Microbiological Techniques in School

Science and Technology Education

Division of Science Technical and Environmental Education

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Microbiological Techniques in School

by

Ronald Westphal
The document series has been established as part of Unesco's Science and Technology Education Programme to encourage an international exchange of ideas and information in the field. The present volume, as part of the "Biology and Human Welfare" theme was produced under the auspices of the Institute for Science Education (IPN), Kiel, Federal Republic of Germany, under contract with Unesco.

This document describes standard techniques of microbiology within the scope of the secondary school curriculum. Sufficient theoretical background is included for easy understanding of the experimental procedures. The text can be used in two ways:

1. The experiments can be easily integrated into existing school curricula. Many interesting biological facts can be expediently and easily demonstrated through the use of micro-organisms by virtue of their high multiplication rates and ease of maintenance.

2. The experiments can serve as the basis for the course concept. The teacher can modify the contents to meet the class organization requirements.

"Microbiological Techniques in School" may help to incorporate the treatment of a modern technology within school biology.
Dr. Ronald Westphal of the IPN is the author of this book. The invaluable help extended to him by his colleagues, in particular, Professor Horst Bayrhuber, Mr. Hans-Joachim Hebenstreit, Mr. Eckard Lucius, Ms. Uta Nellen and Ms. Patricia Nevers in developing this book, is gratefully acknowledged. Thanks are also due to Ms. Maria Fries for the technical assistance; Mr. Lawrence Allen for translating the original text produced in Germans into English; and Mr. Peter Trott for carefully reading the manuscript.

The opinions expressed in the following pages are those of the author, and not necessarily those of Unesco.
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Bacteria and their spores are omnipresent. Even the brief opening of a Petri dish can cause contamination. It is therefore imperative that all equipment, containers and media be sterilized before use. All procedures with micro-organisms must be carried out in such a manner that the cultures can be neither contaminated nor serve as a source of contamination. The usual execution of these procedures under laboratory conditions can be found in every manual for microbiological practice at the university level. However, in the classroom conditions are usually quite the opposite. Too many students crowd around the work area; lab smocks are not always available and the tables are not only used for microbiological work, but for work with plant and animal preparations and the occasional snack as well. The lab work must all too often be accomplished under time pressure since the experiment(s) must be completed and the area cleaned up before the bell rings for the next class.

These conditions demand compromises. The responsible educator can not tolerate any procedure that could endanger the students. The teacher should attempt to estimate whether the conditions in his or her school will allow basic sterile work by conducting the following pre-experiments:

1. Nutrient media for liquid cultures and agar plates should be prepared according to the instructions given below.

2. Nutrient broth should then be inoculated with a drop of a micro-organism suspension and sterilized.

3. The agar should be poured into petri dishes and placed with the liquid cultures together in an incubator at 30° C.

After incubating for two days the nutrient solution should be free of contamination and the petri dishes without any indication of micro-organism growth. Since extraneous microbes can multiply rapidly, a sterilization must be absolutely reliable for the use of nutrient solutions. Small amounts of contamination can be tolerated in dish
cultures since colonies of contaminants can be recognized by their form or colour.

Know-How

1. Work Area

The work area must be free from all apparent impurities. Surfaces should be wiped with a solution of at least 70 % alcohol prior to commencing work. It is advantageous to conduct all operations between two Bunsen burners. (Fig. 1.1.)

Fig 1.1. Working between two burners

2. Equipment

Dry glass utensils can be sterilized by placing them in a drying cabinet at 180° C for 30 minutes. In principle, glass equipment can be
sterilized by holding it in the flame of a Bunsen burner for 2 - 3 seconds. However, this process is not suitable for measuring containers nor for glass types that can easily crack. After opening a glass container with sterile or microbiological contents to remove a sample, the container opening and stoppers should be briefly passed through a flame before reclosing.

3. Solutions

When an autoclave is available: Containers filled to a maximum of two thirds can be heated at 120° C for 20 minutes. **CAUTION:** Some nutrients can not tolerate high temperatures. Solutions of such substances must be sterilized by means of a sterile filtering process. This process, however, is not necessary for the experiments described in this text.

When a pressure cooker is available: The solutions must be heated in saturating steam according to the manufacturer’s specifications.

Tyndallization (Pressureless Sterilization): If pressureless sterilization is to be carried out for want of proper equipment, the solutions must be boiled in water for 45 minutes, allowed to stand at room temperature for 4 hours and finally boiled again for 45 minutes. After 45 minutes the solution can be boiled for a third time, for greater certainty.

The autoclave cooling process can be shortened by opening the pressure valves, thereby allowing the steam to escape. When the pressure decreases too rapidly, however, the agar solutions readily demonstrate the phenomenon of delayed hailing i. e., they froth up and the contents spill over the container. The mere vibrations that arise when pouring culture dishes can stimulate a further seething process when the temperature of the agar solution is great enough. Therefore the autoclave pressure must be reduced very slowly, if it is at all desirable to shorten the cooling process under these circumstances.
Disposal of cultures

Old cultures must be sterilized before discarding in order to avoid all risk of contamination or infection. This antiseptic process can be carried out according to paragraph 3 for liquid cultures. Agar plates can be sterilized in the autoclave or pressure cooker. Contaminated plates can also be covered with disinfectant solution. The manufacturer's instructions should be adhered to regarding the concentration and time necessary for desinfectant solutions to function.

Know-Why

1. Burners produce a steady current of rising heat. This thermal current prevents microbes in the air from sedimenting and helps to keep them from precipitating into culture mediums and containers. (Fig. 1.2.)

Fig. 1.2. Ascending air current near the burner
However open flames in the laboratory require very cautious behavior on the part of the children. The teacher must decide from case to case what the pupils can safely handle.

2. The temperature of 180° C is necessary since spores can survive dry heat at temperatures up to 160° C and at normal pressure.

3. The purchase of pressure cooker is strongly recommended when an autoclave is not available. Pressure cookers are relatively inexpensive, can be used with gas or electric hot plates and sterilize reliably. All cells are killed with the Tyndallization technique described here, and in contrast to many spores they cannot survive a temperature of 100° C. Surviving spores germinate in the following 4-hour period and are killed by the second process. Problems with this process are mainly concerned with the time span selected between boiling processes. If too little time is allowed, the nongerminated spores will still be present. If too much time is taken, then sporiinating bacteria will have been able to develop new spores. If the instructions given here do not produce sterile results, the time allotted between boiling processes should be increased. Time spans of up to 24 hours are recommended in the literature.

4. Cultures which may contain human pathogenic bacteria must, of course, be destroyed before discarding. Even though one would never knowingly work with micro-organism in school which cause disease, dangerous bacteria can find their way into cultures of harmless bacteria and multiply. Therefore, as a precautionary measure, every culture should be sterilized before discarding.
Fig. 1.3. Tyndallization procedure

1. Before the first boiling

2. After the first boiling = before incubation

3. After 4 - 24 hours incubation

4. After the second boiling
2. Recommended Micro-organisms for Use in School

2.1. Cultivation of Bacillus megaterium

In 1884, the German botanist Anton de Bary described an unusually large bacterium that he had discovered on a cooked cabbage leaf. He called it *Bacillus megaterium* due to its size. Many authors, beginning with F. Loehnis in 1911, recognized in *B. megaterium* an excellent organism for laboratory uses due to its size which also facilitates the identification of cell compounds. Furthermore, *B. megaterium* can be easily cultivated, multiplies rapidly and presents no danger to humans. It exists in nature as an saprophyte participates in the decomposition of organic matter and requires no unusual nutrients for growth.

**Properties**

- rod-shaped, peritrichous
- 3 - 6 x 10 \(^3\) mm long (max. 10 x 10 \(^3\) mm), 1.5 - 2 x 10 \(^3\) mm wide.
- spores: 1.0 x 1.6 x 10 \(^3\) mm
- gram positive
- aerobic
- contains oxidase, catalase, amylase and protease
- does not produce dihydrogensulfide
- some strains can reduce nitrate to nitrite
- metabolizes glucose to organic acid(s)

*B. megaterium* consistently grows according to expectations under the cultivation conditions described below. Cultures which become visible after a few days always have traces of *Bacillus subtilis*. Furthermore, the development of pathogenic germs cannot be excluded with certainty. However, *B. megaterium* has been employed for 75 years for practical school applications without a single report of illness. The instructions up to step 4 must be followed if the teacher desires to avoid all risks. After step 4, the culture plates are sealed with tape and not reoponed. The cultures can then be used for purposes of demonstration. Further experiments must be carried out with cultures
acquired from microbiological laboratories that guarantee the purity of their strains.

**Know-How**

**Equipment**

1. 1 paring knife
2. 1 Bunsen burner with tripod and wire screen
3. 1 250 ml beaker
4. 1 pair of tweezers
5. 1 sterile petri dish

**Materials**

1 carrot

**Time Requirements**

30 minutes
Procedure

1. Fill the beaker half full with tap water and bring the water to a boil.

2. Peel the carrot and cut it into slices approximately 5 mm thick.

3. Place the carrot slices in the boiling water.

4. Two slices are removed from the beaker after three, four and five minutes and placed in a petri dish. The plate should be opened only briefly for inserting the carrot slices.

5. The filled petri dishes are then placed in an incubator for a few days at 30°C.

6. After three to five days, whitish-yellow, smooth edged colonies of bacteria can be seen on some of the carrot slices. (Fig. 2.1.)

Know-Why

2. If the carrot slices have a large diameter, then three slices per plate are sufficient. It is important that the petri dish is full enough to eliminate the risk of dehydration. Some authors recommend placing the carrot slices on moistened filter paper. However, this measure may be an additional source of contamination.

3. Cells introduced to the carrot surfaces by peeling and slicing are killed by this process; however, the spores survive. The carrot cells are also killed, which helps accelerate decomposition by bacteria. The time intervals listed were determined empirically. The optimal treatment time can be achieved by one of the three possible trials.
Problems and Questions

1. A single bacterium has the dimensions $5 \times 10^{-3}$ mm x
   $2 \times 10^{-3}$ mm x $2 \times 10^{-3}$ mm.
   How many bacteria fit into 1 mm$^3$ volume?
   A square form for the bacteria should be assumed to simplify calculations.
   (Calculation: Volume of one bacterium
   \[ V = 5 \times 10^{-3} \text{ mm} \times 2 \times 10^{-3} \text{ mm} \times 2 \times 10^{-3} \text{ mm} \]
   Number of bacteria in 1 mm$^3$
   \[ N = \frac{1 \text{ mm}^3}{2 \times 10^{-8} \text{ mm}^3} = 5 \times 10^{-7} \text{ mm}^3 \]
   1 mm$^3$ contains 50,000,000 bacteria.)

2. How long would a chain of these bacteria be if one placed them end to end?
   (5 x $10^{-3}$ mm x $5 \times 10^7$ = $2.5 \times 10^5$ mm = 250 mm.)

3. Why can B. megaterium and B. subtilis survive periods of brief heating?
   (The spores are heat resistant.)

4. Why should the carrot slices not be incubated at 37° C? (To reduce the risk of development of pathogenic micro-organisms.)

2.2 Cultivation of Bacillus subtilis

Bacillus subtilis is another bacterium present in the environment and found on plant matter. B. subtilis is also known as a hay bacillus since it can be obtained from hay compost. However, in as much as a myriad array of micro-organisms, not to mention algae and protozoans, are present on compost, a mere selective method is recommended here.
Properties

- rod-shaped, peritichous
- 2 - 4 x 10^3 mm long, 10.0-1.2 x 10^3 mm wide
- spores: 0.6 x 0.8 x 10^-3 mm
- gram positive
- aerobic
- contains oxidase, catalase, amylase and protease
- does not produce dihydrogensulfide
- no nitrate to nitrite reduction
- metabolizes glucose to organic acids

Know-How

Equipment

1. 1 paring knife
2. 1 Bunsen burner with tripod and wire screen
3. 1 250 ml beaker
4. 1 pair of tweezers
5. 1 sterile petri dish

Materials

1 potato

Time Requirements

1 - 4: 30 minutes
8: 15 minutes
When desired, and in accordance with safety precautions, a selective agar culture can be prepared from individual colonies that arise. This measure improves the purity of the culture. Additional equipment is necessary for pouring plates (see section 6) and the following materials are required: soluble starch, peptone, salt and agar.

**Procedure**

1. Fill the beaker half full with tap water and bring the water to a boil.

2. Peel a potato and cut it into 1 cm slices.

3. Place the potato slices in boiling water for 15 minutes.

4. After boiling, remove the slices from the boiling water with a tweezers and place them in the petri dish. The dish should be opened only briefly for inserting the potato slices.

5. The petri dish is then placed in an incubator for 3 - 5 days at 30° C.

6. After a few days, a coating will form on the potato slices which is composed mainly of colonies of B. subtilis. (Fig. 2.2.).

7. If the cultures are to be purified with the help of a selective medium, the nutrient must be prepared according to the following recipe:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>2 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>3 g</td>
</tr>
<tr>
<td>Salt</td>
<td>70 g</td>
</tr>
<tr>
<td>Agar</td>
<td>18 g</td>
</tr>
<tr>
<td>Destilled water</td>
<td>18 g</td>
</tr>
<tr>
<td>Destilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The pH value must be set at 7.3.
8. A small portion of the coating is removed from the potato slice with a sterile inoculating loop and a fractionated streak is made (see Fig. 6.3.)

9. The petri dishes are then incubated for three days at 30° C.

**Know-Why**

The explanatory remarks necessary for understanding this experiment are for the most part the same as those of the previous experiment. Accordingly, only the deviations from the prior procedure need to be noted here.

**Notes**

3. The spores of *B. subtilis* are heat resistant and can withstand heating for 15 minutes at 100° C. Conversely, many undesirable spores are killed off by this measure, which thus promotes the purity of the resultant culture.

7. Contrary to other micro-organisms *B. subtilis* can tolerate saline solutions of high concentration. The selective medium restricts the growth of other cultures by means of its high salt concentration.

**Problems and Questions**

1. The selective medium employed here contains 70 g table salt per liter. For comparison: How much table salt does saltwater contain? (saltwater contains 30 g/l.)

2. In what manner do the two types of bacteria studied here primarily differ? (They differ in size and in the heat resistance of the spores.)
2.3. The Use of Baker’s Yeast

Baker’s yeast (Saccharomyces cerevisiae) is for many reasons an excellent micro-organism for schools. It can be easily acquired, many students are familiar with it from home and it is not dangerous to humans. Commercial baker’s yeast is pure enough for all the experiments presented in this book in spite of the lactose fermenting bacteria included in such preparations.
Properties

- oval shaped cells
- 10.15 \times 10^{-3} mm long, 7 \times 10^{-3} mm wide
- spores 6 \times 10^{-3} mm long, 2.5 \times 10^{-3} mm wide
- develops cell aggregates in nutrient medium
- ferments various carbohydrates, e.g. glucose, saccharose and maltose

Producing Suspensions

When a yeast suspension is needed, water should not be used since the cells will due to the osmosis in water for the suspension. Instead a diluted table salt solution should be employed (c = 0.085 \%). A 1 \% suspension should be employed for experiments with baker’s yeast for 100 ml suspension.

2.4 Enrichment of Wild Yeast strains

The conditions mentioned in Section 2.1. are valid for yeast cultures purposes derived from natural isolates: They can be produced without hesitation for of demonstration purposes. However, for safety reasons they should not be used for further experiments.

Know-How

<table>
<thead>
<tr>
<th>Equipment</th>
</tr>
</thead>
</table>

1. petri dishes
Material
malt extract
peptone
agar
an unwashed apple or another piece of fruit with sugar content

Time Requirements
45 minutes

Procedure
1. Malt agar plates are prepared according to the instructions in Section 6.

2. A piece of unwashed fruit is then rolled across the surface of the chilled agar; afterwards the dishes are immediately closed.

3. The plates are then incubated for a few days at 30° C. An array of yeast types, some brilliantly coloured, can be observed along with mold cultures which can be recognized by their cotton-like texture.

Fig. 2.3. Cultures of assorted yeast types from an apple skin
3. **Preparing a Culture in a Liquid Medium**

When working with micro-organisms, it is often desirable to have a large number of different types readily available, some for example, to study their physiological properties and growth and other simply to provide an ample supply for course work. This situation can be alleviated through establishing liquid cultures. Different media are necessary for bacterial cultures as opposed fungal cultures.

**Know-How**

**Equipment**

1. 1 100 ml Erlenmeyer flask per experiment
2. 1 10 ml graduated cylinder
3. 1 Bunsen burner
4. 1 tripod
5. 1 wire net
6. 1 inoculating loop
**Materials**

aluminium foil  
For Bacteria  
dried nutrient broth commercial or home made broth prepared according to appendix 13.3.  
For Yeast  
malt extract  

**Time Requirements**

preparation: 45 minutes  
incubation: 24 hours  

**Procedure**

1. **For bacteria**  
   Twenty ml nutrient broth are prepared in an 100 ml Erlenmeyer flask according to the manufacturer’s instructions or according to Appendix. The solution must be clear.  
   
   **For Yeast**  
   Twenty ml malt broth are prepared in an 100 ml Erlenmeyer flask. This amount contains 17 g malt per titer. To pH value must be lie between 5.0 and 5.6; otherwise, hydrochlorid acid or sodium hydroxide must be used for correction.  

2. The cylinder is covered with aluminium foil and sterilized according to one of the methods in Section 1.  

3. The solution is inoculated after cooling. A small amount of material is removed from a micro-organism culture with the tip of a heat sterilized needle and stirred into the nutrient broth.  
   This step should be performed between two burners (see p.6).
4. The flask is again covered with aluminum foil and incubated overnight at 30°C. The presence of many organisms can be recognized by the turbid appearance of the solution, by the sediment on the bottom of the flask and by the surface slime on the solution. (Fig. 3.1.)

![Diagram of liquid culture with surface slime and sediment](image)

**Fig. 3.1. Formation of surface slime on a liquid culture**

**Know-Why**

1. The solution may be cloudy when tap water is used instead of distilled or demineralized water as recommended by the manufacturer. In this case it is difficult to observe the multiplication of the organism. If the flask is filled with too much nutrient broth, not enough oxygen can diffuse to the
deeper layers. When this situation occurs, the solution must be stirred or shaken to ensure that the bacteria receive the oxygen necessary for their development.

Problems and Questions
1. By what means can bacterial suspensions without sediment and surface slime be obtained. (By stirring and shaking.)

2. Why is it dangerous to keep a liquid culture for an extended period of time? (The longer the culture is maintained the greater the risk that unknown micro-organisms unintentionally introduced into the culture can multiply and contaminate it).

3. \textit{B. megaterium} divides approximately every 50 minutes at 37° C. How long would it take, given an indefinite possibility for reproduction, for one bacterium to propagate one million? (less than 17 hours.)

4. By what means does the growth of bacteria cease? (Through an increase in toxic metabolic products or through lack of nutrients.)

4. Preparing a Permanent Culture in Slant Agar Tubes

If one wishes to work with various different micro-organisms, it is helpful, to have permanent cultures available rather than maintaining the culture by means of repeated purification procedures and enrichment cultures. Permanent cultures in slant agar tubes are well suited for this purpose. However, such cultures can present some problems. Contaminants can develop over a long period of time which, in the course of repeated inoculations, can lead to enriched cultures of their contaminants. Mites than can feed on agar are another concern. They carry spore: that have adhered to their bodies into the culture and can consequently render entire collections unusable.
Furthermore, the risk of contamination must be taken into consideration. For this reason in many countries it is not allowed to prepare permanent cultures in school.

Whether it is worthwhile to establish a micro-organism collection in the school according to the process described here must be carefully considered. *B. megaterium* and *B. subtilis* can subsist for long periods on standard nutrient agar. If cultures of different organisms are to be established it is then absolutely necessary to determine the needs of each organism with respect to the composition of the nutrient agar in applicable reference books. The use of homemade nutrient agar is not recommended in such cases.

Stab agar tubes are a convenient alternative to slant agar tubes for the establishment of a permanent culture. (See Section 5.)

**Know-How**

**Equipment**

1. 1 test tube per experiment and test tube caps
2. 1 Erlenmeyer flask (volume depends on desired amount of agar - about 6 ml per test tube)
3. 1 beaker as water bath, volume large enough to hold
all the test tubes used.

4. 1 autoclave, pressure cooker or Bunsen burner with tripod and wire screen.

5. 1 inoculating loop

6. I funnel

Materials

For Bacteria
Dried nutrient agar (commercial) or home made nutrient agar (see appendix 13.3.)

For Yeast
malt agar according to appendix 13.3.

Time Requirements

45 minutes (without inoculation)

Procedure

1. Prepare agar according to the manufacturer’s instructions or according to section 13.3.

2. Using a funnel, pour roughly 6 ml agar into each test tube and close them with a cap.

3. Heat the test tubes in an autoclave in saturated steam at 121º C for 20 minutes or heat them for 30 minutes in a pressure cooker according to the manufacturer’s instructions. Tyndallization according to Section 1 must be employed in the absence of an autoclave or pressure cooker.

4. The test tubes are then placed at an angle at which the liquid surface reaches approximately the halfway point of the tube to allot the agar to congeal properly. (Fig. 4.1.)
5. After the agar has congealed, the slant agar tubes are placed in an incubator for two to three days at 30° C.

6. Tubes which prove to be sterile are then inoculated. For this step the inoculating loop covered with the desired micro-organism is drawn along the surface of the agar from the bottom to the top in a zig-zag motion. Care must be taken not to press too hard in order to prevent damaging the agar surface. (Fig. 4.2.)

7. The openings of the test tube and the caps are passed through the hot flame of a Bunsen burner, after which the tubes are immediately reposed.

8. After incubating, the cultures can be stored in a refrigerator for two days at 30° C.

9. Cultures that are to be maintained over extended periods of time must be reinoculated into fresh slant agar tubes every four weeks. Each time, is done, a small portion of the material should be placed in an agar plate in order to check the purity of the culture.
**Know-Why**

4. The nearer the agar surface is to the opening of the tube, the greater the danger that extraneous micro-organism will invade the agar through the moisture film between the glass and the stopper, thereby, rendering the culture worthless.

5. The sterile condition of the tubes is verified by this means. Contaminants that may have infiltrated the tubes reproduce during incubation and can easily be recognized. This test is especially important when neither an autoclave nor a pressure cooker is available.

7. Micro-organisms that have come into contact with utensils during the inoculating procedure are destroyed by this brief heating process.

9. A four-week interval is a safe period of time for maintaining colonies in slant agar tubes, and under certain circumstances they will keep even longer. However it is recommendable to check the condition of the cultures at least every four weeks. If, colonies are observed anywhere that vary from the norm in colour and growth, then the culture is no longer usable. Such
colonies must be handled as pathogenic types, and they must there-
fore be treated with the usual commercial disinfectants or ste-
rilized according to the instructions in Section 1.

Problems and Questions

1. Test how long various cultures can be stored in a refrigerator
   without developing visible alterations.
   (Cultures can keep for many months.)

2. How long do various cultures maintain their infective
   capacity?
   (Cultures can remain infectious for many months.)

5. Investigating Oxygen Requirements in Stab Agar Tubes

One can differentiate between aerobic and anaerobic bacteria through
the use of stab agar tubes. They are furthermore a suitable alternative to
slant agar tubes for the preparation of a permanent culture.

Know How

<table>
<thead>
<tr>
<th>Equipment</th>
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<tbody>
<tr>
<td>[Image of equipment]</td>
</tr>
</tbody>
</table>
1. 1 test tube per experiment, test tube caps or alternatively absorbent paper wadding (not for permanent cultures)
2. 1 funnel
3. 1 Erlenmeyer flask, volume sufficient to hold 15 ml nutrient agar per test tube.
4. 1 beaker as test tube holder
5. 1 autoclave, pressure cooker or Bunsen burner with tripod and wire screen.
6. 1 inoculating loop

### Materials

**For Bacteria**
dried nutrient agar (commercial) or home made nutrient agar according to appendix 13.3.

**For Yeast**
mal agar according to appendix 13.3.

### Time Requirements

45 minutes (without inoculation)

---

**Fig. 5.1. Pouring liquid agar into a stab agar tube**
Procedure

1. Nutrient agar is prepared according to manufacturer’s instructions or Appendix 13.3.

2. Using a funnel, fill each test tube with agar to a level of roughly 5 cm. The test tubes are then closed with caps or absorbent paper wadding.

3. Heat the test tubes in an autoclave in saturated steam at 121° C for 20 minutes or heat them for 30 minutes in a pressure cooker according to manufacturer’s instructions. Tyndallization according to Section 1 must be employed when neither of these appliances is available.

Fig. 5.2 Inoculating a stab agar tube
4. The test tubes are incubated for two to three days at 30° C.

5. Tubes that prove to be sterile are inoculated. Dip the tip of an inoculating needle into the selected bacteria or yeast culture. Then press the needle vertically 3 - 5 times into the agar as deep as possible move it up and down and finally remove it.

6. The openings of the test tube and the caps are passed through the hot flame of a Bunsen burner, after which the tubes are immediately reposed.

7. The growth of the culture is examined after incubating for five to seven days at 30° C.

8. When extended storage periods are desired, the cultures must be sealed airtight to prevent contamination and then refrigerated. Every year new stab tubes should be made as a quality control. Cultures in stab agar tubes can keep for over a year under favourable conditions.

**Know-Why**

2. The use of a funnel helps to prevent the agar from being deposited on the upper half of the test tube.

3. Problems of sterile producers are discussed in section 1.

4. By this means the tubes should be examined to ensure that they are free from contamination.

5. The bacteria should be pread over the entire inoculating area.

7. The oxygen requirements of the micro-organisms can be determining by examining the growth of the same culture. (Differentation between aerobic and anaerobic micro-organisms).
8. Permanent cultures must be protected against dehydration; otherwise the agar will slowly shrink.

Problems and Questions

1. What causes the turbidness in the area of the growth zones? (Light refraction at the border surfaces between the many cells and air or agar.)

2. What are the oxygen requirements of bacteria which display the following growth form in a stab agar tube? (The culture is facultatively aerobic, or it is possibly a mixed culture of aerobic and anaerobic bacteria.)

Fig. 5.3. Possible growth pattern of a culture in a stab agar tube
6. Preparing Petri Dish Cultures

Plate cultures are prepared to maintain bacteria for a short time (one to six weeks), to isolate bacteria to determine the viable cell titer in a culture and basically to demonstrate the existence of micro-organisms in the air, water, or soil or on objects.

Know-How

**Equipment**

1. 1 sterile petri dish per experiment
2. 1 Erlenmeyer flask volume great enough for 15 ml agar per petri dish
3. 1 autoclave pressure cooker or Bunsen burner with tripod and wire screen.
4. 1 0.1 ml pipette
5. 1 Drigalski spatula
6. 1 inoculating loop

**Materials**

*For Bacteria*
- dried nutrient agar (commercial or home made according to appendix 13.3.)

*For Yeast*
- malt agar according to appendix 13.3.
45 minutes (without inoculation)

**Procedure**

1. Prepare nutrient agar according to the manufacturer’s instructions or section 13.3.

2. Heat the test tubes in an autoclave in saturated steam at 121 °C for 20 minutes or heat them for 30 minutes in a pressure cooker according to the manufacturer’s instructions. Tyndallization according to Section 1 must be employed when neither of these appliances is available.

3. The agar must be poured while it is still hot. The petri dish lids must be opened only briefly and only enough to pour the agar. The agar must completely cover the bottom of the dish. Close the plate immediately. (Fig. 6.1.)

4. Placed on the top of the previously filled dish. The next plate is placed and filled in the same manner. This procedure is continued until stacks of ten dishes each are produced. The plates must not be moved until the agar has completely congealed.

5. The plates are incubated for two to three days at 30° C.

6. Plates that prove to be sterile are inoculated. The following methods may be employed:

**6.1. Fractionated Inoculation Streak**

The tip of an inoculating loop that been passed through a flame is dipped into the bacterial culture and then drawn across the dish according to the figure. The inoculating loop is resterilized in the flame between the first line (1) and the second zig-zag line (2). (Fig. 6.2)
6.2. Pipetting a Sample of a Suspension

A maximum of 0.1 ml of the test culture is applied to the dish, after which the suspension is spread as quickly and as evenly as possible across the entire surface of the dish. The agar is allowed to absorb the liquid and then the dish may be turned over.
6.3. Preparing Cultures by Replication

The surface of the agar is rubbed lightly across the surface to be tested for microbiotic contamination. Samples should not be taken from facilities intended for human hygienic use (toilets, drains, etc.) due to the danger of enrichment of pathogenic micro-organisms. The petri dishes must be sealed with tape after inoculation to prevent their unintentional re-opening after incubation.

Know-Why

3. There is always some risk that micro-organisms in the air e.g., Micrococcus luteus) or their spores might settle on the agar during the pouring process and cause the development of undesired colonies. Working between two burners is a useful preventive measure. (See Section 1.).

4. Condensed water forms on the inside of the lid if the petri dish with agar is not allowed to stand alone. This development is detrimental as it obstructs the observation of the colonies. Furthermore, water from the lid that drops onto sugar can cause an undesirable mixing of the colonies by washing away individual bacteria. If the dishes are stacked one on top of the other, however, each lid is warmed by the dish on top resulting in condensation in the top dish only.

5. This seems to test whether the plates are free of contamination.

6.1. By this zig-zag motion the streak is extended and the amount of bacteria deposited on the agar gradually decreases. Intervals between this bacteria are obtained large enough so that individual colonies can grow independently of one another. For inoculating purposes it is often desirable to employ a single colony. The individuals of a colony are the descendants of a single cell and for this reason, except for occasional mutations, genetically identical.
Resterilizing the needle after the first application line helps to reduce the number of bacteria distributed by the second application. The bacteria distributed by the second check are those taken up when crossing the first streak. (Fig. 6.3.)

Fig. 6.3. Cultures on an inoculated nutrient agar dish after incubation

6.2. Working quickly necessary to prevent the solution from being absorbed 12.- the agar before it has been evenly spread
across the entire surface. Swift procedure is especially necessary with older plates that have lost moisture due to evaporation. If too much solution is added, then it takes longer for it to be absorbed by the agar. Spores diffuse into the superfluous liquid so that too many colonies result.

6.3. Opening cultures obtained by replication is always risky. One can never know beforehand what micro-organisms have been collected. Therefore cultures of this kind should always be handled as if they contain pathogenic micro-organisms. For this reason samples must never be taken from facilities intended for human hygienic use. Cultures obtained by simple replication methods must not be opened after incubation.

Problems and Questions

1. Estimate how many bacteria are contained in a colony with a diameter of 3 mm and an average height of 0.5 mm assuming that a bacterium has a diameter of 1/1000 mm. (A few billion.)

2. Pure cultures of B. megaterium sometimes exhibit two kinds of colony forms. How can one experimentally prove that they are truly two colony forms of the identical sort, and not colonies of two non-identical types? (Prepare a new culture from each sample and compare their growth forms.)

7. Preparing a Dilution Series

7.1. Usual Laboratory Procedure

Dilution series are produced to reduce and/or determine the number of reproductive micro-organisms in a suspension. The determination of the number of viable cells through direct distribution of a sample on
an agar dish is usually not possible since the number of cells is so great that confluent growth (a lawn) is produced rather than individual colonies.

**Know-How**

**Equipment**

- 16 nutrient agar plates (according to section 6)
- 6 1.0 ml pipettes (sterile)
- 1 10.0 ml graduated pipette (sterile)
- 1 1.0 ml pipette (sterile)
- 6 test tubes (sterile)
  - 1 test tube rack
- 1 Drigalski spatula
- 7 1 400 ml beaker
- 8 2 Bunsen burners
- 9 1 waterproof felt tip marker

**Materials**

- sterile tap water
- approximately 50 ml 94 % ethanol (methylated)
- overnight cultures, e. g., *B. megaterium* or baker’s yeast

**Time Requirements**

90 minutes
Procedure

1. Six test tubes are labelled as follows:
   \(10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}\)

2. The bottom of the lower half of a series of plates is labelled as follows:
   \(K, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}\)
   Two such series are prepared.

3. The beaker is filled with roughly 50 ml ethanol and the Bunsen burners are lit.

   The following steps are conducted between the two burners. DANGER: Ethanol is highly flammable. If it ignites, it can be extinguished by covering the container with a glass plate.

4. From the test culture 0.5 ml are pipetted into the test tube labelled “\(10^{-1}\)” and 4.5 ml sterile tap water is added. Thorough mixing should be undertaken by shaking and rolling the test tube back and forth between the palms of the hands. Some micro-organisms do not survive when water is used for dilution. In this case a \(10^{-3}\) M solution of magnesiumsulfate is employed instead of water.

5. An aliquot of 0.5 ml suspension is removed from the test tube labelled “\(10^{-1}\)” and transferred to the test tube labelled “\(10^{-2}\)”\), to which 4.5 ml sterile tap water is added. The contents are carefully mixed again.
Fig. 7.1. Pipette procedure for the production of a dilution series
6. Proceed in the same manner until all six test tubes are filled, each with a sample of 0.5 ml from the previous tube and 4.5 ml sterile tap water. A fresh pipette is used for each step. (Fig. 7.1.)

7. The Drigalski spatula is now sterilized by heating it in a flame. The spatula is removed from the ethanol filled beaker in which it was placed at the beginning of the experiment and allowed to drip thoroughly. Remaining alcohol is completely burned off by rotating the spatula in the flame. Be careful to prevent burning alcohol from dripping. Hold the spatula downwards to prevent burning alcohol from trickling down the handle and burning your fingers. As a control 0.1 ml sterile water is applied to the plate labelled «K» and spread evenly over the surface of the dish with the sterilized spatula.

Both movements in one sweep

Fig. 7.2. Proper use of Drigalski spatula
8. The two plates labeled “10⁻⁶” are inoculated with suspension from the test tubes labeled “10⁻⁶”. The remaining dishes are inoculated in the same manner in the order of increasing concentration. The dish labelled “1” is inoculated last with undiluted test culture.

9. The plates are incubated for one to two days at 30°C. If a significant number of colonies are then apparent in the dishes labeled “K”, then the water used for dilution was not sterile and the experiment results are invalid.

10. The colonies are counted as soon as they can be seen. The dishes that contain 50 - 200 colonies are selected for counting. An average is calculated from the results of each of the two equivalent plates.

11. The number of colonies is divided by the dilution factor. This sum, multiplied by 10, yields the number of cells capable of reproducing in 1 ml of the test culture. Example: On the plates labeled “10⁻⁴”, 84 and 98 colonies were counted. (Fig. 7.3.)

   - Determine the average: \( x = \frac{84 + 98}{2} = 91 \) cells per 0.1 ml suspension applied
   - Multiply by 10: \( x = 91 \times 10 = 910 \) cells per 0.1 ml suspension
   - Divide by the dilution factor \( c = \frac{910}{10^{-4}} = 9.1 \times 10^{-6} \) cells per ml suspension
   - The test culture contains 9,100,000 cells per ml.

**Know-Why**

1., 2. Inadequate labelling is a common cause of mistakes, especially when working with young students. Therefore teachers must always insist on careful and adequate labelling. Simplified symbolization e. g., A, B, C, ..., should be avoided. This method requires a deciphering key, the production and
Fig. 7.3. Calculation of the viable cell titer in a liquid culture

- Average
  \( x = 91 \) Colonies

Multiply by 10: \( x = 910 \) cells/1.0 ml suspension
Divide by the dilution factor: \( c = 910 \) cells/10\(^{-4}\) ml
\( = 9.1 \times 10^6 \) cells/ml
The test culture contains 9,100,000 cells per ml
use of which can also lead to careless mistakes. Labels should always be written on the underside of the plate since lids can be exchanged by mistake.

3. Since the risks of contamination during this phase are immense, conducting the procedures of pipetting and inoculating between two burners is strongly recommended. (Compare Chapter 1).

4. As with yeast, bacteria are also transferred in conglomerates. They must be separated from each other since they will otherwise not disperse evenly in the suspension. The mixing may well be the main cause of error in this experiment as the importance of the mixing process and the separation of the lumps and their inherent practical difficulties are often underestimated by pupils. It is advisable to practice the mixing process beforehand with 0.5 ml stain and 45 ml water.

7., 8. A single 0.1 ml pipette is sufficient if the dishes are inoculated in the order of increasing concentration. Possible contamination by residence that remain in the pipette can be avoided by using a fresh pipette for each application when enough pipettes are available.

10. If colonies less than 50 are counted, the effects of random variation are too great. When there are more than 200 colonies counting is complicated since numerous colonies tend to grow into one another.

Problems and Questions

1. What is the difference between the procedure for determining the number of viable cells in a suspension described here and the optical density measurement described previously? (Only the cells capable of reproduction are counted in the suspension dilution process while all cells including dead cells are counted by optical density measurements.)
2. What kind of error can be expected when the number of micro-organisms in soil is to be determined but the type of micro-organism is unknown?
(One must keep in mind that not all types of micro-organisms reproduce equally well on a standard nutrient agar).

3. Why is the determination of micro-organism titers in drainage water especially dangerous?
(Sewer water most likely contains pathogenic micro-organisms.)

4. How many yeast cells does 1 g of baker’s yeast contain?
(One can expect many billions of cells.)

7.2 Preparing a Dilution Series

The methods described above correspond to standard microbiological laboratory procedure. They should be employed when the main goal of the course is to prepare students for careers or college studies by teaching them the safe and proper use of glassware. If, however, the primary goal of the course is to obtain experimental results, and concessions can be made with regard to accuracy, then this experiment can be simplified in a time-saving manner that greatly decreases the amount of time and glassware required. Furthermore, the problems involved in the mixing procedure are reduced considerably since only 10 drops of liquid per experiment that is less than 1 ml are employed in this process. A single dilution series per student or group, without a parallel experiment, is conducted in order to keep time requirements to a minimum. The result of the various groups are tallied together for the final results. The quality of the final result depends on the number of single results that are tallied together.
Know How

Equipment

1. 8 nutrient agar plates (according to section 6)
2. eye dropper (sterile)
3. 1 10 ml graduated cylinder ;
4. 6 test tubes (sterile)
5. 1 400 ml beaker
6. Bunsen burners
7. 1 waterproof felt tip marking pen

Materials

sterile tap water
approximately 50 ml ethanol (methylated)
overnight culture, e. g., B. megaterium or baker’s yeast

Time Requirements

1 - 8: 45 minutes

Procedure

1. Six sterile test tubes are labelled in series as follows:
   \(10^{-1}, \ 10^{-2}, \ 10^{-3}, \ 10^{-4}, \ 10^{-5}, \ 10^{-6}\).
2. The undersides of the agar dishes are labelled in series as follows:
   \[ K\ 1\ 10^{-1}\ 10^{-2}\ 10^{-3}\ 10^{-4}\ 10^{-5}\ 10^{-6} \]

3. The beaker is filled with roughly 50 ml ethanol and the Bunsen burners are lit.
The following steps are conducted between the two burners. DANGER: Ethanol is highly flammable. If it ignites, it can be extinguished by covering the container with a glass plate.

4. Nine drops of water are pipetted into each test tube.

5. The Drigalski spatula is now sterilized by flame heating. The spatula is removed from the ethanol filled beaker in which it was placed at the beginning of the experiment and allowed to drip thoroughly. Remaining alcohol is completely burned off by rotating the spatula in the flame. Be careful to prevent burning alcohol from dripping. Hold the spatula with downwards to avoid the danger of burned fingers from burning alcohol trickling down the handle. A drop of sterile water is applied the plate labeled «K» and spread evenly over the surface of the dish with the sterilized spatula.

6. One drop to the test tube labeled "10^{-1}" to Of the overnight culture is added. Thorough mixing should be undertaken by shaking and by rolling the test tube back and forth between the palm of the hands. One drop of overnight culture is then applied to the plate labelled "1" and spread evenly with the resterilized spatula (according to step # 5). Finally, the pipette is rinsed with sterile water.

7. One drop from the test tube labeled "10^{-1}". is transferred to the test tube labelled "10^{-2}" and carefully mixed again.
   Another drop of the 10^{-1} suspension is distributed evenly on the petri dish labeled "10^{-1}".
8. Proceed in the same manner until all 6 test tubes are filled, each with one drop of suspension from the previous tube and 9 drops of water and until all 8 petri dishes are inoculated according to their respective labels.

9. The dishes are then incubated for one to two days at 30° C.

10. The colonies are counted as soon as they can be easily recognised. Plates with 50 - 200 colonies are counted completely.

11. In order to determine the titer of the test culture one must determine the volume of one drop. For this purpose a small graduated cylinder is filled with drops (n) until a volume of 2 ml is reached. The volume (V) of a drop is derived according to the following formula:

\[
V = \frac{2}{n} \text{ ml.}
\]

Example: 32 drops are necessary to attain a volume of 2 ml. The volume of a drop is therefore:

\[
V = \frac{2}{32} \text{ ml} = 0.06 \text{ ml.}
\]

12. It a set of identical eyedroppers is available, it is sufficient to allow a student who works quickly to determine the drop volume in order to save time. The other groups can use these results. The number of the counted colonies is divided by the dilution factor. The results indicate the number of viable cells in one drop of the test culture. When this number is subsequently divided by the drop volume (V), the number of viable cells in 1 ml of the test culture is obtained. Example: 186 colonies were counted on the plate labelled $10^{-4}$. The volume of a drop is 0.06 ml. The number of colonies is divided by the dilution factor:
\[ N = \frac{186 \text{ cells}}{10^{-4}} \]

- Divide by the volume (V) of drops:

\[ N = \frac{1.86 \times 10^{-6} \text{ cells}}{0.06 \text{ ml}} = 3.1 \times 10^{-7} \text{ cells per ml} \]

- The test culture contains 31,000,000 cells per ml.

**Know-Why**

Notes for this experiment are similar to those of the previous experiment. Remarks, specific to this experiment only, are noted here.

5. It is important to begin with the control sample since the sterile water is subsequently needed for rinsing and consequently becomes contaminated.

11. This procedure is possible since the volume of a drop in a pipette remains on the whole constant as long as the viscosity of the liquid being used remains constant.

**8. Making Fixed Slide Preparations**

Bacteria are almost always observed with fixed preparations with the exception of a few unusual cases since it is difficult to focus sharply on living bacteria. Furthermore, fixed bacteria can be easily stained.
**Know-How**

**Equipment**

1. 1 microscope slide
2. 1 cover slip
3. 1 eye dropper
4. 1 Bunsen burner

**Materials**

ethanol

**Time Requirements**

30 minutes

**Procedure**

1. Carefully remove grease from a microscope slide with a lint free towel or piece of tissue soaked in ethanol.

2. Place a drop of bacteria or yeast suspension in the middle of the microscope slide. The drop should flow out evenly and must not remain in a globular form. (Fig. 8.1.)
3. Place a cover slip at a 45° angle on the microscope slide in such a manner that the solution is collected in the space between the slide and slip and held by the properties of adhesion and cohesion. (Fig. 8.2.).

Fig. 8.1. Spreading a drop of liquid on a lubricated and on a non-lubricated microscope slide

Fig. 8.2. Placing a cover slide on a microscope slide
4. Push the cover slip evenly across the entire surface of the microscope slide. By this means the suspension is spread across the slide and the film of liquid becomes thinner.

5. Allow the smear prepared in this manner to air dry.

6. Fix the bacteria to the slide by briefly heating the slide in a flame. This step can be done in one of two ways:
   a. with a low flame such as the pilot flame of a Bunsen burner and the coated side of the slide oriented downwards, or
   b. with the high flame of a Bunsen burner with the coated side of the slide up. The slide is passed through the flame three times at a speed of roughly 30 cm per second.

**Know-Why**

1., 2. The suspension will eventually flow back together even when only traces of grease are present on the slide. When this occurs, it not only lengthens the drying time, but allows thick layers of bacteria to develop as well. Consequently it may no longer be possible to observe individual microorganisms. NOTE: If attempts to remove grease from the slide are unsuccessful, then a drop of extremely diluted bacteria suspension, allowed to air dry, can be used in the
place of the smear technique. For younger students this method is easier than executing a smear with the delicate cover slip. (Fig. 8.1.)

4. It is important to pull and not push the suspension across the slide with the cover slip in order to ensure that the thickness of the coating decreases evenly.

5. This drying step must absolutely not be accelerated by heating. The bacteria’s structure alters when heated in water.

6. The first method is recommended. Due to the poor heating qualities of glass, the effect of heating on the bacteria is difficult to estimate with the second method. The protein coagulated in the cells by heating and organisms adhere to the slide surface.

9. Staining Technique

9.1. Making an India Ink Preparation

In the first process explained here the organisms themselves are not stained here, but rather the background which is uniformly stained black through the use of India ink. This staining produces the effect that only the organisms are illuminated in the microscope and appear in bright contrast to the background. This process is especially suited for beginners. It leads to clearly recognizable results and allows organisms and dust particles to be easy distinguished. Simple school microscopes are satisfactory for the observation of these preparations.
**Know-How**

**Equipment**

1. 2 microscope slides
2. 1 cover slip
3. 1 eye dropper

**Materials**

India ink
etheral

**Time Requirements**

30 minutes

**Procedure**

1. Two microscope slides are thoroughly cleaned by wiping them with a lint free towel or tissue soaked in ethanol.

2. A small drop of water is placed on the microscope slides. The drop must spread out otherwise further measures are necessary to remove grease from the slide.

3. A drop of India ink is mixed with the evenly spread drop of water with a glass rod.
Fig. 9.1. Spreading a drop of liquid on a lubricated and on a non-lubricated microscope slide

4. Place a cover slip at a 45° angle on the microscope slide in such a manner that the solution is collected in the space between the slide and slip and held by the properties of adhesion and cohesion. (Fig. 9.2.).

Fig. 9.2. Placing a cover slide on a microscope slide

5. Push the cover slip evenly across the entire surface of the microscope slide. The suspension is thus spread across the slide. The thickness of the film of liquid decreases (Fig. 9.3.).

6. Allow the smear prepared in this manner to air dry.
A second smear is made using a drop of bacteria or yeast suspension instead of a drop of water.

Both smears are then compared under a microscope set at 400 x magnification. The condenser is opened all the way and the brightest possible light source is selected.

Know-Why

1. - 7. Remarks for these steps correspond to those of Section 8. It is recommended to refer to that section where necessary.

8. Students who observe bacteria with a microscope for the first time should definitely prepare a control slide for comparison since beginners often have difficulty interpreting microscopic images.

9.2. Staining with Methylene blue

Methylene blue is employed in the biological and medical fields to stain cell nuclei for histological purposes; to stain ganglion cells according to Nissl: for vital stainings of nerves; as an ingredient in Giemsa-staining and for staining bacteria in general. Methylene blue is an ionic compound which accounts for its solubility in water.
Methylene blue will be used in this experiment to stain bacteria in a smear preparation. This staining is generally necessary to enhance the contrast of the image when light microscopes without phase contrast are used. This process is especially necessary when normal secondary school microscopes are used, since contrast with such instruments is otherwise not sufficient. (Fig. 9.4.).

![Poor contrast without staining](image1) ![Enhanced contrast through stain](image2)

**Fig. 9.4. Increased contrast as a result of staining**

**Know-How**

<table>
<thead>
<tr>
<th>Equipment</th>
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1. microscope slide with heat fixed smear prepared according to section 8
Materials

0.2 - 1.0 % methylene blue solution

Time Requirements

30 minutes

Procedure

1. The smear is generously coated with methylene blue solution and allowed to soak for eight minutes. (Fig. 9.5.).

   Fig. 9.5. Preparing microscope slides with methylene blue stain. Placing the slides on glass rods over a Petri dish is not necessary, it helps prevent possible spotting from stray drops of stain.

2. The stain is then carefully rinsed off under dripping water until clear drops run off. Possible dried stain on edges should not be removed completely.
3. The preparation is now allowed to air dry. If the preparation is to be investigated under the microscope right away, a cover slip must be set properly in place.

4. The finished preparation can be observed in the microscope at 400 x magnification.

**Know-Why**

Concerning Materials: The concentration of the methylene blue solution must not be exact. High concentration can be used since methylene blue does not tend to stain too intensely. The given soaking time (No. 1) is valid for following composition of the solution:

- 0.3 mg methylene blue
- 30.0 ml ethanol
- 100.0 ml destined or demineralized water

**9.3. Gram Staining**

Bacteria of many different types can be stained by the solution described by the Danish doctor Hans Christian GRAM in 1884. Bacteria which react to Gram’s staining technique are called «gram positive» and bacteria that do not react are called «gram negative». *B. megaterium*, *B. subtilis* and *Micrococcus luteus* are good examples for gram positive bacteria and *Escherichia coli* (E. coli) is a good example for a gram negative bacterium. The technique is based on the varying solubility of an iodine stain complex in the walls of bacteria cells. The decisive structural component of the cell wall which determines whether or not a cell can be stained is peptidoglycane (Murein). Peptidoglycane is a macromolecular compound made up of monosaccharides containing nitrogen and amino acids. This compound has the same function in the bacterial cell wall as cellulose in the plant cell wall. Crystal violet is a suitable stain for cell walls. This readily water-soluble stain can easily permeate the wall of a bacteria cell. The term «gentian violet» is used in many manuals. In more recent literature this term appears as synonymous to crystal violet.
Older literature often includes descriptions of assorted mixtures of methylated compounds which contain penta- and tetramethyl compounds along with hexamethyl compounds. Since the composition of gentian violet varies among manufacturers, its suitability for Gram staining is noted in their catalogues. If such a product is not available, it is recommended to use crystal violet.

The staining procedure is divided into the following steps:

1. **Staining with crystal violet**

The cell wall of all bacteria, gram negative ones as well, are dyed violet in this step.

![After treatment with crystal violet: All cells violet]

2. **Treatment with iodine**

Iodine forms a bond with the stain in the cell walls and develops a crystal violet iodine complex that is not easily soluble. Cell walls remain stained violet.

![After treatment with iodine: All cells violet]
3. Differentiation with ethanol

When ethanol is applied, it washes the crystal violet iodine complex out of the cell walls of gram negative bacteria. In the microscope, the gram positive bacteria appear violet while the gram bacteria appear colourless.

4. Counterstaining with Phenol red

Gram negative bacteria are made visible again by means of a red stain. **NOTE:** Rather high demands are made on the students’ ability for visual discrimination. They should have had prior experience with other staining techniques. Teachers should provide an assortment of prepared slides for comparison.
Know-How

Equipment

1. 1 microscope slide with heat fixed smear prepared (according to section 8)
2. 1 100 ml beaker

Materials

- solution of gentian or crystal-violet
- iodine solution
- safranin or carbol-fuchsin solution
- ethanol 96% (phenol, iodine and potassium iodide for preparing solutions)

Time Requirements

60 minutes

Procedure

1. Preparation of the stain

NOTE: Brown bottles and sterile water should always be used for stain solutions.

a. **Gentian or crystal violet solution**.

   Basic solution:
   10 g of gentian violet or crystal violet are combined with ethanol and vigorously mixed or stirred. The mixture should be shaken often over the course of a few days and afterwards the residue should be removed by filtration.
Staining solution:

5 ml of the basic solution combined with 95 ml phenol solution (2.5 % in water).
DANGER: Phenol is poisonous and dangerous to the skin!

b. Iodine solution:

1 g iodine and 2 g potassium iodide are dissolved in 300 ml water. Iodine solutions should always be prepared fresh since these solutions can be maintained only for short times,- even in brown bottles.

c. Safranin solution:

0.25 g safranin are combined with 10 ml ethanol to which 100 ml water are then added.

d. Phenol red solution:

Basic solution:

5 g fuchsin are combined with 100 ml ethanol and vigorously shaken or stirred. The mixture should be shaken often over the course of a few days and afterwards the residue should be removed by filtration.

Staining solution:

10 ml of the basic solution are combined with 90 ml phenol solution (5 % in water).

2. The microscope slides with heat fixed smears are generously coated with gentian or crystal violet solution and allowed to soak for two minutes.

3. The stain (#2) is poured off laterally and iodine solution is applied without intermittent rinsing with water immediately.
4. After one minute the iodine solution is poured off and a new coating of the iodine solution is immediately applied again without rinsing in between. The slide is allowed to soak two minutes and then the solution is poured off. The time allowed for soaking should be extended if this procedure results in insufficient staining.

5. Excess stain is removed by lightly shaking the slide until the runoff is clear (a few seconds) in a beaker filled with alcohol.

6. Residue ethanol is rinsed from the slide with a small amount of safranin or phenol red solution. Afterwards the slide is coated with safranin for 30 seconds or with phenol red solution for 60 seconds.

7. Finally rinse the slides with tap water and allow to air dry.

8. Microscopic investigation must be performed at least 400x magnification. Gram positive bacteria appear dark violet, and gram negative bacteria appear red.

Know-Why

1. Brown bottles are used since solutions decompose in light.

2. The microscope slide must be completely and generously coated with stain. If only a few drops are used, then the solution dries from the edge inwards and forms crystals of dye that make observations difficult.

3. With the first iodine application, superfluous crystal violet solution is washed away while at the same time a very less soluble iodine stain complex begins to be formed.

4. The soluble violet stain that is present in the cell walls is transformed into the barely soluble iodine stain complex through application of the iodine solution. All bacteria are dyed violet.
5. Ethanol, an organic solvent, can dissolve the iodine stain complex out of the walls of certain bacteria. The thicker the bacteria wall and the greater its peptidoglycane content, then the more difficult it is to dissolve the iodine stain complex with an organic solvent. Therefore, only gram positive bacteria remain stained.

6. An additional stain, that can easily be distinguished from violet, is employed to render the gram negative bacteria visible again. While this additional dye stains all bacteria present, it cannot surpass the sharp violet of the original procedure. Therefore the red gram negative and the violet gram positive bacteria remain easily distinguishable.

10. Determining the Titer of a Bacterial Suspension

The titer of micro-organisms in a liquid culture can be determined in three ways:

a. directly, by counting the individual cells with the help of a microscope.

b. indirectly, by plating dilutions of the suspension on nutrient agar counting colonies after incubation and then calculating the titer of the original suspension from the volume applied to the plate and the dilution factor.

c. through measurements of optical density.

10.1. Determining Titers with a Microscope and Counting Chamber

Samples of the culture to be investigated are placed on microscope slides, fixed, stained and counted. This procedure does not permit differentiation between living and dead cells. Furthermore foreign organisms in the suspension may be confused with the bacteria.

Professional laboratories use counting chambers. Counting chambers are microscope slides in which fine grid lines have been etched.
Since counting chambers are seldom used in the classroom, among other things due to their expense, a process is presented here which can be conducted with the means usually available to a school. A drop of a bacteria suspension is placed on a clean microscope slide. The total area of the dried sample is then determined. All the bacteria in the section that can be seen under the microscope are counted. If the area of this section is determined and its size relative to the total sample area is calculated, then the total number of bacteria in the sample can be determined. The number of bacteria present in this sample is thus equal to the number of bacteria in one drop. The number of bacteria in 1 ml of the suspension in question still remains to be determined. First the number of drops per millilitre suspension are obtained by counting. This number of bacteria per drop is multiplied by this factor to give the total number of bacteria in one millilitre.

**Know-How**

**Equipment**

1. - microscope slides
2. - cover slips
3. 1 eye dropper
4. 1 10 ml graduated cylinder
5. 1 ruler (metric measurement)
6. - equipment for heat fixing and staining with methylene blue according to sections 8 and 9
**Materials**

objects with fine and regular textures, e.g., feathers, blades of grass, etc. millimeter paper (or graph paper) with 5 mm units

**Time Requirements**

1.1.: 10 minutes  
1.2.: 60 minutes

**Procedure**

1. Determine the area of the illuminated field on the microscope slide. This value depends upon the power of magnification selected. A ruler can be used to assist in this determination up to a power of about 100 x. Objects with fine structure elements are necessary to serve as a standard of measurement for magnification in greater powers. (e.g. a feather.)

![Ruler under microscope](image)

Fig. 10.1. Picture of a ruler under a microscope. The distance between two marks is equivalent to 1 mm.
1.1. Determining the area with a ruler

Instead of a slide a ruler is placed under the microscope and the diameter (d) can be read directly. The view seen in the microscope may appear as follows (Fig. 10.1.).

From this picture it can be concluded that, with the objective/ eyepiece combination employed, the diameter (d) of the illuminated field is approximately 1.7 mm. Therefore the area (A) of this field is:

\[ A = \pi \times \frac{d^2}{4} \]

The approximate area of the illuminated field in our example is therefore:

\[ A = 3,14 \times \frac{1,7^2}{4} \text{ mm}^2 \]

\[ = 2,3 \text{ mm}^2 \]

1.2 Determining area with a feather.

The method described in 1.1. cannot be used when magnification powers greater than 100 x are employed, e. g. 400 x. At the greater magnification two subsequent marks on the ruler cannot be seen together since the diametre of the section observed is less than 1 mm. This situation calls for the use of a finer and regulary graduated object to serve as a standard of measurement, for example, a feather.

1. A section of a fine and regulary structured feather is placed on a microscope slide and covered with a slip.

2. The length (l) of the feather section which exhibits a regular structure is determined with a ruler. (Fig. 10.2.).

In this example, the value of the length (l) is 6 mm.
3. The number \( n \) of repetitive structural elements present in the section with the length \( l \), are counted under the microscope at low magnification. The unit of length is the distance between such repetitive structures, in this case two branches of the feather. In our example the number of elements in a section of length \( l \) is 15. \( (n = 15) \).

4. The unit \( u \) should then be used as the unit of length when using the microscope at magnifications powers greater than 100 x. The length of such a unit can be calculated as follows:
\[
u = \frac{1}{n}
\]
In our example, the length of the standard unit is:

\[
u = \frac{6 \text{ mm}}{15} = 0.4 \text{ mm}
\]

5. Now the diameter of the illuminated field is determined. The feather is observed with the microscope at the same magnification as that with which the bacteria are to be counted. This step is taken in order to determine how many basic units of length appear in the illuminated field. The length of a unit not completely included in illuminated field can be estimated. The number of basic units obtained by this means is referred to as the factor \(x\).

Fig. 10.4. The length of a distance (d) in a microscope picture
(Enlarged view of the section of feather shown in 10.3.)

From this microscope picture (Fig. 10.4.) the following factor is obtained:

\[x = 1.1\]

The diameter (d) of the illuminated field is the product of the factor \(x\) and the length of basic unit (s):

\[d = x \cdot u\]

In our example:

\[d = 1.1 \times 0.4 \text{ mm} = 0.44 \text{ mm}\]
6. The area \((A)\) of the section in the illuminated field can be calculated according to paragraph 1.1 with this value. In this example the value of the area is:

\[
A = 3.14 \times \frac{(0.44)^2}{2} \text{ mm}^2
\]

\[
= 16 \text{ mm}^2.
\]

7. The next step is to determine the volume \((V)\) of a drop from a pipette. The number of drops \((D)\) from a given pipette required to fill a graduated beaker to the 1 ml mark are counted. The volume \((V)\) of a drop \((D)\) corresponds to

\[
V = \frac{1 \text{ ml}}{D}.
\]

If, for example, it takes 22 drops to obtain a volume of 1 ml, then the volume of a drop is:

\[
V = \frac{1 \text{ ml}}{22}.
\]

\[
= 0.045 \text{ ml}.
\]

8. A drop of bacteria suspension is placed on a microscope slide with the pipette calibrated according to step No. 7. The drop is spread carefully by tilting the slide back and forth, allowed to air dry and fixed according to Section 8.

9. The area \((AD)\) of the drop is determined by placing the slide on graph paper and counting the number of squares covered by the dried preparation. (Fig. 10.5.)
The approximate area in our example is: \( A_D = 1.102 \text{ mm}^2 \).

10. After determining the area the preparation is stained with methylene blue. (see Sec. 7.)

11. When the stained preparation has dried, all the bacteria for ten different sections of the preparation in the illuminated field are counted. These ten values are used to determine the mean value \( m \).

12. The titer \( C \) of the test culture can be calculated. The number \( b \) of bacteria in one drop of the solution can be derived from the following calculation:

\[
\frac{m \times A_D}{A} = b.
\]

When the value for \( b \) is divided by the volume \( V \) determined with the calibrated pipette, the number \( K \) of bacteria in 1 ml of solution can be obtained:

\[
K = \frac{b}{V}
\]

13. Example: The following results were obtained from counting the bacteria in various sections of preparation:

- 78, 93, 112, 86, 122, 83, 101, 90 and 104 bacteria.
- The area of the preparation was determined as 1,102 mm\(^2\).

A section with an area of 0.16 mm\(^2\) can be observed at 400x magnification. Therefore:

Mean: \( m = 96.3 \) bacteria

Area of preparation \( A_D \): \( a = 1012.0 \text{ mm}^2 \)

Volume of a drop: \( V = 0.045 \text{ ml} \)

Area of section: \( A = 0.15 \text{ mm}^2 \)

The number of bacteria in a drop of the test culture is therefore:

\[
b = \frac{96.3 \text{ bacteria} \times 1012 \text{ mm}^2}{0.15 \text{ mm}^2}
\]
The number of bacteria in 1 ml of the suspension is derived as follows:

\[
K = \frac{649704 \text{ bacteria}}{0.045 \text{ ml}}
\]

\[
= 1.4 \times 10^7 \frac{\text{bacteria}}{\text{ml}}
\]

**Know-Why**

1. In planning this chapter it was assumed that a micrometer would not be available to the class. This method has the advantage that the students can work with objects from their environment.

13. The calculation of the number of bacteria in a drop suggests a precision in measurement which is by no means guaranteed in this experiment. However, since the value obtained can be considered an intermediate value, there is no need to round it off.

**10.2. Determining the number of viable cells by plating procedures.**

The method explained in Section 7 can be used to determine changes in the titer of a culture. The titer is determined in regular intervals. As opposed to method a), only viable cells are considered and the absolute number of these cells is determined. However, this method does not allow continual observation of the culture. (Sec. 10.3). The titer should be determined every thirty minutes for six hours when B. megaterium, B. subtilis or yeast is used for this experiment. The amount of equipment and high degree of organisation required for this procedure are serious obstacles so that this procedure is probably only suitable for science clubs or other extramural groups.
10.3. Determining Titers by Measuring Optical Density

The absolute number of bacteria in a culture can be determined by measuring optical density. However, this requires calibration methods that are extremely time consuming and these are not appropriate for use in the secondary school. Optical density measurements are useful for following the growth of a culture of cells. The method explained here entails the use of an easily constructed home-made photometer. (See Sec. 13 for assembly instructions.)

The beaker in which the bacteria grow is directly illuminated by light. If a commercial photometer is available, then measurements are made in the range of 600 to 650 mm with a cuvette that is filled with a sample of the culture in the beaker.

For measurement a bundled ray of light is directed through the bacterial suspension. The light is diffracted on the surface of the cells. This diffraction increases with the number of bacteria in the suspension and thus less light enters the photometer. The relation between apparent absorption and the titer of bacterial suspension is almost linear when the titer is not too high.

The light intensity can be read directly from commercial photometers. When using a home-made photometer the amperemeter must be used to determine current strength (I) as described in section 13.

Extinction is a term used in connection with solutions which absorb light. Here, however, diffraction, not absorption, is the decisive event.

The results that can be expected depend upon how long the experiment is conducted. (Fig 10.6.)

Since there is not an exact linear relationship between the intensity of light and the intensity of the current, the curve represents only an approximation of the changes in titer that occurs (compare section 13). The photometer must be employed in conjunction with titration by plating procedures to obtain exact data about the titer. However, this process is not necessary for normal secondary school use.
Phases I - IV in figure 10.6. correspond to the following physiological states:

I. Lag-phase
   In the first phase, micro-organisms from an overnight culture that is depleted of nutrients but rich in metabolic waste products must adjust to the rich nutrient supply of the fresh growth medium. In the beginning only very few cells are in a physiological state
conducive to cell division. During the lag phase more and more cells attain this state.

II. Log-phase
In this second phase, the maximal growth of the culture is attained and the titer increases exponentially.

III. Stationary phase
In this phase the growth rate and death rate are equivalent. The total number of living cells remains constant.

IV. Death phase
The number of viable cells decreases, possibly due to depletion of nutrients or toxic metabolic waste products.
**Know-How**

### Equipment

1. 1 light source that emits bundled ray e. g., slide projector
2. 1 400 ml beaker
3. 1 stand with clamp for beaker
4. 1 thermometer
5. 1 eye dropper
6. 1 Bunsen burner
7. 1 Erlenmeyer flask to hold the eye dropper in ethanol

### Materials

- dried nutrient broth (commercial or home made nutrient broth according to appendix 13.3.)
- aluminium foil
- overnight culture of the bacteria to be examined, e. g., *B. megaterium*
- ethanol or yeast

### Time Requirements

- preparation of experiment: 45 minutes
- for each measurement in 30 minute intervals: 10 minutes
Procedure

1. An overnight culture is prepared according to Section 3 on the evening before the experiment. If yeast is to be used, then a fresh suspension is prepared with 1 g yeast and 100 ml water.

2. When setting up the photometer, remember to leave enough room under the beaker for the Bunsen burner. The optical axis must describe a straight line from the midpoint of the light source to the midpoint of the cell in the photometric box, and it must pass through the middle of the culture. The beaker must be neither shifted nor removed from its holder during this experiment.

3. Three hundred ml. of nutrient broth warmed to 35° C are poured into the beaker. The light source is turned on and the current adjusted to 3 mA on the amperemeter.

4. The overnight culture is added to the beaker until the amperemeter displays a current reading of 2.7 mA. The suspension must be carefully stirred during this process. Then the beaker is covered with aluminum foil and the potential is increased until the amperemeter reads 3 mA. The value 3 mA is the starting value for the following measurement series and the potential must not be altered for the rest of the experiment.

5. A measurement is recorded every 30 minutes. For this purpose the suspension is carefully stirred with a glass rod and the light source and power supply from the photometer are turned on.

6. The temperature of the suspension should also be registered at 30-minute intervals. This recording is best made immediately subsequent to step 5. If the thermometer reads 30° C or less, then the suspension is warmed while carefully being stirred until a temperature of 35° C is reached again. After both measurements have been successfully accomplished, the beaker is again covered with aluminium foil.
7. At the end of the experiment the values measured are recorded in a current vs. time graph.

Know-Why

The use of a beaker rather than a cuvette with parallel surfaces makes it difficult to obtain reproducible results.

2. The glass walls of the beaker are not flawless and therefore during the entire experiment the optical density of the glass is not uniform. The beaker should not be shifted or removed from its holder, for example, to put it in an incubator or on a magnetic stirrer. Nevertheless standard commercial cuvettes with a width of 1 cm are not recommended since the path of the light through the suspension is much too short for sufficient differentiation. The optimal situation calls for a cuvette of at least 5 cm width. However, these cuvettes are somewhat expensive. Furthermore, it is important to have an adequately high column of liquid for measuring in order to avoid the possibility of distorted measurements from the influence of reflection from either the surface of the liquid or the bottom of the glass.

3. The temperature of a full 400 ml beaker falls from 35 C to 25° C in less than 30 minutes at a room temperature of 20Q C. Therefore the average temperature during this period is 30o C.

4. In order to obtain predictable curves, it is wise not to always use the same volume of overnight culture for inoculating but rather to use the same number of bacteria, as determined by the increase in current. When using B. megaterium, the amount of inoculation listed allows the stationary phase to be reached in the course of a morning (Fig. 10.7.). The beaker is covered with aluminum foil between measurements to prevent contamination from airborne spores.
5. The resistance of the cell increases with extended illumination times and therefore the light source should be switched off between measurements.

11. Techniques for Demonstrating the Physiological Abilities of Micro-organisms

11.1. Carbohydrate Metabolism

Carbohydrates are an important source of energy for micro-organism. Carbohydrates are present in the environment in greatest abundance as dead vegetable matter, known biochemically as starch or cellulose. Roughly 30% of all carbohydrates found in micro-organism organic compounds are therefore present in the environment as cellulose. Through the degradation of carbohydrates, mono- and disaccharides are formed which are further metabolized.

11.1.1. Oxidation / Fermentation Test for Glucose Degradation

According to HUGH and LEIFSON

Monosaccharides are metabolized in different manners according to their oxygen content and the type of micro-organism.

a. In the presence of oxygen:
   - complete oxidation into carbon dioxide and water (respiration)
   - incomplete oxidation to organic acids e.g. acetic or citric acid with participation of oxygen from the air (aerobic fermentation).

b. In the absence of oxygen:
   - incomplete oxidation to organic acids, ethanol or other compounds in which oxygen from the air is not involved (fermentation).
The oxidation/fermentation test is used to determine in what manner unknown bacteria metabolize. This test plays an important role in identifying and classifying unknown micro-organisms. With the help of indicator plates this method tests whether or not acids are produced by the metabolism of sugar under aerobic and anaerobic conditions.

**Know-How**

**Equipment**

1. 2 test tubes per experiment and test tube caps
2. 1 inoculating loop
3. 1 funnel
4. 1 100 ml graduated cylinder
5. 1 Erlenmeyer flask for preparing nutrient broth (10 ml per experiment)

**Materials**

- Peptone made from casein
- Yeast extract
- Salt
- Dipotassiumhydrogenphosphate (K₂HPO₄)
- Glucose (or another monosaccharide or disaccharide)
- Agar
- Sodium hydroxide (c = 0.02 mol/l)
- Bromothymol blue
The substances listed above can be disregarded if a ready-made agar plates are purchased for the O/F test.

paraffin oil

pH paper (range 7 - 8)

cultures of the bacteria to be examined, e. g., \textit{B. megaterium}.

\begin{center}
\begin{tabular}{|l|}
\hline
\textbf{Time Requirements} \\
\hline
45 minutes (without prior preparation) \\
\hline
\end{tabular}
\end{center}

\textbf{Procedure}

Tip: Preparation steps I - 4 should be conducted a day ahead of time.

1. Preparing bromthymol blue solution:
   180 g bromthymol blue are dissolved in 100 ml sodiumhydroxide (c = 0.02 M).

2. Preparing nutrient indicator plates
   2.0 g casein, 1.0 g yeast extract, 5 g salt, 0.3 g dipotassium-hydrogenphosphate and 5.0 g glucose (or another monosaccharide) are dissolved in 1000 ml water, to which 15 g agar and 10 ml basic bromthymolblue solution are added. The pH value must be at 7.5.

3. The paraffin oil is sterilized (see Section 1); around 3 ml per experiment will be needed.

4. The nutrient agar is sterilized according to Section 1.

5. The test tubes are filled with 10 ml nutrient agar each by means of a previously sterilized funnel to keep the upper test tube sides free of agar. (Fig. 5.1.)

6. After the agar has congealed, the test tubes are inoculated with an inoculating needle. The needle should be inserted
until it reaches the test tube bottom. Two test tubes are needed for each bacterial species to be examined.

7. One of each of the test tubes is layered with around 2 cm of para-fin oil after inoculating.

8. The test tubes are incubated at 30° C until a visible change in colour occurs, at the longest for four days.

**Know-Why**

1. Bromthymol blue is an indicator dye. When dissolved in water it produces blue colour at pH value over 7.6 and yellow for values under 6.0. Variations in colour from yellow-green to green to blue-green occur between these two values. It is used because the critical pH value for change in colour is in the range of week acids, so that the production of acids by bacteria can be observed and furthermore the dye does not harm the bacteria in the concentration used.

2. It is important that the pH value be set at exactly 7.5 from the commencement of the experiment since even small amounts of acids produced by bacteria cause colour changes. Therefore the pH value must be checked with pH paper after the addition of the agar. Sodiumhydroxide must be added as necessary to adjust the value. Plates made with bouillon cubes are not suitable for this experiment. Such cells often contain sugar based dyes and consequently innumerable amounts and types of carbohydrates. In place of the glucose noted here, other mono- or disaccharides can be investigated for their degradation properties; e. g., lactose or saccharose.

   J. MOND’s experiment for lactose metabolism through E. coli remains very meaningful for bacterial genetics.

7. All possible extremal oxygen sources are sealed off by this measure.

8. In the presence of an unlimited oxygen supply aerobic bacteria degrades glucose to carbondioxide and water. Many
bacterial types develop small amounts of acids in addition, e. g. B. megaterium and B. subtilis. The type of acid produced depends on the bacterial type. Streptobacillus and Lactobacillus generate lactic acid. E. coli can produce carbon dioxide and water as well as lactic acid.

This experiment can produce the following results (Fig. 11.1.):

Fig. 11.1. Possible results of oxidation/fermentation tests (according to BIRKENBEIL, 1983)

a. The bacteria are aerobic; therefore only in $a_1$ can the bacteria reproduce, not in $a_2$. The species also produces acids under aerobic conditions.

b. This species possesses an anaerobic property as shown by $b_2$, that is, it produces acids in the absence of oxygen, and, as $b_1$ demonstrates, it can also produce acids in the presence of oxygen.
c. It can only be concluded from this result that no acids have been produced. It may well be possible that oxidative degradation has occurred in $c_1$, i.e. carbon dioxide and water have been generated. A pH neutral compound such as ethanol may have been produced in $c_2$. In the case of a complete oxidative degradation in $c_1$, an acid is indeed produced according to the reaction:

$$\text{CO}_2 + 2\text{H}_2\text{O} \rightarrow \text{H}_2\text{O} + \text{HCO}_3^-,$$

However, this acid is too weak to cause a visible change in the indicator.

11.1.2. Starch Degradation

Starches are metabolized by many kinds of bacteria and fungi. Each type possesses a specific enzyme for the metabolism of the macromolecule. L-glucose is always the final product of the starch degradation and can be further converted to carbon dioxide and water. The presence of starch can be demonstrated by means of Lugol’s solution or viscometry. Both processes are described in this section.

11.1.2.1. Proof of Starch Degradation with Indicator Solution (Lugol’s Solution)

**Know-How**

**Equipment**

1. 1 petri dish per experiment
2. 1 inoculating loop
**Materials**

- dried agar, glucose free (ready made)
- starch, soluble
- Lugol’s solution (or iodine and potassium iodide)
- cultures of the bacteria to be examined (Bacillus subtilis degrades starch particularly fast)

**Tip:** Peptone from casein, salt and agar-agar are necessary for homemade nutrient agar plates.

**Time Requirements**

1. 1 - 2: 45 minutes
2. 5: 15 minutes

1. 15 ml nutrient agar are prepared per experiment according to Section 6 and 0.2 % starch is added.

2. After congealing, the agar is inoculated by crosswise streaking with the inoculation loop.

3. The inoculated plates are incubated for 2 - 3 days at 30° C. Step 4 must be accomplished in the meantime.

---

**Fig. 11.2. Streaking on starch-agar**
4. Preparation of Lugol’s solution.
Dissolve 1 g iodine and 2 g potassium iodide in 300 ml water.
Since the concentration must not be exact, it is sufficient to dis-
solve a small amount of iodine (the tip of spatula) and three such
aliquots of potassium iodide in 100 ml water.
DANGER: Iodine is poisonous!

5. Lugol’s solution is poured carefully into the incubated dishes
until the entire surface is covered to a depth of about 1 mm. Spots
where starch is still present are tinted blue-black.

Know-Why

1. Only commercial agar plates may be used in which the manufac-
turer has not used glucose or other mono- or disaccharides becau-
se in the presence of sugar many bacteria do not metabolize
starches. For this reason, the use of broth cubes according to sec-
tion 13 cannot be recommended since bouillon cubes often
contain assorted types of carbohydrates as a colouring. The follo-
wing recipe is recommended if the agar plates are to be made
from individual ingredients:

3.0 g peptone from casein
70.0 g salt
15.0 g agar
2.0 g soluble starch
1000.0 ml distilled water.

4. Lugol’s solution should be prepared a day ahead. The iodine crys-
tals take a while to completely dissolve. The use of a stirrer or
agitator is helpful.

5. Starch and iodine form a blue-black compound. All areas on the
dish where starch is still present turn dark blue immediately after
coating with the iodine solution. Light brown zones (the colour of
the Lugol’s solution) develop along the edges of the inoculating
strip if the micro-organisms have degraded starch.
Fig. 11.3. Results after incubation and coating with Lugol’s solution. The micro-organisms have partially metabolized the starch.

11.1.2.2. Viscometric Proof of Starch Degradation

Starch solutions have a higher viscosity than glucose solutions. Starch solutions therefore flow much slower from a pipette than solutions of the products of their degradation. The metabolism of the starch can be followed by continual determination of flow times.

The measurement process described here is based on the principle of a viscometer described by OSTWALD-UBBELOHDE. The time a definite amount of liquid takes to pass through a capillary is measured by this method. The time taken varies according to the viscosity of the liquid. Any container that can hold 20 ml of solution and has a narrow spout can serve as a viscometer. A simple viscometer can be made from a funnel, clamp valve, and a length of rubber tube. The clamp valve is adjusted so that the starch solution drains in 30 - 60 seconds. The flow time is stopped from the point when the pipette is filled to the point when the meniscus of the draining liquid passes a given mark.

A graduated pipette of at least 20 ml capacity is a suitable alternative for such a viscometer. Differences in solutions of varying viscosity can readily be registered from this volume and up. A few ml of the solution should be retained in the pipette in the end since the
draining speed decreases due to the degree in the hydrostatic pressure of the liquid column. When other pipettes are used, a mark can be made a few cm over the mouth of the pipette.

Fig. 11.4. Construction of a capillary viscometer

Fig. 11.5. Construction of a homemade viscometer
Know-How

Equipment

1. 1 100 ml Erlenmeyer flask
2. 1 100 ml graduated cylinder
3. 1 10 ml graduated cylinder or pipette
4. 1 pipette (at least 20 ml) or funnel with clamp valve and rubber tube
5. 1 stopwatch

Materials

- starch
- overnight culture of the bacterial species to be examined
- dried nutrient broth (glucose free, commercial)

Time Requirements

2 - 3: 45 minutes
4: 15 minutes

Procedure

1. An overnight culture of the desired micro-organism is prepared according to Section 3 on the day prior to this experiment.
2. 4 g starch are mixed with 90 ml water in an Erlenmeyer flask, dissolved by brief boiling and cooled to 30° C. 10 ml of the overnight culture are then added.

It is of no great significance for the success of this experiment whether the starch comes from wheat corn, rice or other grain. However, soluble starch is not suitable. Before undertaking this experiment for the first time, it is recommended to test the viscosity of 4 g starch and 100 ml water after boiling and cooling in pretrial experiment. The resultant solution should take at least twice as long as water to flow out.

3. After thorough stirring, a certain volume of the suspension is taken up in the pipette (use a pipette helper). The time is then measured that it takes for this volume of suspension to flow out. After this has been registered, the Erlenmeyer flask is sealed and incubated at 30° C. The flow time of a similar amount of water is measured for comparative purposes.

4. The flow time of the suspension is measured and recorded daily. The experiment is stopped at the end of one week at the longest.

Know-Why

1. Nutrient bouillon which contains carbohydrates may not be used in this experiment. (For explanation see Sec. 1.2.1.)

2. Soluble starch is not suitable because its molecule chains are degraded so much in the manufacturing process that its viscosity is not much more than that of water.

3. Measurements must be taken at each step of the experiment with the same equipment to ensure that the same flow conditions exist. Furthermore measurements must always be made at the same temperature since the viscosity of a liquid is dependent on temperature.
4. The experiment is stopped within one week to avoid the danger of generating undesirable micro-organisms from possible contamination over a longer period of time.

11. 2. Liquifying a Gelatine Gel with Proteases

Proteins are present in and on the earth in the form of dead plant and animal matter. These protein molecules are broken down to amino acids by micro-organisms possessing the appropriate enzymes. Liquifying a gelatine gel demonstrates the activity of such bacterial enzymes.

Fig. 11.6. The protein molecules composed of amino acids divide the liquid into small enclosed areas. The released amino acids float freely in the liquid.
Liquification occurs through the process of the enzymes that degrade the proteins to their basic components, the amino acids. The water molecules enclosed by the makromoleculees are released through the enzymes which degrade these protein macromolecules which causes the gel to liquify. (Fig. 11.6.)

Gelatine is used for a protein in this experiment because of its unusually high gelatinizing ability. Gelatine is an hydrolysis product of ossein, a cartilage protein that belongs to the group of collagens found in bone. Gelatine is made up mainly of the amino acids glycine, proline and hydroxyproline. The molecular weight varies from 40,000 to 100,000 depending on the manufacturing source and process. Gelatine solutions with a concentration greater than 1% congeal. Congealing is dependent not only upon temperature but upon other components of the solution as well, most especially its content of dissolved salts.

**Know-How**

<table>
<thead>
<tr>
<th>Equipment</th>
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1. 1 test tube per experiment
   1 test tube for the control experiment and test tube caps
2. 1 Erlenmeyer flask for preparation of nutrient gelatine
3. 1 inoculating loop
Materials

- nutrient broth commercial or home made according to appendix 13.3.
- gelatine
- Litmus paper or stabs
- cultures of *B. megaterium* or other bacteria to be examined

Time Requirements

45 minutes

Procedure

1. About 10 ml nutrient broth is necessary for each test tube. The broth is prepared in an Erlenmeyer flask to which 15 g gelatine per 100 ml solution is added. The gelatine is allowed to soak for ten minutes, then dissolved by heating. The solution’s pH value should lie between 7.0 and 7.2. A few drops of diluted hydrochloric acid or sodium hydroxide must be added as necessary to adjust the pH value. Afterwards, the solution is sterilized according to one of the methods explained in Section 1.

2. While still hot the solution is poured into the test tubes in roughly 10 ml portions. A funnel is used to keep the upper portions of the test tubes clean. The test tubes are then stored in a cool place until the solution has congealed. Congealing can be greatly accelerated by storing the tubes in a refrigerator.

3. One test tube serves as a control. The other tubes are inoculated by means of a heat sterilized inoculating needle with a small amount of bacteria. The needle is inserted about 5 cm into the congealed gelatine.
4. The inoculated tubes and the control tube are stored at 20 - 22° C. They are checked daily to see if the gelatine has liquified. The experiment is stopped after four weeks. Liquification begins within a few days when B. megaterium has been inoculated. If the control solution liquifies, this can have two causes:

   a. The storage temperature may have been too warm. The gel can liquify at a temperature of 26° C. In this situation, the first thing to do is to see if the gel congeals again at temperatures between 18 and 22° C.

   b. The gelatine solution was not sterile when poured into the test tubes. The gel has been liquified by bacteria that have invaded and contaminated the control solution.

**Know-Why**

The high protein content (15 %) of the solution used here was selected in order to obtain a gel solid enough so that the test tubes could be inverted to check the progress of liquification. The tubes should not be stored at too warm a temperature or else the gel will liquify. The maximum tolerable temperature is indicated by liquification of the control. The experiment should be conducted a few degrees below this temperature since the upper temperature limit of the gels employed will vary.

**Problems and Questions**

1. Why do gels liquify at higher temperatures? (The protein structure can withstand the pressure caused by the kinetic energy of the water molecules only to a certain degree.)

2. What are the names of the most important proteases produced in the human body and where are they produced?
3. The poison of many snakes contains proteases. How do they work? (They lead to the destruction of cells; for example they attack the blood cells in the animal or human which has been bitten.)

4. What is the ecological importance of the ability of micro-organisms to metabolise proteins?
(The protein molecules of dead plant and animal matter are recycled. The degradation of proteins to amino acids is the first step. The amino acids are then further metabolised. This further metabolism of the amino acids makes their nitrogen content available to the ecosystem again.)

11.3. Degradation of Hydrogenperoxide (H₂O₂) with Catalase

Large amounts of hydrogenperoxide are poisonous for living things. For example, hydrogenperoxide oxidizes -S-H groups that are found in coenzyme A and many other protein molecules. A molecule loses its biological function, for example, its enzymatic action through oxidation. Hydrogenperoxide is generated by assorted biochemical reactions. Acetic acid bacteria can oxidize glucose directly to gluconic acid (Fig. 11.7.). The hydrogen released is absorbed by a molecule of the prosthetic group FAD (flavin-adenin-dinucleotide).

Fig. 11.7. Oxidation of glucose into gluconic acid
Flavin containing enzymes can transfer hydrogen directly to oxygen whereby hydrogen peroxide is formed (Fig. 11.8.).

\[
\text{Enzyme} \quad \text{FAD} \quad \text{H} \quad + \quad \text{H}^+ \quad \text{O}_2 \quad \rightarrow \quad \text{Enzyme} \quad \text{FAD} \quad + \quad \text{H}_2\text{O}_2
\]

Fig. 11.8. Genesis of hydrogen peroxide

Other metabolic processes are known by which hydrogen peroxide is produced. All organisms capable of this metabolic process possess an enzyme that degrades hydrogen peroxide to water and oxygen:

\[2\text{H}_2\text{O}_2 \rightarrow \rightarrow 2\text{H}_2\text{O} + \text{O}_2\]

This enzyme is called catalase and will be demonstrated in the following experiment.

**Know-How**

<table>
<thead>
<tr>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 1 10 ml graduated cylinder</td>
</tr>
<tr>
<td>2. 1 eye dropper</td>
</tr>
<tr>
<td>3. 1 50 ml Erlenmeyer flask</td>
</tr>
</tbody>
</table>
slant agar with the microorganism to be examined, e. g., Bacillus megaterium (see section 4)
a sterile slant agar tube as a control
hydrogenperoxide solution (30 %)

<table>
<thead>
<tr>
<th>Time Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: 15 minutes</td>
</tr>
<tr>
<td>3: 15 minutes</td>
</tr>
</tbody>
</table>

**Procedure**

1. Slant agar tubes are inoculated with the bacteria whose ability to metabolise hydrogenperoxide is to be examined (see Sec. 4). These tubes are then incubated for 1 - 2 days at 30°C.

2. One slant agar tube is not inoculated and reserved as the control tube.

3. 1 ml of 30 % hydrogenperoxide solution is mixed with 9 ml water on the day of the experiment. This combination produces a 3 % solution.

DANGER: Concentrated hydrogenperoxide is extremely caustic. If hydrogenperoxide comes in contact with the skin, the affected area must be immediately rinsed with large amounts of water. Protective gloves and glasses must be worn.

The slant agar tubes are filled half way with the 3 % hydrogenperoxide solution. The immediate welling up of bubbles, or foaming from stronger reactions demonstrates that
oxygen is being released by catalysis of hydrogenperoxide. Bubbling should not occur in the control tube.

**Know-Why**

1. Ideally the culture should be in the logarithmic growth phase (see Sec. 10). Catalase activity is greatest in this phase.

2. Hydrogenperoxide cannot only be degraded by catalase producing organisms. Mangandioxide is the best known example of an anorganic catalysator. Therefore, nutrient agar ingredients must be carefully examined to determine whether or not they have a catalytic effect. A standard commercial agar must be used when necessary.

3. The 3 % hydrogenperoxide solution is prepared on the day of the experiment since, as opposed to the 30 % solution, it decomposes quickly, which results in water and oxygen being produced from the hydrogenperoxide.

**Problems and Questions**

1. Look for another important biochemical compound that has an -S-H group in other applicable reference books. (Another compound of this type is, for example, the amino acid cystein.)

2. What function do -S-H groups have in protein molecules? (They serve to stabilize the tertiary and quaternary structure of the protein)

3. What metabolic processes cannot occur without intact coenzyme A? (The citric acid cycle and degradation of fatty acids belong in this group.)

4. Use reference books to look for an important coenzyme that transfers hydrogen to other molecules. (NAD, nicotinamid adenin-dinucleotide, ist the most common hydrogen transit fer-ring coenzyme in animals, plants and bacteria.)
12. Testing Antibiotics

Many fungi release antibiotics into their environment. These are bactericidal compounds which provide fungi with a competitive advantage over bacteria. Their medical value for combatting diseases caused by bacterial in humans was recognized by FLEMING in 1928. Penicillin has been mass produced since 1940. Many other antibiotics have been discovered in the meantime. Their effectiveness can be observed by means of a paper disk test.

Know-How

**Equipment**

1. petri dishes
2. 1 paper punch (or scissors)
3. 1 pair of tweezers
4. 1 Bunsen burner
5. 1 small beaker
6. 1 large beaker as water bath
7. needles
8. corks and beakers or test tubes with caps drying cabinet

**Materials**

overnight culture, e. g., *B. megaterium* or *B. subtilis* or a yeast suspension (1 g yeast per 100 ml water)
nutrient agar (commercial or home made according to appendix 13.3.) ingredients for malt agar according to appendix 13.3. where required penicillin, streptomycin or another antibiotic filter paper ethanol alumi-nium foil

<table>
<thead>
<tr>
<th>Time Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 4: 60 minutes</td>
</tr>
<tr>
<td>5 - 6: 15 minutes</td>
</tr>
<tr>
<td>7 - 10: 30 minutes</td>
</tr>
</tbody>
</table>

Procedure

1. Nutrient agar is prepared according to the instructions in Section 6. 10 g agar per liter is sufficient for homemade plates. Plates for bacteria must always be prepared while malt agar plates need only be prepared when one wants to demonstrate that antibiotics are ineffective against yeast.

2. The plates are sterilized according to one of the methods explained in Section 1, then cooled to 40° C in a water bath and maintained at that temperature.

3. 1 ml overnight culture or yeast suspension is added to the still liquid nutrient agar. The Erlenmeyer flask is then sealed and shaken well.

4. The inoculated nutrient agar is poured into the petri dishes and allowed to congeal.

5. Five paper disks are punched with the paper punch or cut with scissors from a sheet of filter paper. Two of these paper disks are impaled on the tip of each needle.

6. The paper disks are sterilized by placing them overnight in a 96 % ethanol solution. Alternatively the pins are inserted into a cork which is then placed in a beaker. The beaker is covered with aluminium foil and heated for 3 hours at 135° C in a drying cabinet. Illumination for a few minutes with ultraviolet light also suffices for sterilisation.
7. A solution of the antibiotic is prepared with sterile water. If pure substances are available, then the concentration should be approximately 50 mg / ml. If a commercial medical product is used, then a 50 mg / ml concentration can be prepared by referring to the instructions for the use of the medicine. It is also possible to dilute tablets just enough so that the entire solution can be absorbed by filter paper. Tablets are ground with in a mortar and pestle and the power is then dissolved in water.

8. The sterilized paper disks are dipped in the antibiotic solution and dried. Drying can best be done by placing the paper disks in a drying cabinet at 100° C. for a minute. At least one of the paper disks is dipped in antibiotic free sterile water to serve as a control.

9. The dried antibiotic paper disks and one control disk are placed on previously inoculated and labelled agar dishes. A flame sterilised pair of tweezers are used for this purpose. Flame sterilising is described in Section 7. One dish without paper disks is reserved as a control.

10. The closed petri dishes are incubated for two days at 30° C. One observes that a confluent lawn of bacteria or yeast have grown in all of the control dishes. A bacteria free zone has formed around the paper holes containing antibiotics. Yeast is not influenced by the antibiotics.

11. All petri dishes containing antibiotics must be sterilised very carefully after the experiment, especially if commercial medical products were used.

Know-Why

1. Penicillin disturbs the formation of bacterial cell walls and activates enzymes that are destructive to cell walls. The selective effectiveness of penicillin is based upon this action.
2. The water bath is necessary since the agar should not be inoculated at temperatures that can damage the organism. If the agar is allowed to cool further while pouring the plates, it solidifies too quickly.

7. Medicines in syrup form cannot be absorbed well by filter paper.

8. The effective substance would begin to diffuse uncontrollably if the paper disks were placed on the agar when still wet.

9. The control disk demonstrates that the filter paper contains no substances that could inhibit bacterial growth.

10. The antibiotics diffuse slowly into the agar. The diameter of the area of growth is a measure of the concentration of the effective substance. When different substances in equal concentration are compared, the diameter of the area of growth is then a measure of the toxicity of the substances for the bacteria being examined.

Problems and Questions

1. Why does a dose of penicillin have no noticeable effect on humans even when it is 1000 times greater than that necessary to kill bacteria? (Human cell walls have no comparable structure to the cell walls of the bacteria upon which penicillin acts.)

2. Can a person be made immune to bacterial infections by taking a dose of antibiotics daily? (No, antibiotic resistant strains develop over time.)
13. Appendix

13.1. Equipment and Alternatives List

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Alternative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclave</td>
<td>Pressure cooker, large glass within limitations</td>
</tr>
<tr>
<td>Beakers, as water bath, size according to need</td>
<td>cooking pots</td>
</tr>
<tr>
<td>Beakers, 100 ml</td>
<td>other small glasses as long as the experiment does not require heating</td>
</tr>
<tr>
<td>Caps for test tube</td>
<td>Aluminium foil</td>
</tr>
<tr>
<td>Cover slips</td>
<td>none</td>
</tr>
<tr>
<td>cylinders, graduated, 10 ml</td>
<td>Pipettes for some experiments</td>
</tr>
<tr>
<td>Drigalski spatula</td>
<td>Homemade from glass rods</td>
</tr>
<tr>
<td>Drying cabinet</td>
<td>for hot air sterilization a baking oven, for incubation, see Section 13.5.</td>
</tr>
<tr>
<td>Erlenmeyer flasks, 100 ml</td>
<td>none</td>
</tr>
<tr>
<td>Eye droppers</td>
<td>Droppers from eye and nose drop medicine bottles</td>
</tr>
<tr>
<td>Felt tip marking pens</td>
<td>none</td>
</tr>
<tr>
<td>Flask, graduated, 100 ml</td>
<td>none</td>
</tr>
<tr>
<td>Glass rods</td>
<td>none</td>
</tr>
<tr>
<td>Incubating cabinet</td>
<td>See Section 13.5.</td>
</tr>
<tr>
<td>Inoculating loops</td>
<td>fine wire with the ends formed into loops</td>
</tr>
<tr>
<td>Knife, paring</td>
<td>none</td>
</tr>
<tr>
<td>Paper punch</td>
<td>Scissors</td>
</tr>
<tr>
<td>Petri dishes</td>
<td>Securely sealable and sterile containers</td>
</tr>
<tr>
<td>Pins</td>
<td>none</td>
</tr>
</tbody>
</table>

List of micro-organisms suitable for use in secondary schools.


**Bacteria:**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acetobacter aceti</em></td>
<td>Micococcus luteus</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>Photobacterium phosphoreum</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>Rhizobium leguminosarum</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Fungus</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>Rhodopseudononas palustris</td>
</tr>
<tr>
<td>Chromobacterium lividum</td>
<td>Spirillum serpens</td>
</tr>
<tr>
<td>Chromatium species</td>
<td>Streptococcus lactic</td>
</tr>
<tr>
<td>Erwinia carotovora</td>
<td>Streptomyces griseus</td>
</tr>
<tr>
<td>Escherichia coli K 12</td>
<td>Vibrio natriegens</td>
</tr>
<tr>
<td>Lactobacillus species</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fungus</td>
</tr>
<tr>
<td>Agaricus bisporus</td>
<td>Physalospora obtusata</td>
</tr>
<tr>
<td>Armillaria mellea</td>
<td>Phytophthora infestans</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>Pytium debaryanum</td>
</tr>
<tr>
<td>Botrytis fabae</td>
<td>Rhytisma acerinum</td>
</tr>
<tr>
<td>Chaemotium globosum</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>Coprinus lagopus</td>
<td>Saccharomyces ellipsoides</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>Saprolefgia litoralis</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>Schizosaccharomyces pombe</td>
</tr>
<tr>
<td>Helminthosporium avenae</td>
<td>Scleotinia fructigena</td>
</tr>
<tr>
<td>Myrothecium verucaria</td>
<td>Sordaria fimicola</td>
</tr>
<tr>
<td>Penicillium roquefortii</td>
<td>Sporobolomyces species</td>
</tr>
<tr>
<td>Phycomyces blakesleanus</td>
<td></td>
</tr>
</tbody>
</table>

NOT suitable for secondary school use:

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Fungus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromobacterium violaceum</td>
<td>Aspergillus nidulans</td>
</tr>
<tr>
<td>Clostridium per fringens</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Penicillium chrysogenum</td>
</tr>
<tr>
<td>Pseudomonas solanacearum</td>
<td>Penicillium notatum</td>
</tr>
<tr>
<td>Pseudomonas tabacci Serratia</td>
<td></td>
</tr>
<tr>
<td>marcescens Staphylococcus aureus</td>
<td></td>
</tr>
<tr>
<td>Xanthomonas phaseoli</td>
<td></td>
</tr>
</tbody>
</table>
The microbiological institutes of universities can be helpful in acquiring micro-organisms. A list of world wide collections can be found in the following text:


13.3. Preparation of Growth Media

Basic nutrient agar is sufficient for an of the experiments presented in this book. Basic growth media contain all the substances absolutely required for the growth of micro-organisms. Conversely enriched media contain supplementary carbon and nitrogen sources as well as many vitamins and minerals. Organisms grow better in enriched media. However, such media are more expensive and spoil much easier.

13.3.1. Nutrient Broth

Nutrient broth can be produced in many ways:

a. By dissolving a commercial product according to the manufacturer’s directions. Most manufacturers of chemicals, especially those in the biological field, offer a standard bouillon for microbiology. These media are suitable for the breeding of simple types of bacteria. These ready-made nutrient broth powders are standardised, relatively inexpensive and can be kept almost indefinitely when stored in a cool, dry and dark location. The use of these media is strongly recommended. One can prepare nutrient broth by combining individual ingredients. However, this process is not only inconvenient but more expensive as well.

b. According to our experience, broth made from bouillon cubes is an inexpensive option for the purpose of cultivating bacteria in the school. The bouillon cube is dissolved in boiling water according to the
manufacturer’s instructions. The medium is allowed to cool and finally filtered. Such solutions made from the products of assorted manufacturers were found to allow the growth of B. megaterium or B. subtilis in overnight cultures.

c. A suitable nutrient broth for fungi can be obtained by dissolving 30 g malt extract in 1000 ml water.

13.3.2. Nutrient Agar

Nutrient agar can be prepared by dissolving commercial agar in water according to the manufacturer’s instructions, or by preparing nutrient broth according to one of the recipes listed above, adding 18 g agar per 1000 ml water, then allowing it to soak for ten minutes, and finally heating the mixture while stirring constantly.

CAUTION: Agar heated over an open flame can easily burn. A safer process for inexperienced students is to heat the agar in a water bath. Malt agar for fungi contains 30 g malt extract, 3 g peptone, and 15 g agar per 1000 ml solution.

13.4. Assembly and Funktion of a Homemade Photometer

Developed by H. Bayrhuber, E. R. Lucius and K. Rausch.

A photometer is useful for determining the concentration of coloured or turbid solutions. Photometers are usually used to:

a. observe the activity of enzymes with the help of suitable indicator solution (e.g. the degradation of urea or starch),
b. demonstrate the presences of anions in water samples (e.g. phosphates or nitrates),
c. determine the density of a growing bacterial culture.
13.4.1. Function and Use

A bundled light ray with diaphragm or slide projector and is aimed into a photoelectric cell that is connected to a power slide with a slit housed in a wooden box. The current is set at 3 mA with a 6V power source. (Fig. 13.1.)

If the light intensity decreases, the light dependent resistance of the photoelectric cell increases and the intensity of the current decreases according to the equation:

\[ U = R \times I. \]

Cuvettes can be placed in a holder between the light source and the photoelectric cell and filled every 15 minutes with samples from bacterial suspension. The increasing density (corresponding to an increase in extinction and decrease in current measured by the photoelectric cell) is a measure of bacterial growth registered every 15 minutes.
Fig. 13.2. Circuit diagram for the direct recording of a growth curve

A linear relationship between the light changes intensity and current does not exist in this apparatus due to the use of a photoelectric cell. Since scattered light is produced by light diffraction from the bacteria, since the concentration of the suspension in very high at the end of the bacterial growth period, and since monochromatic light is not used, the Lambert-Beer rule for photometry is not valid here. Actual titer values can only be determined from current measurements or plotter results with the help of a calibration curve (values measured at given concentrations).

The employment of a phototransistor (e.g., BGY 62) in the place of a photoelectric cell allows finer resolution of assorted concentrations. However, the light ray must strike the exact centre of the small lens of the transistor. Therefore adjustment problems must be overcome for practical use of the phototransistor that do not occur with photoelectric cells.
13.4.2. Material

a. plywood, 1 cm thick
   2 side panels (14 x 7 cm)
   1 back panel (9 x 7 cm)
   1 front panel (9 x 7 cm with a 1.2 wide x 4.5 long slit in the center)

b. board (15 mm thick)
   1 bottom panel

c. square wooden slats
   2 pieces (10 x 12 mm), 8.5 cm long
   1 piece (10 x 12 mm), 6.0 cm long
   1 piece (12 x 12 mm), 3.5 cm long

d. 1 iron metal rod, 10 mm circumference, 12.5 cm long

e. 1 photoelectric cell “LDR 07”

f. 2 clips

g. 10 screws, 3.5 mm, 25 mm long

h. wood glue, solder, black enamel paint, clear lacquer

Cost of materials: $ 15.00.

13.4.3. Assembly Instructions

The photometer is assembled from the separate parts according to the figure below. (Fig. 13.3.) The walls and bottom are put together with screws. A 9.5 cm hole is bored in the venter of the underside panel in which the iron rod is secured as the photometer stand. The square wood slats support the photoelectric cell (inside Venter, 6 mm) and serve as the cuvette holder on the front wall and are held in place with glue. The clips are screwed into place left and right of
the wooden block on the rear wall. The photoelectric cell is soldered into place between the clips with the lined side facing forward. The inside of the housing is painted black and the outside coated with clear lacquer. Finally the top is put into place and held with screws.

Fig. 13.3. A simple photometer displayed without top
13.5. Homemade Incubator


The incubator is compatible for incubating nutrient base solutions and liquid cultures at temperatures between 29 and 42° C. (Fig. 13.4.)

13.5.1. Function and Use

The incubator housing is made out of styrofoam. A 15 watt light bulb is attached securely at the bottom of the incubator. A constant temperature in the incubator is maintained by convection. The inside
temperature increases until the heat generated by the light bulb and the heat released to the surrounding are balanced.

The inside temperature can be controlled by means of holes bored in the top of the incubator that can be opened or closed with corks as necessary. Two thermometers are used to determine the optimal temperature. The incubator should not be placed in sunlight. Warmth from sunlight can cause undesirable temperature fluctuations.

13.5.2. Material

2 styrofoam panels (1 m² / 6 cm strength) cut to:

- 6 cm -
  A: 2 pieces, 50 x 44 cm
  B: 2 pieces, 50 x 27 cm
  C: 2 pieces, 32 x 27 cm

- 2 cm -
  A: 1 piece, 37.5 x 32 cm
  B: 2 pieces, 24.5 x 12 cm
  C: 1 piece, 28 x 12 cm
  D: 2 pieces, 24.5 x 5.5 cm
  E: 2 pieces, 25.5 x 9.0 cm

1 fibreboard (0.3 cm thick, one side painted white, (32 x 26 cm)

16 beech dowels, 0.5 cm diameter, 32 cm long

1 15 watt light bulb

1 light socket (held in place by a 15 cm long steel metal tube, through which the power cord passes

2 glass thermometers
1 strip of clear adhesive tape, 4 cm wide, 50 cm long
1 can of styrofoam glue

Cost of materials: $25.00

13.5.3. Assembly Instructions

The styrofoam frame is glued together first. Two side panels (B), the top panel (C) and the bottom panel (D) are glued perpendicular to each other on the rear panel (A). After the glue has dried, seven 2 cm thick styrofoam plates (E - H) are glued onto the inside walls (B). These plates will serve as the shelf supports. The lamp area, which is separated from the main portion of the incubator by the fibreboard, has a height of 12 cm. The styrofoam sections (E - F) for this area are glued to the side and rear walls. The shelf supports are glued into place in 0.5 cm intervals, first the 5.5 cm supports (G), then the 9 cm supports (H).

The incubator door (A) is layered with a sheet of 2 cm thick styrofoam (D) cut so that it fits the inside dimensions of the incubator housing and so that the inside is completely sealed when the door is closed.

For thermometer access two 0.7 cm holes are bored into the right side wall 21.5 and 40 cm from the bottom. A 1.0 cm hole is bored 9 cm above the bottom for the light socket.

The fibreboard shelves are inserted between the first and second shelf supports so that the incubating area is separated from the light

---

1 Most adhesives dissolve styrofoam; therefore, only adhesives specially made for styrofoam may be used.
bulb. The dowels are glued in 3 cm intervals into the notches of the second and third styrofoam shelf supports (Fig. 13.5. G - H).

Six 2.3 cm holes are bored at a distance of 4 cm from each other in the top of the incubator (C). Six 2.3 cm holes are bored at a distance of 4 cm from each other in the incubator door a few centimeters above the lamp area. The door is then assembled onto the incubator housing; the strip of tape is applied to the right side of the housing and the right side of the door, and serves as a hinge that allows the door to be opened and closed easily.

Fig. 13.5. Exploded diagram of the styrofoam incubator (left) with door (right) without interior equipment
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11.4. Construction of a capillary viscometer

11.5. Construction of a homemade viscometer

11.6. The protein molecules composed of amino acids divide the liquid into small enclosed areas. The released amino acids float freely in the liquid.

11.7. Oxidation of glucose into gluconic acid

11.8. Genesis of hydrogen-peroxide

13.1. Sketch of the experimental set-up

13.2. Circuit diagram for the direct recording of a growth curve

13.3. A simple photometer displayed without top

13.4. Incubator, opened and closed

13.5. Exploded diagram of the styrofoam incubator (left) with door (right) without interior equipment