

## PREPARATION OF MEDIA

### I. OBJECTIVES

- To become familiar with the necessary nutritional and environmental factors for culturing microorganisms in the laboratory.
- To understand the decontamination or sterilization process using an autoclave.
- To learn the procedures used in preparing media needed for culturing microorganisms.

### II. INTRODUCTION

Microorganisms depend on a number of factors such as nutrients, oxygen, moisture and temperature to grow and divide. In the laboratory, except for the above factors, the culture medium should be sterile and contamination of a culture with other organisms should be prevented. Let us briefly discuss a few of the more important factors for the growth of microorganisms.

#### Nutrients

A microbiological culture medium must contain available sources of hydrogen donors and acceptors, carbon, nitrogen, sulfur, phosphorus, inorganic salts and, in certain cases, vitamins or other growth-promoting substances. These were originally supplied in the form of meat infusions that were, and still are in certain cases, widely used in culture media. Beef or yeast extracts can replace meat infusions. The addition of peptone provides a readily available source of nitrogen and carbon.

Peptone is used in culture media to mainly supply nitrogen. Most organisms are capable of utilizing the amino acids and other simpler nitrogenous compounds present in peptone. Thus, in many cases, the complicated infusion media can be replaced by simpler media prepared by using the proper peptones in place of the meat infusions.

Certain bacteria require the addition of other nutrients, such as serum, blood, etc. to the culture medium upon which they are to be propagated. Carbohydrates may also be desirable at times, and certain salts such as calcium, manganese, magnesium, sodium, and potassium seem to be required. Dyes may be added to media as indicators of metabolic activity or for their selective inhibitory powers. Growth promoting substances of a vitamin-like nature are essential or assist greatly in the development of certain types of bacteria. Many of these substances are given for individual bacteria in Bergey's Manual of Determinative Bacteriology (Incidentally, this is a reference text that you should familiarize yourself with when working with microorganisms.)

## Oxygen

Most bacteria are capable of growth under ordinary conditions of oxygen tension. Certain types, however, are capable of deriving their oxygen from various substrates. The aerobic organisms require the free admission of air, while the anaerobes grow only in the absence of atmospheric oxygen. Between these two groups are the microaerophiles that develop best under partial anaerobic conditions and the facultative anaerobes that develop under aerobic as well as anaerobic conditions.

It is easy to provide oxygen to aerobic and facultative anaerobic and even microaerophilic organisms; however, special gadgetry is required to exclude the atmospheric oxygen and provide an anaerobic condition. Such conditions are obtained by:

- Addition of a reducing substance to the medium
- Displacement of the air by carbon dioxide
- Absorption of the oxygen by chemicals
- Removal of oxygen by direct oxidation of readily oxidizable substances such as burning a candle, heating of copper, phosphorus or other readily oxidizable metals
- Incubation in the presence of germinating grain or pieces of potato
- Inoculation into the deeper layers of solid media, or under a layer of oil in liquid media or
- A combination of the above methods.

## Moisture

Proper moisture conditions must prevail in the culture media for the growth of microorganisms. A moist medium and atmosphere are necessary for the continued luxuriant growth of cells. For example, if a medium in a plate is inoculated with an organism and wet cotton is placed in the plate and sealed, the organism will show profuse growth. The same organism might fail to show growth if the medium plate is not sealed and is too dry.

## pH

The pH of the culture medium, expressed as hydrogen ion concentration  $[H^+]$ , is extremely important for growth. The majority of microorganisms prefer culture media that are approximately neutral, while others may require a medium that is distinctly acidic.

## Temperature

Every organism shows a rather general curve of growth as affected by temperature. Such a curve shows 1) a minimum temperature below which growth stops, 2) an optimum temperature at which growth is luxuriant and 3) a maximum temperature above which the organism dies.

Microorganisms are divided into three main groups (mesophilic, psychophilic and thermophilic) as far as optimum temperature requirements are concerned. The usual range of temperature suitable for the growth of mesophilic microorganisms lies between 15-43 °C. Psychophilic microorganisms have, however, been known to grow and multiply at 0 °C. Thermophilic organisms may grow at temperatures even greater than 80 °C. In general, the

pathogenic organisms have a temperature requirement of around 37 °C (body temperature) while saprophytes have a much broader latitude.

### **Medium Support**

The consistency of a liquid medium may be modified by the addition of agar, gelatin or albumin in order to change it into a solid or semisolid state. In the early 19th century, infusions of plant and animal tissues, solutions of organic compounds, and gelatin (as a solidifying agent) were employed as media for the growth of microorganisms. However, gelatin had two main disadvantages; being liquid at 37 °C (incubation temperature), and being liquefied or digested by many bacteria.

Bacteriology as a science began with the development of methods for the cultivation of bacteria, and the introduction of agar by Hesse in the 1890's was a step of greatest importance. Agar is actually credited to Fanny Hesse, wife, technician and assistant of the German physician Walter Hesse. Agar-agar, long used as an agent in preparing fruit jellies was suggested by Mrs. Hesse as a replacement and became the standard solidifying agent in microbiology.

The properties of agar which make it ideal in bacteriology are 1) solid agar melts (dissolves) at 100 °C, 2) remains solid at all incubation temperatures, 3) is transparent, 4) is not heat-labile and therefore easily sterilized, and 5) is unaffected by almost all bacteria. Liquid agar solidifies at 42-44 °C which is useful because sterile, heat-labile components such as antibiotics, blood, serum, carbohydrates and even bacterial cultures may be added before allowing the medium to solidify. Solid media generally contain agar at a concentration of 1.5%. Semi-solid media contain 0.05-0.3% agar and are useful in culturing anaerobic and microaerophilic organisms because such media form an oxygen gradient in test tubes, allowing all degrees of oxygen tension to exist in the culture vessels.

### **Sterile Conditions & Autoclaving**

The media upon which microorganisms are grown must be sterile or free from all other forms of microbes. The usual method for sterilization of culture media is by means of the autoclave in which steam under pressure is the sterilizing agent.

Autoclave sterilization for 15 minutes at 15 pounds of pressure and at 121 °C is recommended for quantities of liquid media up to one liter (1 L). These settings are called the standard autoclaving conditions. If larger volumes are to be sterilized in one container, and if the medium is not hot when placed in the autoclave, a longer period should be employed. The medium is prepared according to formula, distributed in tubes or flasks which are then plugged with nonabsorbent cotton or loosely capped before being placed in the autoclave. Plugs should fit neither too loosely nor too tightly. Screw cap tops or metal covers may also be used to close the tubes or flasks. Tubes should be placed in racks or packed loosely in baskets. Flasks should never be more than two-thirds full.

After the sterilization period has been completed, the source of steam is cut off and the autoclave is allowed to return to atmospheric pressure. Pressure should not drop too rapidly or the media will boil over, blowing the plugs from the tubes or flasks. Pressure should, however,

drop rapidly enough to prevent excessive exposure of the media to heat after the sterilization period.

The usual procedure for using the autoclave is as follows:

1. Open door, and place items to be sterilized into the autoclave chamber. Be sure that anything containing fluid is plugged with styrofoam, cheesecloth, cotton, a Morton cap or else screw caps are slightly loose.
2. Close door. Push down door lock lever until door studs are completely in place.
3. Turn hatch wheel clockwise until it is secured tightly.
4. The temperature of the autoclave is set at 121°C. If not, set the temperature by sliding the upper (yellow) arrow to the desired temperature. Do not touch the bottom indicator arrow. If you adjust to any temperature other than 121 °C, return it to 121 °C at the end of the run.
5. Set timer by turning the large knob just below the hands to the desired setting. DO NOT TOUCH the hands, they break very easily!
6. Set cycle selection knob to desired setting. Remember, all liquids MUST be done at SLOW EXHAUST. Dry materials can be done at Fast Exhaust or Fast Exhaust and Dry.
7. Crank operating handle around to the Sterilize position till the red steam light goes on.
8. If you are the first person to use the autoclave that day, it is a good idea to wait and be sure the chamber reaches the proper temperature and pressure.
9. The Slow Exhaust and Fast Exhaust & Dry cycles both take 12-15 minutes longer than the time set to finish.
10. At the end of the run the white STERILE light will go on, and a loud, obnoxious buzzer will come on:
  - a. Turn the cycle knob to MANUAL.
  - b. Rotate the operating handle all the way to OFF. Check that the chamber pressure is zero, and the temperature is below 100 °C.
  - d. Turn hatch wheel counterclockwise, push up door lock lever and slowly open door. Watch out for steam!
  - e. Use heatproof gloves to remove materials.
11. Allow liquid materials to cool before tightening caps.

A maximum of 15 minutes is recommended for the sterilization of carbohydrates media in tubes to be used for fermentation studies. Oversterilization or prolonged heating will change the composition of the medium. For example, oversterilization results in the breakdown of lactose in lactose-containing media.

Agar media on prolonged sterilization, heating or repeated melting are apt to show a precipitate. Media containing agar may also form a flocculent precipitate if the liquid medium is held in the water bath at 43-45 °C for longer than 30 minutes. Reheating the medium, however, may disperse this flocculent agar precipitate. Excessive heating of media may also result in an increase in acidity. The reaction of the media will become more acidic as heating is prolonged.

Culture media that may be harmed by autoclaving are sometimes sterilized by the discontinuous or intermittent method. This procedure consists of heating the medium in a chamber of flowing steam for a period of 20 or 30 minutes on several successive days. Liquid media may be sterilized by filtration through membranes, molecular filters or unglazed porcelain.

### **Storage of Media**

Media should always be stored in a cool moist atmosphere to prevent evaporation, preferably in screw-capped tubes or bottles. Prolonged storage of sterile media cannot, however, be recommended unless stability is established. If tubes of media have been kept for any length of time, they should be reheated just before use. Liquid media should be heated in a boiling water bath or in flowing steam for a few minutes, to drive off dissolved gases, and then cooled quickly in cold water without agitation just prior to inoculation. Agar tubes should be melted and allowed to solidify in order to secure a moist surface that is desired by most microorganisms. These precautions for both liquid and solid media are extremely important for the initiation of growth of highly parasitic organisms such as those encountered in blood culture work.

### **Types of Media**

Culture media may be divided into two main categories, complex (undefined) and synthetic (defined). In a defined medium, all components are known to the investigator such as a synthetic medium containing glucose as the sole carbon source, inorganic salts as sources of sodium, phosphate and many other required minerals such as Fe<sup>++</sup> or Mg<sup>++</sup>, and an ammonium salt as a source of nitrogen. Some bacteria are able to grow on media like that described above, while others require growth factors that they cannot synthesize for themselves (i.e., they are fastidious).

A complex medium contains animal or plant tissue extracts such as beef extract or yeast autolysate. These extracts provide a large variety of nutrients in the form of lipids, hydrolyzed proteins (a source of nitrogen as amino acids), carbon sources and vitamins and other cofactors. The exact components of these extracts are unknown; therefore any medium containing them is called undefined. Other sources of these necessary growth factors are brain or heart tissue infusions, whole blood, serum, etc.

Throughout the semester you will be using several types of general complex growth media, both in broth and solid agar form including: Brain-Heart Infusion (**BHI**), Nutrient Agar (**NA**), Trypticase Soy Agar (**TSA**), Luria or Luria-Bertani agar (**L** or **LB**) and Sheep's Blood Agar (**SBA**) which is actually TSA + 5% sheep's blood.

In today's experiment, you will be making Nutrient Broth (**NB**) and Nutrient Agar (**NA**), the most common standard complex media for culturing many microorganisms.

### III. LABORATORY SUPPLIES

Flask, 1 L	1/table
Graduated cylinder, 1 L	1/table
Glass stirring rod	1/table
Spatula	3/lab
Weigh boats	as needed
Beef extract, bottle	1/table
Bacto peptone, bottle	1/table
Agar, powder	1/table
pH paper, 6.5-10, box	1/table
HCl, 1N 25 ml/dropper bottle	1/table
NaOH, 1N 25 ml/dropper bottle	1/table
Test tube rack	2/table
Test tubes, 18 x 150 mm	20/table
Morton closure, 16 mm clear	10/table
Morton closure, 16 mm metal	10/table
Slant racks	2/ table
Labeling tape, roll	1/room
Heatproof gloves	1/room
Autoclave	RESERVE
Water baths, 48 °C	1/room
Petri plates	10/table
Cheesecloth square	1/table
Cotton or Styrofoam plug	1/table

### IV. PROCEDURE

**Note 1:** The students at each table will work together as a group.

**Note 2:** Check the BlackBoard site for a flow chart of this lab procedure.

1. Wipe down lab bench carefully with Disinfectant to help prevent contamination of your media.
2. Measure approximately 250 ml of distilled water (located in 60°C water bath) in a 1 L graduated cylinder and pour into a 1 L flask.

3. Weigh out 1.5 g beef extract and 2.5 g peptone and add into the flask. Wash your spatula between bottles and wipe dry. DO NOT return excess material that is weighed out to the container - discard. Use approximately 100 ml of the water to rinse any powder stuck to the side of the flask down into the mixture.
4. Stir over gentle heat from a bunsen burner to dissolve completely.
5. Pour the mixture into the 1 L graduated cylinder and add warm water to the 500 ml mark. Pour back into the flask.
6. Check the pH of the medium and adjust to pH 7.0, if necessary, using the HCl and/or NaOH. Adding the agar in the next step will not appreciably change the pH.
7. Using a 10 ml pipette, dispense 10 ml of the mixture into each test tube. Make 10 tubes and place in a test tube rack.
8. Add 6.0 g of agar to the flask and label it NA. Heat to just boiling for 1-2 minutes while stirring constantly. The agar will not dissolve unless it is boiled; the solution will become completely clear when it has dissolved. Allow agar to cool until there is no danger of you being burned and then dispense into the tubes using a 10 ml pipette. Make ten 10 ml tubes.
9. Close the flask with a Styrofoam plug covered with cheesecloth and tape it on top of the flask. Use another piece of tape to go around the neck of the flask and pass over the first tape. Cap all the tubes with Morton closures. These should be pushed down completely or else they will be forced off during the autoclave's exhaust cycle. They are still self-venting when pushed down all the way.
10. Keep one tube of each type in a drawer until next period to demonstrate the need for sterilization. Continue to observe growth for one more period.
11. Autoclave the flask and the tubes for 15 minutes at 121 °C and 15 lb/in<sup>2</sup> pressure at the slow exhaust mode. Watch your instructor for the use of the autoclave.
12. After removing the media from the autoclave, allow the broth tubes to cool, and store for later use. Place the flask in the 48°C water bath. Quickly lay the tubes of NA on the slant racks on the center table so that the medium forms a long slant and a short butt, and allow them to cool and solidify. Do not allow the agar to reach the top of the tube. Allow them to cool completely before returning to the rack. Store for later use. Label rack.
13. Lay your petri dishes on the bench. The cover should be on top. Light your bunsen burner, then remove the NA flask from the water bath. Carefully wipe the bottom dry to prevent the dripping water from contaminating the plates.
14. Remove the tapes and cotton plug from the flask. Carefully flame the neck of the flask, open the plate cover about half way and fill the plate about 1/2 full. The plates have a full

- line on the side; fill to that or slightly above. Put in a little too much rather than too little. If there is not enough medium in the plate, it will dry up in the incubator.
15. Flame the neck of the flask between each plate. Each student must pour at least two plates. IMMEDIATELY rinse the excess agar out of the flask with hot tap water and place on the discard cart. Allow plates to solidify completely, which will take 15 minutes. Then invert, label and incubate at 37 °C overnight to dry off excess moisture and check for contamination.
  16. Clean all glassware and leave on paper towels beside sink.

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*Use of any section of this Lab Manual without the written consent of Dr. Eby Bassiri, Dept. of Biology, University of Pennsylvania is strictly prohibited.*



### Results of Preparation of Media

NAME \_\_\_\_\_ DATE \_\_\_\_\_ GROUP NAME \_\_\_\_\_

Description of non-sterilized media:

Session one

Session two

Description of sterilized media: