Preparation and Sterilization of Microbiological Media

Safety Considerations

- Several types of heat that can cause severe burns are used in this experiment.
- Do not operate the autoclave without approval from your instructor.
- Always where heat resistant gloves when unloading the autoclave or when handling media that is being heated over a Bunsen burner or hot plate.
- Boiling agar can cause severe burns if spilled on your hands.
- Be especially careful with Bunsen burners, hot plates and boiling water baths. All are potentially hazardous and can cause burns.
- The tripod and metal screen will get very hot! Do not touch these until they have cooled back down to room temperature. They may look cool to the touch, but can still be very hot. Be extremely careful.
- Do not fill a flask with more than half its total volume with microbiological media.
- Never heat a flask or tube with a cap on the top. This can be an explosion hazard.
- Always have heat resistant gloves or hot mitts ready when you need to remove a flask from the burner or hot plate.

Materials per Group of Students

24- to 48-hour tryptic soy broth culture of *Escherichia coli* (ATCC 11229)
autoclave for the entire class
petri plates
culture tubes
test-tube rack or wire basket
test-tube caps
components to make Tryptic Soy Broth (TSB), a complex culture medium
agar to make Tryptic Soy Agar (TSA) from TSB
Erlenmeyer flasks
10-ml pipettes with pipettor
weighing paper or boats
balance
heat-resistant fabric gloves
water bath set at 48° to 50°C
boiling water bath
aluminum foil
pH test strips
stirring rod or plate with stirring bar
Bunsen burner, tripod, metal screen or hot plate

Learning Objectives

Each student should be able to:

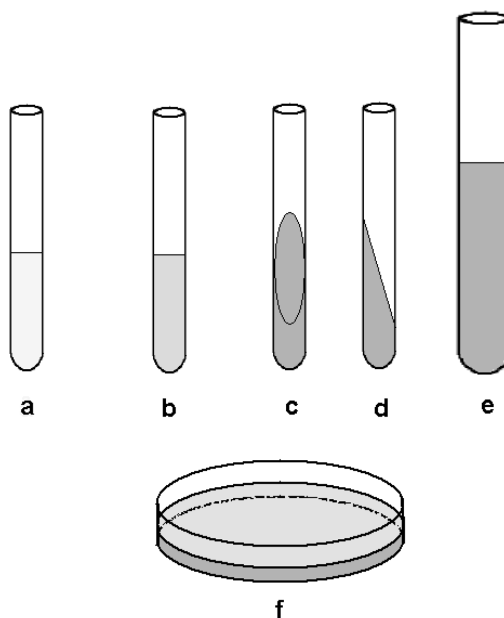
1. Describe the different types of culture media and their composition, and give several examples of what each is used for.
2. Describe the various ways culture tubes are capped
3. Describe how to prepare and transfer culture media
4. Prepare defined and undefined media, and prepare agar plates
5. Describe the concept of sterility
6. Describe how various media, supplies, and equipment can be sterilized
7. Understand the principles of sterilization by the autoclave.

Microbiological Culture Media

The survival and growth of microorganisms depend on available nutrients and a favorable growth environment. In the laboratory, the nutrient preparations that are used for culturing microorganisms are called media (singular, medium). Microorganisms may be cultivated either in a liquid nutrient medium (infusion or broth) or on the surface of a medium hardened or solidified by a gelling agent such as gelatin or, more commonly, agar. Cells may also be grown in semisolid media containing lower amounts of the solidifying agent, typically agar, than is used for more solid media. The medium may vary from a complex array of nutrients obtained by extraction of cellular material (yeast extract, beef extract) to simple combinations of pure chemical substances. It is common when cultivating a variety of organisms whose nutritional requirements are unknown to use a medium containing partially hydrolyzed protein (peptone), extract of yeast, and a carbohydrate. There is, however, no universal medium in which all types of microorganisms will thrive. Complex media are often inhibitory or toxic to many organisms adapted to habitats normally low in organic matter, and simple chemically defined media will not support the more fastidious organisms. Consequently a variety of media are available to be used when the occasion demands. The choice of an appropriate cultivation medium requires knowledge of the general biology and habitat of the organisms with which one is dealing. During the course of this semester you will learn the general composition and appropriate use of several of the more commonly employed culture media.

Liquid media, such as nutrient broth, tryptic soy broth, or brain-heart infusion broth, can be used to propagate large numbers of microorganisms in fermentation studies and for various biochemical tests. Semisolid media can also be used in fermentation studies, in determining bacterial motility, and in promoting anaerobic growth. Solid media, such as nutrient agar or blood agar, are used (1) for the surface growth of microorganisms in order to observe colony appearance, (2) for pure culture isolations, (3) for storage of cultures, and (4) to observe specific biochemical reactions.

While in the liquefied state, solid media can be poured into either a test tube or petri plate (dish). If the medium in the test tube is allowed to harden in a slanted position, the tube is designated an agar slant; if the tube is allowed to harden in an upright position, the tube is designated an agar deep tube; and if the agar is poured into a petri plate, the plate is designated an agar plate. Agar pours (the same as Agar deeps) containing about 15 to 16 ml of media are often used to prepare agar plates.



(a) liquid broth tube; (b) semisolid agar deep; (c) solid agar slant tube, front view; (d) solid agar slant tube, side view; (e) agar deep; (f) petri dish with solid agar medium (plate)

Microorganisms may be cultured using two different types of media. Chemically defined, or synthetic, media are composed of known amounts of pure chemicals (Table 1). Such media are often used in culturing autotrophic microorganisms such as algae or nonfastidious heterotrophs. In routine bacteriology laboratory exercises, complex, or nonsynthetic, media are employed (Table 2). These are composed of complex materials that are rich in vitamins and nutrients. Three of the most commonly used components are beef extract, yeast extract, and peptones.

Table 1. A Chemically Defined Medium	
Ingredient	**Quantity**
Dipotassium phosphate, K_2HPO_4	7 g.
Potassium phosphate, monobasic, KH_2PO_4	2 g.
Hydrated magnesium sulfate, $MgSO_4 \cdot 7H_2O$	0.2 g.
Ammonium sulfate	1 g
Glucose	5 g
Distilled water	1 liter

Table 2. Tryptic Soy Broth (TSB) a complex (chemically undefined) medium	
Ingredient	**Quantity**
Casein peptone *	17 g.
Soybean peptone *	3 g.
NaCl	5 g.
Dipotassium phosphate, K_2HPO_4	2.5 g.
Glucose	2.5 g
Distilled water	1 liter[1]

[1]More or less media can be made using proportional amounts of the components,

* The exact chemical composition of these components is unknown, so the medium is chemically undefined or “complex”.

The preparation of media from commercial dehydrated products is simple and straightforward. Each bottle of dehydrated medium has instructions for preparation on its label. For example, to prepare a liter of tryptic soy broth, suspend 30 g of the dehydrated medium in l,000 ml of distilled water. Mix thoroughly in a 2-liter Erlenmeyer flask (always use a flask that holds twice the volume of media you are preparing). Dispense and sterilize for 15 to 20 minutes at 121 ^{o}C (15 lbs pressure per square inch, or 15 psi). As noted, the amount of powder for 1,000 ml of water will be indicated. You can prepare proportionately more or less medium as needed.

If the medium lacks agar, the powder will usually dissolve without heating. If it contains agar, you must heat the medium until it starts to simmer or boil in order to completely dissolve the agar. Specific heating instructions are given for each type of medium. For example, to prepare a liter of Vogel-Johnson agar, suspend 61 g of the dehydrated medium in a liter of distilled water. Mix until a uniform suspension is obtained. Heat with con- stant agitation and simmer for 1 minute. Dispense in l00-ml amounts into 250-ml flasks and sterilize by autoclaving at 121 oC for 20 minutes.

Most of the exercises you will be doing in this manual will involve the use of sterile media in culture tubes. Normally, 18 x 150 mm, 16 x 125 mm, or 13 X l00 mm bacteriologic culture tubes will be used. These tubes must be capped in order to maintain media sterility. This can be accomplished by using cotton plugs, plastic foam plugs, or plastic or metal caps (e.g., Morton closures or Bacti Capalls). All of these caps keep cultures free from contamination while allowing air into the culture tube, and at the same time, minimize evaporation. It is sometimes desirable to use screw-cap culture tubes. This is

especially true when the culture, such as in the case of slants, may be sealed and stored for long periods.

Culture broth can be dispensed with a pipetting machine, an automatic syringe, or a regular pipette. One can also pipette the proper volume of broth or agar into one culture tube and then pour approximately the same volume of media (using the initial tube as a guide) into a number of other tubes lined up in the same test-tube rack. This approach is fast, convenient, and relatively accurate.
After sterilization of slant tubes, the tubes are removed from the autoclave while the agar is still melted and carefully laid on a table with a piece of wood, vacuum tubing, or metal elevating the capped ends. Some test-tube racks are also specifically set up for this. The tubes are then allowed to remain undisturbed until the agar has cooled and hardened. Slants should be stored in a vertical position.

Agar deep tubes can be stored after sterilization for use in preparing petri plates when needed. Some agar deeps may be stored at room temperature for several days before use. If longer periods of storage are required, they should be placed in the refrigerator in order to prevent drying of the agar. When petri plates are needed, the agar deeps are melted either in a boiling water bath or by bringing them to 121 ^{o}C in an autoclave for 30 to 60 seconds and then releasing the steam under slow exhaust. After the agar has melted, the pours are transferred to a 48° to 50 °C water bath and kept there for at least 5 to 10 minutes before use. The agar deeps should be cooled to about 50 °C before they are used to minimize the amount of steam condensation on the petri plate lids after the agar has been poured. Agar does not solidify until its temperature drops to about 42 °C. When the deeps have reached 50 °C, one is taken from the bath *and the outside is dried with a paper towel, to prevent contamination by organisms that may be in water drops on the sides of the flask.* Its cap is removed and the top is briefly flamed using a Bunsen burner. The agar is immediately poured into a sterile, dry petri plate while holding the top carefully above the petri plate bottom in order to avoid contamination. Replace the top, allow the agar to cool and harden, and store the petri plates in an inverted position . When storing petri plates, do not stack them more than three high, or use a special petri plate storage holder.

Sterilization of Media and Equipment

Sterilization is the process of rendering a medium or material free of all forms of life. There are three basic ways in which sterilization of media and supplies can be achieved. The most useful approach is autoclaving, in which items are sterilized by exposure to steam at 121°C and 15 psi for 15 minutes or longer, depending on the nature of the item. Under these conditions, microorganisms, even endospores, will not survive longer than about 12 to 13 minutes. This method is rapid and dependable. Modem autoclaves are designed to ensure that all of the air has been expelled and only steam is present in the autoclave chamber. They are carefully temperature controlled as well. Almost all media and anything else that will resist 121°C temperatures and steam can be sterilized in this way.

Often, dry glassware such as pipettes and petri plates must be sterilized. Steam tends to etch glassware and also leaves it damp. Therefore, such items are generally dry-heat sterilized. The glassware is placed in an electric oven set to operate between 160 ° and 170 °C. Since dry heat is not as effective as wet heat, the glassware must be kept at this temperature for about 2 hours or longer. The oven temperature must not rise above 180 °C or any cotton or paper present will char.

Sometimes media must be made from components that will not withstand heating at 121°C. Such a medium can be sterilized by passing it through a bacteriological filter, which physically removes bacteria and larger microorganisms from the solution and thereby sterilizes them without heat. Scintered glass filters with ultrafine, fritted disks (0.9 to 1.4 µm pore size) and Seitz asbestos-pad filter funnels (3 mm thick with 0.1 µm pores) are both quite effective in sterilizing solutions. However, if pore sizes greater than 0.22 µm are used, there is an exceedingly high chance that the filtrate will not be sterile. By far, the most useful and popular approach is the use of specially prepared sterile, cellulose- or polycarbonate-based membranes of the appropriate pore size. Generally, membranes with 0.22 µm pores are employed in sterilization. A large number of different devices are commercially available for

membrane sterilization of both large and small volumes. For example, one can use a filter flask with a vacuum or syringe with positive pressure to force liquid through a special membrane filter holder.

Two other sterilization techniques use ultraviolet radiation and ethylene oxide. Ultraviolet (UV) radiation around 260 nm is quite lethal to many microorganisms but does not penetrate glass, dirt films, water, and other substances very effectively. Because of this disadvantage, UV is used as a sterilizing agent only in a few particular situations. For example, UV lamps are sometimes placed on the ceilings of rooms or in biological safety cabinets to sterilize the air and any exposed surfaces.

Many heat-sensitive items such as disposable plastic petri dishes and syringes, sutures, and catheters are now sterilized with ethylene oxide gas. Ethylene oxide is both microbicidal and sporicidal and kills by covalently attaching to cell proteins. It is a particularly effective sterilizing agent because it rapidly penetrates packing materials, even plastic wraps.

Procedure

Preparing a Complex Medium, (Tryptic Soy Broth and Tryptic Soy Agar)

1. Prepare 500 ml of tryptic soy broth according to the recipe outlined in Table 2 (remember Table 2 is for 1 liter or 1000 ml of medium; you should use a proportional amount of the components based on the total volume to be prepared. **Your instructor may direct you to make a different volume.** Again proportional amounts of the components should be used corresponding to the total volume to be made.

2. Add 375 ml (or a proportional amount) of distilled water to a l-liter Erlenmeyer flask and add the ingredients individually (e.g. use half the amounts given in Table 2 if making 500 ml of medium); mix after each addition.
   - **Be sure to thoroughly clean up the balances and balance area.** Return all supplies and equipment to their proper location. Your mother is not enrolled in this course. Clean up is the student's responsibility and reflects good lab technique.
   - You may have to heat the medium with occasional stirring or swirling to dissolve all of the components.
   - ***Make sure the flask is not closed***.
   - **Do not let the medium boil over; be ready to remove the flask from the Bunsen burner or hot plate in a moment's notice, by having someone wearing a heat-protective glove standing by and playing close attention.**
   - **Do not let medium components char on the bottom of the flask.**

1. Add the remaining 125 ml (or proportional amount) of water to rinse the sides of the flask.

2. Using pH test strips, adjust the pH to 7.3 by adding just enough HCl or NaOH dropwise.

3. Dispense 5 ml of the broth into each of 8 tubes (16 mm dia.; 8 tubes per table) and loosely cap them. Place the tubes in a test-tube rack or basket, and place in the autoclave.

4. To the remaining broth (e.g. 460 ml if you started with 500 ml), add sufficient agar to give an agar concentration of approximately 1.5%. Cautiously bring the preparation to a boil to dissolve the agar. Watch and stir or swirl gently to avoid burning and boiling over.
   - Swirl the contents of the flask carefully and *frequently* to completely dissolve the agar.
   - ***Make sure the flask is not closed***.
   - Do not let the medium boil over; be ready to remove the flask from the Bunsen burner or hot plate in a moment's notice, by having someone wearing a heat-protective glove standing by and playing close attention.
   - **Do not let medium components char on the bottom of the flask.**

- Once the agar is dissolved completely, remove from the burner or hot plate (turn off the Bunsen burner) and place the flask on some paper towels that have been folded over. This acts as an insulator to prevent the agar from cooling too quickly on the lab bench.

5. Dispense the proper volume of TSA agar into the appropriate tubes using a wide tip agar pipette. Caution: you will need to work quickly to prevent the agar from solidifying too early. Organize your workstation so that everything you need is nearby and ready to use. Your instructor will outline how many agar deeps and agar slants need to be made from each batch of TSA (e.g. for agar slants dispense 6.5 ml of melted TSA to 16 mm dia. tubes, 12 slant tubes per table; for agar deeps, dispense 16 ml TSA into 18 mm dia. tubes, 4 deep tube per table). Slant tubes may be placed on a slant rack. After autoclaving the tubes will solidify at an angle due to the angle of the shelves on the slant rack. Alternatively a normal tube rack can be lifted to the desired angle with some type of block until the tubes solidify. Deep tubes will be stored in racks that can accomodate the larger diameter tubes.
6. The remaining TSA medium will be transferred to a smaller (500 ml) flask. Be sure to do this before the agar solidifies in the large 1-liter flask. This will be used in a future lab session to pour TSA plates in petri dishes.
7. Immediately rinse out the large flask with a large quantity of warm water to prevent solidification of remaining agar. This facilitates clean up. NEVER pour melted agar into the sink. Dilute melted agar with large volumes of hot water before you wash it down the drain. WHY?
8. **Label** your smaller Erlenmeyer flask and the test tube racks.
9. All liquid and agar tubes, deeps, slants and flasks will be autoclaved by the Prep Lab staff.

Procedure for Autoclaving

1. Your instructor will demonstrate the use of the autoclave.
2. Load the autoclave with the freshly prepared culture media.
3. Close and lock the autoclave door.
4. Set the autoclave time for 15 minutes or longer and select a slow rate of exhaust.(“liquid cycle”
5. Make certain that the autoclave temperature is set to 121°C.
6. Start the autoclave by pushing the start button.
7. When the period of sterilization is completed and the pressure in the chamber reads 0, carefully open the door and remove the containers, using heat-resistant gloves. Watch out for residual steam escaping from the autoclave door. “Crack” the door open for a few minutes to let residual steam escape, before opening the door all the way.

Additional Hints and Precautions for Autoclaving

(1) Don’t overload the autoclave chamber. Provide ample space between baskets, racks or flasks of media to allow circulation of steam. (2) You should bring media to a boil and then, using heat-resistant gloves, quickly remove the media from the Bunsen burner or hot plate to prevent boiling over. Do not shake or swirl the flask as you removed it from the heat because such shaking may cause the media to suddenly and violently boil over. (3) Before opening the door to the autoclave, you should always wear heat-resistant cloves, stand at arm’s length, and slowly open the door. This will prevent two problems from occurring: (a) the trapped steam will dissipate toward the ceiling in a controlled fashion without burning the skin, and (b) the media will not boil out of the stoppered containers because of a too rapid change in internal pressure in the flask.

Preparing Agar Plates

As outlined previously, use some of the sterilized tryptic soy agar to prepare agar plates.

1. After autoclaving, or melting the agar in the flask, cool the flask of sterile agar in a 48° to 50 °C water bath. Line up the desired number of sterile petri plates on the bench top. (You may want

to label the bottoms or sides of the plates at this time.). Plates that are poured too hot will end up with a lot of condensation on the lid.

2. Remove the flask from the water bath and dry the sides of the flask with a paper towel. Place the flask on a few pieces of paper towel to keep the agar from solidifying prematurely. Be ready to pour the plates almost immediately thereafter. You do not want the agar to solidify during the pouring operation.
3. Remove the cap from the flask and briefly flame the flask's neck.
4. Lift the top of each plate, pour about 15 ml of agar (if you pour the plate about 7/8^{ths} full, that should be approximately the right amount), and quickly replace the top. Gently swirl the plates on the bench top in a Figure 8 pattern to distribute the agar evenly across the bottom of the petri dish. Do not swirl too vigorously otherwise some agar may end up on the lid or be spilled.
5. Your instructor will demonstrate the proper plate pouring technique. Aseptic procedures must be followed to prevent plate contamination.
6. Pour all plates without stopping. When all are poured, set them aside and leave them undisturbed until solidified.
7. Immediately rinse out the Erlenmeyer flask with a large quantity of warm water to prevent solidification of remaining agar. This facilitates clean up.
8. If you need to set the agar flask down between plates, use a piece of folded paper towel as an insulator so that the agar does not solidify too quickly. Loosely cap the flask when not in use.
9. Plates should be labeled with the type of medium on the bottom or the sides of the plates either before the plates are poured or after the plates are solidified. Do not label the lid, in case it accidentally becomes separated from the bottom.
10. Alternatively, after dissolving the agar medium, dispense 15-ml portions into 18 x 150 mm tubes instead of transferring to another flask; cap and autoclave the tubes. Cool them in a 48° to 50 °C water bath and pour the agar (one tube per plate).
11. When the plates are cool (agar solidified), invert them to prevent condensing moisture from accumulating on the agar surfaces. Stack them upside down in storage trays or plastic bags for later use.
12. All plates and tubes should be incubated for at least 24 hours to ensure sterility before you use them.

Microbiological Culture Media Preparation and Sterilization

1. After at least 24 hours of incubation, do your prepared plates and broths appear to be sterile? Explain your answer.

2. List the steps you would go through to make tryptic soy agar slants.

3. List the steps you would go through to make Vogel-Johnson agar plates (refer to the *Difco Manual* or the *BBL Manual).*

4. Provide the requested information using a *Difco Manual* or *BBL Manual.*
   a. Quantity of starch in Mueller-Hinton agar

   b. Quantity of lactose in Levine eosin methylene blue agar

   c. Percent of sodium chloride in mannitol salt agar

   d. Percent of agar in MacConkey agar

   e. Quantity of beef extract in nutrient broth

5. Using a *Difco Manual* or *BBL Manual,* find the following:
    a. The purpose of Endo agar

    b. The way in which bismuth sulfite agar selects for *Salmonella* and *Shigella* species

    c. The use of Sabouraud dextrose agar and the role of pH in this selectivity

    d. The roles of thioglycollate and methylene blue in thioglycollate medium

    e. The function of autoclaving in the preparation of Salmonella-Shigella agar

Review Questions

1. What are the three main types (in terms of their physical forms) of microbiological culture media?

2. Define culture medium, defined or synthetic medium, and complex or nonsynthetic medium.

3. Why are culture media sterilized before use?

4. Describe three ways for sterilizing culture media and supplies.

5. Why are petri plates inverted after they cool?

6. Why is culture medium cooled to about 48° to 50°C before it is poured into petri plates?

7. What is a buffer? What is the buffer system used in this exercise?

8. What is the source of carbon in the chemically defined medium in Table 1 ? The source of nitrogen?