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RESEARCH ARTICLE

BACTERIOLOGY LABORATORY HANDBOOK

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ABSTRACT

This handbook is prepared to meet basicneeds of our laboratory and hopefully, it gives highlights onlaboratory procedures to work with a variety of importantgroups of bacteria. It provides comprehensive information and logically organized, instructive, and progresses through laboratory safety, sterilization and disinfection, use of the microscope, sampling techniques, manipulation of microbes, cultures, staining and observation of microorganisms, identification of unknown microbe, maintaining cultures. With this in mind, this handbook is prepared to meet basic needs of our laboratory and hopefully, it gives highlights on laboratory procedures to work with a variety of important groups of bacteria.

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INTRODUCTION

Of the tens of millions of species of living organisms on Earth, only about 1.75 million have been scientifically described. The vast majority of undescribed species are prokaryotic (bacteria, archaea) and eukaryotic microorganisms (algae, protozoa, fungi). This reservoir of organismal diversity remains largely unexplored despite a range of colonizable habitats, biochemical and molecular processes, genomic variation, and consortial/symbiotic behavior far greater than that shown in larger, multicellular organisms. Prokaryotic and eukaryotic microbes are key elements of food webs, may inhibit or trigger significant ecological events (e.g. harmful algal blooms), and are responsible, directly or indirectly, for the health or diseases of larger organisms. Microorganisms produce numerous bioactive compounds, some of which are the basis for novel pharmaceuticals or other commercially useful products. Microbial communities are known to play fundamentally important roles in biogeochemical cycles. Studies of microbial evolution at the genetic and genomic levels provide important clues about how microbial attributes appear, and are exchanged among cells and species in nature. To discover and understand the diversity of microorganisms, their interactions and novel processes remain major challenges in biology. These are:

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- Discovery of large numbers of as yet undescribed microorganisms and microbial consortia from diverse habitats; and
- Characterization of novel biochemical, metabolic, physiological, genomic and other properties and processes of newly described or poorly understood microbes and microbial communities.

Microbial diversity research include but are not limited to:

- Studies to determine the phylogenetic, physiological, metabolic and genomic properties and mechanisms responsible for microbial growth, adaptation and survival in natural environments;
- Studies of the mechanistic basis of interactions among microbes in communities and multispecies biofilms, and of microbes with co-habitating non-microbial species, including mechanisms for the exchange of genetic material;
- Studies of the diversity of microbial processes for anaerobic and aerobic flow of energy and cycling of nutrients, including aquatic, soil/rhizosphere, and sediment ecosystems;
- Studies patterns of microbial distribution in time and space, and in response to specific environmental gradients; and
- Integrative studies of the diversity, physiology, biochemistry and genomics of microorganisms and

microbial communities and the processes that they carry out in the environment.

An initial aim of all microbiologists is the reproducible growth of their microbial culture, no matter whether the microorganisms are of natural origin or have been genetically engineered. Reproducible growth requires defined environmental conditions with respect to energy source, temperature, pH and nutrients. The Manual includes a number of procedures and appendixes encompassing in the field of bacteriology to achieve the following few objectives:

- To understand the appropriate laboratory methods to be employed for sample collection, isolation, identification and preservation of bacteria targeted to conserve their genetic material for sustainable utilization
- To be able to serve as a liaison between our laboratory and other Laboratories working on bacterial genetic resources.

With this in mind, this handbook is prepared to meet basic needs of our laboratory and hopefully, it gives highlights on laboratory procedures to work with a variety of important groups of bacteria.

SECTION I. LABORATORY SAFETY

Laboratory Hazards

The laboratory environment is a hazardous place to work. Walk through any laboratory door and you are confronted with a wide array of chemicals, biologics, and instrumentation. Nearly every common laboratory technique, practice, or procedure carries some risk of exposure or mechanical injury. Appreciating what these risks are and how to work safely with them is the focus of this manual.

Lab Hazards

- Biological
- Chemical
- Ionizing Radiation
- Physical

The Most Hazardous "Dirty Dozen" Chemicals*

- Organic azides
- Perchlorate salts of organic, organometallic, and inorganic complexes
- Diethyl ethers
- Lithium aluminum hydride
- Sodium, potassium
- Potassium metal
- Sodium-benzophenone ketyl still pots
- Palladium on carbon
- Heat generated from exothermic reactions
- Ethers with alpha hydrogen atoms
- Carbon monoxide
- Organic peroxides

Note: Laboratorians should consult with OHS before working with these chemicals. Laboratorians should never assume that they are performing all tasks in a safe and correct manner just because they have never had a laboratory accident. Both the hazard and the route of transmission should be known before beginning any laboratory procedure. Unfortunately, shortcuts are taken, materials are viewed as non-hazardous the longer they are worked with, and equipment is assumed to be functioning properly when they are turned on.

Basic Safety Measures

- Be patient while you are working. Do not start an experiment unless you ascertain all the steps.
- Do not use equipment unless you ascertain how to use. Use the laboratory equipment properly. If you do not know how to use, see the equipment user's manual or ask others.
- Wear gloves and a laboratory coat while you handle any bacterial sample/isolate, toxic or carcinogenic chemicals (e.g., EtBr, CsCl, Phenol, etc.).

- **Do not touch any equipment or place with contaminated gloves.**

- Wear eye or face protection under UV.
- If any bacterial sample/isolate, toxic chemicals, antibiotics or carcinogens is spilt on body or clothes, immediately remove the contaminated clothing and wash small spills under water for at least 5 min. Spills on legs can be rinsed in the sink. Do not hesitate to use the lab shower in the laboratory on large spill. Do not rinse contaminated skin with alcohol.
- After you use any chemical, enzyme or solvent, etc., bring it back to the place where it was.

- **Eating and drinking are strictly prohibited in the laboratory and during laboratory work time.**

- Pencils, pens, fingers, and other objects must be kept out of the mouth.
- Laboratory tables and desks should be free of books, clothing, and other personal items during laboratory work.

- Wipe down the bench surface with disinfectant before and after each laboratory work.
- Don't walk around the laboratory with contaminated loops, open culture tubes or Petri plates, or other externally contaminated materials.
- Discard disposable contaminated materials, such as slides and Petri dishes, in the covered buckets in each laboratory

- **Wash your hands thoroughly after handling inoculated cultures and before leaving the laboratory.** Do not remove cultures from the laboratory area.

SECTION II. STERILIZATION AND DISINFECTION

A. Sterilization

Introduction

Sterilization is the complete destruction or elimination of all viable organisms (in or on an object being sterilized). There are

no degrees of sterilization. An object is either sterile or not. Sterilization procedures involve the use of heat, radiation or chemicals, or physical removal of cells.

Methods of Sterilization

Heat

Heat is the most important and widely used. For sterilization always consider type of heat, 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

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

Boiling

Boling is at 100^{o} for 30 minutes. Kills everything except some endospores (Actually, for the purposes of purifying drinking water 100^{o} for five minutes is probably adequate though there have been some reports that Giardia cysts can survive this process). To kill endospores, and therefore sterilize the solution, very long or intermittent boiling is required.

Autoclaving (steam under pressure or pressure cooker)

It is at 121^{o} for 15 minutes. Good for sterilizing almost anything, but heat-labile substances will be denatured or destroyed.

Dry heat (hot air oven)

It is at 160^{o}/2hours or 170^{o}/1hour. Used for glassware, metal, and objects that won't melt.

Irradiation

Usually destroys or distorts nucleic acids. Ultraviolet light is usually used (commonly used to sterilize the surfaces of objects), although x-rays and microwaves are possibly useful.

Filtration

It involves the physical removal (exclusion) of all cells in a liquid or gas, especially important to sterilize solutions which would be denatured by heat (e.g. antibiotics, injectable drugs, amino acids, vitamins, etc.)

Chemical and gas

Toxic chemicals (formaldehyde, glutaraldehyde, ethylene oxide) kill all forms of life in a specialized gas chamber.

B. Disinfection

Introduction

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. For example, sodium hypochlorite (chlorine), as added to water is safe for drinking, but "chlorox" (5% hypochlorite), an excellent disinfectant, is hardly safe to drink.

Disinfectants

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

Antiseptics

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

Procedures

1. General

- Use aseptic techniques for handling test waters, eluates and cell cultures.
- Sterilize apparatus and containers that will come into contact with test waters and all solutions that will be added to test waters unless otherwise indicated.
- Thoroughly clean all Items before final sterilization using laboratory standard operating procedures.
- Sterilize all contaminated materials before discarding.
- Disinfect all spills and splatters.

Special

a. Solutions

- Sterilize all solutions, except those used for cleansing, standard buffers, hydrochloric acid (HCl), sodium hydroxide (NaOH), and disinfectants by autoclaving them at 121 C for at least 15 min.

b. Autoclavable Glassware, Plasticware, and Equipment

- Lay large vessels on their sides in the autoclave, if possible, to facilitate the displacement of air in the vessels by flowing steam.
- Cover the openings into autoclavable glassware, plasticware, and equipment loosely with aluminum foil before autoclaving. Autoclave at 121 C for at least 30 min. *Glassware may also be sterilized in a dry heat oven at a temperature of 170 C for at least 1 h.*2.
- Sterilize stainless steel vessels (dispensing pressure vessel) in an autoclave at 121 C for at least 30 min.
- Vent-relief valves on vessels so equipped must be open during autoclaving and closed immediately when vessels are removed from autoclave.
- Presterilize 1MDS filter cartridges and prefilter cartridges by wrapping the filters in Kraft paper and autoclaving at 121 C for 30 min.

c. Chlorine Sterilization (0.1% chlorine /HOCl/)

- Add 19 mL of household bleach (Clorox, The Clorox Co.) to 900 mL of dH_2O and adjust the pH of the solution to 6-7 with 1 M HCl. Bring to 1 liter with dH_2O

- Sterilize pumps, plasticware (filter housings) and tubing that cannot withstand autoclaving, and vessels that are too large for the autoclave by chlorination. Prefilters, but not 1MDS filters, may be presterilized with chlorine as an alternative to autoclaving.
- Sterilize filter apparatus modules, injector tubing and plastic bags for transporting injector tubing by recirculating or immersing the items in 0.1% chlorine for 30 min. Filter apparatus modules should be disinfected by sterilization and then cleaned according to laboratory standard operating procedures before final sterilization.
- Drain the chlorine solution from objects being sterilized. Dechlorinate using a solution containing 2.5 mL of 2% sterile sodium thiosulfate per liter of sterile dH_2O.
- Thoroughly rinse pH electrodes after each use to remove particulates. Sterilize
- Before and after each use by immersing the tip of the electrode in 0.1% chlorine for at least 1 min. Dechlorinate the electrode as in Step 2a above. Rinse with sterile dH_2O.

General Precaution

- Do not add antibiotics to media or medium components until after their sterility has been demonstrated.
- Inoculate 1 mL portions of the material to be tested for sterility into tubes containing 9 mL of broth medium by stabbing the inoculum into the broth. Incubate at 36.5 ± 1 C.
- Examine the inoculated broth daily up seven days to determine whether growth of contaminating microorganisms has occurred.
- Incubate either the entire stock of prepared media or portions taken during preparation that represent at least 5% of the final volume at 36.5 ± 1 C for at least one week before use.
- Visually examine and discard any media that lose clarity. A clouded condition that develops in the media indicates the occurrence of contaminating microorganisms.
- Autoclave contaminated materials for at least 30 min at 121 C. and be sure that steam can enter contaminated materials freely.
- Disinfect spills and other contamination on surfaces with either a solution of 0.5% iodine in 70% ethanol (5 g I per liter) or 0.1% chlorine. The iodine solution has the advantage of drying more rapidly on surfaces than chlorine, but may stain some surfaces.

Note: If any biological/biomedical waste has been treated by Incineration in an approved incinerator; steam sterilization at sufficient temperature and for sufficient time to destroy infectious agents in waste; or chemical disinfection where contact time, concentration and quantity of the chemical disinfectant are sufficient to destroy infectious agents in waste, it is rendered to be harmless and biologically inert.

Always have in mind that physical Agents such as applications of heat, sterilization (boiling, autoclaving, hot air oven) pasteurization, low temperature (refrigeration and freezing), drying (removal of H_2O), irradiation (microwave, UV, x-ray) and chemical agents are methods commonly used to control of microbial growth in the laboratory.

SECTION III. USE OF THE MICROSCOPE

Introduction

The microscope is absolutely essential to the microbiology laboratory. Most microorganisms cannot be seen without the aid of a microscope. And, of course, there are some microbes which cannot be seen even with a microscope, unless it is an electron microscope. You will be using an assigned light microscope for a variety of laboratory work, everything from viewing samples to identication of unknown bacterium. Therefore, it is extremely important that to understand how to use the microscope effectively and how to use different types of microscopy such as brightfield, phase-contrast, and darkfield.

1. Procedures before putting a slide on the microscope stage

- Find all of the structures on the microscope (diagrams below) being sure that you know their functions.
- Rotate the condenser so that you see all of the settings (white letters are engraved into the front of the condenser dial).
- Move the iris diaphragm left and right so you can see the effect on the amount of light.
- Start with brightfield microscopy always. The brightfield condenser has a 0 etched in white.
- Raise the condenser stage all the way up. There is a special knob for the condenser stage under the mechanical stage. The condenser gathers all available light from the lamp and directs it up to the stage. We always have the condenser stage closest to the mechanical stage when viewing microorganisms.
- Turn the brightness control knob all the way up, and then back off 1/4 of a turn. This is where the control knob will stay (do not touch it again). Your light amount coming up through the condenser is controlled by the iris diaphragm.
- Rotate the revolving nosepiece until the low power 10Xobjective lens snaps into place.
- Bring the stage all the way up, using the coarse adjustment knob. Keep an eye on the distance between the slide and the lens to make sure that you do not crash the lens into the stage.
- Clean all lenses (oculars, objective lenses, and lens on condenser) with lens paper.
- Set the ocular lenses to the correct distance for your face (the oculars can be moved apart or closer together for your own needs). These ocular eyepiece lenses are both 10X magnification.

2. Procedures to view a sample

- Place the wet mount or prepared smear on the stage, and secure it inside of the stage clips.
- Try to guesstimate where the sample is located on the slide, and place it in the center of the hole allowing light through the stage.
- While looking through the ocular eyepiece, lower the stage slowly using the coarse adjustment knob. Be sure that you are looking through the binocular head of the microscope with both eyes.

- As soon as you see the sample, stop using the coarse adjustment, and switch over to the fine adjustment knob. After focusing at the beginning with the coarse adjustment knob, it is not touched again. All focusing will now be done with the fine adjustment knob.
- Change the objective by rotating the 40x lens in place, making sure that it snaps into place. Your sample should still be seen in the field of vision, but 4 times larger now. Use your fine adjustment knob to clarify the objects.

If your field of vision is fuzzy, and no amount of focusing brings the object into view, you probably have oil residue on the 40xobjective. It has to be cleaned well with lens paper.

3. Procedures to move into oil immersion, 100 x magnifications

- Do not move the focus knobs or the stage knobs.
- Swing the 40x objective (high dry) out of the way.
- Place a single drop of immersion oil on the slide right over where the light is coming through the stage, and rotate the 100x objective (oil immersion) into place. The lens will actually go into the oil drop.
- Now look through the oculars, increasing your light with your iris diaphragm lever. Your object should still be in the field of vision, probably out of focus. Use the fine adjustment knob to focus clearly.
- Once you have gone into oil immersion, do not go back to the 40x objective. The objective will get oil on it, and you will have to really clean it to get the oil off. The 10x can be returned to, since the lens should not touch the slide anyway.
- Once through with the microscope, use the lens paper to wipe the oil from the 100x objective lens.

Note: once you are looking at your object using brightfield, you can easily switch to another type of microscopy: just rotate the condenser knob. However, darkfield is used for wet mounts, using 10x and 40x (100x will not show well). Be sure that your iris diaphragm is open all the way. Phase-contrast is used for wet mounts also, although sometimes it is helpful for delineating subtle shapes and colors that cannot be readily seen using brightfield. Be sure that you are using the correct condenser setting for that particular objective lens**.** Two devices that are used to measure size of organisms with the compound light microscope are an ocular micrometer and a stage micrometer. The stage micrometer gives greater accuracy than is required for this class. An ocular micrometer is a glass disc with an etched scale that fits into the eyepiece. This scale changes for each objective lens. An important concept to keep in mind is that the eyepiece magnification does not affect your calculations. The objective image and the micrometer scale are focused at the same point in the eyepiece so the eyepiece magnifies both by the same amount. The particular micrometers installed in our microscopes have 10 mm / 100 divisions. You will calculate the apparent width of each division (W) for the objectives that are most useful in this course (40X and 100X). Once you have these values you can use them to measure organisms.

W = actual width of each division/ magnification of objective

W with 40X objective =_____ (Give answers in millimeters and micrometers)
W with 100X objective=_____

Observe the prepared slide at this station and give its length and width in micrometers. Notice that the user can turn the eyepiece with the micrometer inside to get the lines superimposed on a particular cell. If size seems to vary include a range in your answer.

Attention:

- You are responsible for your microscope!
- Wrap the cord around the cord holder on the arm.
- Make sure that the lens is in place!
- Turn the coarse adjustment knob so that the stage is far from the lens.
- Place your microscope back in its numbered position in the correct place or cabinet.

SECTION IV. SAMPLING TECHNIQUES

A. General

Introduction

The value of bacteria isolation depends to a considerable degree on the care and skill with which samples are taken, stored, and transported to the laboratory.

Materials

- Sterile plastic bags
- Screw top tubes/sealed sterile tube
- Disposable, screw-capped 25 ml bottles
- Glass bottle
- Anaerobic swab/Port–a–Cul system
- Swabs
- Transport media
- Scissors
- Labeling Markers
- Labels and stickers
- Field data sheet
- Safety equipments
- Decontamination equipments and chemicals
- Compass
- Coolers
- Sample boxes/containers

Procedures

- Select samples judiciously!
- Soil samples should be collected aseptically and placed in sterile plastic bags or heat sterilized containers. Seal tightly. Do not use chemically disinfected containers, or plastic gloves or sleeves.
- Water samples are best collected or submitted an in screw top tubes/sealed sterile tube or sample bottle. Never submit fluids or other specimens in EDTA blood tubes, as EDTA is highly toxic to bacteria.
- Samples for isolation of anaerobic bacteria require special care. Anaerobic bacteria die in the presence of oxygen and

should be shipped in a reduced container, such as anaerobic swab or Port–a–Cul system.
- Label all submissions with the location and sample of origin. The same bacterial species may be highly significant or a meaningless contaminant, depending on the place from which the sample was obtained. Also, depending on the origin, different culture requirements may be necessary to isolate and identify specific bacteria.
- Some samples, such as nasal swabs must be delivered to the laboratory within 12 hours of collection.
- Fastidious microorganisms require special media for transport to the laboratory.
- When collecting large numbers of samples aware the laboratory for scheduling. This permits the laboratory to have personnel and media available for prompt processing.
- Keep samples cold or as their natural status when it is needed from the time they are collected until they arrive at the laboratory.

B. Special

1. Soil sampling

Materials

- Wide topped jars or whirl-pak bags
- Clean trowel, spade or auger
- Sterile disposable gloves
- Esky for storing filled sample bottles
- Coolers
- Any equipment needed for taking on-site tests (thermometer, conductivity meter, pH meter, etc)
- Sampling record sheet

Procedures

- Divide your field into areas which have the same soil type, color, slope, degree of erosion and crop history.
- Take 15-20 cores from each uniform soil area in relatively uniform areas of 20 acres ($0.06km^2$). Use surface samples to eight inches and subsurface samples to 48 inches s sample: 0-8 inches; subsurface sample: 8-24 inches and deep subsurface sample: 24-48 inches)
- Mix them thoroughly in a clean plastic or paper container.
- Fill the soil sample bag one-third to one-half full from this representative sample (max. 2kg + 2 kg /duplicate sample/).
- Scrape away surface litter, and sample to plow depth for all row crops.
- On permanent pastures, sods, lawns, and turf areas, sample four inches deep.
- Label each sample bag with your name and sample identification. The label information should correspond to the sample identity listed on your information sheet.
- Avoid taking samples from areas such as lime piles, fertilizer spills, gate areas, livestock congregation areas, poorly drained areas, dead furrows, fertilizer bands, old fence rows, or any other unusual area
- Do not use galvanized, soft steel or brass equipment if trace metal analyses are desired.
- If samples are excessively wet dry it on air to a workable condition before packaging.
- Place sample bags in a sturdy, spill proof container and pack tightly to prevent opening and spillage during transportation.

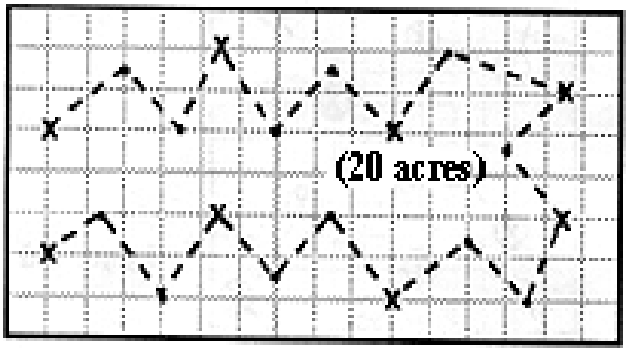
Note:

soil core is an individual boring or coring at one spot in the field. Soil cores can be collected at random in the sample area or in a grid pattern.

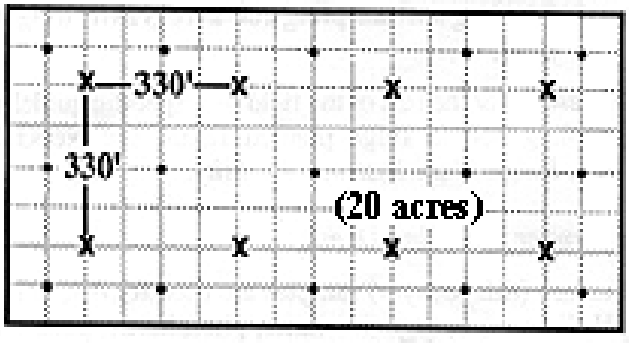
- Collection at random may save some time; however, cores need to be collected from the entire area to obtain the most reliable estimates.
- Collecting soil cores in a grid pattern may require more time to establish the grid; however, it does ensure that the entire area is represented in the sample. Less variation from year to year is expected when samples are collected in a grid pattern from the same areas each year.
- As soil cores are collected, the entire core for the desired depth should be placed in a plastic pail for mixing. Separate pails are needed for surface cores and each subsurface depth sampled. Soil cores in each pail are then thoroughly mixed and a subsample placed in a separate bag or box which has been labeled for the sample area in the field, and for the depth of sample.

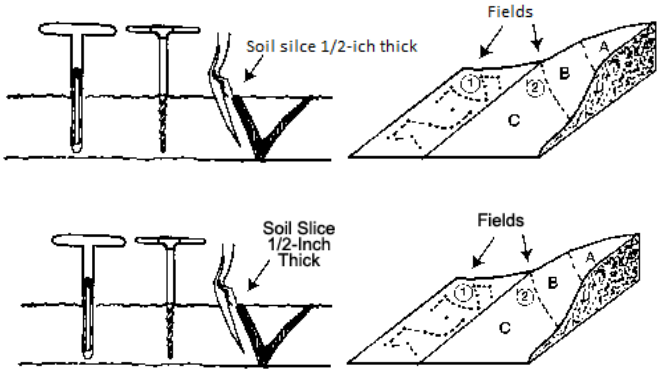
Soil Samples collection strategy

a. Samples collected at random

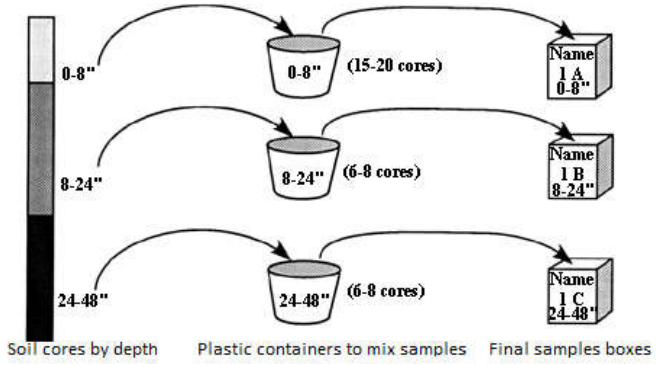


b. Samples collected in grid pattern = Surface samples only, x= Surface + subsurface samples





c. Mixing and pacing of samples



2. Water sampling

Materials

- wide mouthed polyethylene screw top jars/ sample bottles or whirl-pak type bags
- Sterile disposable gloves
- Esky for storing filled sample bottles/bags
- Coolers
- Any equipment needed for taking on-site tests (thermometer, conductivity meter, pH meter, etc)
- Sampling record sheet

Procedures

- Sample directly into the sampling bottle or jar unless a mixed sample is necessary, in which case ensure that the larger mixing container has been prepared in the same manner as the sampling container.
- Use disposable gloves in polluted waters.
- When collecting bacterial samples use clean hands, disposable gloves and sterile collection procedure - that is, only break the seal just prior to collecting the sample, hold lid and bottle opening facing downwards, do not put the lid down on a surface, ensure hand is held drown stream of the bottle, cap the sample immediately after collecting.
- When sampling water in streams or dams, submerge the bottle carefully. The mouth of the bottle should be held at least 10 cm below the surface.
- Do not rinse the sampling container in the sample stream when sampling waters. If it has been correctly prepared the sample container should not contain any contaminants.
- When sampling waterbodies avoid dislodging benthic algal mats, scraping against walls or floors of waterbodies, or otherwise disturbing the sediment. Care should be taken so that no larger animals or algae mats are included in the sample, unless that is what you specifically wish to sample.
- In flowing water, always ensure that your hand is downstream of the collecting bottle's mouth and that the mouth of the bottle is facing into the current. Ensure that the sample is taken midstream.
- Record relevant details on the sampling record sheet, and also on the chain-of-custody record for samples being sent away for testing.
- Samples should be chilled immediately, and stored in a freezer before dispatch to the testing laboratory in an ice-filled esky.
- Samples for bacteria require an air space.
- All samples should be packed in an esky with adequate packing to prevent damage to the bottles. Ice should be packed with the samples.

Note: The choice of sampling sites is important when testing surface waters, because the samples must be representative of the site being tested. Sampling in creeks and dams should take place at a point where there is some depth obtainable, without the sampler causing disturbance to the sediment. It may be necessary to place a structure out into the water if the shallows are less than 50 cm deep. Always attempt to conduct sampling during relatively calm weather, so as to avoid sampling resuspended sediments. If samples are being taken over many days, they should ideally be taken at the same time of day, especially for parameters affected by photosynthesis, such as dissolved oxygen and pH. It is necessary that the person taking the samples use their discretion while collecting, so as not to take a non-representative sample. When sampling waters, flowing or well mixed water is preferable to use for sampling, but this may not always be available, particularly when very still conditions cause stratification in dams or lakes. In this case, attempt to collect three equally sized samples, from the surface, midzone and deeper waters mix them together and sub-sample the mixture.

a. Specific Procedures for stream-water samples

- Clean the equipment coming in contact with the water with dilute nonphosphate, laboratory-grade detergent, rinse it three times with hot tap and three times with deionized or distilled water, and sterilize it (preferably by autoclaving).
- Prepare a separate set of sterile equipment (bottles, nozzles, and caps) for sampling at each site.
- Collect approximately 800 mL of stream water for bacterial indicators and coliphage.
- Leave at least an inch of headspace in the bottle to allow adequate mixing and aeration.
- Process the samples for *Escherichia coli (E. coli*) and (or) *Enterococci* within 6 hours. Store sample on ice in cooler or refrigerator before processing.
- For coliphage and *Clostridium perfringens,* transport samples (at least 500 mL) on ice to the laboratory to arrive within 48 hours after collection.

b. Specific Procedures for ground-water samples

- Clean the sample bottle and equipment coming in contact with the water with dilute nonphosphate, laboratory-grade

detergent, rinse it three times with hot tap water and then three times with deionized or distilled water, and sterilize it by autoclaving.
- Prepare a separate set of sterile equipment for sampling at each site.
- Fill a clean and sterile sample bottle directly from the tap or line
- Collect approximately 800 mL for bacterial indicators. Two 100-mL aliquots are recommended plating volumes for ground water.
- Collect a 1-L sample volume for coliphage
- Leave at least an inch of headspace in the bottle to allow adequate mixing and aeration.
- Process the samples for total coliforms, *E. coli*, and (or) enterococci within 6 hours.
- For *Clostridium perfringens*, send samples on ice to the laboratory to arrive within 48 hours after collection. A 500-mL to 1-L plating volume is recommended.
- Transport the sample on ice to the laboratory to arrive within 48 hours after collection.

Note: Stream or ground water samples for viruses can be analyzed by a molecular technique such as reverse-transcriptase-polymerase chain reaction (RT-PCR) and (or) a cell-culture method (U.S. Environmental Protection Agency, 1996). A method that incorporates both techniques is integrated cell culture-polymerase chain reaction (ICC-PCR) (Reynolds and others 1996). Molecular techniques target specific viruses, but cannot be used to detect the infectious state of a virus; it is usually a presence or absence method. The cell culture method detects infectious viruses, but does not identify the type of virus. Cell culture methods require about 2 weeks, whereas RT-PCR results are available in a few days.

3. Plant Sampling

Plant tissue

Materials

- Sterile plastic bags
- Scissors
- Sterile disposable gloves
- Esky for storing filled sample bottles/bags
- Coolers
- Disinfectants
- Any equipment needed for taking on-site tests (thermometer, conductivity meter, pH meter, etc)
- Sampling record sheet

Procedures

- Before taking tissue samples ensure that timing and location of samples correlates with the purpose of analyses
- Be sure to use a clean container. Never use a metal container as the metal may contaminate the sample.
- Take sufficient amount to conduct an analysis
- Collect samples from both a "good" and a "bad" area often helps in determining analyses.
- collect samples from at least 10 plants
- Never send fresh samples in sealed plastic bags unless kept cool.
- Never freeze samples.

Alternatively

- Observe carefully the affected plants and collect plants that exhibit various stages of seriousness of the disease.
- Collect plants from the center, middle and margins of an affected area of plants and keep the collections separate.
- Collect entire plants. Often leaf or stem symptoms are caused by problems in the root system. If the root system is not included in the sample, the cause cannot be determined.
- Collect only that particular part several samples, not just one, if a disease or disorder obviously affects only a particular part of the plant such as leaves, stems, fruits.
- Retain the soil or potting mix around the root system for entire plants. Keep the root system with in a plastic bag and seal the bag around the main stem of the plant.
- Wrap moist paper towels or cloth around the rooted end prior to packing young rooted plants.
- Place samples plant parts (leaves, stems, fruits), in paper bags. It may be desirable to press leaves flat between two pieces of cardboard.
- Never moisten the above ground parts, particularly the leaves, and then place them in a plastic bag prior to transport.
- Tag each sample and include the completed information.
- Transport samples as soon as possible after collection and by the fastest means

SECTION V. MANIPULATION OF MICROBES

A. Aseptic technique

Introduction

A series of operations have been developed to limit the risk of contaminating sterile media and pure cultures materials during manipulations. These operations are known as aseptic techniques. They also help protect investigators from infecting themselves or releasing the organisms into the environment. Learn the rules and procedures and understand how each requirement contributes to maintaining asepsis.

1. General Procedures

- Work on a clear tabletop. Put all unnecessary items away.
- Wear a lab coat; wash hands before performing any manipulations and after you are through.
- Disinfect the bench-top with an appropriate disinfectant before you begin working and after you are through.
- Keep all cultures closed and tubes upright in a rack until ready for use.
- Work quickly without disturbances.

2. Special procedures

Preparation of Agar Plates

- Melt sterile agar and place the container in a water bath at 45-50°C. Make sure that there is enough water to cover the

agar. If necessary, use a lead doughnut to prevent the agar-containing vessel from tipping.
- Place sterile petri plates on a disinfected bench-top.
- Remove the lid from the agar-containing vessel and pass the mouth through a flame to destroy any contaminating organisms. Hold the container at an angle and not vertical.
- Pour the agar into the petri dishes. Gently swirl the plate to distribute the agar to cover the bottom of the plate.
- If any agar remains in the container, pass the mouth through a flame and close the vessel. Return it immediately to the water bath.
- Allow the plates to solidify. Label the plates with the type of media they contain and the date they were poured.
- If plates are being stored for more than 48 hours, seal them in a plastic bag.

Transfer of microorganisms

Taking/removal from Broth Cultures or Solid Media

- Microbes can be taken/ removed from broth with an inoculating loop or a pipette; they can be removed from solid media with an inoculating loop or needle.
- Inoculating loops and needles are made from metal wire and can be sterilized by flaming until red-hot.
- The entire length of the metal wire must be sterilized. This is done by flaming from the handle end to the tip (avoiding aerosolizing liquid in the loop). Flaming incinerates all organisms that are present. Once sterile, the loop is inserted into a tube of broth and liquid is lifted out. The needle is touched to the region of growth on the solid medium.
- Pipettes are either purchased sterile and disposable or are sterilized in canisters prior to use. Remember to keep the pipette canister on its side.
- Do not stand it up. When lifting a pipette from the canister, touch only at the mouthpiece and only one pipette. Close the canister when you have removed the pipette. Never return pipettes to the canister. A pipetter is always used to remove a portion of the broth. Handle only the upper portion of the pipette when inserting it into the pipetter. If the tip should inadvertently touch another surface, do not use the pipette. Take a new one.
- Micropipettors with sterile tips can also be used.

Transfer to Broth Media in Flasks or Tubes

- After the lid is removed, flame the mouth of the flask or tube.
- Holding the vessel at an angle, introduce the loop or needle into the liquid and agitate gently. If a pipette is used, a measured volume of liquid can be released using the pipetter.
- Flame the mouth of the flask or tube before returning the cover. Flame the loop or needle after use to incinerate any remaining organisms. Dispose of all contaminated pipettes or tips in the appropriate containers.

Transfer to Agar Slants

- After the lid is removed, flame the mouth of the tube.
- Holding the tube at an angle, place the loop or needle toward the bottom of the slant and draw it up over the agar surface.
- Flame the mouth of tube before returning the cover. Flame the loop or needle after use to incinerate any remaining organisms.

Transfer to Agar Plates

- Pour an agar plate.
- Transfer a loop of sterile water to two tubes of sterile Columbia base Blood Agar (BA)
- Transfer 0.1 ml of sterile water to two tubes of sterile BA using sterile pipettes.
- Incubate these tubes at room temperature for 48 hours.
- Tape the agar plate to prevent the lid from falling off.
- Examine for growth. If there is any growth in the broth or on the plate, you have not mastered aseptic transfer technique. Repeat the procedure.

Note: Assess your performance of

1) Pouring an agar plate

- **Before pouring**

- Was the bench-top disinfected?
- Was the agar at 45-55C (did they check)?
- Was the mouth of the vessel flamed? Was it at an angle?
- Was the lid held so that it wasn't contaminated while it was off the vessel?

- **Pouring**

- Was the agar poured to just fill the bottom of the plate?
- Was the petri plate lid held when agar was poured?
- Was the mouth of the vessel flamed again if it contained more agars?

- **After Pouring**

- Was the agar returned to the water bath immediately?
- Was the plate allowed to solidify before it was incubated?
- Is the plate sterile after incubation?

2) Transferring a loop of sterile water to BA

- **Before transfer**

- Was the bench top disinfected?
- Were the tubes labeled properly?
- Were the tubes labeled legibly?
- Was the entire length of the loop sterilized until red hot?
- Was it sterilized from the handle end to the tip?
- Was the lid of the sterile water held so that it wasn't contaminated while it was off the vessel?
- Was the mouth of the tube of water flamed after the lid was removed?
- Was the loop held so that it wasn't contaminated prior to transfer?
- Transfer

- Was the loop inserted into the tube of water held at an angle?
- Was the lid of the water tube placed back after removal of sample?
- Was the lid of the TSB tube held so that it wasn't contaminated while off the vessel?
- Was the mouth of TSB tube flamed after the lid was removed?
- Was the loop inserted into the BA tube held at an angle?
- Was the lid of the TSB tube placed back on after transfer of the sample?
- Was the loop flamed to incinerate any materials on it?
- Were the tubes incubated at the recommended temperature?
- Was the BA sterile after incubation?

3) Labeling

- **Label on the bottom of the plate**
  - In waterproof marker
  - Date
  - Name/initials Media
- **Label around the edge**
  - identification of sample/use
  - legible

B. Working with dilutions

Introduction

It is a common practice to determine microbial counts for both liquid and solid specimens. Most specimens have high enough numbers of microorganisms that the specimen has to be serially diluted to quantitate effectively. The following is a step-by-step procedure to solve working dilution problems, and includes some practice problems at the end.

Principles

The standard formula is

$$\frac{\text{Colony count (CFUs) on an agar plate}}{\text{Total dilution of tube X Volume plated}}$$

To work the problem, you need 3 values

- a colony count from the pour or spread plates
- a dilution factor for the dilution tube from which the countable agar plate comes
- the volume of the dilution that was plated on the agar plate

Procedures

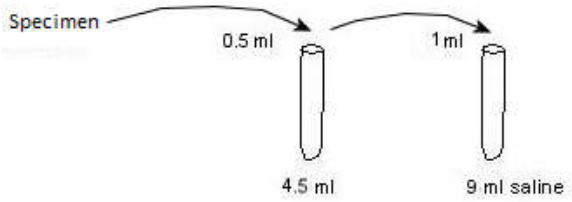
1. Determine the appropriate plate for counting

- Look at all plates and find the one with 30-300 colonies
- Use the total dilution for the tube from where the plate count was obtained.
- If duplicate plates (with same amount plated) have been made from one dilution, average the counts together.

2. Determine the total dilution for the dilution tubes

- Dilution factor = amount of specimen transferred divided by the total volume after transfer (amount of specimen transferred + amount of diluent already in tube).
- Determine the dilution factor for each tube in the dilution series.
- Multiply the individual dilution factor for the tube and all previous tubes.

To calculate this dilution series



- Determine the dilution factor of each tube in the set.

Dilution factor for a tube = $\frac{\text{amount of sample}}{\text{Volume of specimen transferred + volume of diluent in tube}}$

But after the first tube, each tube is a dilution of the previous dilution tube.

So: Total dilution factor = previous dilution factor of tube X dilution of next tube

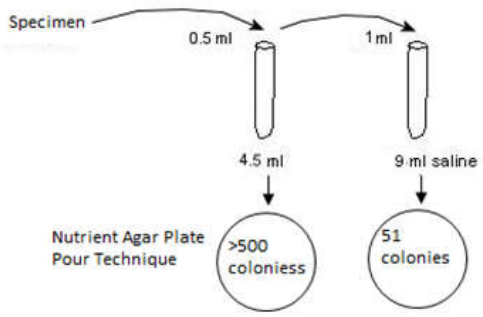
For the above dilution series

0.5 ml added to 4.5ml = 0.5/5.0 = 5/50 = 1/10 for 1st tube 1ml added to 9ml = 1/10 (2^{nd} tube) X previous dilution of 1/10 (1^{st} tube) = total dilution of 1/100 for 2^{nd} tube.

3. Determine the amount plated (the amount of dilution used to make the particular pour plate or spread plate).

There is nothing to calculate here: the value will be stated in the procedure, or it will be given in the problem.

4. Solve the problem



- The countable plate is the one with 51 colonies.
- The total dilution of the 2nd tube from which that pour plate was made = $1/10^2$

- The amount used to make that pour plate = 0.1ml (convert to 1/10 - it is easier to multiply fractions and decimals together).

51 colonies = 51 X 10^3 = **5.1 X 10^4** (scientific notation) OR **51,000 CFUs/ml**
$1/10^2$ X 1/10

45 colonies = 45 X 10^4 = **4.5 X 10^5** (scientific notation) OR **450,000/ml**
$1/10^3$ X 1/10

C. Pure culture techniques

Introduction

Microbes could be spread across the solid surface and separated from each other. When these individual organisms reproduced, they formed colonies. When each colony-forming unit (CFU) represents the progeny of a single bacterium; it is a pure culture. Today's widely used solidifying agent is agar, a more stable algal product. Only with pure cultures can the properties of individual types of microorganisms be examined and understood. Bacteria can be separated from each other by quadrant streak, spread plate or pour plate.

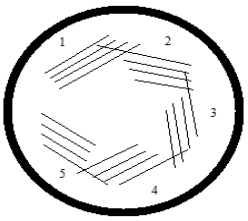
Materials

- Appropriate agar medium
- Petri dishes
- Glass spreading rods
- Alcohol
- Micropipettors (instructions on page 4-7)
- Loop
- Mixed cultures at 10^8/ml for quadrant streak
- Mixed cultures in broth for spread and pour plates at concentrations of ~10^3/ml

Procedures

1. Quadrant streak plate

- Flame the loop and wire until it is red hot. Do not contaminate the loop during this procedure
- Take a colony.
- Spread the over a small region on the edge of the plate
- Flame the loop and let it cool for a few seconds.
- Streak from the end of region 1 across the edge of the plate forming region 2.
- Flame the loop and let it cool for a few seconds.
- Streak from the end of region 2 across a quarter of the plate forming region 3.
- Flame the loop and let it cool for a few seconds.
- Streak from region 3 across the remaining portion of the plate forming region 4.
- Flame the loop before setting it down.
- Incubate the plate for 24 hours in an inverted position. Look for isolated colonies



2. Spread plate

- Remove the cap from the culture tube and flame the mouth of the tube. Do not contaminate the cap during this procedure.
- Take 0.1 ml of culture broth.
- Flame the mouth again and cover the tube.
- Place culture broth in the center of the plate.
- Dip the bent glass rod in the alcohol and shake off excess liquid. Keep the alcohol container away from the flames.
- Carefully flame the rod. When all the alcohol has burned, allow to cool for a few seconds. You may be sure the rod is cooled by placing it on the agar surface at the edge of the plate.
- Use the rod to spread the organisms over the surface of the plate while rotating the plate on the desktop.
- Return the glass spreader to the alcohol.
- Incubate the plate for 48 hours in an inverted position. Look for isolated colonies.

3. Pour plate

- Pipette 0.1 ml of the mixture into individual sterile Petri dishes. Be sure to flame the mouth of the culture tube before and after you pipette.
- Flame the mouth of a bottle of appropriate agar and carefully pour 10 to 20 ml of agar into the Petri dish. Flame the mouth of the bottle, cover it and return it to the water bath. Do not keep the agar out of the water bath for any extended period of time.
- Mix the contents of the Petri plate by carefully moving the dish in a figure eight motion on the bench top.
- Allow the agar to solidify and incubate for 24 hours in an inverted position to prevent condensation.
- The microorganisms will grow as individual colonies within the agar.

Note: performance Assessment

- **Streak plate**

- Is the streak spread over four quadrants?
- Are there isolated colonies in the fourth quadrant?
- Is the level of the agar no more than half of the height of the plate?
- Is the plate labeled correctly?

- **Spread plate**

- Are there isolated colonies uniformly spread throughout the plate?
- Is the level of the agar no more than half of the height of the plate?
- Is the plate labeled correctly?

- **Pour plate**

- Are there isolated colonies uniformly spread throughout the plate?

- Is the level of the agar no more than half of the height of the plate?
- Is the plate labeled correctly?

SECTION VI. CULTURES

A. Isolation

Introduction

The single most important step in analyzing a sample containing bacteria is to obtain isolated colonies of bacteria that arise from single cells. Attempts to identify bacteria in a field sample cannot be done unless isolated colonies are used. To obtain well-isolated colonies, it is essential to disperse the inoculum (sample) on the surface of an enriched agar plate so that individual bacteria are well separated from each other. It is better to make the primary isolation on sheep blood agar, a rich medium that supports the growth of many types of microorganisms. The appearance of colonies and red blood cell lysis are important identification/ diagnostic features.

Materials

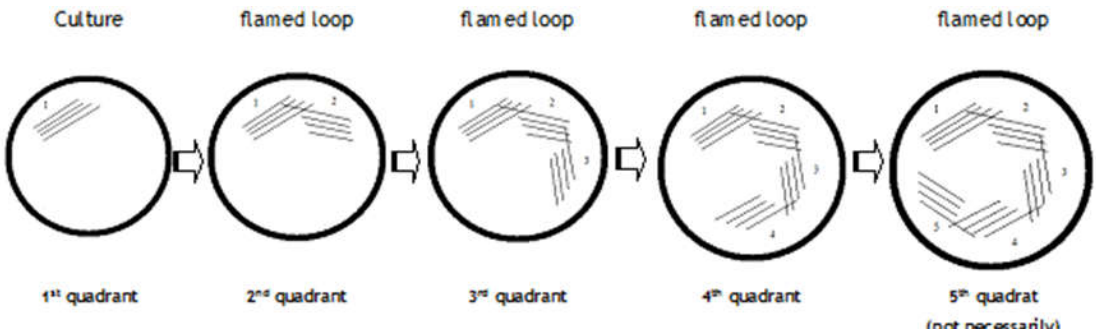
- Agar plates
- Wire loops
- Bunsen Burner
- Field sample
- Labeling marker
- Incubators

Procedures

- With the loop, spread the inoculum back and forth across the upper 1/4 of the plate, keeping the lines of inoculation very close together. Isolated colonies are not expected in this area.
- Do not use strong pressure, which will break the surface of the agar. Use the end of the loop, not its side when streaking.
- Dispose of the loop in the biohazard bucket on the bench.
- Turn plate approximately 90 degrees. Streak the plate across about 1/4 of the plate.
- Dispose of the loop.
- Turn the plate 90 degrees again, using the loop streak into the second area only a couple of times and then zig-zag across the remaining open area of the plate - being sure not to cross into areas 1 or 2 as this will put too many bacteria into this area that should hopefully contain isolated colonies.
- Repeat the above procedure to have 4 to 5 streaked area
- Stab the first streak area a couple of times to accentuate hemolysis.
- Label plates and incubate inverted at 37 C.
- Single colonies should appear in the third to fifth area.

Note: lines should be closest together in streak 1 and progressively further apart in succeeding streaks.

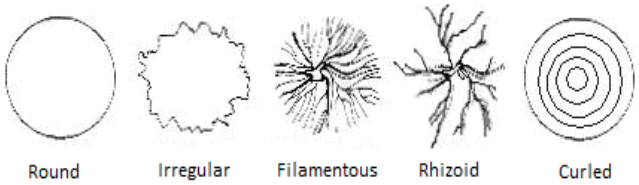
Streak for Isolation



Colony Morphology

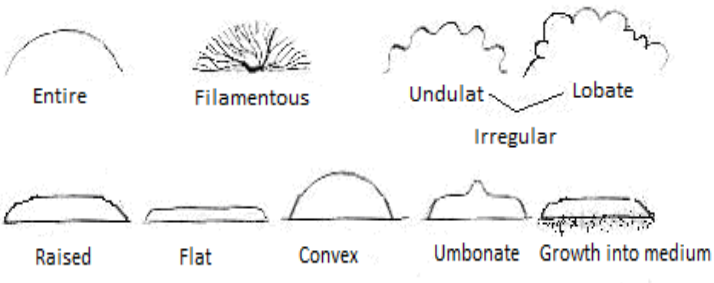
It is the evaluation grew colonies on a plate:

- Colony size (punctiform, small, medium/moderate, large)
- Colony shape (round, irregular, filamentous, rhizoid, curled)



- **Type of margin/edge**

(Entire, erose/irregular, crenote/round-toothed or scalloped edge, undulate/wavy surface or edge, lobate, ciliate, fimbriate, lacerate, ramose/branched



- **Colony elevation**

(Effuse, low convex, raised, convex, convex papillate, convex rugose, raised with concave beveled edge, umbonate, pulvinate)

- **Colony texture (surface appearance)**

- Shiny to dull
- Smooth to wrinkled
- Rough
- Granular (finely granular, coarsely granular)
- Mucoid

- **Optical property**

(transparent, translucent, opaque, wavy interlaced, arborescent)

- Complete (transparent)
- Through intermediate (translucent)
- Through completely lacking (opaque)

- **Colony pigmentation**

- Non-pigmented (cream, tan, white)
- Pigmented (purple, yellow, red, black, etc)

Determine and record the identity of your colonies

Determination	Colony 1	Colony 2	Colony 3	Colony 4
Size				
Shape				
Margins				
Elevation				
Surface appearance/texture				
Optical property				
Pigmentation				

B. Monitoring of Bacterial Growth

There are a number of ways that we can measure the growth of bacteria in our culture. The most direct is to collect the bacteria at various times and either weigh or count them.

1. Measuring cell mass

Introduction

Bacteria are denser than the medium in which they are grown. They fall to the bottom of the culture tube. We can speed up this process by centrifugation**;** centrifugation increases effective gravitational field the cells experience and so reduces the time it takes for them to fall to the bottom of the test tube. The method is used to measure the mass of bacteria in a known volume of culture at various times.

Materials

- 24-hour 10ml broth culture of bacteria
- Pipettes with pipetter
- Eppendorfs
- Centrifuge (low speed and high speed)
- Analytic balance

Procedures

- Take 5 samples in Eppendorf tubes (max 1.0ml)
- Use centrifugation to separate the bacteria from the culture medium
- Mark and weight each tube before proceed
- Fill a tube with 0.5ml of culture
- Centrifuge the tubes using microfuge by setting the speed and time. As the microfuge rotor spins bacteria, which are denser than the medium in which they are growing, are thrown to the bottom of the centrifuge tube
- When the rotor comes to rest the bacteria will have been removed from solution and compacted into a pellet
- Remove the liquid with a pipette. To get an accurate measure of mass, it is important not to disturb the pellet.
- Place the tubes, with their top open into a second machine with a low speed centrifuge attached to a vacuum pump.
- Close the centrifuge top, apply a vacuum; over time the liquid will evaporate away and the dried bacteria will remain at the bottom of the tube. Close the lids of the microfuge tubes to prevent the loss of the dried bacteria.
- Now weigh the tube again. The increased weight of the tube should be due almost entirely too dried bacteria. After choosing a time, determine the grams biomass/ml of culture medium Take a microfuge tube out of the carton, mark it and weigh it. At the end, weight the same tube, which now contains dried bacteriaSubtract the original weight of the tube from the weight of the tube+ dried bacteria. This number, multiplied by to (remember, we started with 0.5 ml), is an estimate of the bacterial biomass per ml of culture

2. Determination of bacterial numbers

The two most widely used methods for determining bacterial numbers are the standard, or viable, plate count method and spectrophotometric (turbidimetric) analysis.

a. Standard plate count method

Introduction

The standard plate count method is an indirect measurement of cell density and reveals information related only to live bacteria. The standard plate count method consists of diluting a sample with sterile saline or phosphate buffer diluent until the bacteria are dilute enough to count accurately. That is, the final plates in the series should have between 30 and 300 colonies. Fewer than 30 colonies are not acceptable for statistical reasons, and more than 300 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct colony-forming units (CFUs). The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). Thus, the number of colonies should give the number of bacteria that can grow under the incubation conditions employed. A wide series of dilutions (e.g., 10^{-4} to 10^{-10}) is normally plated because the exact number of bacteria is usually unknown. Greater accuracy is achieved by plating duplicates or triplicates of each dilution.

Materials

- 24-hour 10ml nutrient broth culture of bacteria
- Sterile 99-ml saline blanks/bottles
- Pipettes with pipetter
- Petri plates
- Agar pour tubes of nutrient agar (plate count agar)
- Water baths
- Bunsen burner
- Tubes of 5ml nutrient broths

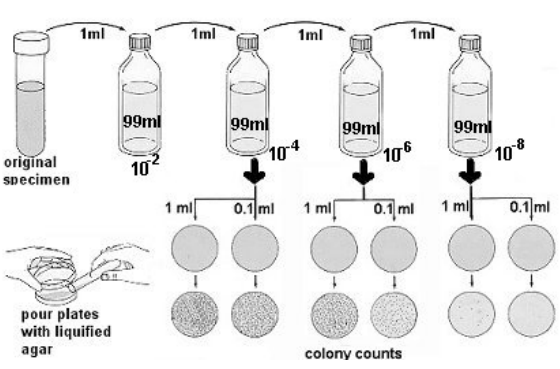
Procedures

- Label six petri plates 1-6. Label four tubes of saline 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}.
- Using aseptic technique, the initial dilution is made by transferring 1 ml of bacterial sample to a 99ml sterile saline blank. This is a 1/100 or 10^{-2} dilution.
- Then shake the 10^{-2} blank by grasping the tube between the palms of both hands and rotating quickly to create a vortex. This serves to distribute the bacteria and break up any clumps.
- Uncap it and aseptically transfer 1ml to a second 99ml saline blank immediately after the 10^{-2} blank has been shaken. Since this is a 10^{-2} dilution, this second blank represents a 10^{-4} dilution of the original sample.
- Shake the 10^{-4} blank vigorously and transfer 1ml to the third 99ml blank. This third blank represents a 10^{-6} dilution of the original sample. Repeat the process once more to produce a 10^{-8} dilution.
- Shake the 10^{-4} blank again and aseptically transfer 1.0 ml to one petri plate and 0.1 ml to another petri plate. Do the same for the 10^{-6} and the 10^{-8} blanks.
- Take/remove one agar pour tube from the 48 to 50C water bath. Carefully remove the cover from the 10^{-4} petri plate and aseptically pour the agar into it. The agar and sample are immediately mixed gently moving the plate in a figure-eight motion or a circular motion while it rests on the tabletop. Repeat this process for the remaining five plates.
- After the pour plates have cooled and the agar has hardened, invert and incubate at 25C for 48 hours or 37C for 24 hours.
- At the end of the incubation period, select all of the Petri plates containing between 30 and 300 colonies.
- Plates with more than 300 colonies cannot be counted and are designated too many to count (TMTC).
- Plates with fewer than 30 colonies are designated too few to count (TFTC)
- Count the colonies on each plate. A colony counter should be used.
- Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquified agar.

Number of colonies = Number of bacteria/ml
Dilution X Amount plated

- Record your results.

Diluting and pouring



b. Spectrophotometric (turbidimetric) analysis

Introduction

Standard curve is determined by spectrophotometery. The amount of light passing through the sample is indicated on a meter and can be read either as percent transmittance or optical density (O.D.). Optical density is a measure of absorbance and is related to transmittance by the following equation:

O.D. = 2 - log of % transmittance

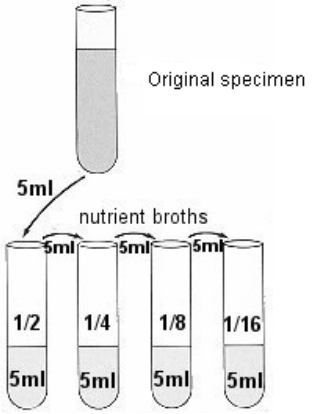
Increased turbidity in a culture is another index of bacterial growth and cell numbers (biomass). By using a spectrophotometer, the amount of transmitted light decreases as the cell population of bacteria increases. The transmitted light is converted to electrical energy, and this is indicated on a galvanometer. The reading indirectly reflects the number of bacteria. This method is faster than the standard plate count but is limited because sensitivity is restricted to bacterial suspensions of 10^{7} cells or greater.

Materials

- 24-hour 10ml nutrient broth culture of bacteria
- Sterile test tubes
- Pipettes with pipetter
- Micro-cuvettes
- Micro-cuvettes holder
- Spectrophotometer

Procedures

- Put the original tube of the specimen and four tubes of the sterile nutrient broth in a test-tube rack. Each tube of nutrient broth contains 5 ml of sterile broth.
- Use four of these tubes (tubes 2 to 5) of broth to make four serial dilutions of the culture.
- Transfer 5ml of the specimen to the first tube of nutrient broth, thoroughly mixing the tube afterwards.
- Transfer 5ml from that tube to the next tube, and so on until the last of the 4 tubes has 5ml added to it. These tubes will be ½, 1/4, 1/8, and 1/16 dilutions.



- Set the wavelength of the spectrophotometer between 550-600nm.
- Standardize the spectrophotometer. The blank used to standardize the machine is sterile nutrient broth: it is called

the blank because it has a sample concentration equal to zero.

- Pipette 1ml of the sterile NB into one of the micro-cuvettes. Place micro-cuvette with "V" facing you into cuvette holder. Place into cuvette chamber, close the cover and read. Save blank to re-standardize the machine to infinity absorbance and zero absorbance before each reading because the settings tend to drift.
- Pipette 1ml of the 1/16 bacterial dilution into a second micro-cuvette. Place in cuvette holder and read.
- When read, discard micro-cuvette into bleach container on your table.
- Next pipette the 1/8 dilution into the third cuvette and read it.
- Repeat this with the 1/4 and ½ dilutions. Last, read the absorbance value for the original tube of the specimen

- **The micro-cuvette must contain 1ml for the spectrophotometer to read the fluid**

- Place micro-cuvette with parallel lines facing you into cuvette holder.
- Close the hatch when reading the spectrophotometer.
- Re-standardize between readings to account for drift.
- Mix the dilutions before pipetting into the micro-cuvette to read absorbance.
- Read to the *nearest* thousandth (0.001) on the absorbance digital display.

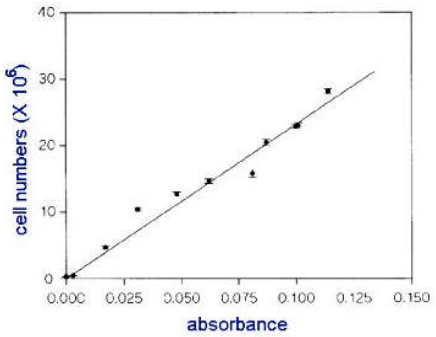
Record your values, along with the dilutions that they came from. Using the plate count data, calculate the colony-forming units per milliliter for each dilution.

Data collection

Specimen dilutions	Absorbance (X)	Number of bacteria (Y)
1/2		
1/4		
1/8		
1/16		

- Fill in your absorbance values for the 5 tubes read in the spectrophotometer.
- Calculate the number of bacteria in the original tube of bacteria, and place that value in the top right cell of the table.
- Calculate the approximate numbers of bacteria in the ½, 1/4, 1/8, and 1/16 by halving the number in the cell above.
- Plot these 5 coordinates on a graph.

An example of a graph



Use of the spectrophotometer

- Light entering a cloudy solution will be absorbed. A clear solution will allow almost all of the light through. The amount of absorbance can be determined by using a spectrophotometer, which measures what fraction of the light passes through a given solution and indicates on the absorbance display the amount of light absorbed compared to that absorbed by a clear solution.
- Inside, a light shines through a filter (which can be adjusted by controlling the wavelength of light), then through the sample and onto a light-sensitive phototube. This produces an electrical current. The absorbance meter measures how much light has been blocked by the sample and thereby prevented from striking the phototube. A clear tube of water or other clear solution is the blank and has zero absorbance. The amount of substance in the solution is directly proportional to the absorbance reading. A graph of absorbance vs. concentration will give a straight line.

Bacterial concentrations can also be estimated using McFarland standards

The McFarland Scale is a scale numbered from 1 to 10 which represents specific concentrations of bacteria/ml. The two most widely used McFarland standards methods are labelled tubes 1 through 10 filled with suspensions of Barium salts and calibrated Turbidimeter.

1. Labelled tubes with McFarland scale number: Each tube approximates the turbidity of bacterial solutions corresponding to the McFarland Scale number.

Conversion of McFarland Scale

McFarland Scale	No. Bacteria ($x10^6$/ml)
1	300
2	600
3	900
4	1200
5	1500
6	1800
7	2100
8	2400
9	2700
10	3000

If you have a culture and wish to quickly determine its approximate population, its turbidity can be visually compared to a set of McFarland Standards.

2. McFarland Turbidimeter: It measures the concentration of bacterial suspensions by scaling in number. The scale number corresponds to concentration of bacteria X 10^6/ml. The advantage of these standards is that no incubation time or equipment is needed to estimate bacterial numbers.

Materials

- 24h pure bacterial culture plate
- Pasteur pipettes/wire loop
- Test tube
- Deionized/distilled water
- Vortex

- McFarland Turbidimeter /labelled tubes with McFarland scale number

Procedures

- Flame the Pasteur pipettes/wire loop until it is red hot. Do not contaminate the Pasteur pipettes/wire loop during this procedure
- Fill test tubes with 5ml Deionized/distilled water
- Take similar size colonies from pure culture plate to test tubes
- Vortex to homogenize
- Clean the surface of the tubes
- Insert the tube into the chamber of the Turbidimeter
- Read the concentration on display and adjust the concentration you want for work by adding colonies or by diluting
- Read the final result
- Calculate according to the McFarland Scale

SECTION VII. STAINING AND OBSERVATION OF MICROORGANISMS

A. Smear Preparation

Introduction

A smear is a dried preparation of cells on a slide. Smears can be prepared from cells in a liquid culture. When the smear is completely dry it is heat fixed to a) kill the bacteria b) make them adhere to the slide and c) coagulate the cytoplasmic proteins to make them more visible.

Materials

- Clean Slides
- cultures/colony plates
- Wire loop
- Bunsen burner
- Dropper jar distilled water
- Labeling marker (magic marker or grease pencil)
- Filter papers

Procedures

1. General

- Sterilize work area
- Gather tools
- Label the bottom of the slides
- Light Bunsen burner

2. Special

a. with liquid culture

- Agitate culture tube slightly to suspend microorganism but do not moisten cap.
- Flame loop and entire wire to red hot. Flame handle adjacent to wire briefly.
- Remove cap with loop hand, hold tube in a slanted position, and briefly flame neck of tube.
- After loop has cooled for 10 seconds remove a loopful of the culture being careful not to touch sides of tube.
- Flame mouths of tube again, recap, and return tube to test-tube rack.
- Smear loopful of microorganisms onto glass slide and spread so it will dry quickly.

b. with culture on agar

- Place a small drop of water on a clean dry slide.
- Sterilize loop.
- Open plate so as to minimize contamination from microorganisms in the air.
- With cooled loop or needle remove small amount of culture from an isolated colony. A very small amount of bacteria will suffice
- Mix culture in water drop and spread it out into a thin film.

In common for step1and 2:

- Reflame loop before placing it down to prevent contamination of work area.
- Circle smear with an 'indelible' marker underneath slide because it may become hard to see.
- Allow the smear to dry on the slide at room temperature/air dry. Do not heat the slide to speed drying because this can distort the cellular morphology or staining properties of the microorganism.
- After the smear has dried, heat-fix the slide. Gently heat the slide by passing quickly through the flame 3-4 times. The slide should not get uncomfortably hot when touched to your wrist. It should be warm but not hot to the touch.

B. Staining

Staining of the bacteria from colonies on laboratory media provide a direct visualization of the morphology of the organisms as well as their reactions to the chemicals present in stains. This is an invaluable and easy-to-use tool for establishing the identity of various microorganisms.

1. Simple staining

A simple stain is a procedure in which only one stain is used to create a contrast between the sample and its background. The goal is to become practiced at staining and observing bacteria without you becoming covered with telltale colored spots.

Ingredients and preparation

- Methylene blue 0.3 gm
- Distilled water 100 ml

Dissolve the dye in water. Filter through a filter paper.

Materials

- Smears
- Staining rack
- Tap water
- Dye
- Timer
- Filter papers
- Oil immersion

- Microscope
- Slide box
- Sharp container

Procedures

- Add just enough methylene blue to cover the smear.
- Wait 30 sec to one min, and then wash with a gentle stream of water from a wash bottle. Do not let water strike smear directly or it may wash off.
- Blot dry with *bibulous paper, being careful not to rub the smear.
- Examine the stained smear with low-power, high-power and oil-immersion objectives.

- **Sketch and label it with the following:**

- name of microorganism
- total magnification used
- color
- shape of individual cells as you saw them and as described in a text
- arrangement of cells
- measured/relative size
- You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

2. Differential staining

a. The Gram stain

This is the most important and widely used differential stain. It permits the separation of bacteria into two general groups referred to as Gram positive and Gram negative. Four reagents are used in the Gram staining procedure. *Crystal violet*, a primary dye, is first applied. The basic group of this dye interacts with the acidic group of the cell wall and cytoplasm. A mordant, *Gram's iodine*, is then applied which forms a chemical complex with the primary stain. A decolorizer, *Ethyl alcohol (EtOH)* is then used to remove the primary stain iodine complex from Gram negative cells. Gram positive cells are resistant to such decolorization. A secondary stain*, saffranin,* is applied after decolorization to replace the primary stain within Gram negative cells. Since those cells which are Gram positive retain the primary stain, they appear violet or deep purple which is the color of crystal violet. Gram negative microbes lose this and are stained red or pink due to the safranin. Prepare Gram stains of cultures provided.

Ingredients and preparation

Crystal violet

Solution A

- Crystal violet 2.0 gm
- Ethanol, 95% 20 ml

Solution B

- Ammonium oxalate 0.8 gm
- Distilled water 80 ml

Mix solutions A and B. Store for 24 hours before use.

Gram iodine

- Iodine crystals 1.0 gm
- Potassium iodide 2.0 gm
- Distilled water 300 ml

Grind the dry iodine and potassium iodide in a mortar. Add water, a few ml at a time, and grind thoroughly after each addition until the iodine and iodide dissolve.

Rinse the solution into an amber glass bottle with the remainder of the distilled water.

Saffranin solution

Stock solution

- Saffranin O 2.5 gm
- Ethanol, 95% 100 ml

Working solution

- Stock solution 10 ml
- Distilled water 90 ml

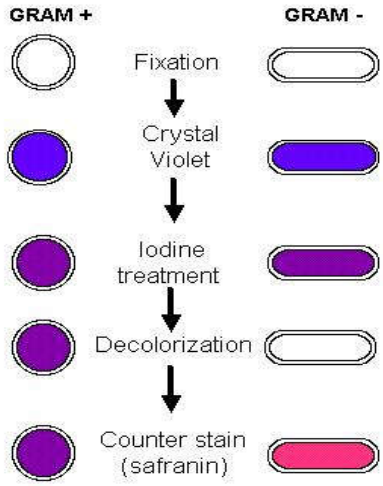
Materials

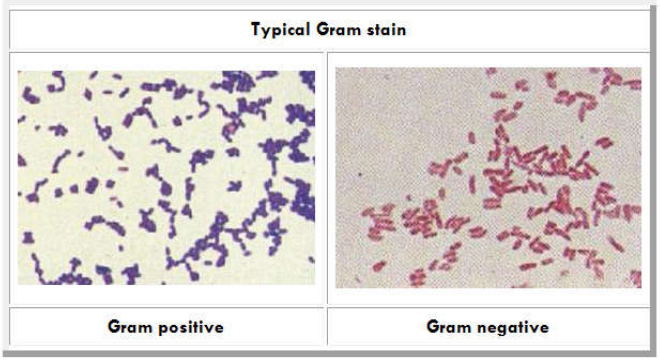
- Smears
- Staining rack
- Tap water
- Dyes
- Timer
- Filter papers
- Oil immersion
- Microscope
- Slide box
- Sharp container

Procedures

- Prepare a heat fixed smear of each of the cultures provided as you did before
- Flood smear with 1 % crystal violet stain for 1 min.
- Rinse off excess stain with water.
- Flood smear with Gram's iodine for 1 min.
- Pour off the iodine. Rinse with water.
- Flood the smear with 95% EtOH for 10 to 30 seconds and quickly rinse with water.
- Flood the smear with 1% saffranin for 20 seconds to1 min.
- Rinse with water.
- Blot with absorbent/filter paper and air dry for a few minutes.
- Examine the stained smear with low-power, high-power and oil-immersion objectives.
- Move the condenser almost all the way up to touching the slide. Do not let the high/dry (40X) lens get into the oil. It will be very difficult to clean.
- Gram-positive organisms will be purple/blue.
- Gram-negative organisms will be pink to red.

Reaction of Gram Staining





- **Sketch and label it with the following:**
- name of organism
- total magnification used
- color
- shape of individual cells as you saw them and as described in a text
- arrangement of cells
- measured/relative size
- gram positive or gram negative
- one interesting fact about this genus or particular species
- You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

b. Capsule staining (negative staining)

When a stain, such as an acid dye, cannot penetrate the outer layers of a microbe, the cell will appear transparent on a colored background. This stain is called a negative or background stain. It is performed by mixing the dye with a suspension of bacteria on a slide and spreading the mixture into a thin layer for viewing. The capsule is a structure surrounding the cell wall that certain bacteria can produce. The ability to form a capsule is genetically and environmentally controlled. Only those microbes with the genes for capsule production have the potential to manufacture this polysaccharide (or polypeptide) surface layer. Special nutrients or other growth factors often are necessary for the genes to be expressed. The role of the capsule is primarily for protection of the bacteria. For example, the capsule affords a seal against dehydration. Many capsules repel white blood cells and thus allow pathogenic invading bacteria to elude one of the primary host defenses. Capsules are not readily stained and therefore are visualized by negative stain techniques. The organisms are prepared as a smear in the presence of an acid dye and allowed to air dry because heat will cause the capsule to shrink. Our procedure will combine a negative stain (which colors the background) and a simple stain to color the bacterial cell. The capsule appears as a colorless layer between the bacterium and the background.

Materials

- India ink or Congo Red
- Methylene blue
- Microscope slides
- Cultures (controls)
- Positive (*Klebsiella pneumoniae* or *Enterobacter aerogenes*)
- Negative (*Corynebacterium xerosis*)

Procedures

- Add one drop of methylene blue onto the smear. Use another clean glass slide to spread the drop into a thin film.
- Allow the dye to react for 1-2 minutes, and then rinse gently with water.
- Shake the excess water off the slide and blot the slide gently with bibulous paper.
- Add one drop of nigrosine onto the smear and spread the dye out using a new clean glass slide.
- Allow to air dry completely. Do not heat fix.
- Examine the smear under oil using the oil immersion lens.
- Diagram the appearance of the microorganisms.

Note: Assess your performance

- Is the Congo red spread uniformly in a thin layer?
- Can individual cells be seen uniformly throughout a single field?
- Is this distribution seen in several fields on the slide?
- Is the capsule easily seen?
- Is the bacterial cell stained and readily observed?

c. Endospore Stain

Spores survive harsh environments, remaining dormant for many years, until conditions for growth become more favorable. They can survive desiccation, high temperatures, UV light, toxic chemicals or a lack of nutrients. Because an endospore's outer coat is an effective barrier to chemicals, endospores generally stain poorly. Endospores can be stained, however, by using very hot dyes. The heat apparently causes the coat to expand so that the dye penetrates. Endospores may be located in the middle of the cells (central), at the end (terminal), or between the end and the middle of the cells (subterminal). Endospores themselves, once stained, may be viewed under oil immersion as round or oval.

Ingredients and preparations

- malachite green

- saffranin

Materials

- Smears
- Staining rack
- Forceps
- Tap water
- Dyes
- Timer
- Filter papers
- Oil immersion
- Microscope
- Slide box
- Sharp container

Procedures

- Cover the dried smear with a small piece of paper towel and then with malachite green. Heat the stain so that it steams and continue to heat for at least 5 minutes. Do not let the stain dry while you are heating it.
- Remove slide from heat and let cool 1 min. Then remove towel with forceps and discard as designated.
- Wash the smear thoroughly with water. Water does not remove the stain from the endospores but will decolorize the vegetative cells so that they become clear.
- Counterstain with saffranin for 1 min.
- Rinse the smear briefly with water. Safranin is a weak dye and is easily removed from vegetative cells by over-rinsing.
- Drain and then blot dry.
- Examine the stained smear with low-power, high-power and oil-immersion objectives.
- You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

- **Sketch and label it with the following**

- name of organism
- total magnification used
- color
- shape of individual cells as you saw them and as described in a text
- arrangement of cells
- State the position of the endospores
- measured/relative size
- one interesting fact about this genus or particular species
- You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

d. Albert staining

This staining is used to demonstrate metachromatic granules in *certain bacteria such as Clostridium*.

Ingredients and preparations

Albert stain A

- Toluidine blue 0.15 gm
- Malachite green 0.20 gm
- Glacial acetic acid 1.0 ml
- Alcohol(95%) 2.0 ml
- Distilled water 100 ml

Grind and dissolve the dyes in alcohol, add water and then add acetic acid. Let the mixture stand for 24 hours and then filter.

Albert stain B

- Iodine 2.0 gm
- Potassium iodide 3.0 gm
- Distilled water 300 ml

Dissolve iodine and potassium iodide in water by grinding in a mortar with a pestle. Filter through a filter paper.

Materials

- Smears
- Staining rack
- Forceps
- Tap water
- Dyes
- Timer
- Filter papers
- Oil immersion
- Microscope
- Slide box
- Sharp container

Procedures

- Cover the heat-fixed smear with Albert stain A. Let it stand for two minutes.
- Wash with water.
- Cover the smear with Albert stain B. Let it stand for two minutes.
- Wash with water, blot dry and examine.
- Examine the stained smear with low-power, high-power and oil-immersion objectives. The granules appear bluish black whereas the body of bacilli appear green or bluish green.
- You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

- **Sketch and label it with the following:**

- name of organism
- total magnification used
- color
- shape of individual cells as you saw them and as described in a text
- arrangement of cells
- State the position of the endospores
- measured/relative size
- one interesting fact about this genus or particular species

You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

e. The acid-fast stain

This is a differential stain which measures the resistance of a stained cell to decolorization by acids. Such resistance to

decolorization is a property of certain bacteria which correlates with their high lipid content. *Carbolfuchsin* is lipid soluble and therefore can penetrate the waxy wall. Steam heating melts the waxes in acid-fast cells and allows the carbolfuchsin to enter. Once the waxes cool, the carbolfuchsin is trapped inside the cell by the wax. *Acid-alcohol* is used to decolorize the cells (although it actually will decolorize only species which are not acid-fast). Cells are counterstained with *methylene blue*. Acid fast organisms stain red and non-acid fast stain blue. The acid-fast stain is primarily used in the identification/diagnosis and study acid-fast bacteria such as *Mycobacteria* and *Actinomycetes*.

Ingredients and preparations

- Carbol fuchsin
- 95% EtOH with 2.5% HNO3
- Methylene blue

Or alternatively

- Carbol fuchsin 1%
- Sulphuric acid 25%
- Methylene blue 0.1%

Materials

- Smears
- Staining rack
- Tap water
- Dyes
- Timer
- Filter papers
- Oil immersion
- Microscope
- Slide box
- Sharp container

Procedures (Ziehl-Neelsen)

- Place a single ply piece of paper towel over the smear (the size of the smear; do not let it overlap edges of slide).
- Cover the paper towel with carbolfuchsin and heat the stain so that it steams for at least 5 minutes. DO NOT ALLOW the stain to boil or to dry out. Let slide cool and remove towel with forceps. Discard towel in container provided.
- Rinse stained smear thoroughly with water and then drain off excess water.
- Hold the slide in a slanting position and decolorize by adding acid-alcohol (95% EtOH with 2.5% HNO3) drop by drop until the slide remains only slightly pink. This requires 10 to 30 seconds and must be done carefully. Do not over-decolorize. At this stage *Mycobacterium* should show a faint pink color unless it is a very thin smear; the other microorganisms will be colorless.
- Quickly wash with water.
- Counterstain with methylene blue for 30 sec (no heat).
- Rinse with water and blot dry.
- Examine the stained smear with low-power, high-power and oil-immersion objectives.
- You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

- **Sketch and label it with the following:**

- name of organism
- total magnification used
- color
- shape of individual cells as you saw them and as described in a text
- arrangement of cells
- measured/relative size
- one interesting fact about this genus or particular species
- You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

Or alternate procedures

- Let the smear air-dry for 15-30 minutes.
- Fix the smear by passing the slide over the flame 3-5 times for 3-4 seconds each time.
- Place the fixed slide on the staining rack with the smeared side facing upwards.
- Pour filtered 1% carbol fuchsin over the slide so as to cover the entire slide.
- Heat the slide underneath until vapours start rising. Do not let carbol fuchsin to boil or the slide to dry. Continue the process up to five minutes.
- Allow the slide to cool for 5-7 minutes.
- Gently rinse the slide with tap water to remove the excess carbol fuchsin stain. At this point, the smear on the slide looks red in colour.
- Decolor the stained slide by pouring 25% sulphuric acid on the slide and leaving the acid for 2-4 minutes.
- Lightly wash away the free stain. Tip the slide to drain off the water.
- If the slide is still red, reapply sulphuric acid for 1-3 minutes and rinse gently with tap water.
- Counter stain the slide by pouring 0.1% methylene blue solution onto the slide and let it stand for one minute.
- Gently rinse the slide with tap water and tip the slide to drain off the water.
- Place the slide in the slide tray and allow it to dry.
- Examine the stained smear with low-power, high-power and oil-immersion objectives.
- You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

Sketch and label it with the following:

- Name of organism
- Total magnification used
- Color
- Shape of individual cells as you saw them and as described in a text
- Arrangement of cells
- Measured/relative size
- One interesting fact about this genus or particular species
- You may save the slide in your slide box for further study or discard the stained smear in the sharps container.

Precaution

- Do not insert more than the "wire" portion of the loop into tubes containing bacteria - keep the handle clean. Dispose of the loops loop-side down into the disposal buckets
- The bacteria on a dried and fixed slide are usually, but not necessarily, dead. Handle such slides with care.
- Alcohol and Gram's iodine will kill bacteria, so properly prepared Gram-stained slides are safe. All staining should be done on the staining racks provided over sinks to prevent spills on the bench tops and ultimately your possessions
- To preserve your social life you may want to wear gloves when staining. Do all staining over dishes provided. Use clothespins to hold slides and lay out paper towels as needed.

SECTION VIII. IDENTIFICATION OF UNKNOWN MICROBE

A. Phenotypic techniques

- Colony morphology
- Cellular morphology/Staining
- Biochemical Tests

Catalase test

Place a drop of 3% hydrogen peroxide on a clean microscope slide. Place a heavy loopful of cells from isolated colonies into the liquid (you may have to pick up four to five colonies if they are small). Immediate generation of gas bubbles constitutes a positive test. Avoid the inclusion of blood cells from blood agar plates as blood contains catalase. Lack of bubbles is a negative test.

$2H_2O_2$ ----------> $2H_2O + O_2$ (bubbles)

- Serological test
- Antimicrobial susceptibility test

B. Genomic Techniques

1. Example for Identification of Unknown Bacteria

Isolating Microbes

- Colony was removed from the primary culture medium and streaked onto nutrient agar plates and incubated at room temperature in the laboratory.
- After 48 hours, colonies of varying shapes and colors grew on the plates.
- One microbe, forming smooth, curly rhizoid colonies, was selected for identification and isolated by streaking on a sterile nutrient agar plate. T
- This culture was then incubated for 48 hours to allow a pure culture to be grown and tested

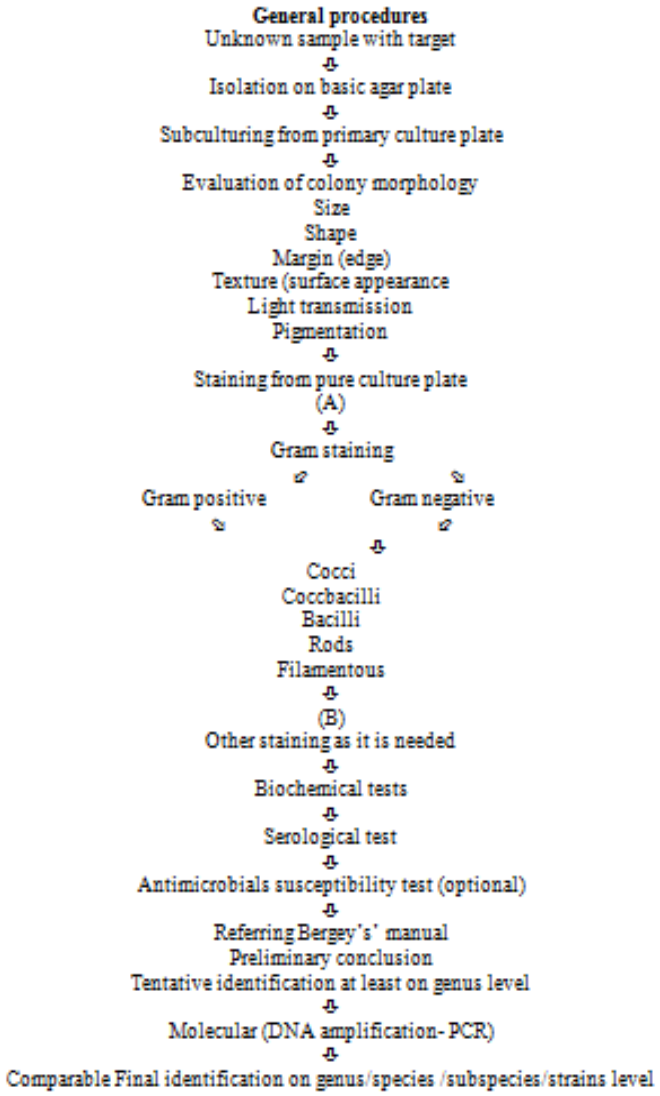
Examining the Morphology

- Slides were prepared for Gram staining and endospore staining and examined under microscope and observed Gram positive bacilli with endospores

Biochemical Testing

Upon isolating and studying the morphology of the unknown microorganism from the primary culture medium, a battery of biochemical tests were performed to determine the type of metabolic processes the unknown bacteria underwent.

The tests were used to help ascertain the identity of the unknown isolate. Some of these test are: fermentation of carbohydrates (lactose, sucrose, and dextrose) catalase Production, hydrogen sulfide Production, indole production, urea digestion, lipid digestion, casein digestion and others.

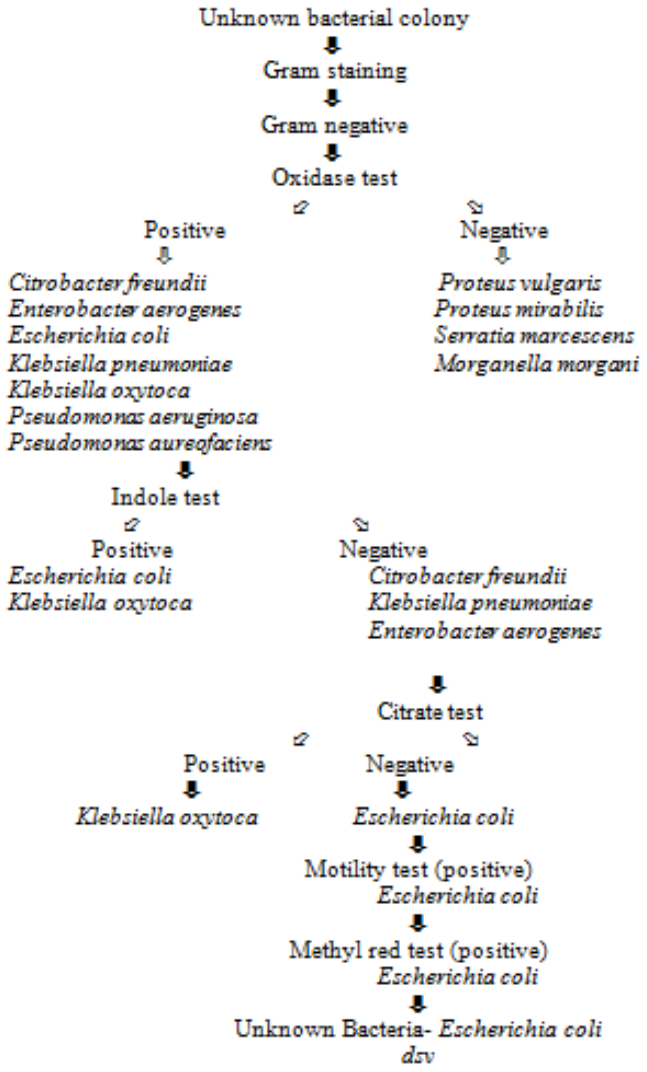


Conclusion

From the morphology studies and the biochemical tests, it has been determined that the unknown isolate is from the Genus *Bacillus*. This genus has many species that are known for their rhizoid colonies, Gram positive bacilli with endospores, motility, and production of catalase.

Bacillus cereus var. mycoides, a common soil bacterium, is well known for its unusual rhizoid colonies.

Example for Identification of Unknown Gram staining Bacteria



SECTION IX. PRESERVATION

Preserving Bacterial Cultures

Refrigeration

Refrigeration can be used for short-term storage. Cultures streaked on agar slants or stab cultures may be viable over several months when stored at 4°C. Plates have to be sealed to prevent their tendency to dry out. To preserve cultures for longer periods of time, two methods are commonly employed:

Deep Freezing

A pure culture of bacteria is suspended in a liquid and quick-frozen (often with liquid nitrogen) at temperatures between -50°C and-95°C. Sensitive microorganisms require the presence of glycerol (end concentration 15-20 %), which acts as an "antifreeze", or extra protein (skimmed milk powder) to protect them. Cultures can be thawed and used up to several years later.

Lyophilization

A suspension of bacteria is quickly frozen and the water removed by means of a high vacuum. The microbes survive in the powder like residue for several years and can be revived at any time by rehydration of the culture in a nutrient medium. Bacterial strains ordered from strain collections are usually delivered in this form.

Cryopreservation of Cell Lines

The protocol below describes the use of passive methods involving an electric -80ºC freezer for the Cryopreservation of cell cultures. ECACC routinely use a programmable rate controlled freezer (Planer Series Two) from Planer Products. This is the most reliable and reproducible way to freeze cells but as the cost of such equipment is beyond the majority of research laboratories the methods below are described in detail. If large numbers of cell cultures are regularly being frozen then a programmable rate controlled freezer is recommended.

Materials

- Freeze medium (commonly 70% basal medium, 20% FCS, 10% DMSO
- 70% ethanol in water
- PBS without Ca_{2+} Mg_{2+}
- 0.25% trypsin/EDTA in HBSS, without Ca_{2+}/Mg_{2+}
- DMSO
- Trypsin/EDTA
- HL60

Equipment

- Personal protective equipment (sterile gloves, Laboratory coat)
- Full-face protective mask/visor
- Waterbath set to 37ºC
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- Heamocytometer
- Pre labeled ampules/cryotubes
- Cell Freezing Device

Procedure

- View cultures using an inverted microscope to assess the degree of cell density and confirm the absence of bacterial and fungal contaminants.
- Bring adherent and semi adherent cells into suspension using trypsin/EDTA as above and re-suspend in a volume of fresh medium at least equivalent to the volume of trypsin. Suspension cell lines can be used directly.
- Remove a small aliquot of cells (100-200uL) and perform a cell count. Ideally the cell viability should be in excess of 90% in order to achieve a good recovery after freezing.
- Centrifuge the remaining culture at 150g for 5 minutes.
- Re-suspend cells at a concentration of 2-4x106 cells per ml in freeze medium.

- Pipette 1ml aliquots of cells into cyroprotective ampules that have been labeled with the cell line name, passage number, cell concentration and date.
- Place ampules inside a passive freezer. Fill freezer with isopropyl alcohol and place at -80ºC overnight.
- Frozen ampules should be transferred to the vapor phase of a liquid nitrogen storage vessel and the locations recorded.

Key Points

- The most commonly used cryoprotectant is dimethyl sulphoxide (DMSO Prod. No. D2650), however, this is not appropriate for all cell lines e.g. HL60 (Prod. No. 98070106-1v1) where DMSO is used to induce differentiation. In such cases an alternative such as glycerol should be used (refer to ECACC data sheet for details of the correct cryoprotectant).
- ECACC freeze medium recommended above has been shown to be a good universal medium for most cell types. Another commonly used freeze medium formulation is 70% basal medium, 20% FCS, 10% DMSO but this may not be suitable for all cell types. Check it works for your cells before using on a regular basis
- It is essential that cultures are healthy and in the log phase of growth. This can be achieved by using pre-confluent cultures (cultures that are below their maximum cell density) and by changing the culture medium 24 hours before freezing.
- The rate of cooling may vary but as a general guide a rate of between -1ºC and -3ºC per minute will prove suitable for the majority of cell cultures.
- An alternative to the Mr. Frosty system is the Taylor Wharton passive freezer where ampules are held in liquid nitrogen vapor in the neck of Dewar. The system allows the ampules to be gradually lowered thereby reducing the temperature. Rate controlled freezers are also available and are particularly useful if large numbers of ampules are frozen on a regular basis.
- As a last resort if no other devices are available ampules may be placed inside a well insulated box (such as a polystyrene box with sides that are at least 1cm thick) and placed at -80ºC overnight. It is important to ensure that the box remains upright throughout the freezing process. Once frozen, ampules should be transferred to the vapor phase of a liquid nitrogen storage vessel and the locations recorded.
- If using a freezing method involving at -80ºC freezer it is important to have an allocated section for cell line freezing so that samples are not inadvertently removed. If this happens at a crucial part of the freezing process then viability and recovery rates will be adversely affected.

SECTION X. MAINTAINING CULTURES

Introduction

Cultures may be maintained on media and stored under conditions that inhibit growth. Long term storage requires other techniques such as lyophilization or storage in liquid nitrogen. In this course, you will be responsible for maintaining viable cultures of control bacteria and your environmental unknown. For every organism that you wish to maintain, you must create a stock and working culture.

Stock culture

The stock culture is an inoculated slant that is incubated under conditions for microbial growth and then stored at a temperature below the minimum required for growth of the culture. Stock cultures should be stored in the refrigerator after its growth on the slant. Stock cultures are not used unless the working culture dies or becomes contaminated. The stock culture must be restreaked whenever it is used or at appropriate intervals (approximately every three weeks) to insure the viability of the organism.

Working culture

The working culture is a slant that is maintained to inoculate media and to make smears for stains. A new working culture should be created every time it is used. Make sure that all cultures are properly labelled and sealed. The name of the organism and other identifying characteristics such as strain type must be legible and written in permanent marker. The date of inoculation and your initials must also be indicated on the tubes. Write the name of the medium and indicate if it is a stock culture. After sufficient growth store the organism in the appropriate refrigerator. Live cultures should never be stored in the same refrigerator as toxic volatile chemicals.

Growth Requirements

1. Nutritional Requirements

Each culture medium is formulated with various macro- and micronutrients that are essential for microbial growth.

Needed in relatively large amounts are:

- proteins
- carbohydrates
- lipids
- nucleic acids

These organic substances provide energy and a source of essential atoms like carbon, hydrogen, nitrogen, oxygen, phosphorous, and sulfur (i.e., CHNOPS). Cells use these to synthesize their own structural and regulatory components. Also essential, but required in smaller amounts, are:

- vitamins (e.g., thiamine, niacin, folic acid)
- metallic elements (e.g., Ca^{++}, Zn^{++}, Cu^{++}, Mn^{++}, Mg^{++}, K^{+}, Na^{+}, Fe^{+++})

These substances are particularly critical for normal enzyme function and maintenance of protein structure.

1. Physical Requirements

Culture media must also provide cells a source of moisture (water), and a particular tonicity (osmotic pressure) and hydrogen ion concentration (pH). These greatly influence

microbial growth and survival. For instance, since most bacteria grow best under isotonic conditions and around a pH of 7 or slightly lower, culture media are chemically adjusted to provide these requirements. While cultivating microbes, atmospheric gases must be considered.

Most microbes are greatly effected by the levels of oxygen and carbon dioxide available to them. Anaerobic bacteria must be cultured in a special anaerobic environmental chamber that removes all oxygen. In contrast, aerobic or facultative anaerobes can be grown in environments where oxygen is more readily available. Finally, microbial growth is dependent on temperature. After inoculation, cultures are routinely placed in an incubator thermostatically set within the microbe's optimal temperature range. The microbes are incubated at 25 ºC, 30 ºC, or 37ºC. After a specified incubation period (usually 18-24 hours), these cultures are stored and preserved by placing them into a refrigerator set around 5ºC. This temperature inhibits growth and prevents cultures from depleting nutrients and accumulating toxic wastes that can cause cells to die off more rapidly.

Culture Media

1. Physical state of Culture media

a. Liquid Media: Often called broths, milks, or infusions, these media have a liquid or fluid consistency that allows them to be easily poured or pipetted from one container to another. A liquid medium is usually prepared in tubes, flasks, or bottles and consists of various solutes dissolved in distilled water. Once inoculated, microbial growth can occur throughout this liquid, transforming a transparent medium into a cloudy (turbid) suspension.

b. Solid Media: These media, usually prepared in tubes or Petri plates, provide a firm surface for microbes to grow on or within. Unlike a liquid medium, bacteria dispersed on a solid medium can grow as a continuous layer or as separate colonies. A solid medium is typically prepared by adding a solidifying agent to a liquid medium.

c. Semisolid Media: A medium having more of a "jellylike" consistency is considered a semisolid medium. Unlike a liquid medium that flows freely, this medium cannot be poured. However, to give it less body or firmness than a solid medium, the solidifying agent used in its preparation is added in smaller amounts. One of the useful features of a semisolid medium is it can be used for determining motility.

Solidifying Agents

A couple of solidifying agents commonly used in microbiology labs are agar and gelatin. Each imparts different traits or attributes to the medium.

a. **Agarz**

This is the most widely used solidifying agent. Agar is a complex polysaccharide extracted from marine red algae. It is typically added to a broth at a concentration around 1.5%. If a firmer medium is desired, higher concentrations can be used.

Useful Properties

- Most microbes are unable to degrade agar. This keeps the medium a solid while microbes grow on it.
- The melting point of agar is between 95-100ºC. While the medium is still relatively warm, it can be poured, pipetted, or inoculated with microbes. As it cools to 42-44ºC, agar begins to solidify the medium. Once solidified, microbes can be incubated at 37ºC (or higher) without the fear of the medium liquefying.

b. Gelatin

This solidifying agent when used at concentrations of 10%-15% creates a firm solid medium. However, its uses are more limited because of its low melting point. Mediums using gelatin liquefy at room temperature.

Useful Property

- Nutrient gelatin is useful in bacterial identification. Gelatin is a protein that can be broken down by enzymes secreted by some bacteria. Bacteria that secrete these enzymes are identified when the nutrient gelatin they are grown on undergoes liquefaction.

2. Chemical Composition of Culture Media

a. Synthetic Media

Whenever media are prepared to exact specifications, they are known as synthetic or chemically defined media. They are composed of highly purified organic and inorganic substances of known amounts and molecular composition. The constituents of these media may include a variety of specific sugars, salts, amino acids, and vitamins. They can be formulated to grow microbes that call for specific nutritional requirements or growth factors. They are particularly useful when studying microbial metabolism.

b. Non-synthetic Media

When media contain one or more ingredients of imprecise composition, they are known as non-synthetic or complex media. The ingredients usually include complex mixtures of organic and inorganic substances prepared from extracts. The extracts are made from yeasts or animal and plant tissues. Present in extracts are various amino acids, vitamins, minerals, and sugars of imprecise quantities. Another possible constituent of a complex medium is peptone. This substance is prepared from the partial digestion of animal or plant proteins. This results in a complex mixture of amino acids and polypeptides. Overall, a complex medium serves as a rich mixture of compounds that is likely to supply the general nutritional requirements for a wide variety of microbes.

3. Functional Types of Culture Media

a. General Purpose Media

These media contain a diverse mixture of nutrients that can support the growth of a broad range of bacteria. Non-synthetic

(complex) media like nutrient agar, nutrient broth, and trypticase soy agar are good examples of general purpose media.

b. Enriched Media

Prepared with special complex nutrients or growth factors (i.e., specific amino acids, vitamins), these media promote the growth of certain species. Bacteria that require such substances are said to be fastidious. For example, adding sheep or horse blood to a culture medium provides several specific growth factors required by various species of streptococci.

c. Selective Media

These media are designed to help in the isolation and growth of certain species of bacteria from a mixed sample. This is accomplished by adding one or more agents to a medium that will promote (or select) the growth of the desired bacteria, while at the same time, inhibiting the growth of the unwanted organisms. Inhibitory agents include such substances as bile salts, NaCl, dyes, acids, and certain antimicrobial drugs. For instance, mannitol salt agar is formulated to a 7.5% NaCl concentration to promote the growth of *Staphylococcus*. This same salt concentration, however, establishes a hypertonic environment that is very inhibitory for most other organisms.

d. Differential Media

Differential media contain substances that are utilized or reacted to in different ways by microbes. These reactions produce specific visible changes in the appearance of the medium (e.g., color changes, formation of gases or precipitates) or the microbial colonies growing on it. These media are very useful in bacterial isolation and identification because they differentiate one species from another according to their particular biochemical traits.

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