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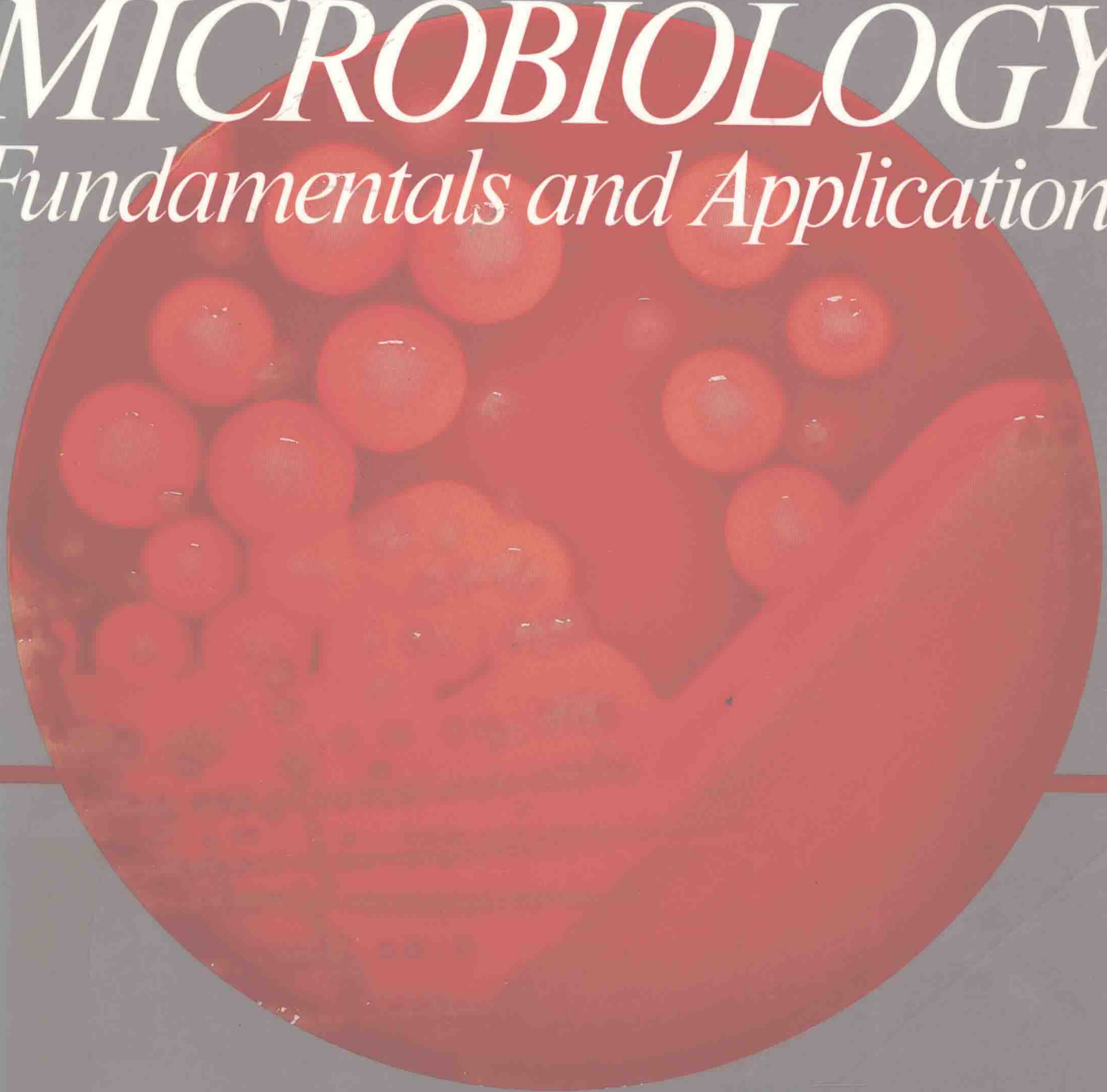
SECOND EDITION

EXPERIMENTAL MICROBIOLOGY

Fundamentals and Applications



2 ND EDITION

EXPERIMENTAL MICROBIOLOGY

Fundamentals and Applications

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PREFACE

Experimental Microbiology: Fundamentals and Applications, 2nd Edition is intended to introduce you to the exciting field of microbiology. This laboratory manual is designed for you, the introductory microbiology student. It particularly addresses your needs as a student who will be dealing with microorganisms and performing many microbiological procedures for the very first time. A laboratory manual should provide sufficient information so that you understand the principles involved in each exercise (what does the exercise mean and why are we doing it) and sufficient detail so that you can perform the experiment independently. We know from personal experience how frustrating it is to follow the procedures carefully only to obtain results that are not consistent with what you expected.

In the second edition we have made some significant changes which we feel will vastly improve the lab manual. We have retained the majority of exercises that comprised the first edition. These are a detailed series of exercises that can be performed with a high degree of probability. The exercises will work, but do not become frustrated if you encounter some failures along the way; always remember that you are dealing with living organisms that exhibit variability, sometimes leading to unanticipated results. The most important aspects of performing laboratory exercises is to record and interpret what you see. We have included a results section at the end of each exercise to aid you in recording and interpreting the results of your exercises. We have also included questions that are relevant to the exercises and give you the opportunity to challenge your understanding of the principles and applications of the information obtained from the experiment.

You will find that the manual is divided into fourteen major sections that span the breadth of the field of microbiology:

Culturing and Handling of Microorganisms
Observing Microorganisms and Microbial Structures
Microbial Metabolism
Microbial Growth
Microbial Genetics
Virology
Microbial Taxonomy
Archaebacteria
Food Microbiology
Industrial Microbiology
Environmental Microbiology
Medical Microbiology
Immunology and Host Defenses Against Disease
Antibiotic Sensitivity

In this edition we have included new sections, on Virology and the Archaebacteria, and have added new exercises to existing sections. The Archaebacteria are a group of bacteria which are an evolutionary link between bacterial and higher cells, and are the subject of intense investigation. We feel that students should be exposed to this group of bacteria to appreciate their unique characteristics and current importance.

We have also designated certain exercises as advanced. These exercises usually require equipment that may not be readily available in every general microbiology laboratory, such as gas chromatographs and electrophoresis equipment, but nevertheless illustrate principles which are of primary importance to microbiology today. These include such techniques as use of radioisotopes, which has been an obligatory necessity to modern biology in revealing the mysteries of cellular structure and metabolism, and modern electrophoretic techniques, which have ushered in the age of recombinant DNA and biotechnology. We strongly urge that these exercises be tried because they will expose the student to many of the contemporary methods that are found in any modern microbiology laboratory.

In the second edition of this manual we have not included specific exercises which require the use of *Bergey's Manual of Determinative Bacteriology*. We have chosen to do this for several reasons. Primarily, this "bible" of bacteriology is presently undergoing a major revision itself and will expand from a single volume to at least four volumes. Two of these volumes will not be in print until probably after this manual is published. Also, other taxonomic keys that exist can be used in the identification of unknown bacterial isolates. What is important for the student in all of these identification schemes, including Bergey's, is the use and application of a dichotomous key for means of identifying unknown bacteria. Therefore, we leave to the discretion of the instructors the choice of how Bergey's manual should be used in each class. We feel the student should be cognizant of Bergey's existence; but this awareness must be in the context of an obvious changing role. What is important for the student to understand is that very logical taxonomic methods exist for identification of bacteria but that these methods may require alteration and updating as research uncovers new information.

Learning to be observant is another key to success in microbiology. Gross observation of colonies on an agar plate, the color change in a broth culture of microorganisms, and the proper use of the microscope are some of the essential observations needed to interpret the results in microbiology experiments. Be sure to prepare in advance for the laboratory exercises you are going to perform: read your laboratory manual before coming to the laboratory. As Pasteur stated, "Chance favors the prepared mind."

By performing the exercises in this manual you will develop an appreciation of the variability of microbial structure and function and an understanding of fundamental aspects of microbial growth and genetics. In addition to developing basic techniques and a fundamental appreciation of microbial diversity, and ecology. Microbiology is a relevant science that impacts our daily lives in numerous ways.

Experimental Microbiology: Fundamentals and Applications, 2nd Edition will provide you with the tools you need to discover the microbial world. Performing experiments in microbiology can be exciting and fascinating, and we hope that you will become motivated to pursue further studies in the dynamic field of microbiology as a result of this introduction to experimental microbiology.

CONTENTS

SECTION ONE

Culturing and Handling of Microorganisms

To examine and study the characteristics of microorganisms, including obtaining organisms for microscopic examination, it is usually necessary to grow the organisms in pure culture. There are many different types of microorganisms, and each type has minimal requirements, tolerance limits, and optimal conditions for growth. In order to grow, microorganisms require a suitable environment, including a growth medium that can support their nutritional needs. Many bacterial species can be grown in the laboratory on a defined medium, which normally contains an organic carbon growth substrate, such as glucose or protein; mineral nutrients, including a source of nitrogen and phosphorus; and water. There are numerous different types of media used for growing bacteria and fungi in pure culture. In addition to a defined growth medium, the culture of microorganisms requires careful control of various environmental factors, including temperature, which normally is maintained within narrow limits by using a temperature controlled incubator. By understanding the growth requirements of a given microbial species, it is possible to establish the necessary conditions *in vitro* to support the optimal growth of that microorganism.

The growth of a microorganism in pure culture further mandates that all other microbial species be eliminated. Since microorganisms are ubiquitously distributed in nature, obtaining and maintaining pure cultures requires the elimination of other microorganisms from the growth medium (sterilization), the separation of the microorganism being cultured from a mixture of microbes (isolation), the movement of the microorganism from one place to another without contamination (aseptic transfer), and the maintenance of the pure culture (preservation).

Every student of microbiology must master certain basic techniques, including (1) preparation and sterilization of a suitable medium that will support microbial growth; (2) aseptic handling and transfer of microbial cells; (3) isolation of microorganisms and the establishment of pure culture; and (4) the establishment and preservation of a stock culture to serve as a source of inoculum for further studies.

EXERCISE 1
Preparation of Culture Media

Any medium for the cultivation of bacteria must provide certain basic nutritional requirements, which include (1) a carbon source that may also serve as an energy source; (2) water; (3) a nitrogen source; (4) a phosphate source; and (5) various mineral nutrients, such as iron and magnesium. Some bacteria are capable of growth on a medium consisting of a single carbon source, such as the carbohydrate glucose; a simple nitrogen source, such as ammonium salts; and inorganic salts, such as phosphates. This kind of medium is termed defined or synthetic because its exact chemical composition is known. For routine laboratory work, however, complex media are employed where the basic nutrients are provided by complex nutrients, such as plant and animal extracts in which the exact composition is not known. For example, beef extract and peptones (hydrolyzed protein) are the basic ingredients of nutrient agar. These materials supply a variety of carbon sources, nitrogen compounds in the form of amino acids, and a mixture of cofactors, such as vitamins. This basic medium can be further enriched to support the growth of more fastidious types of bacteria by the addition of carbohydrate sources, yeast extract, and materials such as plasma or blood, which provide a variety of complex nutritional factors. A broth medium is one in which the components are simply dissolved in water. The addition of agar-agar (a complex carbohydrate extracted from seaweed) results in a solid medium. Agar is an ideal solidifying agent for microbiological media because of its melting properties and because it has no nutritive value for the vast majority of bacteria. Solid agar melts at 90–100° C; liquid agar solidifies at about 42° C.

Because microbes are ubiquitously distributed in the environment, during the preparation of any culture medium, bacteria are introduced from many sources such as glassware, dry medium components, air, and so on. These bacteria would eventually grow and flourish if the medium were not sterilized, that is, if these unwanted microbes were not destroyed.

Sterilization procedures eliminate all viable microorganisms from a specified region. Culture dishes, test tubes, flasks, pipettes, transfer loops, and media must be free of viable microorganisms before they can be used for establishing pure cultures of microorganisms. The culture vessels must be sealed or capped with sterile plugs to prevent contamination. There are various ways of sterilizing the liquids, containers, and instruments used in pure culture procedures; these include exposure to elevated temperatures or radiation levels to kill microorganisms and filtration to remove microorganisms from solution.

Media preparation for the microbiology laboratory involves the use of an autoclave for sterilization, which permits exposure to high temperatures for a specified period of time. Generally, a temperature of 121° C (achieved by using steam at 15 lb/in^2) for 15 minutes is used to heat-sterilize bacteriological media. Much of the time spent in preparation for the bacteriology laboratory involves cooking the media for growing bacteria; that is, mixing and sterilizing the growth media in suitable sterile culture vessels.

In this exercise you will prepare both a defined and a complex medium. You will learn how to mix the proper constituents to support the growth of microorganisms and how to sterilize the medium.

References

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Gerhardt, P. (ed.). 1981. *Manual of Methods for General Bacteriology.* American Society of Microbiology, Washington, D.C.
Sirockin, G., and S. Cullimore. 1969. *Practical Microbiology.* McGraw-Hill Publishing Co., Ltd., London.

A. PREPARATION OF A COMPLEX MEDIUM

Materials

Beef extract
Peptones
Agar
Yeast extract
Glucose
Flasks (1 L)
Graduated cylinder (1 L)
Balance
pH meter or pH indicator paper
Test tubes, 16 x 125 mm; 20 x 150 mm
Test tube rack
Sterile petri plates
Autoclave
Hydrochloric acid, HCl (1 *N*)
Sodium hydroxide, NaOH (1 *N*)
Pasteur pipettes
Heat-proof gloves

Procedures

1. Deliver 500 mL of distilled water to a 2-liter Erlenmeyer flask. Weigh out 3 g of beef extract and 5 g of peptone; dissolve these in the water by agitation. (Note: heating may be necessary.) After complete dissolution, bring the volume to 1000 mL by adding distilled water.
2. Divide this broth solution into three flasks as shown in Figure 1-1.
   (a) Flask A (nutrient broth)—make no further additions.

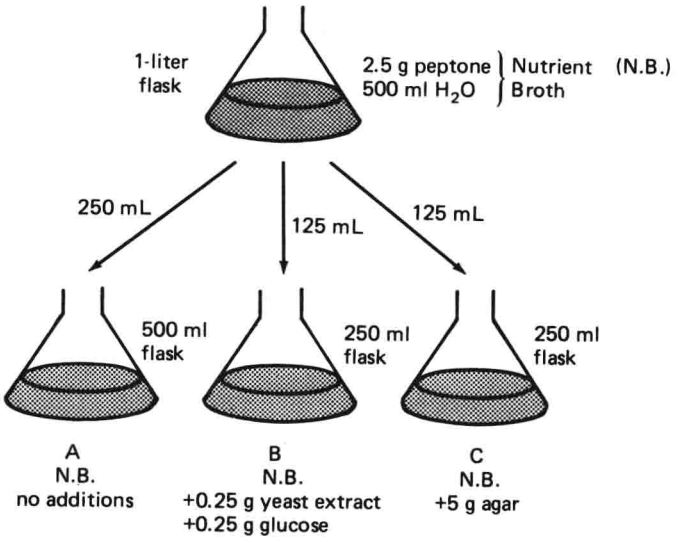

FIGURE 1-1. Preparation of complex media.

(b) Flask B (nutrient broth supplemented with yeast extract and glucose)—add 0.25 g of yeast extract and 0.25 g of glucose.
(c) Flask C (nutrient agar)—add 5 g of agar. Heat to boiling and swirl to effect solution. (**Caution:** do not allow the agar to boil over!)

3. Cool each flask to about 55–60° C and then check the pH. Adjust the pH to 7.0 if necessary by adding 1 *N* NaOH or 1 *N* HCl. Make sure to clean the pH electrode with water.

4. Dispense the broth medium into 16 x 125-mm test tubes, adding 5 mL of broth to each tube. Dispense the agar medium into the 20 x 150-mm test tubes, adding 15 mL of medium to each tube. Label the tubes.

5. Cap each test tube, do not tighten excessively, and place them into the autoclave.

6. To demonstrate the necessity of the sterilization step, do not autoclave 1 or 2 tubes of the nutrient broth enriched with yeast extract plus glucose; simply allow these tubes to remain stoppered until the next lab period.

7. After the autoclave is loaded, lock the door. Most autoclaves have an automatic cycle—set the autoclave for liquids and the timer for 20 minutes. Start the autoclave cycle by pushing the start button in the automatic cycle mode or in the manual mode by turning the selection lever to the fill position. The sterilization cycle involves filling the jacket, allowing steam to enter the chamber, holding temperature for the amount of time that you have set, and venting the chamber. In the automatic mode the chamber will begin to fill with steam after the jacket pressure reaches 15–20 lb/in^2; in the manual mode you must move the selection lever to the fill chamber position when the jacket pressure reaches this level. In manual operation you must move the selection lever to the vent position after the chamber has been at 121° C for 20 minutes. Slow venting is required to prevent liquids from boiling over. Only after complete venting of the chamber can you open the autoclave door and remove your material. Autoclaving for 20 minutes actually takes about 40 minutes when you include the time required for heating and cooling the chamber. When removing material from the autoclave, use heat-proof gloves—the material is still hot! Also be aware that liquids can boil over during removal. Be very cautious when swirling hot liquids!

8. After removal from the autoclave, allow the broth tubes to cool and store them for use in later exercises.

9. Allow half of the nutrient agar tubes to cool and solidify in the upright position. These can be used for nutrient agar pours or stab cultures in later experiments.

10. Carefully tilt the remaining nutrient agar tubes so that the medium forms an incline of about 30–45° (Figure 1-2). The tubes can be placed on an inclined plane or the capped ends of the tubes can be rested on a pencil or other object to establish the proper degree of tilt. Be careful not to allow the agar to reach the tops of the tubes. Allow the agar to cool and solidify, being careful not to move the tubes once you have set them at the proper angle. After the agar has solidified, store these nutrient agar slants for use in later experiments.

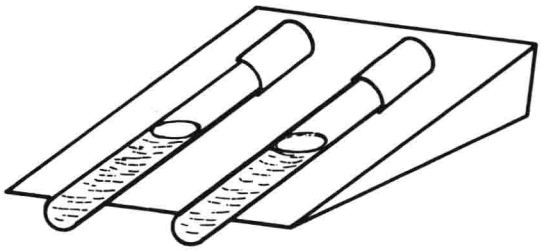

FIGURE 1-2. Preparation of nutrient agar slants.

B. PREPARATION OF A DEFINED MEDIUM

Materials

Agar
Glucose
Potassium phosphate, K_2HPO_4
Magnesium sulfate, $MgSO_4$
Ammonium chloride, NH_4Cl
Ferric chloride, $FeCl_3 \cdot 6H_2O$
Nicotinic acid
Flasks (2 L)
Graduated cylinder (1 L)
Balance
pH meter or pH indicator paper
Sterile petri plates
Autoclave
Hydrochloric acid, HCl (1 *N*)
Sodium hydroxide, NaOH (1 *N*)
Pasteur pipettes
Heat-proof gloves

Procedures

1. Glucose-mineral salts agar; deliver 900 mL of distilled water to a 2-L flask. Weigh out the following amounts of each component and dissolve them in the water.

Glucose	1.0 g
NH_4Cl	0.5 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$FeCl_3 \cdot 6H_2O$	0.005 g
Agar	15.0 g

2. Carefully adjust the pH to 7.0 with either NaOH or HCl.
3. Bring the volume to 1 L and boil to dissolve the agar. (Caution: do not allow the agar to boil vigorously.)
4. Glucose-mineral salts agar supplemented with the growth factor nicotinic acid. After the agar is dissolved, pour half (500 mL) of the glucose-mineral salts agar into a separate flask and add 0.05 g of nicotinic acid. To the remainder (500 mL), make no further additions. Stopper the flasks with cotton or foam plugs and autoclave the media.
5. After each of the media prepared above has been sterilized, allow it to cool until you can pick up the flask without burning your hand (approximately 50°C). Do not let it cool too much or the agar will solidify. To dispense the medium into sterile petri plates, flame the mouth of the flask and, while carefully lifting the lid of a petri plate, pour about 15–20 mL of agar into the plate (enough medium to

cover the bottom of the plate). Replace the lid and continue filling additional plates until all of the medium is dispensed (Figure 1-3). Never completely remove the lids of the Petri plates or the plates will become contaminated with bacteria and fungi from the air. Work quickly to minimize contamination, but carefully to prevent accidents. You can periodically reflame the mouth of the flask to reduce contamination. Allow the agar plates to cool. After the agar has solidified, store the plates for use in a later laboratory experiment.

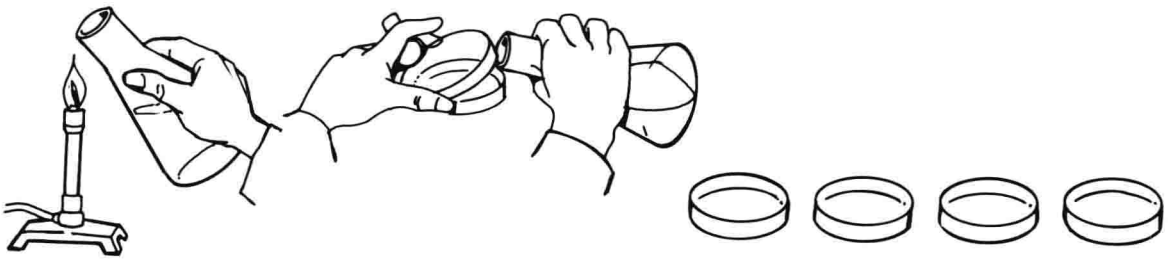

FIGURE 1-3. Flame the mouth of the flask and aseptically pour the medium into a sterile Petri plate. To maintain aseptic conditions, keep the cover of the plate over the bottom of the dish while you are pouring the medium.

EXERCISE 1:
Preparation of culture media

Results and Questions

Name _____ Date _____ Section _____

RESULTS

What changes did you notice in the unsterilized medium compared to the sterilized medium?

_____

_____

_____

QUESTIONS

1. Why are media for culturing bacteria adjusted to a neutral pH?

_____

_____

2. What is the difference between a synthetic and a complex medium?

_____

_____

3. What nutrients are provided by the beef and yeast extracts?

_____

_____

5. What kinds of organisms could be expected to grow in the synthetic medium? What kinds in the complex?

_____

_____