Preparing Culturing Media

Lab VII-2

EQUIPMENT AND MATERIALS

You'll need the following items to complete this lab session. (The standard kit for this book, available from *www.thehomescientist.com*, includes the items listed in the first group.)

MATERIALS FROM KIT

- Goggles
- Agar
- Bottle, polypropylene, 125 mL
- Centrifuge tubes, 15 mL
- Petri dishes
- Spatula
- Stirring rod
- Test tubes
- Test tube rack
- Thermometer

MATERIALS YOU PROVIDE

- Gloves
- Aluminum foil
- Balance (or measuring spoons)
- Bottle, 500 mL soda (clean and empty)
- Broth, chicken (or bouillon cube)
- Cotton balls
- Marking pen
- Measuring cup, 500 mL (microwave-safe)
- Microwave oven
- Pressure cooker (optional; see text)
- Refrigerator
- Sodium chloride (table salt)
- Spray disinfectant (Lysol or similar)
- Sprayer bottle filled with water
- Sucrose (table sugar)
- Tape
- Water (distilled)

BACKGROUND

All living things require a suitable "life support" environment if they are to grow and flourish. Just as people need air, water, food, and protection from temperature extremes, bacteria and other microorganisms grow only in environments that supply their essential requirements.

The process of encouraging growth of particular organisms by providing conditions optimal for those organisms is called *culturing*. For example, growing plants and livestock is called *agriculture*. Fish farming is *pisciculture*, a form of *aquaculture*. In each case, human activity is directed at modifying the environment to suit the needs of the organism.

Culturing microorganisms is no different. We provide everything the microorganism needs to grow and flourish, including nutrients, suitable temperatures and other environmental factors, and a place to grow undisturbed and unthreatened. That place to grow is a gel or a liquid, and is called a *culturing medium*.

Gels are usually based on agar, which when dissolved in hot water and allowed to cool forms a semisolid gel much like dessert gelatin. (Gelatin is seldom used for general bacterial culturing because many bacteria species eat gelatin, turning it into a runny mess. With the minor exceptions of a few marine bacteria, no common microorganisms eat agar.)

Gel media have the advantage of providing a firm, flat surface for bacterial growth. If a mixture of two or more bacteria species is placed on the gel (a process called *inoculation*), those species form separate and distinct *colonies*, the form, color, and other characteristics of which are useful in identifying the particular species, or at least narrowing down the possibilities. Also, because colonies each contain only one species of bacteria, it's easy to obtain a specimen of that single species and use it to inoculate another culturing vessel, thereby producing a *pure culture* of that single species.

Liquid culturing media do not separate growing bacteria into colonies. If multiple bacteria species are introduced into a liquid culturing medium, they grow willy-nilly throughout the medium. But liquid media have the advantage of allowing bacteria to grow much faster than gel media, because bacteria grow throughout the entire volume of the liquid, rather than being limited to just the surface of a gel. For that reason, liquid culturing media are used almost exclusively for producing pure cultures.

The primary characteristic of a culturing medium is the mix of nutrients included. For bacteria, which are what we'll be culturing, a typical nutrient mixture contains some form of carbohydrate (usually a saccharide such as glucose or sucrose, but sometimes glycerol or another carbon source) and nitrogen in the form of proteins or inorganic nitrates. Other components may be added to encourage or discourage the growth of particular classes (or even species) of bacteria.

Such media are called *selective media*, because they favor the growth of one type or species of bacteria at the expense of others. For example, eosin-methylene-blue (EMB) agar contains methylene blue, which is toxic to most Gram-positive bacteria and therefore favors the growth of only Gram-negative bacteria. Conversely, mannitol-sugar agar (MSA) favors the growth of Gram-positive bacteria.

Some media, called *differential media*, provide a visual indication of the types of bacteria growing on the medium, usually by including a dye that changes color when exposed to the waste products of particular types of bacteria. For example, McConkey (MCK) agar is differential for lactose fermentation. It includes the dye neutral red, which is yellow at pH 8.0 or higher and red at pH 6.8 or lower. When it is prepared, McConkey agar is slightly basic, and therefore has a pale yellow color. If McConkey agar is inoculated with lactose-fermenting bacteria, as those bacteria grow and produce acidic waste products, the agar turns red. If the bacteria growing on the McConkey agar are not lactose fermenters, the agar remains a pale yellow color.

Another important distinction in culturing media is whether the identities and quantities of nutrients are known:

Defined medium

A *defined medium* (also called a *simple medium*) contains only known amounts of chemicals whose identities are known exactly. Carbohydrates (the carbon source) are usually supplied as known amounts of glucose, sucrose, or another sugar. The nitrogen source is usually a known amount of a simple ammonia compound or nitrate salt, such as ammonium nitrate or urea. Trace nutrients may be provided in the form of tiny amounts of inorganic salts.

Undefined medium

An *undefined medium* (also called a *complex medium*) contains complex chemicals from plant and/or animal sources. For example, an undefined medium may contain yeast extract or beef broth, both of which contain mixtures of many organic chemicals whose exact identities and amounts are unknown.

The most important advantage of defined media is that they can be tailored to provide optimal growing conditions for specific bacteria or other microorganisms. The most important disadvantage of defined media is that they cannot be used for most microorganisms because those microorganisms require resources that cannot be provided by defined media. (For example, viruses can be cultured only in the presence of the cells that host them.) Even most bacteria cannot be cultured on defined media, simply because we don't know all of their requirements. Conversely, most microorganisms can be cultured on undefined media, if they can be cultured at all, but at the cost of not knowing exactly what we're giving them to eat.

Culturing media are used in vessels that protect the growing cultures from contamination. Liquid media are used in test tubes (culture tubes) or flasks, which are simply filled, stoppered (usually with sterile cotton balls, but sometimes with solid rubber stoppers or screw-on caps), and sterilized. The most common vessels for gel media are the *Petri dish* (also called a *Petri plate*) and the *slant tube*.

Petri dish

A Petri dish is simply a shallow, flat, circular dish with a slightly larger lid. To fill a Petri dish with gel media, you pour the warm, liquid gel into the Petri dish to a depth of a few millimeters, cover the dish, sterilize it in an autoclave or pressure cooker, and allow it to cool until the gel sets.

Petri dishes are available in reusable glass and disposable presterilized plastic versions. The plastic versions are inexpensive individually—typically $0.50 each or less, depending on size—but because they are single-use, their cost adds up quickly if you run many cultures. Conversely, glass models can be sterilized and reused repeatedly, but they cost anything from a couple dollars to $10 or so each. They also tend to suffer breakage.

Slant tube

A slant tube is simply a test tube that functions as a miniature Petri dish. To fill a slant tube, you pour warm, liquid gel into the tube to about one-third full, plug the tube with a sterile cotton ball, sterilize it in an autoclave or pressure cooker, and then place the tube at an angle while the gel cools and sets. Allowing the gel to set with the tube angled increases the surface area of the gel available for culture growth.

Autoclaving is an important preparation step, because it kills every living thing in the culturing vessel. You might think that boiling the medium during preparation would accomplish that, but it doesn't. The problem is that the boiling point of water—100 °C at standard conditions—is sufficient to kill only bacteria, protozoa, viruses, and fungi. So what's left? Spores, which some microorganisms form when exposed to adverse conditions, such as high temperatures or extreme dryness.

Spores are not killed quickly at 100 °C, a temperature they can survive for anything from several hours to days or more. Fortunately, temperatures just a bit higher are sufficient to kill them quickly, which is where autoclaving comes in. In an autoclave (or pressure cooker), water is boiled under pressure higher than atmospheric pressure, which increases the boiling point. In a standard pressure cooker, water boils at 121 °C, which is sufficient to kill spores after only 15 minutes' exposure, called the *15-15 rule*.

> Most pressure cookers operate at 15 PSI (just over one standard atmosphere) higher than atmospheric pressure, a standard established by the US Department of Agriculture during WWI as being safe for home canning. Some pressure cookers operate at lower pressures, typically 10 to 12 PSI, which produces maximum temperatures of 115 °C to 118 °C.

In fact, this "rule" is not a rule at all, because the actual time needed for sterilization varies with the size of the container being sterilized, its composition, the degree of contamination of the material being sterilized, and other factors. For example, a 250 mL flask takes much longer to sterilize than a test tube because it takes longer for the liquid in the flask to reach the maximum temperature in the pressure cooker. Similarly, a metal tube sterilizes faster than a glass tube because the metal conducts the heat faster, and a polypropylene tube takes longer still because the plastic insulates the liquid inside the vessel. In short, there is no hard and fast rule about how long sterilization requires.

You might think the easy answer would be simply to autoclave or pressure-cook culturing vessels for a long time to ensure they're in fact sterile. The problem with that is that heating the medium too long may destroy nutrients. So, we shoot for a happy medium, so to speak, by autoclaving long enough to kill all or nearly all of the spores present while not cooking the nutrients to death.

Kitchen/lab-size pressure cookers (and autoclaves) were not invented until the late 19th century. So what did biologists do back then to sterilize their culturing media? Well, mostly they just sterilized with boiling water, hoped for the best, and didn't worry too much about a few spores germinating in their "sterilized" media.

But in 1876 an Irish physicist named John Tyndall devised a cunning plan. As usual, he boiled his medium to kill all of the bacteria and most of the spores, but then he allowed the medium to sit undisturbed for a day, during which time many of the spores germinated. He then reboiled the media, killing all the germinated spores. He repeated this for a third day to kill any late-germinating spores. His procedure, called *Tyndallization*, often killed all of the spores in the medium. A few sometimes survived, but even such imperfect sterilization was much better than simple boiling.

Tyndallization is still sometimes used to sterilize materials, such as some seeds, that cannot tolerate the higher temperature in a pressure cooker or autoclave. In fact, it's often not necessary to reach even 100 °C to kill the bacteria of concern on such materials.

We've written this lab session on the assumption that you have (or can borrow) a pressure cooker. Even an inexpensive model from Walmart is fine. Although ordinarily we strictly segregate lab equipment from food-preparation equipment, there is, by definition, no risk to using a pressure cooker both for ordinary cooking and for sterilizing media. The pressure cooker kills anything present inside it, whether you're pressure-cooking a roast or a bunch of culture tubes.

You can also use the pressure cooker to kill live cultures in Petri dishes, slant tubes, and culturing flasks, as long as the containers are glass. When you're finished with cultures, simply autoclave them to kill anything present. The other benefit of this method is that agar gel re-liquefies, making it much easier to clean out the dishes and tubes. Wait until the culturing container has cooled sufficiently to be quite warm to the touch, and then just pour the sterile gel down the drain.

With a pressure cooker, there's no need to follow aseptic procedures while making up the media, filling glass culturing vessels, and so on, because sterilization is the final step. If you don't have a pressure cooker, or if you're using plastic culturing vessels that cannot be autoclaved (as we are in this lab session), aseptic procedure becomes much more important.

Use the following guidelines to keep your culturing media as uncontaminated as possible:

- Turn off the central heat or air conditioning. You don't want the fan circulating air while you're working with culturing media and vessels.
- Immediately before you begin work, sterilize your work surface by misting it with Lysol or a similar disinfectant spray.
- Use a spray mister filled with tap water to mist the air around your work area. The tiny water droplets capture airborne particulates and settle out.
- When you prepare culture medium in a beaker, keep the beaker covered as much as possible with a loosely fitting cap of aluminum foil, plastic wrap, or waxed paper to prevent airborne contaminants from entering the beaker. (Do this whether you are simply boiling the culture media or pressure-sterilizing it. In the latter case, use aluminum foil, because plastic wrap or waxed paper won't survive being pressure-cooked. If you are boiling the medium in a microwave oven, use plastic wrap or waxed paper to avoid damaging the oven and possibly causing a fire.)
- Wear gloves at all times while handling culturing media or vessels. It does no harm to use Lysol or hand sanitizer on the gloves as well to kill any microorganisms present.

If you do not have a pressure cooker to sterilize the culture media, culture plates and tubes should be used as soon as possible after you make them up, and certainly within 24 hours. If you use a pressure cooker, agar plates and tubes remain usable for several days and broth tubes for a week or more. In any event, store unused culture vessels in the refrigerator until you are ready to use them, and then allow them to come to room temperature before inoculating them.

There are also times when you want to preserve a live bacteria culture but not encourage growth. For example, in the following lab sessions we use a purchased mixed culture of three species of bacteria. We would prefer to purchase that culture only once, so we'll preserve the culture by transferring a small amount of it to a tube of sterile *isotonic saline* (also called *normal saline*) solution, in which many species of bacteria can survive for extended periods. This solution is essentially a dilute solution of ordinary table salt. It provides no nutrients, so the bacteria we inoculate into it will not grow (reproduce). Some, however, usually survive by going dormant, essentially putting themselves into hibernation until a food source is again available.

In this lab session, we'll prepare a nutrient broth based on chicken stock (as a nitrogen source) and table sugar (for carbohydrates). We'll use that nutrient broth to prepare broth culturing tubes as well as agar culturing plates and agar culturing slant tubes. We'll also prepare and sterilize isotonic saline tubes that we'll use in the next lab session to preserve our purchased bacteria culture for later use. We'll need about 10 mL of nutrient medium for each broth culture tube, about 5 mL for each agar slant tube, and about 35 mL for each 90 mL Petri dish.

Again, if you do not have a pressure cooker, make up only enough medium for your immediate needs. If you do have a pressure cooker, you can save time by making up additional medium for later use. That's what we'll do in these instructions. Modify them as necessary if you do not have a pressure cooker.

In science, volumes and masses usually matter a great deal. In this and the following lab sessions, neither are critical.

PROCEDURE VII-2-1: PREPARING NORMAL SALINE AND NUTRIENT MEDIA

1. Label a clean, empty 500 mL soda bottle "Normal Saline" and transfer 500 mL of distilled water to it.

2. Weigh out 4.50 g (a slightly rounded half teaspoon) of sodium chloride (table salt) and transfer it to the bottle. Cap the bottle and invert it several times to dissolve the salt and mix the solution thoroughly.

Retain this normal saline solution for use in later lab sessions. Although it is not sterile, it may often be used in nonsterile form. When you do require it in sterile form, you can simply sterilize as much as you need for immediate use, as we do next.

3. Transfer about 10 mL of the normal saline solution to each of two labeled 15 mL centrifuge tubes. Cap the tubes loosely and place them in the test tube rack for now.

Plastic centrifuge tubes are are autoclavable, but never screw the caps down tightly until the tubes have been autoclaved. Autoclaving a tightly capped tube may cause it to burst. Before autoclaving, place the caps on the tubes and turn them just a fraction of a turn—enough to keep the cap from falling off, but not enough to form a gas seal. Once the autoclaving is complete and the tubes have cooled, you can screw the caps down tightly.

4. Calculate the amount of nutrient medium you need for the number of broth culturing tubes, agar slant tubes, and Petri dishes you intend to fill.

For immediate use, we decided to make up enough nutrient broth to fill four broth tubes (4 X 10 mL = 40 mL), and enough nutrient agar to fill two slant tubes (2 X 5 mL = 10 mL), and two Petri dishes (2 X 35 mL = 70 mL). That requires a total of 120 mL of nutrient broth. To save time later, we also decided to make up an extra 125 mL of nutrient agar, which we'll store in the 125 mL polypropylene bottle and autoclave. That medium will remain usable for at least several weeks if refrigerated, and is sufficient to fill four 90 mm Petri dishes, which we'll use in a later lab session. Accordingly, we'll make up 250 mL of nutrient broth.

Use the 125 mL polypropylene bottle from the kit that contains the nitrogen-free fertilizer concentrate A. If you haven't used all of that solution by the time you start this lab session, simply transfer the fertilizer concentrate to another container and label it. Peel off the original label from the 125 mL polypropylene bottle and relabel the bottle with its current contents.

1. Dissolve a bouillon cube (or dilute canned chicken broth) in twice the recommended amount of warm tap water. Use a spoon or paper towel to skim off or blot up any oil or grease floating on the surface of the solution.

2. Transfer 250 mL of the defatted broth to the measuring cup.

3. Add two teaspoons of table sugar to the broth, and stir until it dissolves.

4. Label four 15 mL centrifuge tubes "Nutrient Broth" and fill them to about 10 mL each. Cap each of the tubes loosely and place the tubes in the test tube rack for now.

5. Make sure there is sufficient broth remaining in the measuring cup to make up the desired amount of agar gel medium. In our case, we needed 70 mL for the two Petri dishes, 10 mL for the two slant tubes, and 125 mL for the bottle we're making up for later use, or a total of about 205 mL. We added a bit extra just to be safe. (If there is too little broth remaining in the cup, simply add a small amount of tap water to make up the necessary volume.)

6. Weigh or measure out 2 g of agar powder (~5/8 teaspoons) per 100 mL of broth and stir the agar powder into the cool broth.

WARNING

Be very careful heating any liquid in a microwave oven. The liquid may superheat, which means its temperature reaches higher than the boiling point without the liquid actually boiling. In this unstable condition, the liquid may suddenly start boiling violently and eject itself from the container.

7. Put the measuring cup in the microwave and heat it until it just boils.

8. When the solution is boiling, carefully remove it from the microwave oven and stir until all of the powder is dissolved and the agar solution is clear, homogeneous, and slightly viscous. Allow the agar medium to cool to about 60 °C (hot to the touch).

9. Carefully pour about 5 mL of the agar medium into each of your two slant tubes. Plug them with cotton balls and place the slant tubes in the test tube rack for now.

10. Fill the 125 mL polypropylene bottle with the warm agar medium and cap the bottle loosely.

11. Prepare your pressure cooker, adding the recommended amount of water. Place the pressure cooker rack in the pressure cooker (if your pressure cooker did not come with a rack, use an oven rack that fits it) and transfer the broth tubes, slant tubes, and polypropylene bottle to the pressure cooker.

If the pressure cooker is large enough, you can leave these tubes in the test tube rack, which is autoclavable. Otherwise, stand the tubes in a beaker or a similar container.

12. Cover the measuring cup loosely with aluminum foil, and transfer it to the pressure cooker.

13. Replace the cover on the pressure cooker, and apply heat until the pressure reaches nominal. Note the time and begin the cycle.

If your pressure cooker operates at 15 PSI (121 °C), allow 30 to 45 minutes for complete sterilization to occur. If your pressure cooker operates at lower pressure, extend the time appropriately. At 115 °C, we recommend doubling the time.

14. Follow the instructions provided with your pressure cooker for cooling. (Some pressure cookers recommend allowing the unit to cool naturally, but others recommend cooling it under cold tap water.)

15. Once the pressure is released, remove the cover from the pressure cooker. Allow the contents to cool sufficiently that you can handle them with your bare (well, gloved...) hands. They should feel quite warm, but not hot enough to burn you. The goal is to remove them while the agar gel is still flowing as a liquid rather than set up.

16. Tighten the caps on the broth tubes and place them in the rack for now. Place the agar slant tubes at an angle sufficient to allow the agar to almost but not quite reach the cotton plugs. Allow them to remain in this position until they have cooled sufficiently for the agar to set.

17. Carefully remove the measuring cup from the pressure cooker, keeping the contents protected from airborne contamination by the aluminum foil.

18. Carefully remove two of the presterilized Petri dishes from the outer sleeve. Note that the dishes are not individually wrapped, and remain sterile only as long as you keep the covers in place. Be careful not to disturb the covers until you are ready to fill the dishes.

19. Working in a sanitized area as described in the introduction, place the first dish flat on your work surface with the measuring cup of agar medium next to it. Reposition the aluminum foil to expose the pouring lip of the cup. Carefully lift the edge of the Petri dish cover just enough to allow you to pour in gel. Pour sufficient gel to cover the bottom of the dish to a depth of about 0.5 cm, and then replace the Petri dish lid immediately. Repeat to fill the second Petri dish.

20. Allow the Petri dishes to sit undisturbed until the agar gel sets. Placing the Petri dishes in the refrigerator will set the gel within a few minutes. Otherwise, allow at least half an hour for the gel to set completely.

We'll use these Petri dishes, slant tubes, and broth tubes in the next lab session. Store them in the refrigerator until then. Store the Petri dishes inverted (with the gel side up). Store the slant tubes and broth tubes upright, either in the test tube rack or in a beaker or similar vessel. Remove the Petri dishes and tubes from the refrigerator and allow them to warm to room temperature before you use them.

REVIEW QUESTIONS

Q1: Is the nutrient media we made defined or undefined? Why?

Q2: Why do we store unused culturing media in the refrigerator?

Q3: How do unselective and selective media differ? Why might you choose an unselective medium?

Q4: What is the defining characteristic of a differential medium?

Q5: Why might you choose a broth medium rather than a gel medium, and vice versa?

Q6: Why is agar so widely used for making up gel culturing media?