be held at a 45 angle so that the bacteria-nigrosin solution

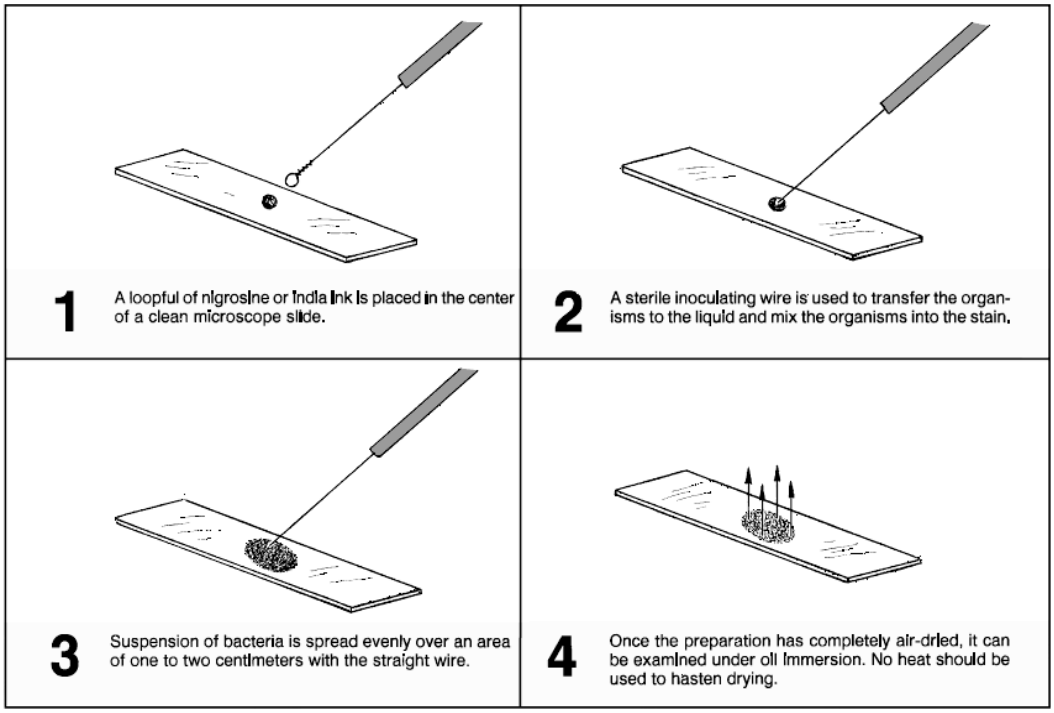
4 Allow the smear to air dry. **Do not heat-fix**!

5. With the low-power objective, find an area of the smear that is of the optimal thickness for observation.

6. Use the oil immersion lens to observe and draw the three bacterial species in the report for exercise.



Negative staining technique, using a spreader slide



A second method for negative staining

2- SMEAR (FILM) PREPARATION

Materials

24- to 48- hour nutrient broth or agar slants of *Bacillus subtilis*, *Micrococcus luteus*, and *Pseudomonas aeruginosa* or any available strains.

Microscope, clean microscope slides, inoculating loop and needle, sterile distilled water, Bunsen burner, Loffler's alkaline methylene blue, crystal violet (1% aqueous solution), Ziehl's carbol fuchsin, immersion oil, lens paper and lens cleaner, slide holder or clothespin, slide warmer

Learning Objectives

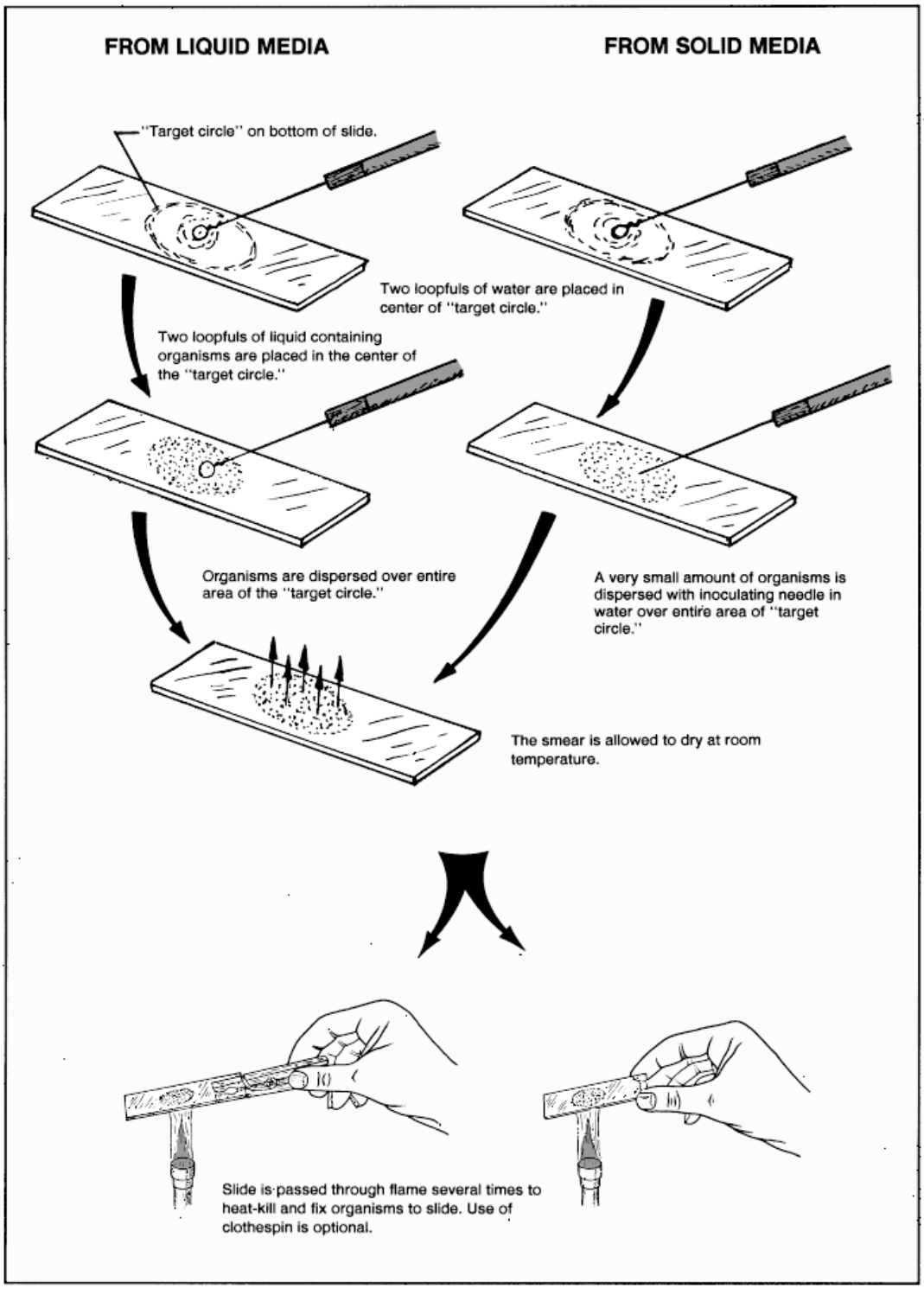
Each student should be able to

1. Learn the proper procedure for preparing a bacterial smear
2. Do several simple staining procedures

Procedure

Smear Preparation

1. For the broth culture, shake the culture tube and, with an inoculating loop, aseptically transfer 1 to 2 loopfuls of bacteria to the center of the slide. Spread this out to about a 1 cm area. When preparing a smear from a slant or plate, place a loopful of water in the center of the slide. With the inoculating needle, aseptically pick up a very small amount of culture and mix into the drop of water. Spread this out as above.
2. Allow the slide to air dry, or place it on a slide warmer
3. Pass the slide through a Bunsen burner flame three times to heat-fix and kill the bacteria.



Procedure for making a bacterial smear

3- SIMPLE STAINING

1. Place the three fixed smears on a staining loop or rack over a sink or other suitable receptacle

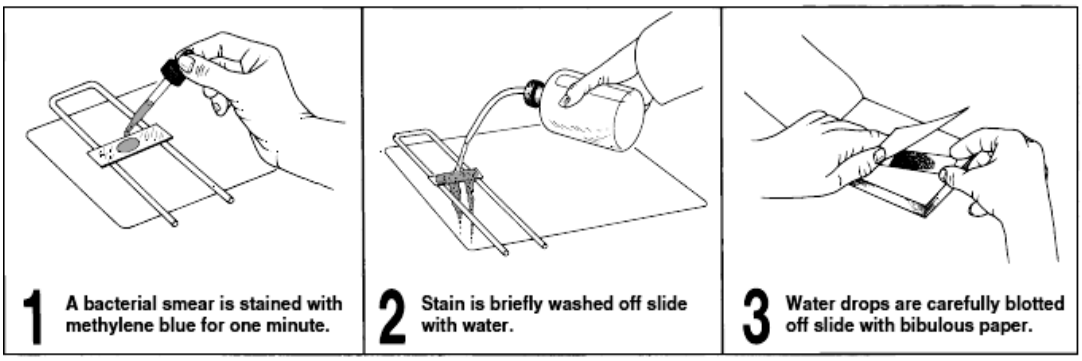
2. Stain one slide with alkaline methylene blue for 1 minute; one slide with carbol fuchsin for 5 to 10 seconds; and one slide with crystal violet for 20 to 30 seconds.

3. Wash stain off slide with water for a few seconds

4. Blot slide dry with bibulous paper be careful not to rub the smear when drying the slide because this will remove the stained bacteria.

5. Examine under the oil immersion lens and draw the bacteria.

6. You may want to treat smears of the same bacterium with all three stains in order to compare them more directly. It is also instructive to cover bacterial smears for varying lengths of time with a given stain in order to get a feel for how reactive they are and the results of over staining or understaining a slide preparation.



Procedure for simple staining

4- GRAM STAINING

- Named after the Danish bacteriologist who originally devised it in 1844, Hans C. Gram.
- The Gram staining method is one of the most important staining techniques in microbiology.

- It is almost always the first test performed for the identification of bacteria.
- It is a four step procedure which uses certain dyes:
    1. Crystal Violet (the Primary Stain)
    2. Iodine Solution (the fixative)
    3. Decolorizer (ethanol with or without acetone)
    4. Safranin (the Counter-stain)
- It is based on the ability of bacteria cell wall to retain the crystal violet dye during solvent treatment.

Materials

18- to 24-hour nutrient broth cultures of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC25922), and a mixture of *S. aureus and E. coli*, solutions of crystal violet, Gram's iodine (2 g potassium iodide in 300 ml distilled water plus 1 g iodine crystals), 95% ethanol and/or isopropanol-acetone mixture (3:1 v/v), and safranin Bismark brown stain (for colour-blind students), clean glass slides, inoculating loop, Bunsen burner, microscope lens paper and lens clean immersion oil, staining rack.

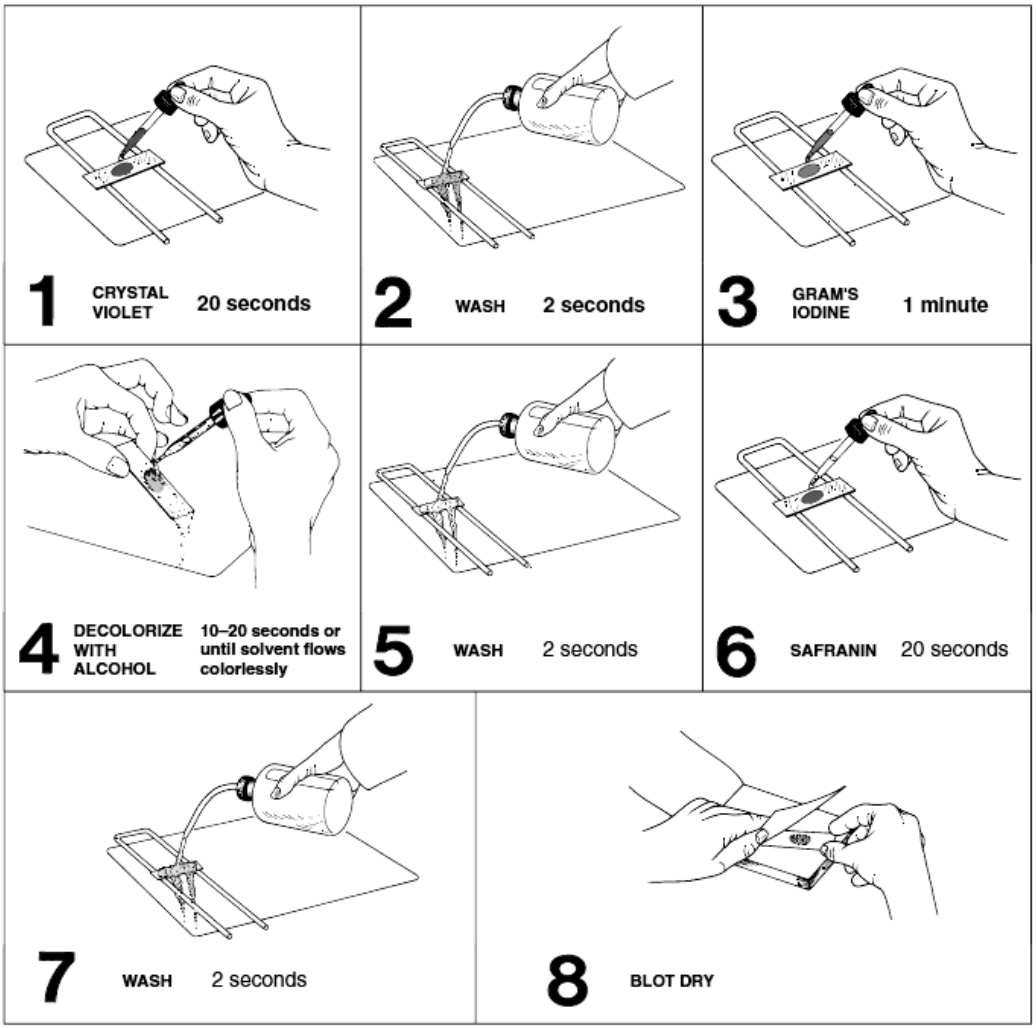
Learning Objectives

Each student should be able to

1. Understand the biochemistry underlying the Gram stain
2. Understand the theoretical basis for differential staining procedures
3. Perform a satisfactory Gram stain
4. Differentiate a mixture of bacteria into gram-positive and gram-negative cells

Procedure for Traditional Gram-Stain Technique

1. Prepare heat-fixed smears of *E. coli, S. aureus,* and the mixture of *E. coli* and *S. aureus*
2. Place the slides on the staining rack.
3. Flood the smears with crystal violet and let stand for 30 seconds.



The Gram-staining procedure

4. Rinse with water for 5 seconds.

6. Rinse with water for 5 seconds.

7. Decolorize with 95% ethanol for 15 to 30 seconds.

Do not decolourize too long. Add the decolourizer drop by drop until the crystal violet fails to wash from the slide. Alternatively, the smears may be decolourized for 30 to 60 seconds with a mixture of isopropanol-acetone.

8. Rinse with water for 5 seconds.

9. Counterstain with safranin for about 60 to 80 seconds. Safranin preparations vary considerably in strength, and different staining times may be required for each batch of stain. (If you are colour-blind, use Bismarck brown stain rather than safranin).

10. Rinse with water for 5 seconds.

11. Blot dry with bibulous paper and examine under oil immersion. Gram-positive organisms stain blue to purple; Gram-negative organisms stain pink to red.

5- ENDOSPORES STAINING (Schaeffer-Fulton or Wirtz-Conklin)

Materials

24- to 48-hour nutrient agar slant cultures of *Bacillus megaterium* (ATCC 12872) and *Bacillus macerans* (ATCC 8244), and old (more than 48 hours) thioglycollate cultures of *Clostridium butyricum* (ATCC 19398) and *Bacillus circulans* (ATCC 4513) or any available cultures, clean glass slides, microscope, immersion oil, wax pencil, inoculating loop, hot plate or boiling water bath with staining rack or loop, 5% malachite green solution, safranin, bibulous paper, paper toweling, lens paper and lens cleaner, slide warmer, forceps

Learning Objectives

Each student should be able to

1. Understand the biochemistry underlying endospores staining
2. Perform an endospores stain
3. Differentiate between bacterial endospores and vegetative cell forms

Principles

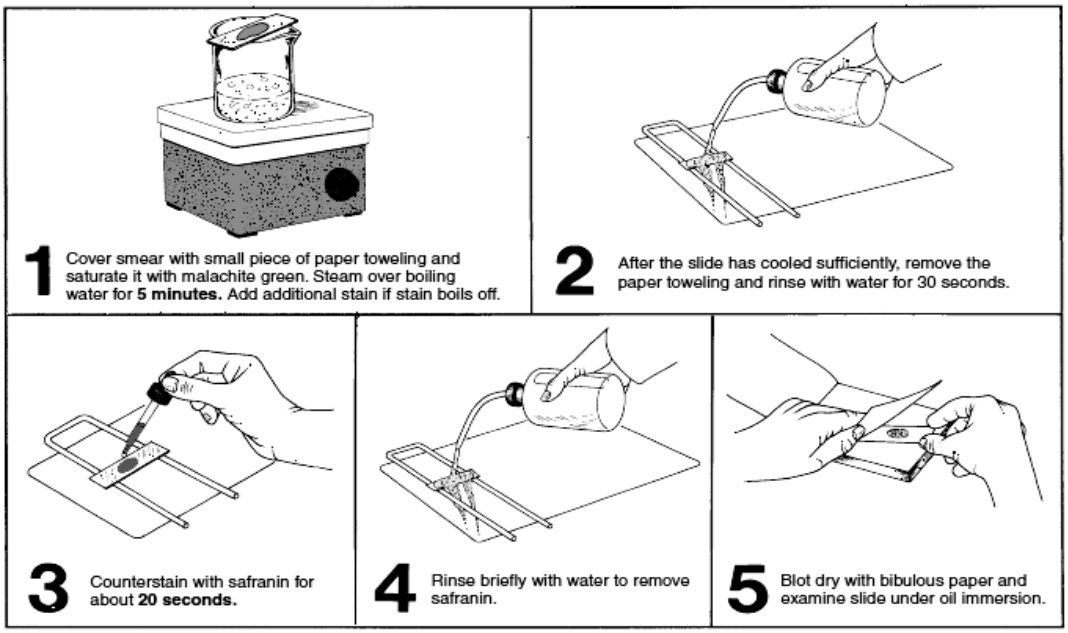
Bacteria in genera such as *Bacillus* and *Clostridium* produce quite a resistant structure capable of surviving for long periods in an unfavorable environment and then giving rise to a new bacterial cell. This structure is called endospores since it develops within the bacterial cell. Endospores are spherical to elliptical in shape and may be either smaller or larger than the parent bacterial cell. Endospores

position within the cell is characteristic and may be central, sub terminal, or terminal.

Endospores do not stain easily, but, once stained, they strongly resist decolourization. This property is the basis of the **Schaeffer-Fulton method** of staining endospores. The endospores are stained with malachite green. Heat is used to provide stain penetration. The rest of the cell is then decolorized and counterstained a light red with safranin.

Procedure

1. With a wax pencil, place the names of the respective bacteria on the edge of four clean glass slides.
2. As shown in figure 14.3, aseptically transfer one species of bacterium with an inoculating loop to each of the respective slides, air dry (or use a slide warmer), and heat-fix.
3. Place the slide to be stained on a hot plate or boiling water bath equipped with a staining loop or rack. Cover the smear with paper toweling that has been cut the same size as the microscope slide.
4. Soak the paper with the malachite green staining solution. Gently heat on the hot plate (just until the stain steams) for 5 to 6 minutes after the malachite green solution begins to steam. Replace the malachite green solution as it evaporates so that the paper remains saturated during heating. Do not allow the slide to become dry.
5. Remove the paper using forceps, allow the slide to cool, and rinse the slide with water for 30 seconds
6. Counterstain with safranin for 60 to 90 seconds
7. Rinse the slide with water for 30 seconds.
8. Blot dry with bibulous paper (figure 10.2*e*) and examine under oil immersion. A cover slip is not necessary. The spores, both endospores and free spores, stain green; vegetative cells stain red.



The Schaeffer-Fulton spore stain method

CULTURING OF BACTERIA

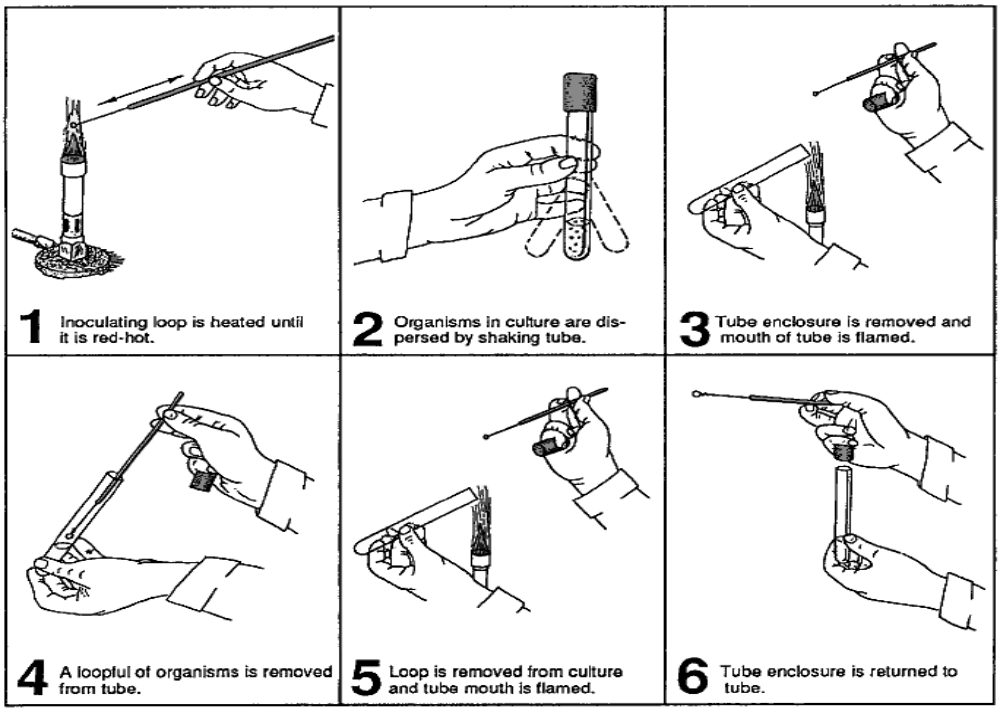
SUBCULTURING

Use an **inoculating needle** for agar deeps and an **inoculating loop** for the agar plate and the broths.

Subculture a broth culture of *Staph. aureus* and *Bacillus subtilus* onto a slant. Subculture a mix of these cultures in a new slant. The goal here is to be able to identify the 2 species of bacteria mixed together, and to isolate them for purposes of purification.

Aseptic technique

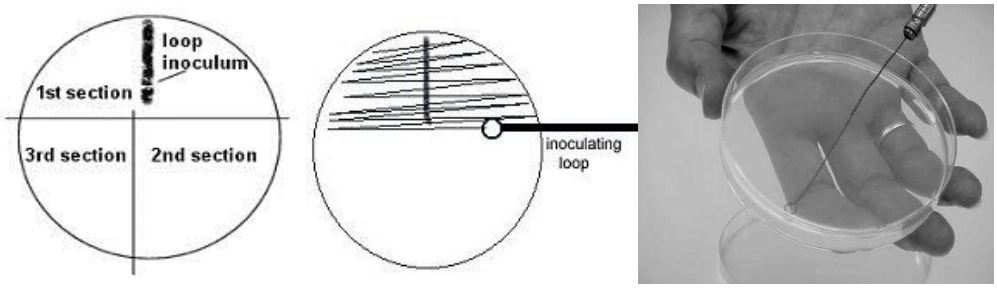
1. Have both the culture that you are taking the inocula from and the new, sterile medium in front of you. Be sure that the new medium is already labeled so you do not confuse the various cultures.
2. Pick up both tubes in the hand.
3. Heat the inoculating wire of the loop or needle until red-hot, and be sure that the entire wire is sterilized. You are now ready to pick the inoculum from the bacterial culture.
4. keeping the sterile inoculation instrument in your hand, remove both tube caps with your little finger.
5. Run the tops of the tubes through the heat to create an updraft (taking air contaminants away from the tube entrance).
6. Go into the tube to take your inoculum and quickly place the inoculum into the new medium tube.
7. Sterilize the tops of the tubes again (to eliminate potential air contamination again) and replace the caps.
8. Incubate the plates and tubes in the 30 ºC incubator.



Procedure for removing organisms from a broth culture with inoculating loop

Streaking an agar plate

Until you become well- acquainted with this procedure, you might want to draw the 3 sections that you will streak inside of, on the back (bottom of plate containing agar medium) with a sharpie pen.



1. Pick up a loopful of your inoculum from either a broth or an agar culture. Using a sterile agar medium plate (lift the lid just enough to insert the loop), streak a vertical line straight down.

2. When streaking the agar, keep the loop horizontal and only streak the surface of the agar:

DO NOT DIG INTO THE AGAR.

3. Move the loop in a zigzag pattern across the agar until 1/3 of the plate is covered, finishing the first section.

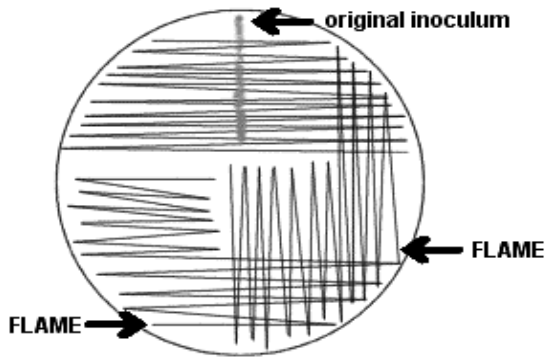
4. Sterilize the loop in the flame and let it cool before continuing to spread the bacteria. You can do this by 1) sticking the hot loop in the agar at the edge of the agar away from the bacteria, or 2) just holding the loop for a few seconds while it cools.

5. Rotate the plate about 90 degrees and spread the bacteria from the first streak into a second area using the same zigzag spread technique.

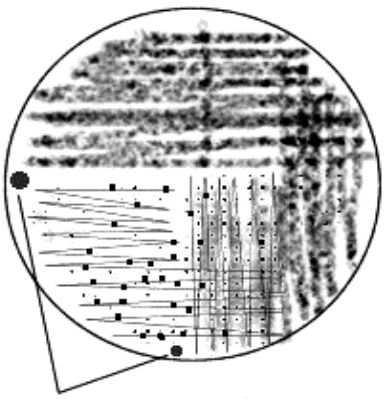
6. Sterilize the loop again. Rotate the plate about 90 degrees and spread the bacteria from the second streak into the 3rd area in the same pattern.

7. Sterilize the loop again. Replace the lid and invert the plate. Incubate the plate.

You should see bacterial cells growing along streak lines and in isolated areas.



original inoculum picked up only once
loop glides over top of agar medium
loop flamed at beginning of new section
3-5 crossovers from new section into previous
then streak only within that section

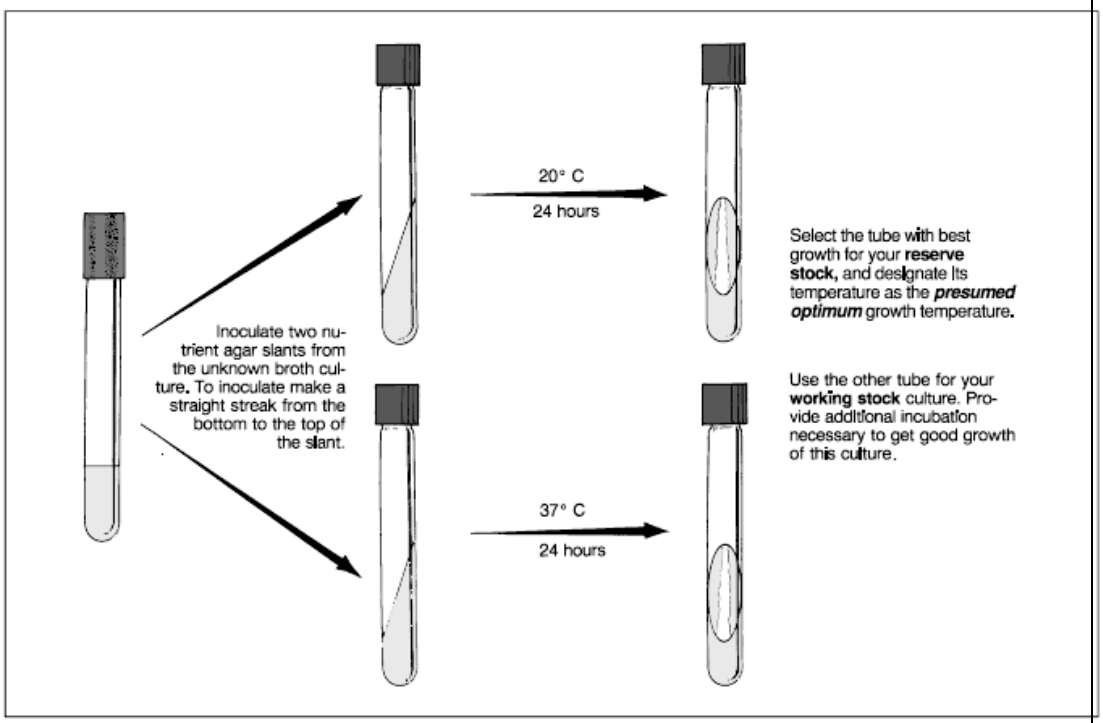


contaminants not on streak lines

well-isolated colonies in 3rd section

PREPARATION AND CARE OF STOCK CULTURES

Different types of organisms require different kinds of stock media, but for those used in this lab, nutrient agar slants will suffice. For each unknown, you will inoculate two slants. One of these will be your reserve stock and the other one will be your working stock. The **reserve stock culture** will *not* be used for making slides or routine inoculations; instead, it will be stored in the refrigerator after incubation until some time later when a transfer may be made from it to another reserve stock or working stock culture. The **working stock culture** will be used for making slides and routine inoculations. When it becomes too old to use or has been damaged in some way, replace it with a fresh culture that is made from the reserve stock.



Stock culture procedure

Note in the above figure that one slant will be incubated at 20° C and the other at 37° C. This will enable you to learn something about the optimum growth

temperature of your unknown. Proceed as follows:

First period

Inoculate two nutrient agar slants from each of your unknowns as follows:

Materials

for each unknown:

2 nutrient agar slants (screw-cap type) gummed labels

1. Label two slants with the code number of the unknown and your initials. Use gummed labels. Also, mark one tube 20° C and the other 37° C.

2. With a loop, inoculate each slant with a straight streak *from the bottom to the top*.

3. Place the two slants in separate baskets on the demonstration table that are designated with labels for the two temperatures (20° C and 37° C). Although the 20° C temperature is thought of as "room temperature," it should be incubated in a biological incubator instead of leaving it out at laboratory room temperature. Laboratory temperatures are often quite variable in a 24-hour period.

Second period

After 24 hours incubation, evaluate the slants made from each of your unknowns, as follows:

1. Examine the slants to note the extent of growth. Some organisms require close examination to see the growth, especially if the growth is thin and translucent.

2. Determine which temperature seems to promote the best growth.

3. Record on the Descriptive Chart the *presumed* optimum temperature. (Obviously, this may not be the actual optimum growth temperature, but for all practical purposes, it will suffice for this exercise.)

4. If there is no growth visible on either slant, there are several possible explanations:

• It may be that the culture you were issued was not viable.

• Another possibility might be that the organism grows too slowly to be visible at this time.

• Or, possibly, neither temperature was suitable! Think through these possibilities and decide what you should do to circumvent the problem.

5. Label the tube with the best growth **reserve stock.** Label the other tube **working stock.**

6. If both tubes have good growth, place them in the refrigerator until needed.

If one tube has very scanty growth, refrigerate the good one (reserve stock) and incubate the other one at the more desirable temperature for another 24 hours, then refrigerate.

7. Remember these points concerning your stock cultures:

• Most stock cultures will keep for 4 weeks in the refrigerator. Some fastidious pathogens will survive for only a few days. Although none of the organisms issued in this unit are of the extremely delicate type, don't wait 4 weeks to make a new reserve stock culture; instead, make fresh transfers every 10 days.

• Don't use your reserve stock culture for making slides or routine inoculations.

• Don't store either of your stock cultures in your desk drawer or a cupboard. After the initial incubation period cultures must be refrigerated. After 2 or 3 days at room temperature, cultures begin to deteriorate. Some die out completely.

DETERMINATION OF MICROBIAL NUMBERS

The most common method of measuring cell numbers is the plate or colony count. This is based on the theoretical relationship of one bacterial cell or a clump of cells giving one colony and ,therefore, the number of colonies on the plate corresponds to the original bacterial count.

Other methods include using membrane filters, direct microscopic counts, turbidimetric methods, chemical estimates (e.g. protein), dry weight and cell volume (using packed cell volume tubes or PCV).

Quantitative plating methods

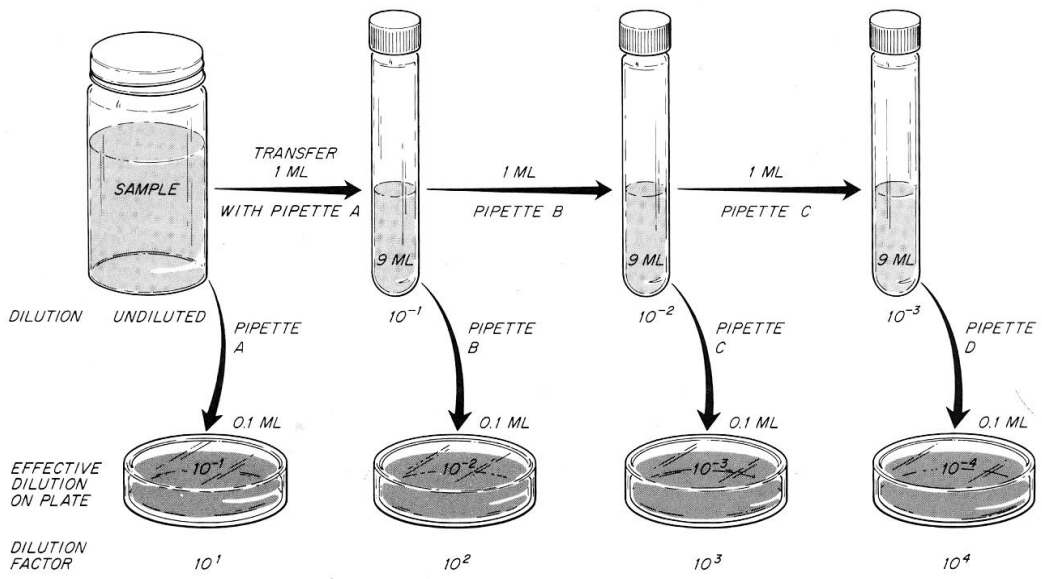
We can determine the bacterial number in a material by calculating the number of colony forming units in the material or (cfu's) since one cell or a group of cells may form a colony in the enumerating medium plate.

Two major quantitative techniques are used: the spread plate and the pour plate methods. In the spread plate we distribute the sample over the surface of a nutrient agar plate (or other media) and in the pour plate we mix the sample with the melted agar medium and then pour it in the plate. In both methods we follow the number of germinating colonies on the plate after an incubation period

To dilute the sample, a 1- g or a 1- ml of sample is diluted stepwise through a series of tubes or bottles containing a known amount of sterile buffer or salt solution.

A) Spread plate.

Dilutions are made stepwise as discussed before (see the following Figure). Be sure to change pipettes with each dilution tube. Amounts of 1/10 ml of each dilution are spread on the surface of agar plates. Then we use the dilution factor to determine the number of organisms in the original material.

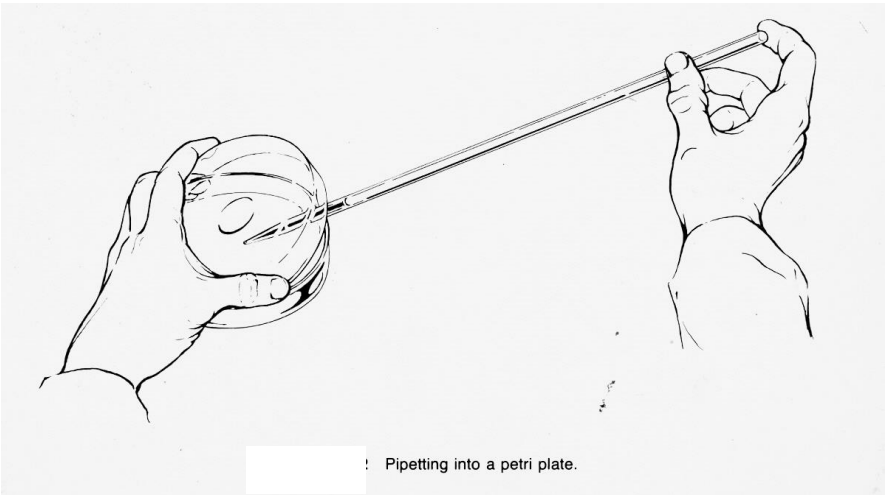


Diluting the sample for the spread plate, a quantitative plating method.

Procedure:

1- Obtain three dilution tubes and four nutrient agar plates. Label the tubes 10^{-1}, 10^{-2}, and 10^{-3}, and the plates 10^{-1} to 10^{-4}.

2- With a sterile pipette, remove 1.1 ml of the original sample. Pipette 0.1 ml on the agar plate and 1 ml to the 10^{-1} dilution tube. Mix thoroughly by shaking or by vortex.



Pipetting into a petri plate.

3- Repeat for the rest of dilutions.

4- Spread the samples on each plate using a sterile glass spreader as illustrated in the Figure

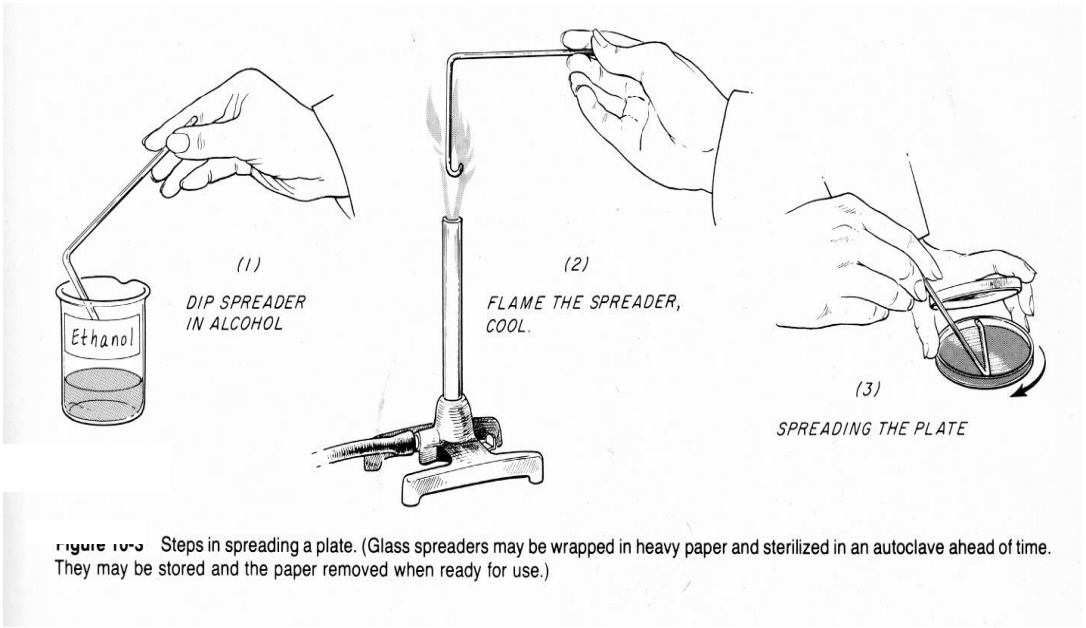


Figure 10-3 Steps in spreading a plate. (Glass spreaders may be wrapped in heavy paper and sterilized in an autoclave ahead of time. They may be stored and the paper removed when ready for use.)

The glass spreader is sterilized by dipping it in alcohol is burned off. Allow the spreader to cool then spread the sample over the surface of the agar plate by revolving the plate on your disk.

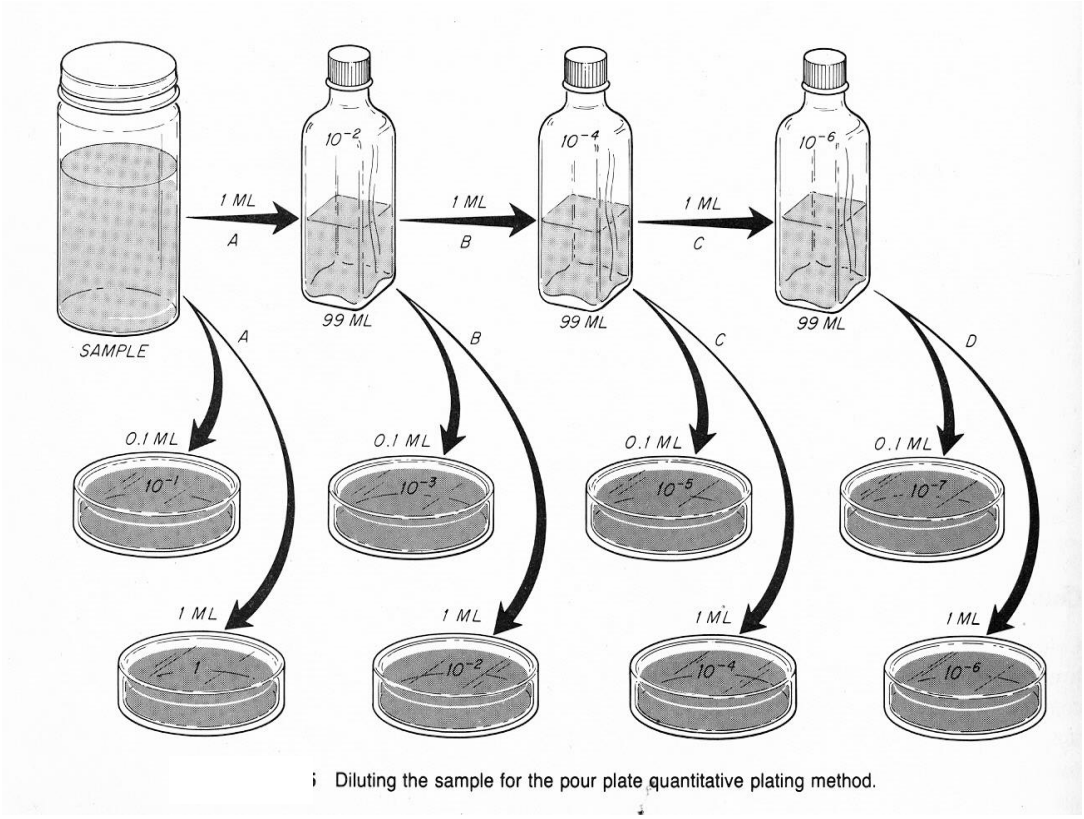
5- Incubate the petri plates at 30 ºC in an inverted position for the defined time.

6- Count the colonies on each plate containing between 20 and 200 colonies.

7- **Calculation of count**. The number of bacteria per ml of the original sample is obtained by multiplying the number of colonies on the plate by the dilution factor. For example if you counted 120 colonies on 10^{-4} dilution , then 120 x 10000= 1200000 cell per ml of the original sample. It is advisable to prepare replicates of each dilution to avoid big errors. The counts of the replicate plates should be averaged.

B) Pour plate

In this exercise we will determine the microbial count in two water samples, A and Z. A is a good quality water containing low bacterial count and Z is an impure sample containing large number of bacteria and must be diluted to obtain countable plates (see the Figure).



Diluting the sample for the pour plate quantitative plating method.

Procedure

1- Melt five tubes of nutrient agar (15 ml each). Cool to 45 ºC and have these tubes ready to use when the dilutions are made. Do not leave the plates more than few minutes before mixing the sample with the melted agar.

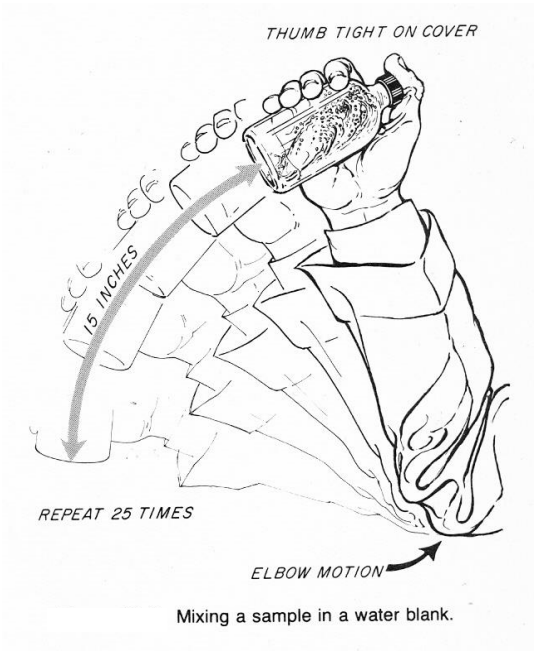
2- With a sterile pipette remove 1.1 ml of A, and place 0.1 ml into a sterile Petri plate. Drain the remaining 1 ml of the same sample into another plate. Mark the first plate A- 10^{-1} and the second A-1.

3- With a sterile pipette remove 1 ml of sample Z and put it into a 99 ml dilution blank in a bottle. This bottle now contains 1 ml of the original sample diluted 100 times. Therefore, 1 ml of this dilution is equivalent to 0.01 ml of the original sample.

4- Shake the bottle vigorously, 25 time (see the Figure) or vortex for few minutes.

5- With another sterile pipette, remove 1.1 ml from the bottle (10^{-2} dilution) and place 0.1 ml in a sterile Petri plate (10^{-3} dilution), mark it Z- 10^{-3}. Place the remaining 1 ml in another sterile plate (10^{-2} dilution); mark it Z- 10^{-2}.

6- With the same pipette take another 1 ml aliquot from the same 1/ 100 dilution bottle and transfer it to another 99 ml sterile dilution blank. Shake as before and transfer 1 ml to a Petri plate. Label this plate Z- 10^{-4}.



Mixing a sample in a water blank.

7- Pour the melted, cooled agar into the plates and mix with the samples thoroughly. This could be done by tilting the plate slightly so a wave of agar and sample travels around. Do this several times but be sure not to spill the agar over the edge of the plate.

8- Set the petri plates on a level surface. When cool, incubate at 30 ºC in an inverted position.

9- Count the colonies on each plate containing between 30 and 300 colonies and then calculate the average number of colonies (cells) in the original samples.

TURBIDIMETRIC ESTIMATION OF BACTERIAL GROWTH

A bacterial culture acts as a colloidal suspension, blocking and reflecting light that passes through it. Within certain limits the light absorbed or reflected by bacterial suspension is directly proportional to the concentration of cells in the culture. Thus by applying one of the following:

1- Nephelometry: measurement of the reflection of light rays to bacterial suspension.

2- Turbidimetry: measurement of the percentage of light absorption to bacterial suspension.

For such determination you will use a **photocolorimeter** which is a source of monochromatic light (that is, light of single wavelength). The light is passed through a bacterial culture, and the amount of light reflected or transmitted is measured by means of a photoelectric cell wired to a galvanometer.

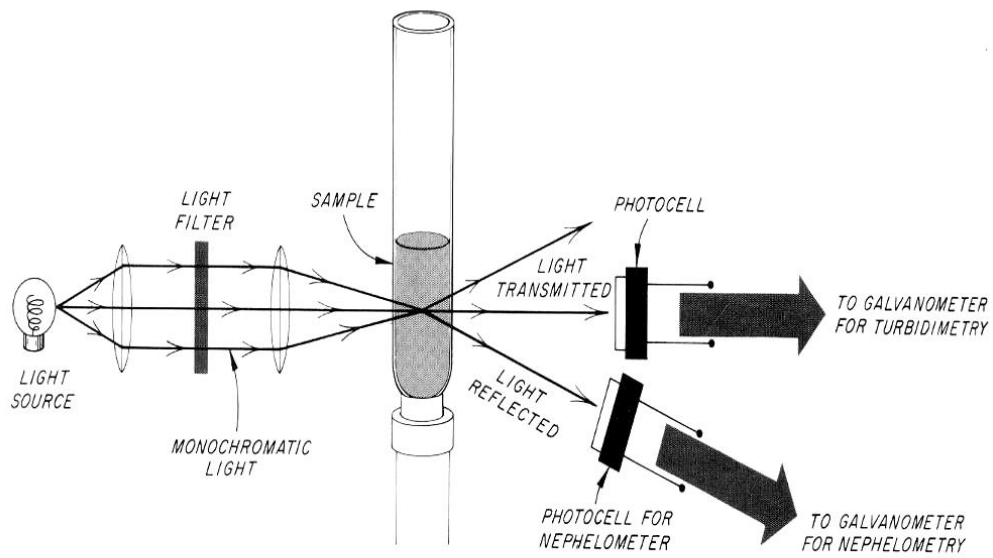
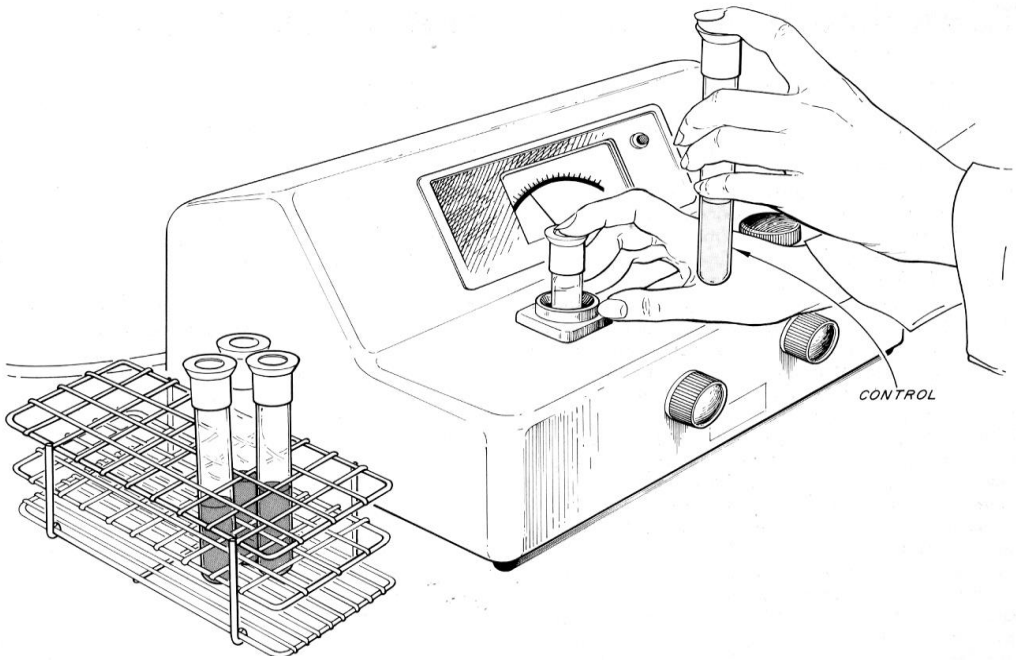


Figure 11-1 Schematic diagram of a photocolorimeter that might function as a nephelometer or a turbidimeter.

In turbidimetry the light-stopping of a culture can be expressed as the percentage of light transmitted. Within limits, this percentage is inversely proportional to the cell concentration. Ordinary, however, it is more useful to express the turbidity as **absorbance** (A), which is directly proportional to the cell concentration (see the following Figure). The absorbance is a function of the negative log of the percent transmission (-log G) that is A= log 100 – log of galvanometer reading.



Reading the absorbancy of test tubes in a turbidimeter.

Procedure

1- Plating 10^{-6}, 10^{-7}, 10^{-8} dilution of *Escherichia coli* and incubate the plates at 37^{o}C for 48h.

2- Take five tubes of sterile nutrient broth and make four serial 1:2 dilutions, of the culture in four tubes of broth, by transferring 5ml of the culture to one of sterile broth tube and transfer 5ml from one to second tube etc.

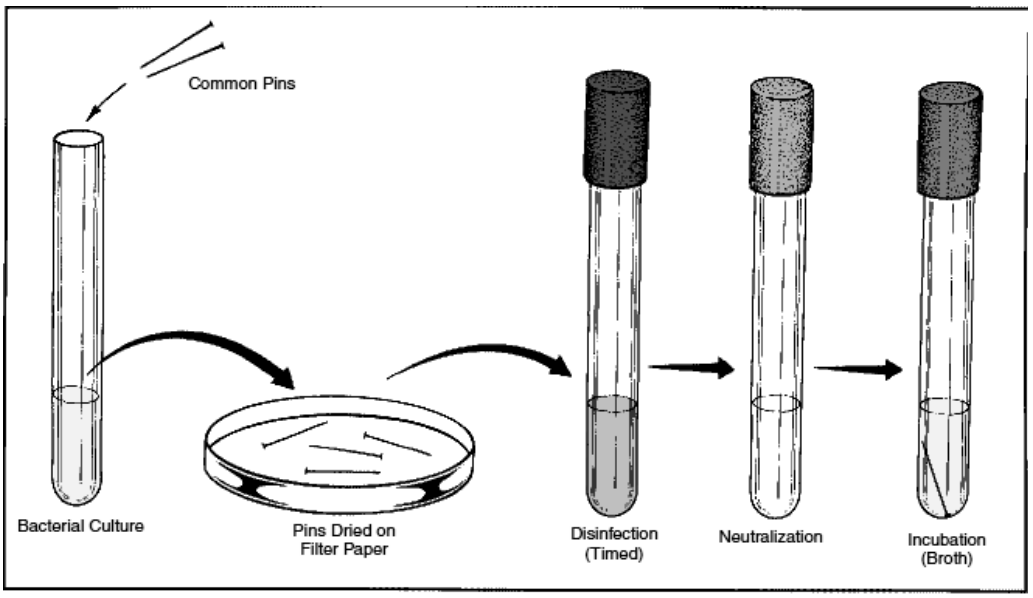
3- Use the photocolorimeter, set the galvanometer to read 100% transmission. Then determine the absorbance of the undiluted 48-hour culture of *Escherichia coli* as well as the four serially diluted cultures.

4- Record the optical density of each tube.

5- After determining the plate count of these cultures, plot the absorbances of the various dilutions against the corresponding actual bacterial count on your report sheet.

EVALUATION OF DISINFECTANTS

The use-dilution method



Procedure for use-dilution evaluation of a disinfectant

Materials:

- 2 tubes of one of the following agents:
- 5% phenol for students
- 8% formaldehyde for students
- 2 tubes of sterile water (about 7 ml each)
- 2 tubes of nutrient broth (about 7 ml each)

- Forceps
- 1 nutrient broth culture of *S. aureus*.
- 1 physiological saline suspension of a 48 hour nutrient agar slant culture of *B. megaterium*.
- 2 sterile Petri plates with filter paper in bottom several forceps and Bunsen burner
- 2 test tubes containing 36 sterile common pins in each one (pins must be plated brass, which are rustproof).

Procedure

1. Get two tubes of the disinfectant, two tubes of sterile water, and two tubes of nutrient broth. Label one of each pair *B. megaterium* and the other *S. aureus* (or other available strains).

2. Pour the broth culture of *S. aureus* into one of the tubes of pins and the saline suspension of *B. megaterium* into the other tube of pins. After decanting the organisms into a beaker of disinfectant, the pins are deposited onto filter paper in separate Petri plates to dry. Plates should be clearly labeled as to contents. Allow a few minutes for the pins to dry. Make certain, also, that a Bunsen burner and forceps are set up near the two dishes of pins.

3. Gently flame a pair of forceps, let cool, and transfer one pin from each Petri plate to the separate tubes of disinfectant. Be sure to put them into the right tubes.

4. Leave the pins in the disinfectant for the length of time indicated by your instructor.

5. At the end of the assigned time, flame the mouths of the tubes of disinfectant and carefully pour the disinfectant into the sink without discarding the pins. Then, transfer the pins into separate tubes of sterile water. Avoid transferring any of the disinfectant to the water tubes with the pins.

6. After 1 minute in the tubes of water, flame the mouths of the water and broth tubes, pour off the water, and shake the pins out of the emptied tubes into

separate, labeled tubes of nutrient broth. At this point, put one pin from each of the Petri plates into separate labeled tubes of nutrient broth to be used as positive **controls** for each organism.

7. Incubate all nutrient broth tubes with pins for 48 hours at 37° C. Examine them and record your results.

EFFECTS OF CHEMICAL AGENTS ON BACTERIA: DISINFECTANTS

Learning Objectives

Each student should be able to:

1. Determine the effectiveness of some chemical disinfectants used in hospitals or homes as antimicrobial agents
2. Calculate a phenol coefficient

Materials

- 20-hour tryptic soy broth cultures of *Staphylococcus aureus* (ATCC 25923) and
- *Pseudomonas aeruginosa* (ATCC 10145)
- 2 sterile screw-cap test tubes
- 1 sterile 5-ml pipette with pipettor
- 12 sterile 1-ml pipettes
- 48 tryptic soy broth tubes (10 ml per tube)
- Sterile water in Erlenmeyer flask
- 12 sterile tubes for making dilutions
- Commercial disinfectants such as 3% hydrogen peroxide, 70% isopropyl alcohol, bleach, or Lysol cleaner or students can bring in their own to test.

If commercial disinfectants are used, note the use, dilution and active ingredients.

- Dilute with normal tap water. The tap water need not be sterilized for commercial disinfectants.
- Phenol (carbolic acid)

- Wax pencil
- 35°C incubator
- Test-tube rack
- Bunsen burner
- Inoculating loop

Principles

Many factors influence the effectiveness of chemical disinfectants and antiseptics. The **microbicidal** (to kill) or **microbiostatic** (to inhibit) **efficiency** of a chemical is often determined with respect to its ability to deter microbial growth. The first part of this exercise will examine this effect of several chemicals. More specifically, the microbicidal efficiency of a chemical is often determined with respect to phenol and is known as the **phenol coefficient (PC).**

The phenol coefficient is calculated by dividing the highest dilution of the antimicrobial of interest, which kills all organisms after incubation for 10 minutes but not after 5 minutes, by the highest dilution of phenol that has the same characteristics.

Chemicals that have a phenol coefficient greater than 1 are more effective than phenol, and those that have a phenol coefficient less than 1 are less effective than phenol. However, this comparison should only be used for phenol- like compounds that do not exert bacteriostatic effects and are not neutralized by the subculture media used. The second part of this experiment will enable you to calculate a phenol coefficient for a select chemical.

Procedure

First Period

Growth Inhibition

1. Select one of the disinfectants and, if necessary, dilute it according to the specifications on the label (the use- dilution).

2. Place 5 ml of disinfectant into two sterile tubes. Add 0.05 ml of *P. aeruginosa* to one tube and 0.05 ml of *S. aureus* to the other.

3. Using the wax pencil, label the tubes with your name and those of the respective bacteria. Mix each of the tubes in order to obtain a homogeneous suspension.

4. At intervals of 1, 2, 5, 10, and 15 minutes, transfer 0.1 ml of the mixture containing the bacteria and disinfectant to separate tubes of tryptic soy broth. Do this for both bacteria. Also, inoculate two tubes of broth with 0.1 ml of both bacteria and mark these "controls."

5. Incubate all tubes for 48 hours at 35°C.

Phenol Coefficient (*See Safety Considerations*)

1. Dilute phenol in sterile distilled water 1/80, 1/90, and 1/100; dilute the Lysol 1/400, 1/450, and 1/500 so that the final volume in each tube is 5 ml.

2. Label 18 tryptic soy broth tubes with the name and dilution of disinfectant, the time interval of the subculture (e.g., 5 minutes, phenol 1/80), and your name. Each dilution should be tested after 5, 10, and 15 minute incubations.

3. Place in order in a test-tube rack, one test tube of each of the different Lysol and phenol dilutions for each time interval.

4. Add 0.5 ml of *S. aureus* to each tube of disinfectant and note the time. Mix each of the tubes in order to obtain a homogeneous suspension and allow the disinfectant to come into contact with the bacteria.

5. Using aseptic technique, at intervals of 5, 10, and 15 minutes, transfer one loopful from each disinfectant tube into the appropriately labeled tryptic soy broth tube.

6. Incubate all tubes for 48 hours at 35°C.

7. The experiment can be repeated with *P. aeruginosa.*

Second Period

Growth Inhibition

1. Shake and observe each of the tubes for growth.

Record the presence of growth as + and the absence of growth as –. Tabulate your results as well as the results of the class.

Phenol Coefficient

1. Shake and observe all tryptic soy broth cultures for the presence (+) or absence (–) of growth.

2. Record your observations in Part 2 of the report.

3. From your data, calculate the phenol coefficient for Lysol. For example, assume a 1/20 dilution of phenol (1 part phenol in a total of 20 parts liquid) kills *S. aureus* within 10 minutes. A 1/300 (1 to 300) dilution of Lysol also kills *S. aureus* within 10 minutes.

$$PC = \frac{300}{20} \text{ or } \frac{1/20}{1/300}$$
$$PC = 15$$

Thus, Lysol is 15 times more effective than phenol in killing *S. aureus.*

The effects of chemical agents on bacteria: antimicrobial agents (kirby-bauer method, see medical microbiology, PART 4)

BACTERIAL METABOLISM

Although fermentation and oxidation represent two different types of energy-yielding reactions, they can both be present in the same organism, as is true of facultative anaerobes.

STARCH HYDROLYSIS

Many bacteria produce enzymes called hydrolases. Hydrolases catalyze the splitting of organic molecules into smaller molecules in the presence of water. This exercise will present the hydrolysis of the carbohydrate starch. The starch molecule consists of two constituents: amylose, an unbranched glucose polymer (200 to 300 units) and amylopectin, a large branched polymer. Both amylopectin and amylose are rapidly hydrolyzed by certain bacteria, using their α-amylases, to yield dextrins, maltose, and glucose, as follows:

$$\underset{\substack{\text{(Large polysaccharide)}}}{\substack{\text{Starch}\\ \text{[Amylose + Amylopectin]}}} \xrightarrow[H_2O]{\alpha\text{-amylase}} \underset{\substack{\text{(Intermediate}\\ \text{polysaccharides)}}}{\text{Dextrins}} + \underset{\text{(Disaccharide)}}{\text{Maltose}} + \underset{\text{(Monosaccharide)}}{\text{Glucose}}$$

Gram's iodine can be used to indicate the presence of starch. When it contacts starch, it forms a blue to brown complex. Hydrolyzed starch does not produce a colour change. If a clear area appears after adding Gram's iodine to a medium containing starch and bacterial growth then, amylase has been produced by the bacteria. If there is no clearing, starch has not been hydrolyzed.

Procedure

1- With a wax pencil, divide a starch agar plate into three straight sections as indicated. Label each with the bacterium to be inoculated.

2- Streak the respective bacteria onto the plate in a straight line within its section.

3- Incubate the plate for 24 to 48 hours at 35°C.

4- Place several drops of Gram's iodine on each of the line streaks on the starch agar plate. If the area around the line of growth is clear, starch has been

hydrolyzed, and the test is positive; if it is not clear or the entire medium turns blue, starch has not been hydrolyzed, and the test is negative.

METHYL RED TEST

All enteric bacteria catabolize glucose for their energy needs. However, the end products vary depending on the enzyme pathways present in the bacteria. The pH indicator methyl red detects a pH change to the acid range as a result of acidic end products such as lactic, acetic, and formic acids. This test is of value in distinguishing between *E. coli* (a mixed acid fermenter) and *E. aerogenes* (a butanediol fermenter). Mixed acid fermenters such as *E. coli* produce a mixture of fermentation acids and thus acidify the medium. Butanediol fermenters such as *E. aerogenes* form butanediol, acetoin, and fewer organic acids. The pH of the medium does not fall as low as during mixed acid fermentation. At a pH of 4, the methyl red indicator turns red—a positive methyl red test. At a pH of 6, the indicator turns yellow—a negative methyl red test.

Procedure

First Period

1. Label each of the MR-VP broth media tubes with the bacterial name (*E. coli, E. aerogenes,* and *K. oxytoca* or other available strains) to be inoculated, your name, and date.
2. Using aseptic technique, inoculate each tube with the appropriate bacterium by means of a loop inoculation.
3. Incubate tubes at 35°C for 24 to 48 hours. For slow fermenters, it may take four to five days.

Second Period

1. Transfer 1/ 3 of each culture into an empty test tube and set these aside for the Voges- Proskauer test.

2. To the remaining 2/3 of the culture, add 0.2 ml (about 4 to 5 drops) of methyl red indicator. Carefully note any colour change (a red colour is positive).

VOGES- PROSKAUER TEST

The Voges-Proskauer test (named after Daniel Voges, German physician, and Bernhard Proskauer, German hygienist, in the early twentieth century) identifies bacteria that ferment glucose, leading to 2,3- butanediol accumulation in the medium. The addition of 40% KOH and a 5% solution of alpha- naphthol in absolute ethanol (Barritt's reagent) will detect the presence of acetoin—a precursor in the synthesis of 2,3- butanediol. In the presence of the reagents and acetoin, a cherry-red colour develops. Development of a red colour in the culture medium, 15 minutes following the addition of Barritt's reagent, represents a positive VP test; absence of a red colour is a negative VP test.

Procedure

Use the aliquot from the methyl red test. While wearing disposable gloves, add 0.6 ml of Barritt's solution A and 0.2 ml of solution B to each culture, and shake vigorously to aerate. (Alternatively, about 15 drops of reagent A followed by 5 drops of reagent B works fairly well and avoids pipetting.) Positive reactions occur at once or within 20 minutes and are indicated by the presence of a red colour.

INDOLE PRODUCTION

The amino acid tryptophan is found in nearly all proteins. Bacteria that contain the enzyme tryptophanase can hydrolyze tryptophan to its metabolic products, namely, indole, pyruvic acid, and ammonia. The bacteria use the pyruvic acid and ammonia to satisfy nutritional needs while indole is not used and accumulates in the medium. The presence of indole can be detected by the addition of Kovacs' reagent. Kovacs' reagent reacts with the indole, producing a bright red compound

on the surface of the medium. Bacteria producing a red layer following addition of Kovac's reagent are indole positive; the absence of a red colour indicates tryptophan was not hydrolyzed, and the bacteria are indole negative.

Procedure

First Period

1. Label each of the SIM deep tubes with the name of the bacterium to be inoculated (*E. coli, P. vulgaris, and E. aerogenes),* your name, and date. (If SIM medium is unavailable, tryptic soy broth is a good substitute for testing indole production.)

2. Using aseptic technique, inoculate each tube by a stab inoculation or with a loopful of culture.

3. Incubate the tubes for about 24 hours at 35°C.

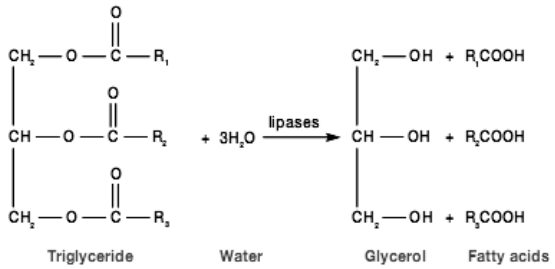
Second Period

Remove the tubes from the incubator and while wearing disposable gloves, add 0.5 ml (about 10 drops) of Kovac's reagent to each tube, and shake the tube gently. A deep red colour develops in the presence of indole. Negative reactions remain colourless or light yellow.

LIPID HYDROLYSIS

Lipids are high molecular weight compounds possessing large amounts of stored energy. The two common lipids catabolized by bacteria are the triglycerides (triacylglycerols) and phospholipids. Triglycerides are hydrolyzed by the enzymes called lipases into glycerol and free fatty acid molecules as indicated in the following diagram. Glycerol and free fatty acid molecules can then be taken up by the bacterial cell and further metabolized through reactions of glycolysis, oxidation pathway, and the citric acid cycle. These lipids can also enter other metabolic pathways where they are used for the synthesis of cell membrane phospholipids. Since phospholipids are functional components of all cells, the

ability of bacteria to hydrolyze host-cell phospholipids is an important factor in the spread of pathogenic bacteria. In addition, when lipase- producing bacteria contaminate food products, the lipolytic bacteria hydrolyze the lipids, causing spoilage termed rancidity.



When these same lipids are added to an agar solidified culture medium and are cultured with lipolytic bacteria, the surrounding medium becomes acidic due to the release of fatty acids. By adding a pH indicator to the culture medium, it is possible to detect the hydrolysis of lipids by a colour change. For example, spirit blue agar with Bacto lipase reagent has a lavender colour. It turns royal blue around lipolytic bacterial colonies due to the acid pH.

Procedure

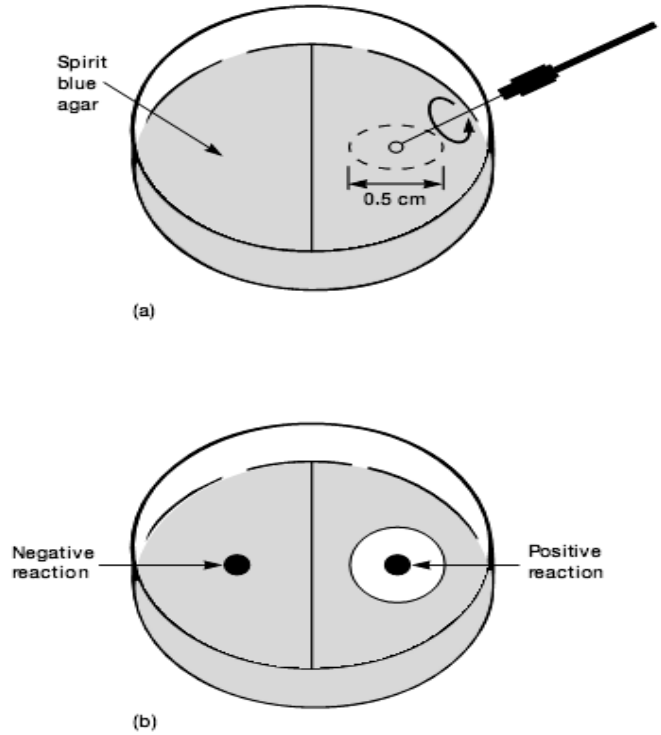
First Period

1. With a wax pencil, divide the bottom of a spirit blue agar plate in half and label half the plate *P. mirabilis* and the other half *S. epidermidis* (or use the available strains). Place your name and date on the plate.

2. Spot- inoculate (a in the figure) the spirit blue agar plates with the respective bacteria.

3. Incubate the plate in an inverted position for 24 to 48 hours at 35°C.

Lipid Hydrolysis. (**a**) Procedure for spot inoculating a spirit blue agar plate. (**b**) Positive and negative reactions.



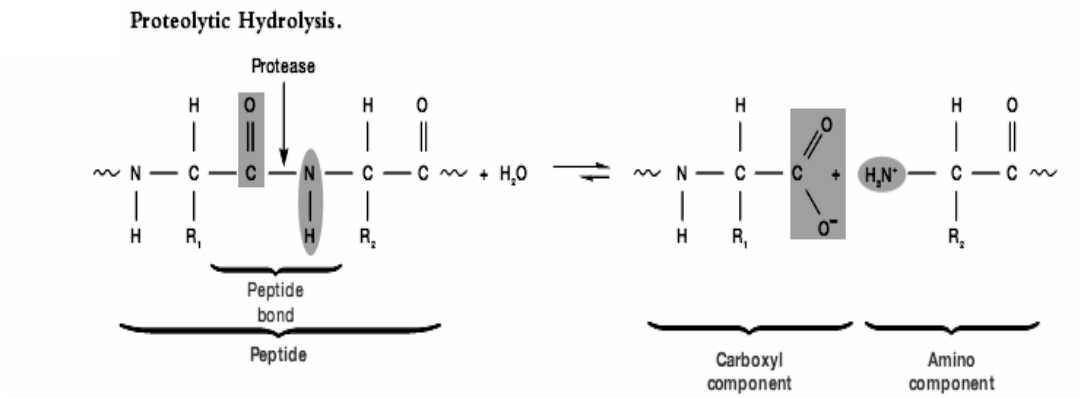
Second Period

1. Examine the plate for evidence of lipid hydrolysis (see the Figure below). Hydrolysis is evidenced by a blue zone around the bacterial growth. If no lipid hydrolysis has taken place, the zone around the colony will remain lavender.
2. Measure the zone of hydrolysis.

CASEIN HYDROLYSIS

Casein is a large milk protein incapable of permeating the plasma membrane of bacteria (Its presence is the reason milk is white.) Therefore, before casein can be used by some bacteria as their source of carbon and energy, it must be degraded into amino acids. Bacteria accomplish this by secreting proteolytic enzymes that catalyze the hydrolysis of casein to yield amino acids, which are then transported into the cell and catabolized. When milk is mixed with plate count agar, the casein

in the milk makes the agar cloudy. Following inoculation of the plate count agar, bacteria that liberate proteases (e.g., caseinase) will produce a zone of proteolysis (a clear area surrounding the colony). Clearing of the cloudy agar (a positive reaction) is the result of a hydrolytic reaction that yields soluble amino acids. In a negative reaction, there is no protease activity, and the medium surrounding the bacterial colony remains opaque.



Procedure

First Period

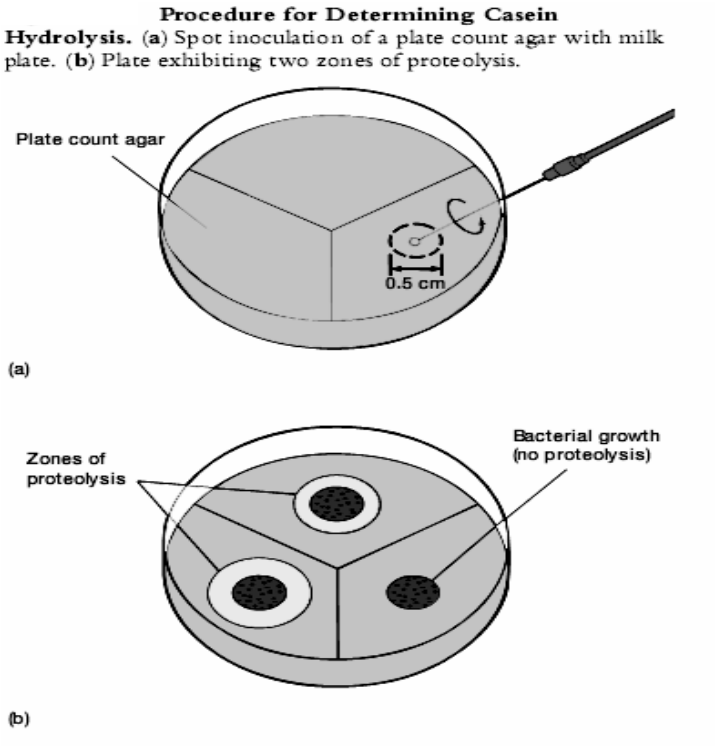
1. Melt the tubes of plate count agar by placing them in the boiling water bath. After melting, place the tubes in the 48° to 50°C water bath for 10 minutes.

2. With a wax pencil, mark the bottom of a petri plate into three sections: label one *E. coli*, the second, *B. subtilis*, and the third, *P. aeruginosa*. Add your name and date to the plate.

3. Pipette 2 ml of warm (48° to 50°C) sterile skim milk into the petri plate. Add the melted agar and mix thoroughly by moving the plate in a circular motion. Allow this medium to gel on a cool, level surface.

4. As shown in the figure, aseptically spot inoculate each third of the petri plate with the appropriate bacterium as per the label. Place a loopful of culture on the center of each section and spread it in a circular fashion to cover an area about the size of 5 to18 mm in diameter or less.

5. Incubate the plate in an inverted position at 35°C for 24 to 48 hours.



Second Period

Examine the plate for the presence or absence of a clear zone (zone of proteolysis) surrounding the growth of each of the bacterial test organisms. You can see the clear zones best against a dark background.

PART (3). INDUSTRIAL MICROBIOLOGY

FACTORS AFFECTING BACTERIAL GROWTH

1- EFFECT OF TEMPERATURE

Materials 1

- Nutrient broth cultures of *Serratia marcescens, Bacillus stearothermophilus,* and *Escherichia coli* or any available strains.
- 2 nutrient agar slants
- 5 tubes of nutrient broth

Procedure

1. Label the tubes as follows:

Slants: Label both of them *S. marcescens;* label one tube 25° C and the other tube 38° C.

Broths: Label each tube of nutrient broth with your other organism and one of the following five temperatures: 5° C, 25° C, 38° C, 42° C, or 55° C.

2. Inoculate each of the tubes with the appropriate organisms. Use a wire loop.

3. Place each tube in one of the five baskets that is labeled according to incubation temperature.

Note: The instructor will see that the 5° C basket is placed in the refrigerator and the other four are placed in incubators that are set at the proper temperatures.

Materials 2

- Slants and broth cultures that have been incubated at various temperatures
- Spectrophotometer and cuvettes
- Tube of sterile nutrient broth

Procedure

1. Compare the nutrient agar slants of *S. marcescens.* Using colored pencils, draw the appearance of the growths.

2. Shake the broth cultures and compare them, noting the differences in turbidity. Those tubes that appear to have no growth should be compared with a tube of sterile nutrient broth.

3. If a spectrophotometer is available, determine the turbidity of each tube.

4. If no spectrophotometer is available, record turbidity by visual observation (as no growth, weak, medium and good growth).

5. Exchange results with other students to complete data collection for experiment.

2- EFFECT OF pH

First period

Materials

- 1 tube of nutrient broth of each of the following pH values: 3.0, 5.0, 7.0, 8.0, 9.0 and 10.0
- Broth cultures of *Escherichia coli, Staphylococcus aureus, Alcaligenes faecalis* and *Saccharomyces cerevisiae*

Procedure

1. Inoculate a tube of each of these broths with one organism.

2. Incubate the tubes of *E. coli, S. aureus,* and *A. faecalis* at 37° C for 48 hours. Incubate the tubes of *S. ureae, C. glabrata,* and *S. cervisiae* at 20° C for 48 to 72 hours.

Second period

Materials:

- Spectrophotometer
- 1 tube of sterile nutrient broth
- Tubes of incubated cultures at various pHs

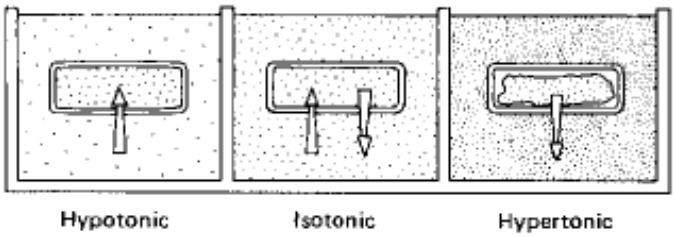
Procedure

1. Use the tube of sterile broth to calibrate the spectrophotometer and measure the %T of each culture. Record your results in tables.

2. Plot the O.D. values in a graph.

3- EFFECT OF OSMOTIC PRESSURE

Organisms that thrive in hypertonic solutions are designated as halophiles or osmophiles. If they require minimum concentrations of salt (NaCl and other cations and anions) they are called **halophiles.** Obligate halophiles require a minimum of 13% sodium chloride. **Osmophiles,** on the other hand, require high concentrations of an organic solute, such as sugar.



Osmotic variabilities

The salt concentrations will be 0.5, 5, 10, and 15%. After incubation for 48 hours and several more days, comparisons will be made of growth differences to determine their degrees of salt tolerances.

Materials:

- 1 Petri plate of nutrient agar containing: 0.5, 5, 10, 15 NaCl.
- Nutrient broth cultures of *Escherichia coli, Staphylococcus aureus* and *Halobacterium salinarium* or any available strains.

Procedure

1. Mark the bottoms of the four Petri plates (strain and concentration).

2. Streak each organism in a straight line on the agar, using a wire loop.

3. Incubate all the plates for 48 hours at room temperature with exposure to light (the pigmentation of *H. salinarium* requires light to develop).

4. Measure growth as described in "turbidity" experiment and write your results.

STANDARD ANALYSIS OF WATER

Because of humanity's increasing numbers and developing technologies, the earth's supply of potable water– long taken for granted– has become a precious resource to be carefully guarded. Everyone today must become aware of the pressing need for an adequate supply of clean water. The standard analysis of water for the presence of coliform bacteria (one of the most important indicators for water pollution) is in three parts:

1- **Presumptive test:** in the presumptive test you look for microorganisms (coliforms) capable of fermenting lactose with gas production.
2- **Confirmed test:** in the confirmed test you culture from those lactose- broth tubes of the presumptive test that show growth and gas production onto media that are selective and differential for the coli aerogenes group.
3- **Completed test:** in the completed test you isolate and grow in pure culture the organisms that gave reactions typical of coliforms on the confirmatory media; these should demonstrate the typical coliform morphology and physiology, including the ability to ferment lactose with gas formation.

Procedure

Presumptive test

1- Inoculate 10 ml portions of water sample into different one of three tubes containing 10 ml of lactose broth of double strength.
2- Inoculate 1ml and 0.1ml of sample into test tubes of lactose broth of single strength.
3- Incubate at 37^{o}C for 48h.

Confirmed test

This test should be applied to all samples giving a positive presumptive test.

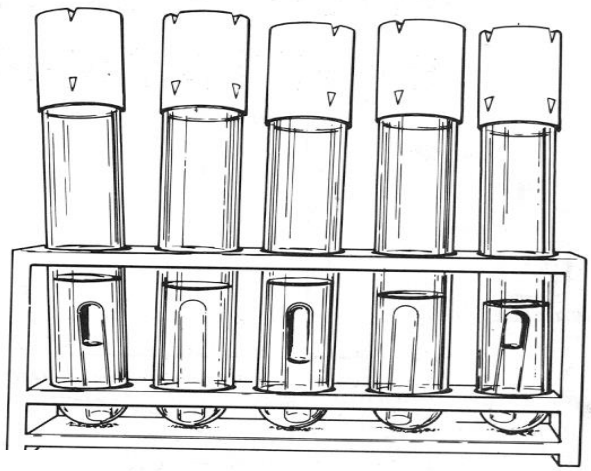
1- From the positive tube of smallest inoculum of water, streak a plate of eosin-methylene-blue (EMB) agar to give well isolated colonies.

2- Incubate at 37°C for 48h. If typical colonies have developed upon the plate within this period, the confirmed test may be considered positive.

Completed test

Coliform organisms on EMB agar form darkish colonies that often exhibit a greenish metallic sheen.

1- Transfer two colonies of coliform into an agar slope and lactose broth fermentation tubes
2- Incubate at 37°C for 2 days.
3- From the agar slope make a Gram stain and a spore stain.



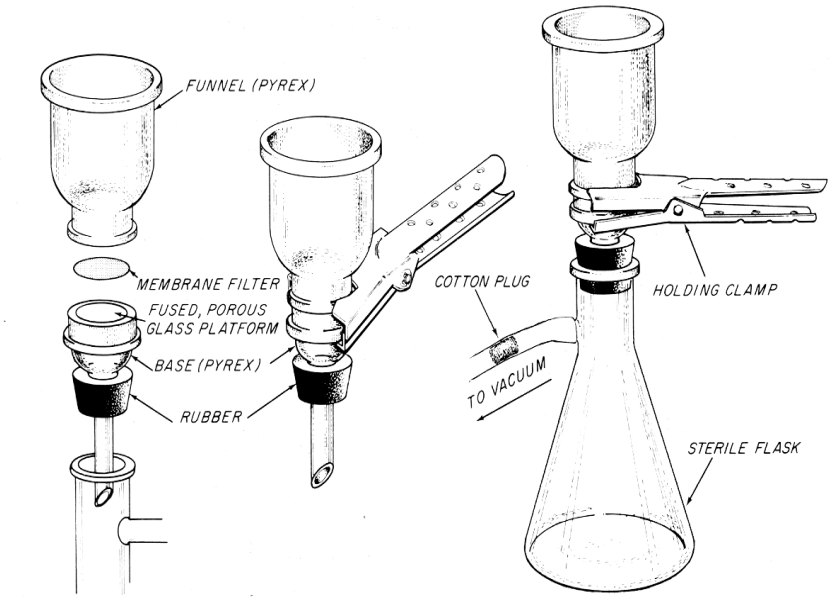
The presumptive test for the coliform group, showing gas production from lactose broth.

MEMBRANE FILTER TECHNIQUE IN WATER ANALYSIS

A membrane filter has been developed to filter bacteria from water and other materials. The trapped bacteria are then grown directly on the filter by placing it on a suitable growth medium. Through the use of particular selective medium, the numbers of coliforms and certain other species of bacteria in the sample can be determined.

Procedure

1- Take the sterile membrane filter apparatus with sterile membrane filter (see the Figure below).



The membrane-filter apparatus.

2- Pour about 20 ml of sterile distilled water into the funnel before adding the sample

3- Add known volume of water sample into the funnel of filter unit

4- Add sterile distilled water equal in volume to the sample to the funnel two times to rinse cells from the measuring vessel.

5- Turn on the vacuum and allow all the liquid to pass through the filter into the flask.

6- Use sterile distilled water equal to the total amount of filtrate to rinse cells and allow the vacuum to run until the filter appear dry.

7- Turn of the vacuum and remove the membrane with sterile forceps to the appropriate medium in a small petri dish.

8- Push the membrane against the far side of the petri dish and onto the medium and roll it on to the medium to avoid entrapment of air bubbles under the membrane.

9- Incubate at the recommended temperature and time.

10- Calculate the numbers of organisms per 100 ml by using the formula:

Indicator count per 100 ml = Total number of colonies counted / number of ml of the sample tested X 100.

Total coliform test

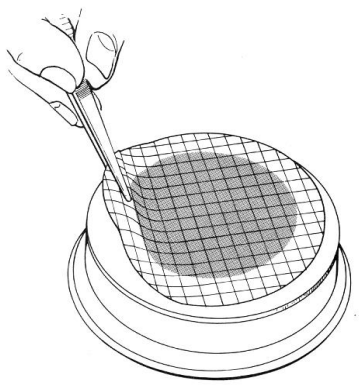
1- Insert a sterile absorbent pad into each of three petri dishes.

2- Add 1.8 – 2ml of M-Endo broth to the surface of each pad.

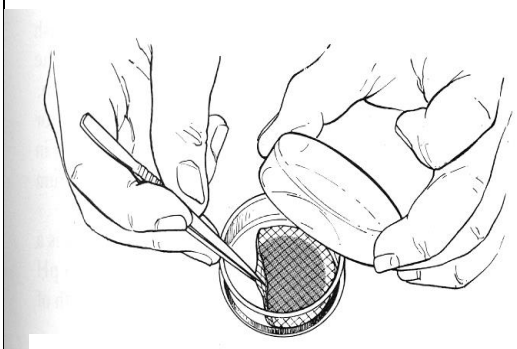
3- Add 1, 4 and 15 ml of water sample to the surface of each pad and label plates with these volumes.

4- Incubate the prepared plates in an inverted position at 37°C for 22-24h.

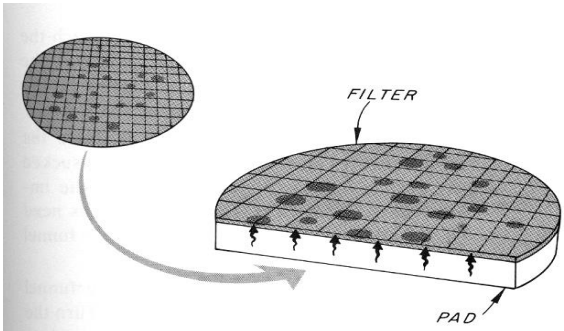
5- Count colonies that are pink to dark red with a golden green metallic sheen. Use a plate containing 20-80 colonies and no more than 200 of all types of colonies.



Removal of filter from the filtering apparatus.



Placing the filter on a pad in a petri plate.



The diffusion of broth from the pad to the filter to support growth.

Fecal coliform test

1- Insert a sterile absorbent pad into each of three sterile petri dishes.

2- Add 1.8 to 2ml M-FC broth to the surface of each pad.

3- Add 1, 2 and 10 ml water samples to petri dishes and label plates with these volumes.

4- Incubate prepared plates in 44.5^{o}C water bath for 22 hours.

5- Count blue-colored colonies with coliform characteristics. Use the plate containing 20-60 colonies.

Fecal *Streptococcus* test

1- Obtain three petri dishes containing KF agar.

2- Add 1, 5 and 25 ml of water samples into petri dishes and label petri plates with these volumes.

3- Incubate the prepared plates for 48 hours at 37^{o}C.

4- Examine the plates for colonies that are light pink and flat and for smooth dark red ones with pink margins. Count the plate that has 20-100 colonies.

$$\text{FC/FS} = \frac{\text{Number of fecal coliform per ml}}{\text{Number fecal streptococci per ml}}$$

- An **FC/FS** greater than 4 shows strong evidence of pollution derived from human waste.
- An **FC/FS** less than 0.7 indicate pollution derived from livestock or poultry waste.
- If **FC/FS** is between 2 and 4, it suggests a predominance of human waste in mixed polluation.

FOOD MICROBIOLOGY

Introduction

Microbiologists have always been aware that foods, especially milk, have served as important inanimate vectors in the transmission of disease. Foods contain the organic nutrients that provide an excellent medium to support the growth and multiplication of microorganisms under suitable temperatures.

Food and dairy products may be contaminated in a variety of ways and from a variety of sources:

1. Soil and water: Food- borne organisms that may be found in soil and water and that may contaminate food are members of the genera including: *Alcaligenes, Bacillus, Citrobacter, Clostridium, Pseudomonas, Serratia, Proteus, Enterobacter,* and *Micrococcus.* The common soil and water molds include *Rhizopus, Penicillium, Botrytis, Fusarium,* and *Trichothecium*.

2. Food utensils: The type of microorganisms found on utensils depends on the type of food and the manner in which the utensils were handled.

3. Enteric microorganisms of humans and animals: The major members of this group are *Bacteroides, Lactobacillus, Clostridium, Escherichia, Salmonella, Proteus, Shigella, Staphylococcus,* and *Streptococcus.* These organisms find their way into the soil and water, from which they contaminate plants and are carried by wind currents onto utensils or prepared and exposed foods.

4. Food handlers: People who handle foods are especially likely to contaminate them because microorganisms on hands and clothing are easily transmitted. A major offending organism is *Staphylococcus,* which is generally found on hands and skin, and in the upper respiratory tract. Food handlers with poor personal hygiene and unsanitary habits are most likely to contaminate foods with enteric organisms.

5. Animal hides and feeds: Microorganisms found in water, soil, feed, dust, and fecal debris can be found on animal hides. Infected hides may serve as a source of infection for workers, or the microorganisms may migrate into the musculature of the animal and remain viable following its slaughter. By enumerating microorganisms in milk and foods, the quality of a particular sample can be determined. Although the microorganisms cannot be identified, the presence of a high number suggests a good possibility that pathogens may be present. Even if a sample contains a low microbial count, it can still transmit infection.

In the laboratory procedures that follow, you will have an opportunity directly and indirectly to enumerate the number of microorganisms present in milk and other food products and to thereby determine the quality of the samples

Like water and eating utensils, food can be sources of diseases caused by microorganisms. Food preservation is used to prevent contamination by using of environmental extremes, such as acidic surroundings or heat, to inhibit or destroy the microorganisms in food.

The standard plate count, as well as the multiple tube test, can be used on foods much in the same manner that they are used on milk and water to determine total counts and the presence of coliforms. To get the organisms in suspension, however, a food blender is necessary.

Samples of different foods will be tested for total numbers of bacteria. The Figure below illustrates the general procedure.

BACTERIAL COUNT OF A FOOD PRODUCT

- **Materials**
- 20 g of hamburger or chicken
- 180 ml of sterile distilled water

- weighing paper
- scale or balance
- Bunsen burner
- colony counter
- plate count agar (Standards Methods Agar)
- 2 X 99-ml sterile saline dilution blanks
- sterile 1-ml pipettes with pipettor
- 5 sterile petri plates
- 35°C incubator
- wax pencil
- boiling water bath
- 48° to 50°C water bath for cooling tubes

Learning Objectives

After completing the tests, you should be able to:

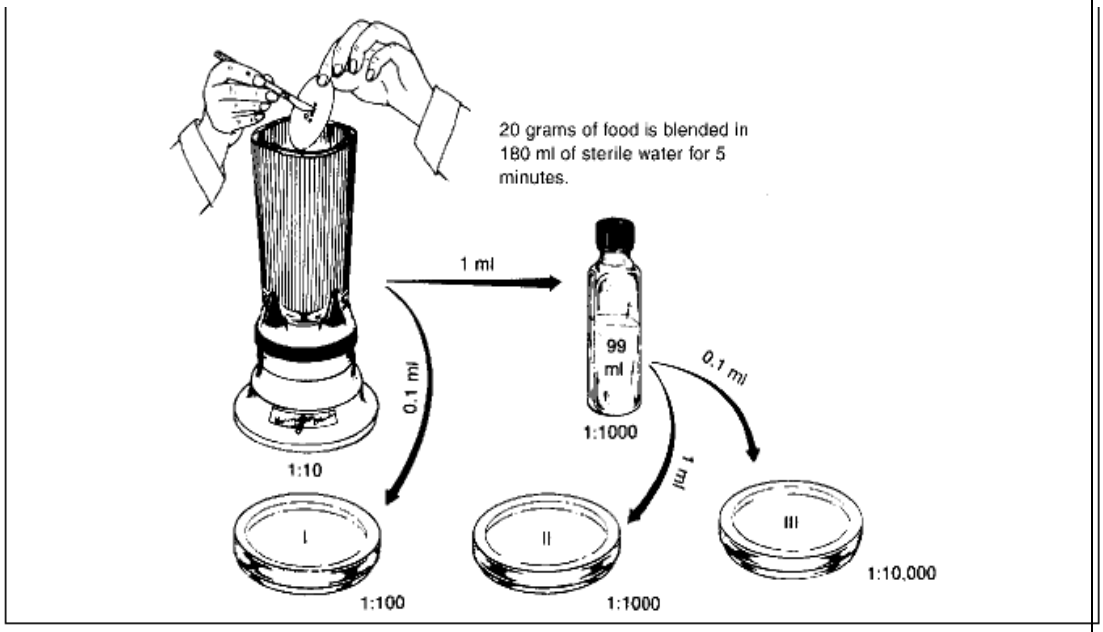
1. Explain why the standard plate count is used in food quality control.
2. Determine the number of bacteria in a food sample by performing a standard plate count.

Principles

The sanitary control of food quality is primarily concerned with testing food products for the presence of specific microorganisms. Food products are the primary vehicle responsible for the transmission of microbial diseases of the gastrointestinal system. For this reason, food products are routinely examined for the presence of bacteria.

The **heterotrophic plate count** can be used to determine the number of viable bacteria in a food sample. The larger the count, the greater the likelihood that specific pathogens capable of causing disease will be present and also that the food will spoil. Normally, raw hamburger should not contain over 10^6 bacteria per gram. One of the limitations of the heterotrophic plate count is that only bacteria

capable of growing in the culture medium, under the environmental conditions provided, will be counted. As a result, a medium that supports the growth of most heterotrophic (requiring organic carbon) bacteria is commonly used. The following Figure illustrates the procedure for performing a heterotrophic plate count on a food product.



Dilution procedure for bacterial counts of food

Materials

- 3 Petri plates
- 1 bottle (45 ml) of Plate Count agar or Standard Methods agar
- 1 99 ml sterile water blank
- 2 1.1 ml dilution pipettes
- Food blender
- Sterile blender jars (one for each type of food)
- Sterile weighing paper
- 180 ml sterile water blanks (one for each type of food)

- Samples of ground meat, dried fruit, and frozen vegetables, thawed for 2 hours

Procedure

1. Using aseptic techniques, weigh out on sterile weighing paper 20 grams of food to be tested.

2. Add the food and 180 ml (or 10 g in 90 ml) of sterile water to a sterile mechanical blender jar. Blend the mixture for 5 minutes. This suspension will provide a 1:10 dilution.

3. With a 1:10 ml dilution pipette dispense from the blender 0.1 ml to plate I and 1.0 ml to the water blank.

4. Shake the water blank 25 times in an arc for 7 seconds with your elbow on the table.

5. Using a fresh pipette, dispense 0.1 ml to plate III and 1.0 ml to plate II.

6. Pour agar (50° C) into the three plates and incubate them at 35° C for 24 hours.

7. Count the colonies on the best plate and record the results.

8. Calculate the average number of bacteria per gram of sample as follows:

$$\text{Number bacteria/gram} = \text{number of colonies} \times \frac{1}{\text{dilution factor}} \times \frac{1}{\text{weight of sample}}$$

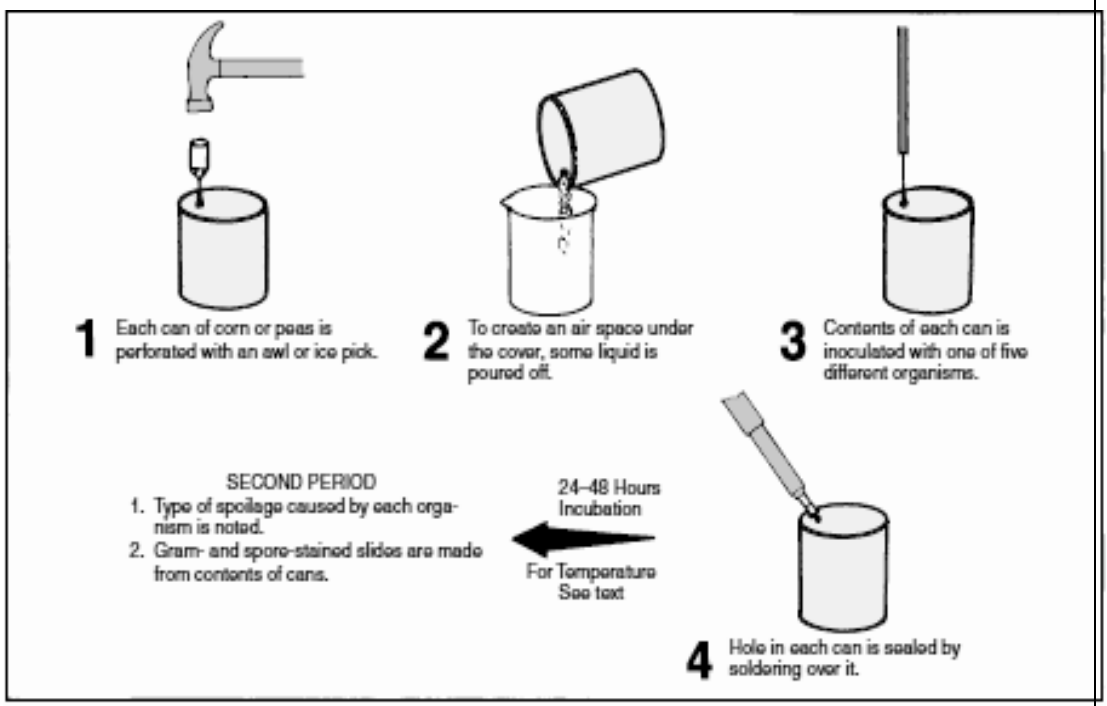
For example, if 200 colonies were counted from a 10^{-3} dilution of a 20 gram sample, the number of bacteria in one gram will be calculated from the following equation:

$$\text{Number bacteria/gram} = 200 \times \frac{1}{10^{-3}} \times \frac{1}{20\text{g}}$$

$$= 10{,}000 \text{ per gram}$$

MICROBIAL SPOILAGE OF CANNED FOOD

Spoilage of heat- processed, commercially canned foods is confined almost entirely to the action of bacteria that produce heat- resistant endospores. Canning of foods normally involves heat exposure for long periods of time at temperatures that are adequate to kill spores of most bacteria. Particular concern is given to the processing of low- acid foods in which *Clostridium botulinum* can thrive to produce botulism food poisoning. Spoilage occurs when the heat processing fails to meet accepted standards. This can occur for several reasons: (1) lack of knowledge on the part of the processor (usually the case in home canning); (2) carelessness in handling the raw materials before canning, resulting in an unacceptably high level of contamination that ordinary heat processing may be inadequate to control; (3) equipment malfunction that results in undetected underprocessing; and (4) defec- tive containers that permit the entrance of organisms after the heat process.



Canned food inoculation procedure

Our concern here will be with the most common types of food spoilage caused by heat- resistant spore- forming bacteria. There are three types: "flat sour," "T.A. spoilage," and "stinker spoilage."

Flat sour pertains to spoilage in which acids are formed with no gas production; result: sour food in cans that have flat ends. **T.A. spoilage** is caused by thermophilic anaerobes that produce acid and gases (CO_2 and H_2, but not H_2S) in low- acid foods. Cans swell to various degrees, sometimes bursting. **Stinker spoilage** is due to spore- formers that produce hydrogen sulfide and blackening of the can and contents. Blackening is due to the reaction of H_2S with the iron in the can to form iron sulfide.

In this experiment you will have an opportunity to become familiar with some of the morphological and physiological characteristics of organisms that cause canned food spoilage, including both aerobic and anaerobic endospore formers of *Bacillus* and *Clostridium*, as well as a non-spore- forming bacterium.

Working as a single group, the entire class will inoculate

10 cans of vegetables (corn and peas or others) with five different organisms. The above Figure illustrates the procedure. Note that the cans will be sealed with solder after inoculation and incubated at different temperatures. After incubation the cans will be opened so that stained microscope slides can be made to determine Gram reaction and presence of endospores.

FIRST PERIOD (Inoculations)

Materials

- 5 small cans of corn
- 5 small cans of peas
- cultures of *B. stearothermophilus, B. coagulans, C. sporogenes,*
- *C. thermosaccharolyticum,* and *E. coli* (or as instructed)
- ice picks or awls

- hammer
- solder and soldering iron
- plastic bags
- gummed labels and rubber bands

Procedure

1. Label the can or cans with the name of the organism that has been assigned to you. Use white gummed labels. In addition, place a similar label on one of the plastic bags to be used after sealing of the cans.

2. With an ice pick or awl, punch a small hole through a flat area in the top of each can. This can be done easily with the heel of your hand or a hammer, if available.

3. Pour off a small amount of the liquid from the can to leave an air space under the lid.

4. Use an inoculating needle to inoculate each can of corn or peas with the organism indicated on the label.

5. Take the cans up to the demonstration table where the instructor will seal the hole with solder.

6. After sealing, place each can in two plastic bags. Each bag must be closed separately with rubber bands, and the outer bag must have a label on it.

7. Incubation will be as follows till the next period:

• **55° C**—*C. thermosaccharolyticum* and *B. stearothermophilus*

• **37° C**—*C. sporogenes* and *B. coagulans*

• **30° C** — *E. coli*

Note: If cans begin to swell during incubation, they should be placed in refrigerator.

SECOND PERIOD (Interpretation)

After incubation, place the cans under a hood to open them. The odors of some of the cans will be very strong due to H2S production.

Materials

- can opener, punch type
- small plastic beakers
- Parafilm
- Gram staining kit
- spore staining kit

1. Open each can carefully with a punch- type can opener. If the can is swollen, hold an inverted plastic funnel over the can during perforation to minimize the effects of any explosive release of contents.
2. Remove about 10 ml of the liquid through the opening, pouring it into a small plastic beaker. Cover with Parafilm. This fluid will be used for making stained slides.
3. Return the cans of food to the plastic bags, reclose them, and dispose in a proper trash bin.
4. Prepare Gram- stained and endospore- stained slides from your canned food extract as well as from the extracts of all the other cans. Examine under brightfield oil immersion.
5. Record your observations.

MICROBIAL SPOILAGE OF REFRIGERATED MEAT

Contamination of meats by microbes occurs during and after slaughter. Many contaminants come from the animal itself, others from utensils and equipment. The conditions for rapid microbial growth in freshly cut meats are very favorable, and spoilage can be expected to occur rather quickly unless steps are taken to prevent it. Although immediate refrigeration is essential after slaughter, it will not prevent spoilage indefinitely, or even for a long period of time under certain conditions. In time, cold- tolerant microbes will destroy the meat, even at low refrigerator temperatures.

Microorganisms that grow at temperatures between 5° and 0° C are classified as being either psychrophilic or psychrotrophic. The difference between the two groups is that **psychrophiles** seldom grow at temperatures above 22° C and **psychrotrophs** (psychrotolerants or low -temperature mesophiles) grow well above 25° C. While the optimum growth temperature range for psychrophiles is 15°–18° C, psychrotrophs have an optimum growth temperature range of 25°–30° C. It is the psychrotrophic microorganisms that cause most meat spoilage during refrigeration. The majority of psychrophiles are Gram negative and include species of *Aeromonas, Alcaligenes, Cytophagia, Flavobacterium, Pseudomonas, Serratia,* and *Vibrio.* Gram-positive psychrophiles include species of *Arthrobacter, Bacillus, Clostridium,* and *Micrococcus.*

Psychrotrophs include a much broader spectrum of Gram positive and Gram negative rods, cocci,vibrios, spore-formers, and non-spore- formers. Typical genera are *Acinetobacter, Chromobacterium, Citrobacter, Corynebacterium, Enterobacter, Escherichia, Klebsiella, Lactobacillus, Moraxella, Staphylococcus,*

and *Streptococcus.*

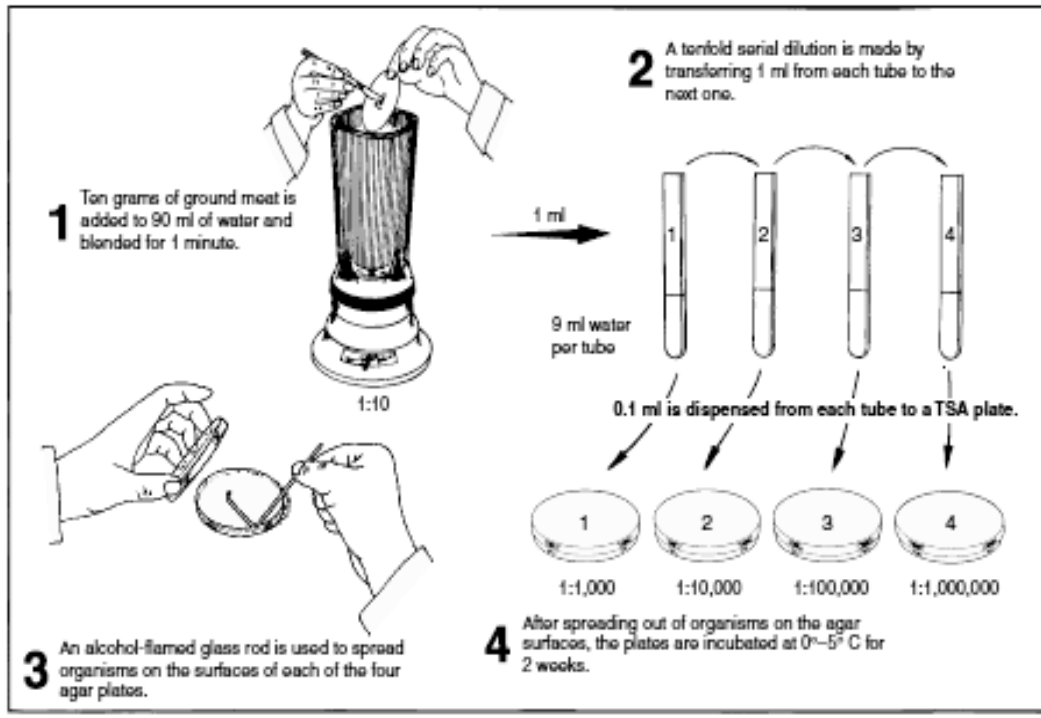
The widespread use of vacuum or modified atmospheric packaging of raw and processed meat has resulted in food spoilage due to facultative and obligate anaerobes, such as *Lactobacillus, Leuconostoc, Pediococcus,* and certain Enterobacteriaceae. Although most of the previously mentioned psychrotrophic representatives are nonpathogens, there are significant pathogenic psychrotrophs such as *Aeromonas hydrophila, Clostridium botulinum, Listeria monocytogenes, Vibrio cholera, Yersinia entercolitica,* and some strains of *E. coli.*

In addition to bacterial spoilage of meat there are many yeasts and molds that are psychrophilic and psychrotrophic. Examples of psychrophilic yeasts are

Cryptococcus, Leucosporidium, and *Torulopsis.* Psychrotrophic fungi include *Candida, Cryptococcus, Saccharomyces, Alternaria, Aspergillus, Cladosporium, Fusarium, Mucor, Penicillium,* and many more.

Our concern in this experiment will be to test one or more meat samples for the prevalence of psychrophilic- psychrotrophic organisms. To accomplish this, we will liquefy and dilute out a sample of ground meat so that it can be plated out and then incubated in a refrigerator for 2 weeks. After incubation, colony counts will be made to determine the number of organisms of this type that exist in a gram of the sample.

The following Figure illustrates the overall procedure.



Dilution and inoculation procedure

FIRST PERIOD

Materials

- ground meat and balance
- sterile foil-wrapped scoopula

- 1 blank of phosphate buffered water (90 ml)
- blender with sterile blender jar
- sterile Petri dish or sterile filter paper
- 4 large test tubes of sterile phosphate buffered water (9 ml each)
- 4 TSA plates
- 9 sterile 1 ml pipettes
- L-shaped glass spreading rod
- beaker of 95% ethyl alcohol

At Demonstration Table

1. With a sterile scoopula, weigh 10g of ground meat into a sterile Petri plate or onto a sterile piece of filter paper.
2. Pour 90 ml of sterile buffered water from water blank into a sterile blender jar and add the meat.
3. Blend the meat and water at moderate speed for 1 minute.

Student

1. Label the four water blanks 1 through 4.
2. Label the four Petri plates with their dilutions, as indicated in the figure. Add your initials and date also.
3. Once blender suspension is ready, pipette 1ml from jar to tube 1.
4. Using a fresh 1 ml pipette, mix the contents in tube 1 and transfer 1 ml to tube 2.
5. Repeat step 4 for tubes 3 and 4, using fresh pipettes for each tube.
6. Dispense 0.1 ml from each tube to their respective plates of TSA. Note that by using only 0.1 ml per plate you are increasing the dilution factor by 10 times in each plate.
7. Using a sterile L- shaped glass rod, spread the organisms on the agar surfaces. Sterilize the rod each time by dipping in alcohol and flaming gently. Be sure to let rod cool completely each time.

8. Incubate the plates for 2 weeks in the back of the refrigerator (away from door- opening) where the temperature will remain between 0° and 5° C.

SECOND PERIOD

Materials

- colony counters
- hand tally counters
- Gram staining kit

Procedure

1. After incubation, count the colonies on all the plates and calculate the number of psychrophiles and psychrotrophs per gram of meat.

2. Select a colony from one of the plates and prepare a Gram-stained slide. Examine under oil immersion and record your observations.

EXAMINATION OF MILK FOR BACTERIA

There are two methods, both with advantages and disadvantages, can be used to determine the number of organisms in milk.

1- **The agar plate method:** is more sensitive, gives more accurate results for milk containing few bacteria.

2- **The direct microscopic count:** is more applicable for milk containing many bacteria.

Milk that contains a large number of growing bacteria will have a lower concentration of O2 (a lower oxidation-reduction potential) compared to milk with few bacteria. This is because growing aerobic and facultatively anaerobic bacteria use oxygen as a final electron acceptor in cellular respiration.

The dye, **methylene blue,** is a redox indicator. It loses its blue colour in an anaerobic environment and is reduced to leuco-methylene blue. As a result, the **methylene blue reductase test** can be used to rapidly screen the quality of milk for the load of coliforms and *Lactococcus* (*Streptococcus*) *lactis,* strong reducers of methylene blue and indicators of contamination. The larger the bacterial load, the more quickly the milk will spoil. The speed at which the reduction occurs and the blue colour disappears indicates the quality of milk as follows:

a. Reduction within 30 minutes—very poor milk quality (class 4 milk)

b. Reduction between 30 minutes and 2 hours—poor milk quality (class 3 milk)

c. Reduction between 2 to 6 hours—fair quality (class 2 milk)

d. Reduction between 6 to 8 hours—good quality (class 1 milk)

Procedure

Coliform Analysis

1. Shake the milk sample 25 times. Make dilutions of the pasteurized and unpasteurized milk samples as indicated in the following figure.

2. Use the wax pencil to label the Petri plates with your name, date, the respective dilution, and either pasteurized or unpasteurized milk.

3. Pipette 1-ml milk aliquots of each dilution into the appropriate plates.

4. Melt and cool the violet red bile agar (VRBA) tubes and add 15 ml of it to each of the plates. Swirl gently on a flat surface and allow the agar to solidify. Afterward, add 5 ml of VRBA to each plate, swirl gently, and allow to solidify.

5. Incubate all plates at 32°C for 24 hours.

Second Period

For accuracy, select the plate that has between 25 to 250 colonies, which are located below the surface, are lens shaped, deep red, and surrounded by a pink halo. Record these as the coliform count **per ml of milk.**

Methylene Blue Reductase Test

1. Label the two screw-cap tubes with your name and pasteurized and unpasteurized, respectively.

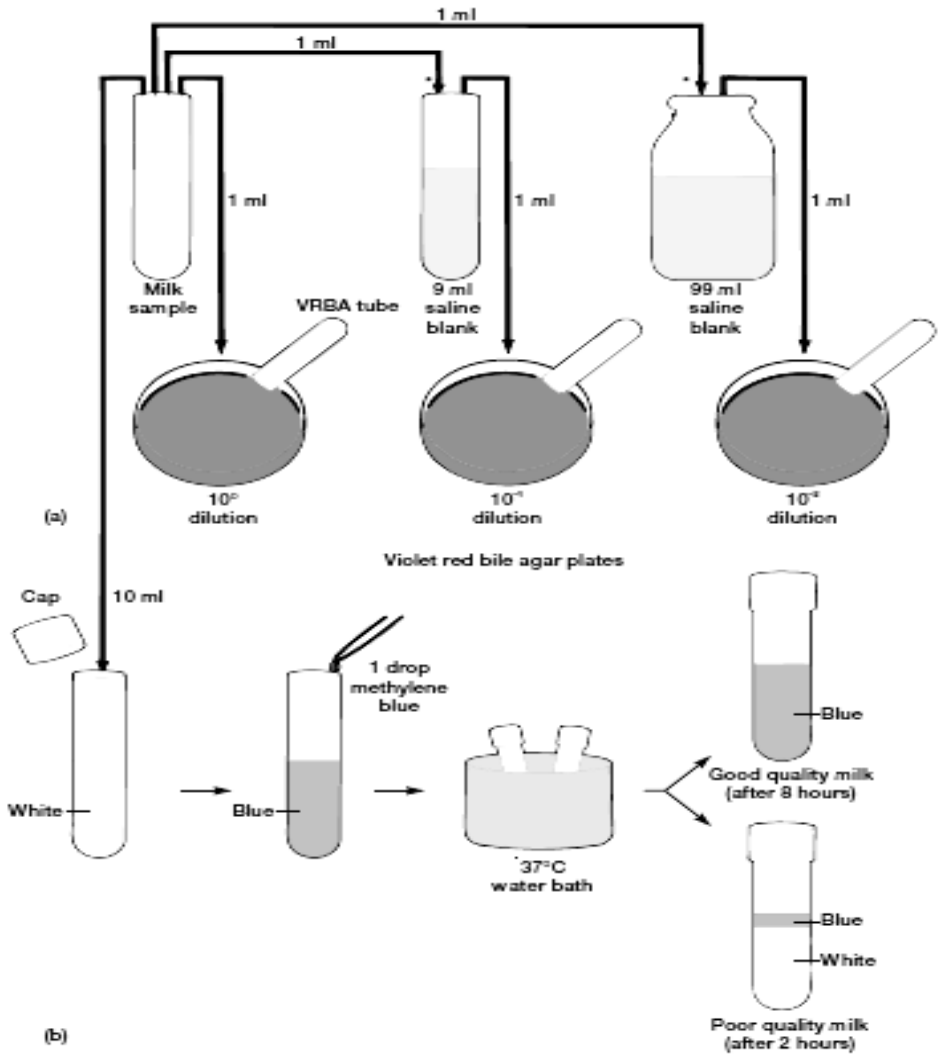
2. Using the 10-ml pipette, transfer 10 ml of unpasteurized milk to one screw-cap tube and, with another pipette, 10 ml of pasteurized milk to the other tube.

3. Add 1 drop of methylene blue to each tube.

4. Cap tightly and invert the tubes several times.

5. Place the tubes in a test-tube rack and place the rack in the 37°C water bath. After a 5-minute incubation, remove the tubes from the water bath and invert several times to mix again.

6. Observe the tubes at 30-minute intervals for 8 hours. Reduction is demonstrated by a change in colour of the milk sample from blue to white. When at least 4/5 of the tube has turned white, the end point of reduction has been reached, and the time should be recorded.



Examination of Milk for Bacteria. (a) Dilution series for coliform analysis of pasteurized and unpasteurized milk samples, (b) Methylene blue reductase test.

Quantitative examination of bacteria in milk (agar plate method 2)

Pasteurization is the heat treatment that destroys all pathogenic organisms in milk without completely sterilizing. Direct plating on desoxycholate agar is both selective and differential medium for coliforms in milk. The lactose fermenting coliform bacteria appear as red colonies that may be readily to distinguished and counted but non lactose fermenters appear as whitish colonies.

This test is applied to determine the relative numbers of coliforms and other bacteria on raw and pasteurized milk. The effectiveness of pasteurization is indicated by the reduction of total bacterial number as well as by the destruction of all coliforms.

Procedures

1- Plate dilutions of 10^{-2}, 10^{-3} and 10^{-4} of the sample of raw milk provided on standard plate-count agar and 10^{-1} and 10^{-2} on desoxycholatr agar.

2- Pasteurize the milk by heating in a water bath to 61.7^{o}C for 30 minutes. Be sure that timing is exact and the temperature is maintained.

3- Plate out the pasteurized milk, using dilution 10^{-2} and 10^{-1} on standard plate count agar, and 1ml and 10^{-1} dilutions on desoxycholate agar.

4- Incubate all plates at 37^{o}C for 24 hours and make counts of the raw and pasteurized milk on each type of agar.

Direct microscopic determination of bacteria in milk

In this experiment you will determine the bacterial count of a sample of high-grade milk, a sample of low-grade milk, and sample of mastitis milk. Milk from animals with mastitis (infection of the udder) characteristically has large numbers of white blood cells, or leucocytes. These show up in the stained film as circular or irregularly shaped cells, stained blue. Their function is to engulf and destroy invading bacteria. Occasionally, a partially devoured chain of streptococci may be seen projecting from leucocyte.

Procedure

1- Draw 0.01 ml of the milk into the pipette and wipe the bottom of the pipette with cheese cloth.

2- Place the tip of the pipette in the center of a 1square cm area of a slide and gently blow the milk sample out to form a drop.

3- By using inoculating needle, spread the milk over the 1sq cm area, outlining first, and then filling the center. Make duplicate smears of the same slide.

4- Dry the films of milk very slowly on a perfectly level surface.

5- Immerse slides with dried films in methylene blue stain for 2 min.

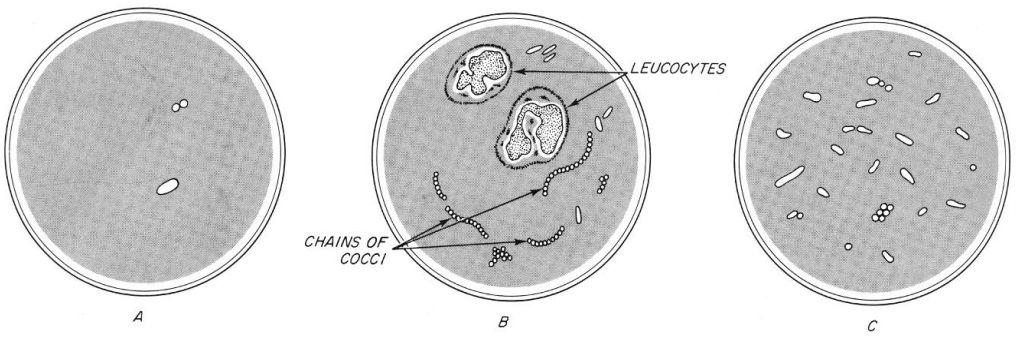
6- Drain excess stain, wash the slides with water, drain, and air dry.

7- By use of the oil immersion objective, determine the number of bacteria per field. Count clusters, clumps, or chains as one bacterial cell since each grouping would give rise to one colony if plated. The greater the number of bacteria in the milk, the smaller the number of fields that must be counted in order to obtain accurate results. A good rule is to count 100 fields for high grade milk, for low grade milk, 10 fields.

8- Estimate the number of bacteria per milliliter of milk. With 0.01ml of milk spread over an area of 1sq cm, and with a microscopic field of 0.16 mm diameter, the number of bacteria per field multiplied by 500,000 gives the number of bacteria per milliliter of original milk.

Total no. in one field X 500,000 = no. per ml

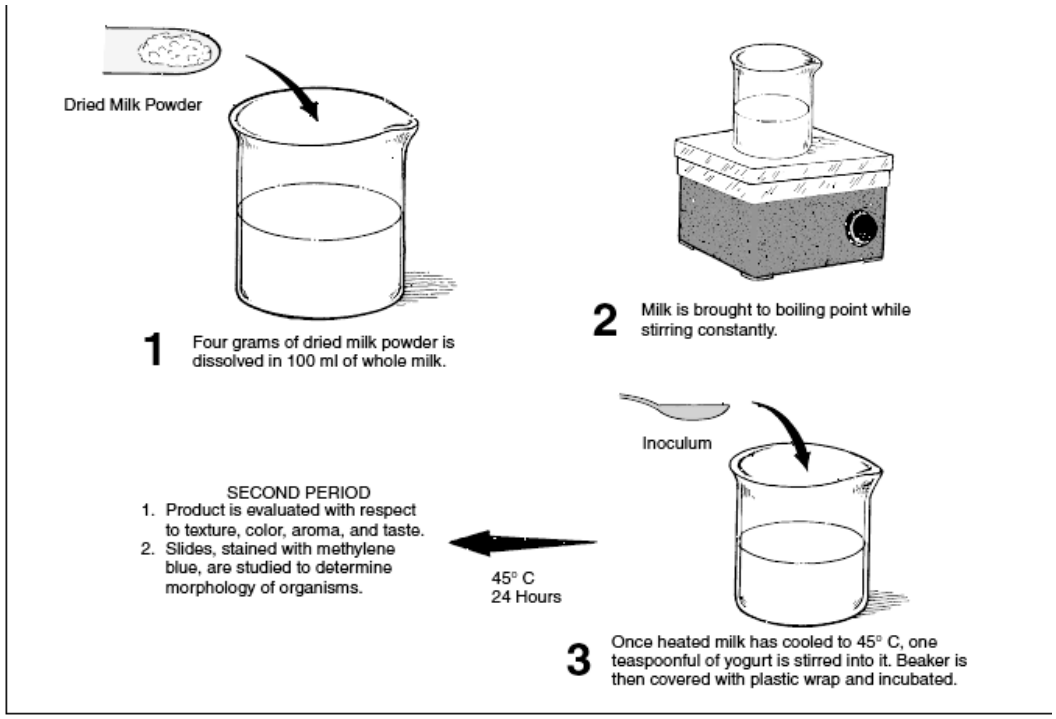
Because of its relative insensitivity, the direct microscopic count is not an official method for the determination of milk quality. It finds considerable use for leucocyte counts in milk from cows with diseases such as mastitis, and for the examination of some raw milk supplies.



Appearance of microscopic fields of different milk samples.
A: Low-count, high-grade milk.
B: Long chains of streptococci and numerous leucocytes often typical of milk from a cow with mastitis.
C: Numerous different bacteria often typical of milk stored in dirty utensils.

MICROBIOLOGY OF YOGURT PRODUCTION

For centuries, people throughout the world have been producing fermented milk products using yeasts and lactic acid–producing bacteria. The yogurt of eastern central Europe, the kefir of the Cossacks, the koumiss of central Asia, and the leben of Egypt are just a few examples. In all of these fermented milks, lactobacilli act together with some other microorganisms to curdle and thicken milk, producing a distinctive flavor desired by the producer. Kefir of the Cossacks is made by charging milk with small cauliflower-like grains that contain *Streptococcus lactis, Saccharomyces delbrueckii,* and *Lactobacillus brevis.* As the grains swell in the milk they release the growing microorganisms to ferment the milk. The usual method for producing yogurt in large-scale production is to add pure cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* to pasteurized milk.



Yogurt production by Method A

METHOD A

First Period

The above Figure illustrates the procedure for this method. Note that 4 g of powdered milk are added to 100 ml of whole milk. This mixture is then heated to boiling and cooled to 45°C. After cooling, the milk is inoculated with yogurt and incubated at 45° C for 24 hours.

Materials:

Dried powdered milk

Whole milk

Commercial yogurt (with viable organisms)

Small beaker, graduate, teaspoon, stirring rod

Plastic wrap

Filter paper (for weighing)

Procedure

1. On a piece of filter paper weigh 4 grams of dried powdered milk.

2. To a beaker of 100 ml of whole milk add the powdered milk and stir thoroughly with sterile glass rod to dissolve.

3. Heat to boiling, while stirring constantly.

4. Cool to 45° C and inoculate with 1 teaspoon of the commercial yogurt. Stir. Be sure to check the label to make certain that product contains a live culture. Cover with plastic wrap.

5. Incubate at 45° C for 24 hours.

METHOD B

First Period

The Figure below illustrates a slightly different method of culturing yogurt, which, due to its simplicity, may be preferred. Note that no whole milk is used and provisions are made for producing a sample for tasting.

Materials

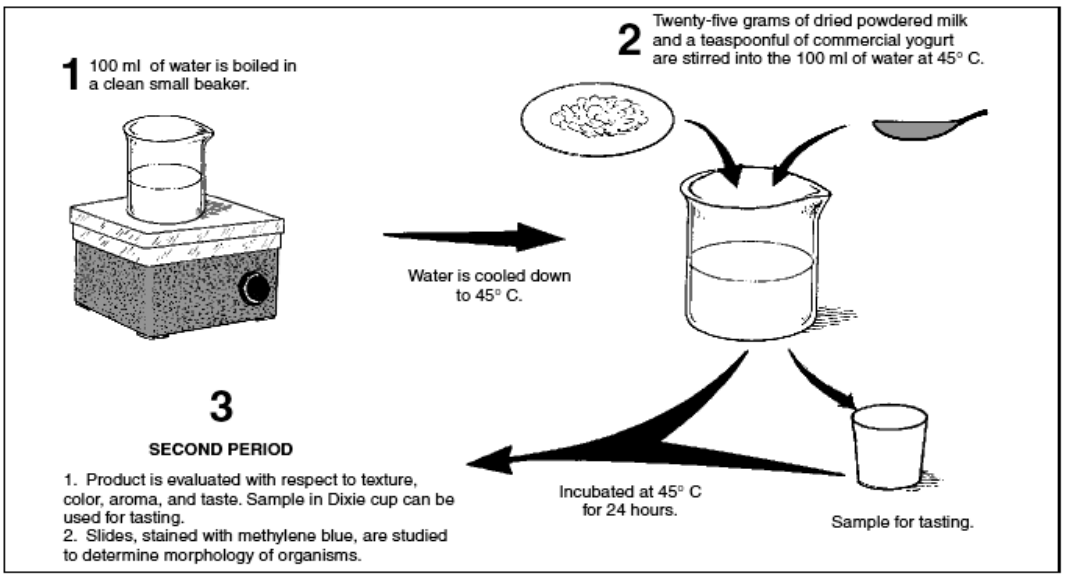
Small beaker, graduate, teaspoon, stirring rod, dried powdered milk, commercial yogurt (with viable organisms), plastic wrap, filter paper for weighing, paper Dixie cup (5 oz size) and cover, electric hot plate or Bunsen burner and tripod.

Procedure

1. On a piece of filter paper weigh 25 grams of dried powdered milk.
2. Heat 100 ml of water in a beaker to boiling and cool to 45° C.
3. Add the 25 grams of powdered milk and 1 teaspoon of yogurt to the beaker of water. Mix the ingredients with a sterile glass rod.
4. Pour some of the mixture into a sterile Dixie cup and cover loosely. Cover the remainder in the beaker with plastic wrap.
5. Incubate at 45° C for 24 hours.

Second period (<u>Both Methods</u>)

1. Examine the product and record the colour, aroma, texture, and, if desired, the taste.
2. Make slide preparations of the yogurt culture. Fix and stain with methylene blue. Examine under oil immersion and record your results.



Yogurt production by Method B

MICROFLORA OF CHEESE

Many microorganisms are responsible for the aromas, flavor and other characteristics of the various types of cheeses. This experiment is to determine the general nature of the characteristics flora of several types of cheese. After being weighed, cheese may be prepared for plating by blending with sterile water in a sterile blender.

Procedure

1- Plate a sample of the emulsified cheese provided on bromo- cresol purple lactose agar in dilutions of 10^{-2}, 10^{-3} and 10^{-4}.

2- Incubate the plates until the next laboratory period (2days) at 30 ^{o}C.

3- Count the colonies and report in terms of organisms per gram of cheese.

4- Store the plates for 1 week.

5- Observe the different types of colonies from the various cheeses and prepare Gram stains from different colonies.

6- Observe the texture, aroma, and flavour of the different cheeses.

ALCOHOLIC FERMENTATION OF FRUIT JUICES

A moist acidic medium containing fermentable carbohydrate is likely to undergo alcoholic fermentation by yeast.

$$C_6H_{12}O_6 \qquad 2C_2H_5OH + 2CO_2$$

Procedure

1. Inoculate a tube of the grape juice medium and a tube of sugarcane juice medium provided with 0.1 ml of a broth culture of *Saccharomyces cerevisiae* (or 5 ml of yeast solution).
2. Incubate at 25^{o}C until the next laboratory period.
3. Note the aroma and flavor of the product.

PART (4) MEDICAL BACTERIOLOGY

Medical, or **clinical, microbiology** deals with the diagnosis, treatment, and prevention of infectious diseases caused by all groups of microorganisms. As such, a complete coverage of these groups is not feasible in a few exercises.

This part is constructed to enable the student to perform some of the routine techniques that are used in a clinical microbiology laboratory. For example, isolating some of the microorganisms that are part of the normal human microbiota by using aseptic technique and selective and differential media.

STAINING WITH ALBERT STAIN (for the detection of *Corynebacterium diphtheriae*)

If diphtheria is suspected a sputum smear should be stained with Albert stain. This stain is used to show the dark-staining volutin granules that appear in *Corynebacterium diphtheriae* bacilli.

Materials and reagents

- Microscope
- Slide rack
- Albert stain (reagent no. 7).

Method

1. Fix the smear as described earlier.
2. Cover the smear with Albert stain for 3–5 minutes.
3. Wash off the stain with clean water and place the slide upright in a slide rack to drain and air-dry.

Microscopic examination

First examine the slide using the 40X objective to see how the smear is distributed and then use the 100X oil-immersion objective.

Corynebacterium diphtheriae appears as green rods (see the Fig. below) containing green– black volutin granules. The rods may be arranged in rows (a) or in V-formation (b), or joined at angles, giving the appearance of Chinese characters (c). The presence of slender rods containing volutin granules is sufficient evidence for starting treatment for diphtheria.

If diphtheria is suspected, a specimen should be sent to the bacteriology laboratory for culture.

Albert stain

Toluidine blue 0.15g

Malachite green 0.20g

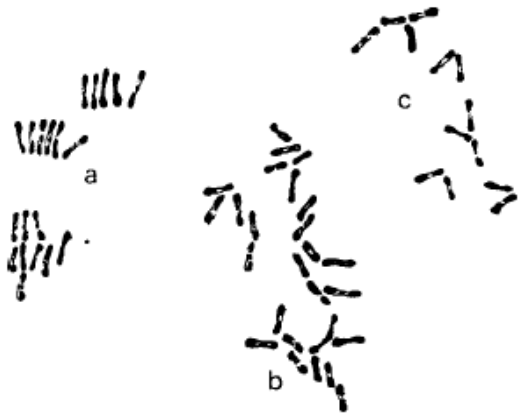
Glacial acetic acid (CH3COOH) 1ml

Ethanol (CH3CH2OH), 96% 2ml

Distilled water: to 100ml

Dissolve the glacial acetic acid in 30 ml of distilled water in a clean 100-ml bottle. Add the toluidine blue and malachite green and mix well. Add the ethanol and make up the volume to 100ml with distilled water. Mix well. Label the bottle "ALBERT STAIN" and write the date. Store at room temperature.

Warning: Glacial acetic acid is highly corrosive.



Corynebacterium diphtheriae C. diphtheriae rods may be arranged in rows (a) or in V-formation (b) or joined at angles, giving the appearance of Chinese characters (c).

STAINING WITH ZIEHL–NEELSEN STAIN (for the detection of acid-fast bacilli)

Ziehl– Neelsen stain is used to identify mycobacteria and oocysts of *Cryptosporidium*

Principle

When mycobacteria and oocysts of *Cryptosporidium* spp. are stained with a hot strong solution of carbol fuchsin, they resist decolorization with a solution of acid or acid– ethanol and stain red. Tissues and other organisms are decolorized by the acid– ethanol solution and are demonstrated by a counterstain such as methylene blue, which stains them blue.

Mycobacterium leprae and oocysts of *Cryptosporidium* spp. only resist decolorization with weak solutions of acid or acid– ethanol. They are demonstrated using the modified Ziehl– Neelsen technique. *Mycobacterium* spp. and oocysts of *Cryptosporidium* spp. are referred to as "acidfast" due to their resistance to decolorization with acid solution. They do not stain well with Gram stain or simple stains such as methylene blue.

Learning Objectives

Each student should be able to

1. Understand the biochemical basis of the acid-fast stain
2. Perform an acid-fast stain
3. Differentiate bacteria into acid-fast and non-acid-fast-groups

Materials

Tryptic soy broth culture of *Escherichia coli* (ATCC 11229) and nutrient agar slant culture of *Mycobacterium smegmatis* (ATCC 19420) or *Mycobacterium phlei* (ATCC 354) or any other available strains—5-day old cultures

Ziehl's carbol fuchsin.

Carbol fuchsin prepared with either Tergitol No. 4 (a drop per 30 ml of carbol fuchsin) or Triton-X (2 drops per 100 ml of carbol fuchsin). Tergitol No. 4 and

Triton-X act as detergents, emulsifiers, and wetting agents, alkaline methylene blue, acid-alcohol, clean glass slides, commercial slides showing acid-fast *Mycobacterium tuberculosis*, inoculating loop, hot plate, microscope, bibulous paper, paper toweling, lens paper and lens cleaner, immersion oil, staining racks and 1-ml pipettes with pipettor.

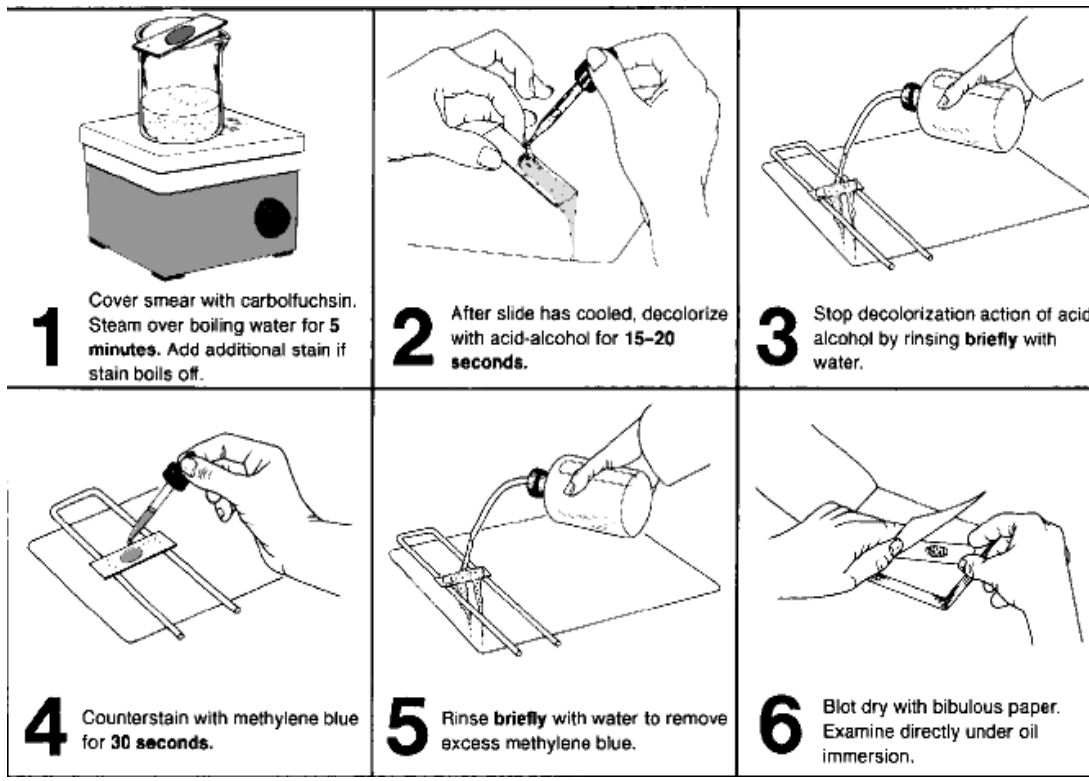
Procedure

1. Prepare a smear consisting of a mixture of *E. coli* and *M. smegmatis.*
2. Allow the smear to air dry and then heat-fix.
3. Place the slide on a hot plate that is within a chemical hood (with the exhaust fan on), and cover the smear with a piece of paper toweling that has been cut to the same size as the microscope slide. Saturate the paper with Ziehl's carbol fuchsin. Heat for 3 to 5minutes. Do not allow the slide to dry out, and avoid excess flooding! Also, prevent boiling by adjusting the hot plate to a proper temperature. A boiling water bath with a staining rack or loop held 1 to 2 inches above the water surface also works well. (Instead of using a hot plate to heatdrive the carbolfuchsin into the bacteria, an alternate procedure is to cover the heat-fixed slide with a piece of paper towel. Soak the towel with the carbolfuchsin and heat, well above a Bunsen burner flame.)
4. Remove the slide, let it cool, and rinse with water for 30 seconds.
5. Decolorize by adding acid-alcohol drop by drop until the slide remains only slightly pink. This requires 10 to 30 seconds and must be done carefully.
6. Rinse with water for 5 seconds
7. Counterstain with alkaline methylene blue for about 2 minutes.
8. Rinse with water for 30 seconds.
9. Blot dries with bibulous paper.
10. There is no need to place a cover slip on the stained smear.
11. Examine the prepared slide of *Mycobacterium tuberculosis.*

Examine under oil- immersion lens. Acid- fast organisms stain red; the background and other organisms stain blue.

Organisms stained by Ziehl– Neelsen stain

Sample	Organism
Sputum	*M. tuberculosis*
	M. bovis
Skin	*M. leprae*
	M. ulcerans
Urine	*M. tuberculosis*
	M. bovis
Stool	*Cryptosporidium* spp.
Gastric lavage	*M. tuberculosis*
	M. bovis



Ziehl-Neelsen acid-fast staining procedure

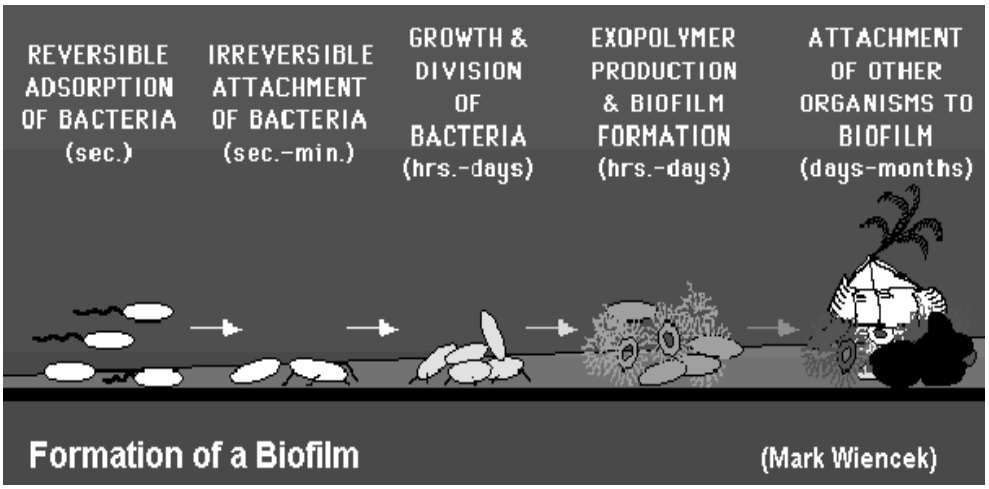
Reporting the number of acid-fast bacilli present

Number of acid-fast bacilli present per microscope field	Result
< 0.1 (< 10 per 100 fields)	Specify number present per 100 fields
0.1–1 (10–100 per 100 fields)	+
1–10	++
> 10	+++

ORAL BIOFILMS

Most of the time in the microbiology lab, we study free-floating bacteria in broths or bacteria in colony forms, and generally in pure culture. However, in the real world bacteria are usually interacting with other species in pretty sophisticated ecosystems. This assemblage of various organisms attached to a surface is called a **biofilm**, and the organisms that comprise it can include bacteria, plants, fungi, protozoa, and even multicellular animals, depending on where the biofilm is.

These biofilm communities attach to teeth, to the inside of a toilet, on a sponge, in a catheter, inside of a blood vessel, in the middle ear, on contact lenses, metal pipes, and so on. Interacting microorganisms act differently than if the microbes were living independent to each other. Different chemical by-products are produced and the surfaces that they grow on can be damaged by these physical and chemical interactions among the various species. In addition, interacting species can produce infections that are difficult to eliminate, possibly because the physical architecture of the biofilm protects the individual organisms from antibiotics (like schooling fish are protected by moving closely around together) or because there is no ONE microbe causing the infection. In the body, the biofilm community can produce inflammation and discomfort.



In this lab exercise, we are interested in oral biofilms- on teeth and on toothbrushes. Plaque is a protective layer that the bacteria live within, and, in fact, produce: It consists of bacteria, and the slime layers that they have around their cells and food particles. Within the plaque they are protected, and they are also free to metabolize and produce erosive by-products which then cause tooth decay, destroying the dentin of the teeth. If the plaque is not eliminated, becoming harder and denser, it causes the gum to recede and the tooth can lose attachment to the jawbone. This is periodontal disease.

NORMAL THROAT MICROFLORA

The mouth and throat of a healthy person contain considerable numbers of bacteria. Most of them are harmless some are potential pathogens, virulent organisms may be present but not producing diseases although the same species may be the cause of diseases on other person.

Organisms that may be isolated from healthy person include *Staphylococcus aureus*, Streptococci of different species, diphtheroid bacilli, pneumococci, gram negative rods, *Klebsiella, proteus, Hemophilus influenza.*

Procedure

1.Melt a tube 12 ml of blood base agar and cool it to 43-45 ^{o}C.

2.Transfer 1ml of blood to the cooled tube of agar and mix with the pipette.

3.Pure blood agar into petri dishes.

4.Swab your throat with one of sterile cotton swab provided.

5.Streak the swab thoroughly on the surface of the solidified blood agar plate.

6.Incubate at 37 for 24h.

7.Examine the plate for colony development and for hemolytic zones.

8.Make gram stains from several typical colonies on the plate.

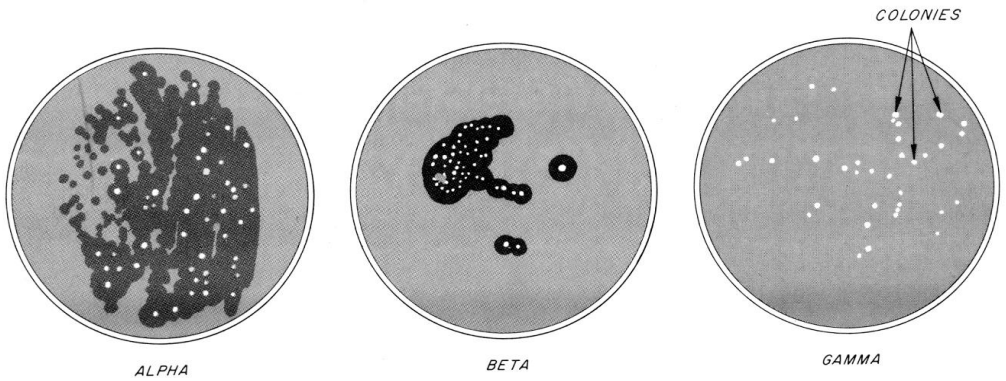


Figure 48-1 Reactions of bacteria on blood agar.

ANTIBIOTIC SENSITIVITY TEST (Kirby- Bauer method)

Antibiotics' are biochemicals secreted by microorganisms which, in low concentration, inhibit the growth or kill other microorganisms, i.e., the antibiotics are 'antimicrobial agents of microbial origin'.

Learning Objectives

Each student should be able to:

1. Appreciate the scope of antimicrobial activity of selected antibiotics
2. Perform the Kirby-Bauer method for determination of antibiotic sensitivity
3. Correctly interpret a Kirby-Bauer plate

Principles

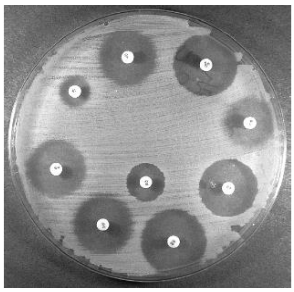
One method that is used to determine antibiotic susceptibility is the sensitivity disk method of **Kirby- Bauer** (named after W. Kirby and A. W. Bauer in 1966). In this method, antibiotics are impregnated onto paper disks and then placed on a seeded Mueller-Hinton agar plate using a mechanical dispenser or sterile forceps. The plate is then incubated for 16 to 18 hours, and the diameter of the **zone of inhibition** around the disk is measured to the nearest millimeter. The inhibition zone diameter that is produced will indicate the susceptibility or resistance of a bacterium to the antibiotic. Antibiotic susceptibility patterns are called **antibiograms.** Antibiograms can be determined by comparing the zone diameter obtained with the known zone diameter size for susceptibility. For example, a zone of a certain size indicates susceptibility, zones of a smaller diameter or no zone at all show that the bacterium is resistant to the antibiotic.

Frequently one will see colonies within the zone of inhibition when the strain is antibiotic resistant. Many factors are involved in sensitivity disk testing and must be carefully controlled. These include size of the inoculum, distribution of the inoculum, incubation period, depth of the agar, diffusion rate of the antibiotic, concentration of antibiotic in the disk, and growth rate of the bacterium. If all of these factors are carefully controlled, this type of testing is highly satisfactory for determining the degree of susceptibility of a bacterium to a certain antibiotic.

The Kirby-Bauer method is not restricted to antibiotics. It may also be used to measure the sensitivity of any microorganism to a variety of antimicrobial agents such as sulfonamides and synthetic chemotherapeutics.

This exercise illustrates the differences in sensitivity of Gram positive and gram negative bacteria to several different antibiotics. The size of inhibition zone caused by the diffusion of the agent into the agar is directly related to the degree of susceptibility of the organism. Inhibition zone size is affected by a number of technical variables including inoculation size, incubation time and

temperature, medium composition, pH, gaseous atmosphere, stability of antibiotics and others.



A Kirby-Bauer Plate. A Mueller- Hinton agar plate inoculated with *S. aureus* and various antibiotics. Notice the diameter of the various zones of inhibition.

Procedure

3- Add 0.1 ml of bacterial suspension over the entire Muller Hinton agar plate surface.

4- Apply the discs to the surface using sterile forceps (see the Figure).

5- Within 15 min. after the discs are applied incubate the plates at 37^{o}C for 16-18h.

6- After incubation period measure the diameters of the zones of complete inhibition using a ruler (see the following Figures). Measure the zones of inhibition to the nearest mm for each antibiotic. For each antibiotic, determine whether the bacteria are resistant or susceptible.

Hints and precautions

(1) If the plate is satisfactorily inoculated, and the inoculum is sufficient, the zones of inhibition will be uniformly circular and confluent growth should be seen over the entire plate. If you see isolated colonies on your plate, then the technique performed is less than adequate, and the procedure should be repeated.

(2) Colonies growing within the zone of inhibition usually result in considering the bacteria drug resistant.

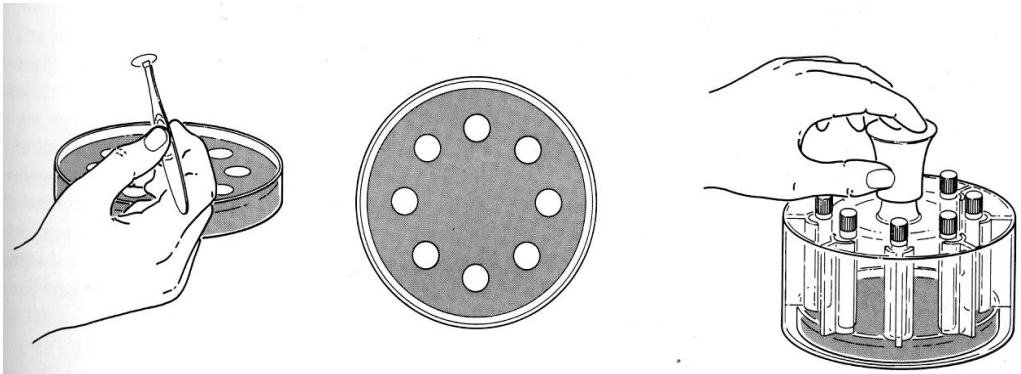
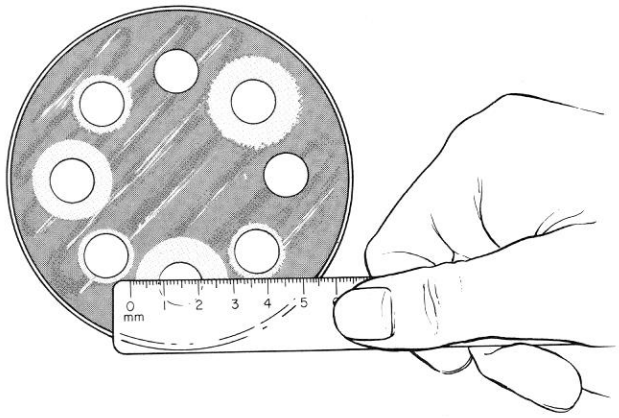


Figure 50-1 Application of antibiotic discs to test culture individually and by automatic dispenser.



ż Zones of growth inhibition due to antibiotics.

THE STAPHYLOCOCCI: ISOLATION AND IDENTIFICATION

Often in conjunction with streptococci, the staphylococci cause abscesses, boils, carbuncles, osteomyelitis, and fatal septicemias. Collectively, the staphylococci and streptococci are referred to as the pyogenic (pus- forming) Gram positive cocci. Originally isolated from pus in wounds, the staphylococci were subsequently demonstrated to be normal inhabitants of the nasal membranes, the hair follicles, the skin, and the perineum of healthy

individuals. The fact that 90% of hospital personnel are carriers of staphylococci portends serious epidemiological problems, especially since most strains are penicillin-resistant.

The **staphylococci** are Gram positive spherical bacteria that divide in more than one plane to form irregular clusters of cells. They are listed in section 12, volume 2, of *Bergey's Manual of Systematic Bacteriology*. The genus *Staphylococcus* is grouped with three other genera in family Micrococcaceae:

SECTION 12 GRAM-POSITIVE COCCI

Family I Micrococcaceae

Genus I *Micrococcus*

Genus II *Stomatococcus*

Genus III *Planococcus*

Genus IV ***Staphylococcus***

Family II Deinococcaceae

Genus I *Deinococcus*

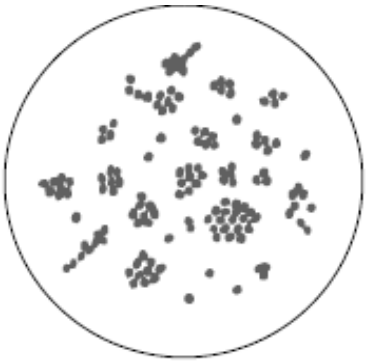
Genus II ***Streptococcus***

Although the staphylococci make up a coherent phylogenetic group, they have very little in common with the streptococci except for their basic similarities of being Gram positive, non-spore- forming cocci. Note that *Bergey's Manual* puts these two genera into separate families due to their inherent differences.

Of the 19 species of staphylococci listed in *Bergey's Manual,* the most important ones are *S. aureus, S. epidermidis,* and *S. saprophyticus.* The single most significant characteristic that separates these species is the ability or inability of these organisms to coagulate plasma: only *S. aureus* has this ability; the other two are coagulase- negative.

Although *S. aureus* has, historically, been considered to be the only significant pathogen of the three, the others do cause infections. Some cerebrospinal fluid infections (2), prosthetic joint infections (3), and vascular

graft infections (1) have been shown to be due to coagulase- negative staphylococci.



In this experiment we will attempt to isolate staphylococci from (1) the nose, (2) a fomite, and (3) an "unknown-control." The unknown-control will be a mixture containing staphylococci, streptococci, and some other contaminants. If the nasal membranes and fomite prove to be negative, the unknown-control will yield positive results, providing all inoculations and tests are performed correctly.

Since *S. aureus* is by far the most significant pathogen in this group, most of our concern here will be with this organism. It is for this reason that the characteristics of only this pathogen will be outlined next. *Staphylococcus aureus* cells are 0.8 to 1.0 µm in diameter and may occur singly, in pairs, or as clusters. Colonies of *S. aureus* on trypticase soy agar or blood agar are opaque, 1 to 3 mm in diameter, and yellow, orange, or white. They are salt-tolerant, growing well on media containing 10% sodium chloride. Virtually all strains are coagulase- positive. Mannitol is fermented aerobically to produce acid. Alpha toxin is produced that causes a wide zone of clear (beta-type) hemolysis on blood agar; in rabbits it causes local necrosis and death.

The other two species lack alpha toxin (do not exhibit hemolysis) and are coagulase- negative. Mannitol is fermented to produce acid (no gas) by all strains of *S. aureus* and most strains of *S. saprophyticus*.The following Table

lists the principal characteristics that differentiate these three species of staphylococcus.

	S. aureus	*S. epidermidis*	*S. saprophyticus*
Alpha toxin	+	–	–
Mannitol (acid only)	+	–	(+)
Coagulase	+	–	–
Biotin for growth	–	+	NS
Novobiocin	S	S	R

Note: NS _ not significant; S - sensitive; R - resistant;
(+) - mostly positive

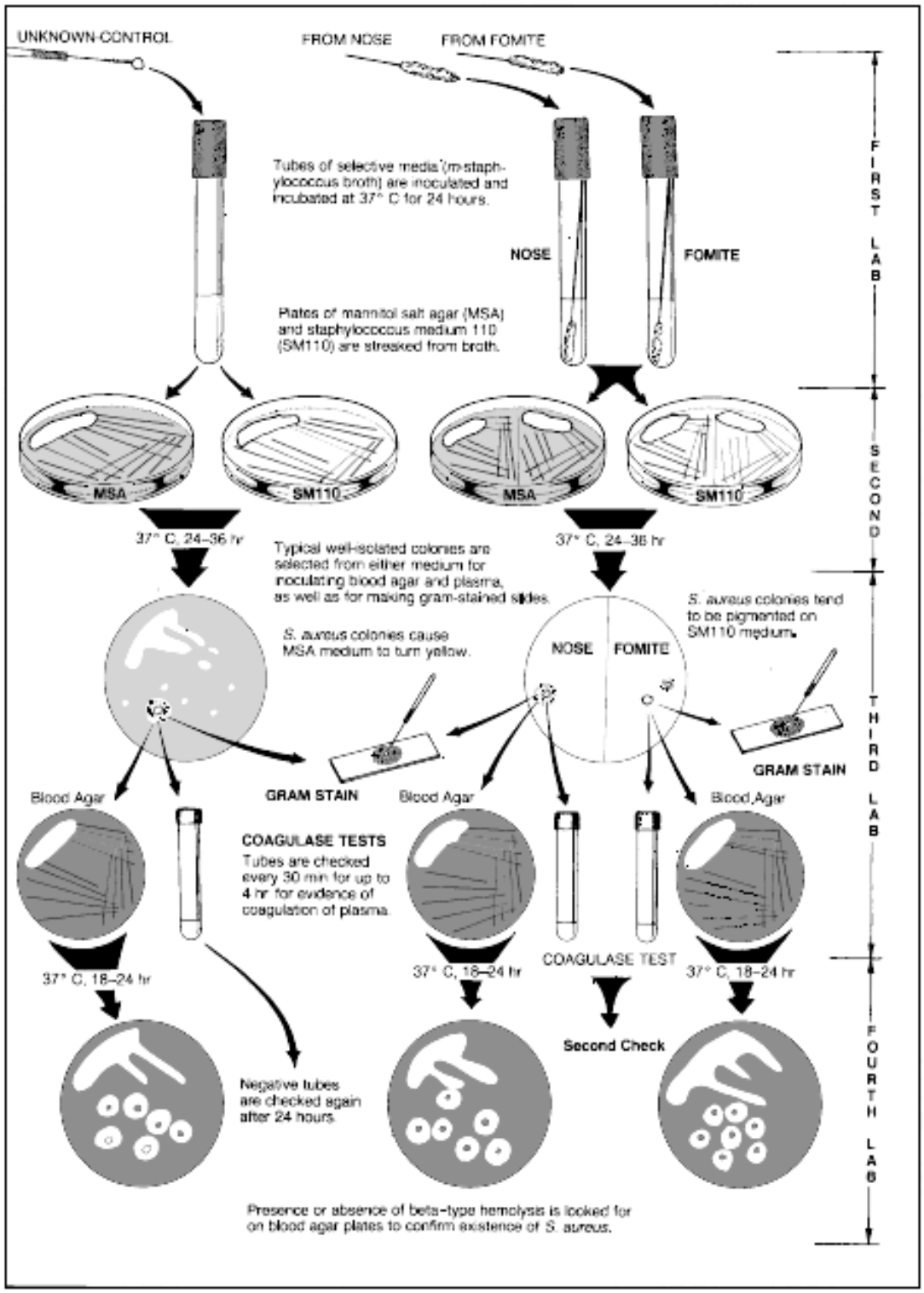
To determine the incidence of carriers in our classroom, as well as the incidence of the organism on common fomites, we will follow the procedure illustrated in the following figure. Results of class findings will be tabulated. The characteristics we will look for in our isolates will be (1) beta- type hemolysis (alpha toxin), (2) mannitol fermentation, and (3) coagulase production. Organisms found to be positive for these three characteristics will be presumed to be *S. aureus*. Final confirmation will be made with additional tests.

FIRST PERIOD (Specimen Collection)

Note in the procedure figure that swabs that have been applied to the nasal membranes and fomites will be placed in tubes of enrichment medium containing 10% NaCl (*m-Staphylococcus* broth). Since your unknown control will lack a swab, initial inoculations from this culture will have to be done with a loop.

Materials

- 1 tube containing numbered unknown- control
- 3 tubes of *m*-staphylococcus broth
- 2 sterile cotton swabs



Procedure for presumptive identification of staphylococci

Procedure

1. Label the three tubes of *m-Staphylococcus* broth NOSE, FOMITE, and the number of your unknown- control.

2. Inoculate the appropriate tube of *m-Staphylococcus* broth with one or two loopfuls of your unknown-control.

3. After moistening one of the swabs by immersing partially into the "nose" tube of broth, swab the nasal membrane just inside your nostril. A small amount of moisture on the swab will enhance the pickup of organisms. Place this swab into the "nose" tube.

4. Swab the surface of a fomite with the other swab that has been similarly moistened and deposit this swab in the "fomite" tube.

The fomite you select may be a coin, drinking glass, telephone mouthpiece, or any other item that you might think of.

5. Incubate these tubes of broth for 4 to 24 hours at 37° C.

SECOND PERIOD (Primary Isolation Procedure)

Two kinds of media will be streaked for primary isolation: mannitol salt agar and *Staphylococcus* medium 110. Mannitol salt agar (MSA) contains mannitol, 7.5% sodium chloride, and phenol red indicator. The NaCl inhibits organisms other than staphylococci. If the mannitol is fermented to produce acid, the phenol red in the medium changes color from red to yellow.

Staphylococcus medium 110 (SM110) also contains NaCl and mannitol, but it lacks phenol red. Its advantage over MSA is that it favors colony pigmentation by different strains of *S. aureus*. Since this medium lacks phenol red, no color change takes place as mannitol is fermented.

Materials

- 3 culture tubes from last period
- 2 Petri plates of MSA
- 2 Petri plates of SM110

Procedure

1. Label the bottoms of the MSA and SM110 plates as shown in the figure. Note that to minimize the number of plates required, it will be necessary to make half- plate inoculations for the nose and fomite. The unknown- control will be inoculated on separate plates.

2. Quadrant streak the MSA and SM110 plates with the unknown control.

3. Inoculate a portion of the nose side of each plate with the swab from the nose tube; then, with a sterile loop, streak out the organisms on the remainder of the agar on that half of each plate. The swabbed areas will provide massive growth; the streaked- out areas should yield good colony isolation.

4. Repeat step 3 to inoculate the other half of each agar plate with the swab from the fomite tube.

5. Incubate the plates aerobically at 37° C for 24 to 36 hours.

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