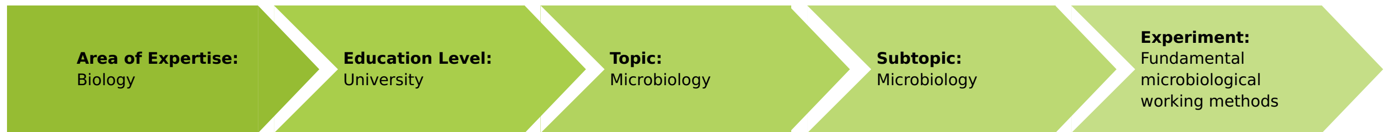
Fundamental microbiological working methods

(Item No.: P4140100)

Curricular Relevance



Difficulty	**Preparation Time**	**Execution Time**	**Recommended Group Size**
Difficult	30 Minutes	2 Hours	2 Students

Additional Requirements:

Experiment Variations:

Keywords:

Microbiological work techniques, Sterility, Sterilisation, Nutrient agar, Moulds, Yeast, Bacteria, Inoculation

Overview

Short description

Principle

In order to prevent nutrient media and cultures from being contaminated with microorganisms that adhere to the working equipment, the equipment, nutrient media, and nutrient solutions must be sterilised. Petri dishes filled with a solidified nutrient medium are called plates in the specialised language of microbiologists. The nutrient medium is poured into the dishes either from test tubes, with one test tube holding the required quantity of ready-made medium for one Petri dish, or from Erlenmeyer flasks if a large number of plates needs to be prepared at the same time.

Fig. 1: Procedure.

Equipment

Position No.	Material	Order No.	Quantity
1	Compact Balance, OHAUS TA 302, 300 g / 0.01 g	49241-93	1
2	Glass beaker DURAN®, tall, 600 ml	36006-00	1
3	Graduated cylinder 100 ml	36629-00	1
4	Tripod,ring d=140 mm, h=240 mm	33302-00	1
5	Microscopic slides, 50 pcs	64691-00	1
6	Graduated pipette 10 ml	36600-00	2
7	Test tube rack for 12 tubes, holes d= 22 mm, wood	37686-10	1
8	Erlenmeyer flask, narrow neck, 500 ml	36121-00	2
9	Test tube, 160 x 16 mm, 100 pcs	37656-10	1
10	Meat extract 10 g	31521-03	1
11	Peptone,dry,from meat 50 g	31708-05	1
12	Sterile stoppers f. id 15mm, 250	39266-00	1
13	Sterile stoppers f. id 29mm, 100	39267-00	1
14	Pipettor	36592-00	1
15	Spatula, double blade, 150 mm	33460-00	1
16	Glass rod,boro 3.3,l=300mm, d=7mm	40485-05	1
17	Wire gauze with ceramic, 160 x 160 mm	33287-01	1
18	pH test sticks 6.5-10, 100 sticks	30301-04	1
19	Bunsen burner, natural gas,w.cock	32167-05	1
20	Safety gas tubing, DVGW, sold by metre	39281-10	1
21	Sodium hydroxide, pellets, 500 g	30157-50	1
22	Water, distilled 5 l	31246-81	1
23	Agar-agar, powdered 100 g	31083-10	1
24	Autoclave with insert	04431-93	1
25	Watch glass, dia.60 mm	34570-00	1
26	Spatula, double blade, 150 mm	33460-00	1
27	Hydrochloric acid,approx.5% 250ml	30315-25	1
28	Tweezers, l = 130 mm, straight, blunt	64610-00	1
29	Universal oven, 32 liters, 230 V	49559-93	1
30	Ethyl alcohol, absolute 500 ml	30008-50	1
31	Petri dish, d 100 mm	64705-00	1
32	pH test sticks 4.0-7.0,100 sticks	30301-03	1
33	Heating + cooking hotplate,230V	04025-93	1
34	Wire loop, streaking	64936-00	1

Tasks

1. Practise the following working techniques:
2. Sterilisation of equipment
3. Preparation of standard nutrient agar for bacteria
4. Preparation of standard nutrient agar for moulds and yeasts
5. Preparation of a standard nutrient solution for bacteria
6. Preparation of slant agar tubes
7. Inoculation of microorganisms

Set-up and procedure

Procedure

Sterilisation of equipment

In order to avoid the contamination of nutrient media and cultures with microorganisms that adhere to the working equipment, the equipment must be sterilised.

Metal equipment is sterilised with the flame of a gas burner. Before and after their use, inoculation loops and needles are heated in the flame of a gas burner (Fig. 2) until they are red hot and then they are allowed to cool. The holder is flamed if it can come into contact with the cultures or any sterile equipment. Small glass objects, such as glass rods, microscope slides, and cover slips are also sterilised by flaming. Prior to flaming, they can be immersed in 96% ethyl alcohol. During this process, the microscope slides and cover slips must be held with a pair of tweezers.

Pipettes are sterilised by filling them twice or three times with 96% ethyl alcohol, by letting the alcohol flow out, by blowing the residual ethyl alcohol into the flame of a gas burner, and by passing the pipette repeatedly through the flame afterwards.

All other equipment parts made of glass are heated up to 160 °C for 1 to 1½ hours in a hot-air steriliser (Fig. 1). To do so, Petri dishes are enveloped in paper in groups of 5, forming one roll. In these packages, the dishes can be transported rather comfortably and, in addition, they can be stored in a sterile manner for longer periods of time. The risk of shattering is also lower, since they can cool more slowly. This is why glass equipment is always placed in a cold steriliser where it is also left to cool after the sterilisation.

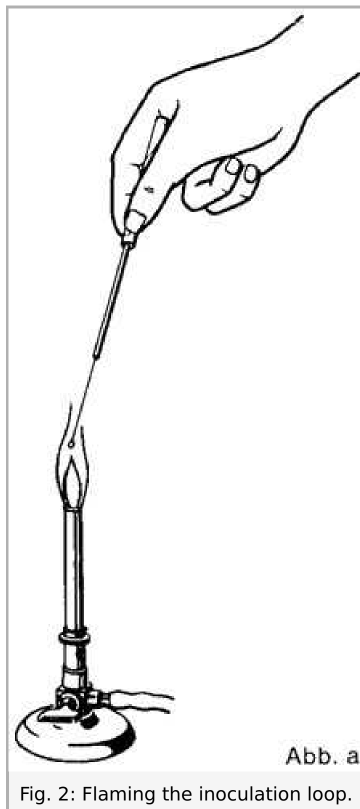


Fig. 2: Flaming the inoculation loop.

Preparation of standard nutrient agar for bacteria

The composition of this nutrient medium corresponds to the nutritional requirements of the majority of non-pathogenic types of bacteria. A major constituent is bouillon. For school purposes, however, this bouillon should not be cooked based on lean beef or horse meat as it is usually done. It is much easier and less time-consuming to use Liebig's meat extract. The gelling agent that is used is agar-agar or short agar, a product made of red algae. Unlike gelatine, agar-agar can be decomposed by only a very small number of types of bacteria.

Gelatine, a scleroprotein that is produced from sinew, skin, and bone, was widely used as a gelling agent for nutrient media when agar was still unknown. However, since gelatine is decomposed by numerous microorganisms, thereby causing it to liquefy, it is only used in special nutrient media nowadays, e.g. in order to prove proteolytic enzymes in microorganisms.

The nutrient medium should include:

Liebig's meat extract 0.3%

Peptone 0.5%

Agar 2.0%

Distilled water is used as the solvent.

Weigh the required quantities of all of the ingredients, fill them into a beaker, and boil them until the agar has completely dissolved. Since agar foams strongly during boiling and, therefore, boils over rather easily, the beaker must be large enough to hold three or four times the quantity of the prepared nutrient medium. This is also why, as soon as the solution boils, the flame must be reduced and the solution must be stirred repeatedly with a glass rod.

It is recommended to weigh the meat extract on a tared microscope slide and to transfer it to the slide with the aid of a glass rod. To do so, unroll the glass rod on the microscope slide (Fig. 3) and flush the meat extract into the beaker with the aid of the water that must be added to the nutrient medium. The microscope slide can also be boiled together with the solution and

removed afterwards.
When all of the ingredients have completely dissolved, the nutrient medium is brought to a pH value of 7.4 to 7.6 by adding drops of a 1% sodium hydroxide solution. While doing so, check the pH value with pH test sticks (pH 6.5 to 10.0). Bacteria grow best in a neutral to weakly alkaline environment, i.e. at a pH value of approximately 7.0 to 7.2. Since the concentration of hydrogen ions will decrease slightly during the sterilisation of the nutrient media, it is adjusted right from the onset to a higher level than the actually desired level.
The prepared nutrient medium is then filled into test tubes; approximately 12 to 15 ml (approximately half full) for plate pouring.

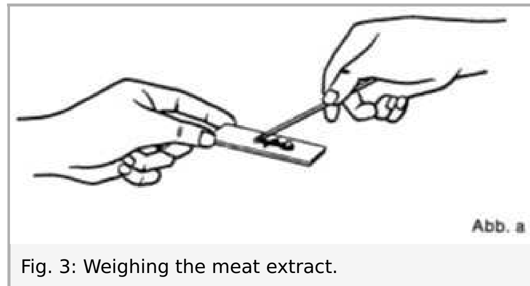


Fig. 3: Weighing the meat extract.

Preparation of standard nutrient agar for moulds and yeasts
Moulds and yeasts grow mainly on slightly acid substrates, which must be taken into consideration when preparing the nutrient media.
The nutrient medium should include:
Syrup (beet juice) 5.0%
Agar 2.0%
Distilled water is used as the solvent.
Fill the required quantities of all of the ingredients into a beaker and boil them until the agar has completely dissolved. Since agar foams strongly during boiling and, therefore, boils over rather easily, the beaker must be large enough to hold three or four times the quantity of the prepared nutrient medium. This is also why, as soon as the solution boils, the flame must be reduced and the solution must be stirred repeatedly with a glass rod.
When all of the ingredients have dissolved, test the pH value of the nutrient medium with a test stick. It should be approximately 5.0-6.0. If the pH value differs from this reference value, add drops of a 1% sodium hydroxide solution or of 1% hydrochloric acid.
The prepared nutrient medium is then filled into test tubes; approximately 12 to 15 ml (approximately half full) for plate pouring and 5 to 6 ml (approximately one quarter full) for the preparation of slant agar tubes. Larger quantities that can be used in one go for pouring plates are filled into Erlenmeyer flasks. Seal the test tubes and Erlenmeyer flasks with sterile stoppers and sterilise them in the autoclave.
Most yeasts and moulds will grow well on this type of nutrient medium.
Proceed in the same manner in order to prepare nutrient media with a different composition for yeasts and moulds.

Preparation of a standard nutrient solution for bacteria
The composition of this nutrient solution corresponds to the nutritional requirements of the majority of non-pathogenic types of bacteria.
The nutrient solution should include:
Liebig's meat extract 0,3% by weight
Peptone 0,5% by weight
Distilled water is used as the solvent.
Heat the weighed ingredients and the required quantity of water in a beaker in order to dissolve them. It is recommended to weigh the meat extract on a tared microscope slide and to transfer it to the slide with the aid of a glass rod. To do so, unroll the glass rod on the microscope slide (Fig. 3) and flush the meat extract into the beaker with the aid of the water that must be added to the nutrient solution. It is also possible to place the microscope slide together with the meat extract into the water, to heat it, and to remove it after the meat extract has dissolved. When all of the ingredients have completely dissolved, the nutrient medium is brought to a pH value of 7.4 to 7.6 by adding drops of a 1% sodium hydroxide solution. While doing so, check the pH value with pH test sticks (pH 6.5 to 10.0).
Fill approximately 6 to 8 ml of the prepared nutrient solution into several test tubes. Seal the test tubes with sterile stoppers and sterilise them in the autoclave.
Proceed in the same manner in order to prepare nutrient solutions with a different composition.

Sterilisation of nutrient media and solutions
The ingredients of the nutrient media and solutions as well as the tools and equipment that are used for their preparation are contaminated with a wide range of microorganisms that would quickly grow and contaminate the prepared nutrient media. This is why they must be sterilised immediately after their preparation. For this purpose, they are placed in an autoclave and sterilised in flowing steam. Nutrient media and solutions must not be sterilised in hot-air sterilisers since, due to the vaporisation, their water content would be reduced, thereby increasing the nutrient concentration.
Autoclaves are steam pots that can be sealed in an air-tight manner (Fig. 1). They enable the sterilisation under overpressure and at temperatures above 100 °C, thereby also killing bacteria spores. However, the killing time strongly depends on the steam saturation in the autoclave (see the table). This is why it must be ensured that, after the water in the autoclave has started to boil, as much air as possible can escape in order to achieve a high level of steam saturation.
The autoclave must be filled with the necessary quantity of water - in general just below the insert - and then the objects to be sterilised are added. Several test tubes are placed in a container as a group. Attach the lid so that the arrow on the lid is aligned

with the middle of a pot handle. Ensure that the orange knob is set to the symbol "pot open" and that the steam release valve is open (as a result, it is easier to close the lid, which puts less stress on the seal). Press the lid down and turn it clockwise until the handles are located above each other. The orange knob is set to the symbol "pot closed". The sterilisation conditions are set by rotating the sterilisation valve until it reaches the stop:
anti-clockwise for 1.4 bar, approx. 125 °C
clockwise for 2.7 bar, approx. 140 °C
After the water in the autoclave has started to boil, which is indicated by steam escaping from the steam release valve, keep the steam release valve open for another 3 minutes until the autoclave has been vented and then close it. The operating pressure will now be reached because of the pressure increase, and steam will escape from the sterilisation valve. Depending on the sterilisation conditions, the needle of the pressure gauge should not fall below the blue or red mark. This means that a little steam should escape the entire time during the sterilisation. After a high pressure of 1.4 bar has been reached, the sterilisation duration is 20 minutes or 15 minutes at 2.7 bar. After the sterilisation, leave the items in the autoclave in order to allow them to cool. Wait until the high pressure has subsided before setting the orange knob to the symbol "pot open". Then, remove the lid. If the pressure is relieved too quickly by opening the steam release valve, there is a risk that liquid sterilisation products will boil out of the vessels.
If the steam saturation is sufficiently high, a one-time sterilisation for 20 minutes at a high pressure of 1.4 bar is sufficient for killing even resistant bacteria spores and, thereby, for freeing the sterilisation products from any microorganisms that are capable of development.

Pouring of plates

Petri dishes filled with a solidified nutrient medium are called plates in the specialised language of microbiologists. The nutrient medium is poured into the dishes either from test tubes, with one test tube holding the required quantity of ready-made medium for one Petri dish, or from Erlenmeyer flasks if a large number of plates needs to be prepared at once. Test tubes with nutrient medium (or nutrient solution) are simply called tubes by microbiologists.
In order to be able to pour plates at any time, it is useful to keep tubes (or Erlenmeyer flasks) with the ready-made nutrient media in stock (in a refrigerator).
Tubes with nutrient agar are boiled in a water bath until their content liquefies. Tubes with nutrient gelatine are heated in a water bath until the gelatine has become liquid. A suitable water bath for this purpose is a 600 ml beaker that is half full with water.
After the nutrient medium has liquefied, take one tube out of the water bath and remove the cotton plug. Then, flame the mouth of the tube briefly in the flame of a gas burner in order to kill any microorganisms that may have adhered to it (Fig. 4).

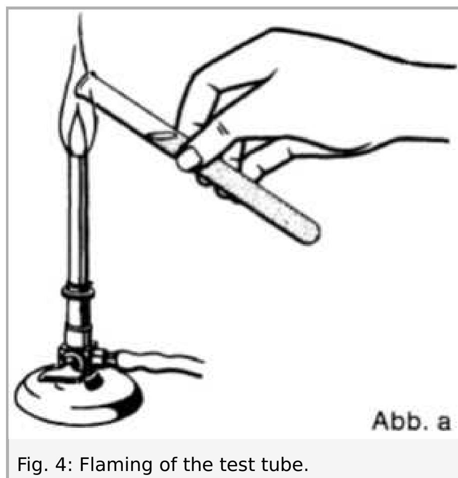


Fig. 4: Flaming of the test tube.

Open the cover of a sterile Petri dish with a diameter of 100 mm to such an extent that the mouth of the tube fits between the upper and lower part without touching them. Then, pour the nutrient medium from the tube into the Petri dish (Fig. 5) and re-attach the cover immediately.
During pouring, the upper part of the Petri dish should cover the lower part as completely as possible in order to prevent any microorganisms in the air from falling into the dish. This is why it must be lifted perpendicularly. Perform a circular movement with the closed Petri dish on the tabletop in order to distribute the nutrient medium evenly over the entire surface of the dish. Then, let the plate rest until the nutrient medium has solidified.
Proceed in the same manner when pouring plates from Erlenmeyer flasks. Plates that are not used immediately can be stored in a refrigerator for some time with the cover facing downwards, i.e. upside down.

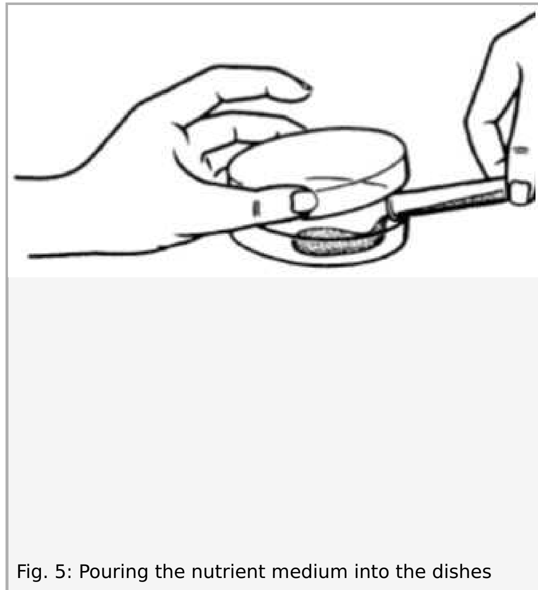


Fig. 5: Pouring the nutrient medium into the dishes

Preparation of slant agar tubes
Slant agar tubes are test tubes in which the nutrient media has solidified with a slant surface. As a result, a greater amount of the nutrient surface is available for inoculation. Since they are rather space-saving, they are often used instead of plates in test series. In addition, pure cultures of microorganisms are kept on slant agar tubes for collection purposes, provided that the culture conditions allow for it.

Prepare the desired nutrient medium, fill 5-6 ml of it into some test tubes (approximately one quarter full), seal them with sterile stoppers, and sterilise them. Immediately afterwards, as long as the nutrient medium is still liquid, place the upper end of the test tubes on a strip of wood that is approximately 12-15 mm high. The tube of a gas burner is also ideally suitable for this purpose since it has the perfect diameter (Fig. 6). As a result, the nutrient medium will solidify in the test tubes and form a long, slant surface. The nutrient medium should reach approximately the beginning of the upper third of the tube.

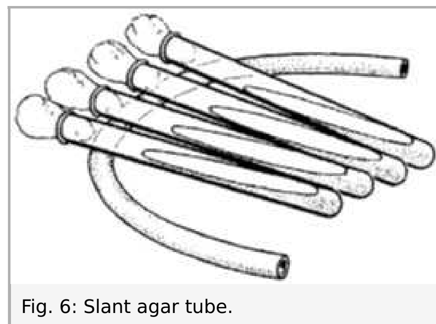


Fig. 6: Slant agar tube.

Inoculation of microorganisms

For the inoculation of microorganisms, special precautionary measures must be taken in order to prevent the nutrient media or cultures from being contaminated with foreign microorganisms.
Prior to the inoculation, all of the doors and windows of the room must be closed. Do not walk around the room during the inoculation. This is to avoid any unnecessary air movement.
Flame the inoculation loop in the flame of a gas burner until it is red hot in order to clean and sterilise it. Then, let it cool. When withdrawing microorganisms from a tube (test tube with nutrient medium), remove the cotton plug from the tube with the hand that holds the inoculation loop by holding it between the little finger (bent towards the palm) and the palm and by twisting it slightly. Hold the stopper in this position (Fig. 7) until it is reinserted into the tube. Flame the open mouth of the tube briefly in the flame of a gas burner. Insert the inoculation loop carefully into the tube, withdraw a little from the culture, flame the mouth of the tube once more, and reinsert the cotton plug. Then, transfer the inoculation material onto or into the new nutrient medium.
If a slant agar tube is to be inoculated, proceed in the same manner when opening the tube as during the withdrawal of the inoculation material.
During the transfer into nutrient solutions, the mass of microorganisms is spread out carefully on the glass wall just below the liquid surface so that it distributes as evenly as possible in the nutrient solution.
If plates are to be inoculated, they must be placed down with the cover facing downwards, i.e. upside down. Then, lift the lower part, turn it around, spread the inoculation material out (Fig. 7), and place the lower part back onto the cover. Proceed in the same manner when withdrawing material from a plate on which microorganisms have grown.

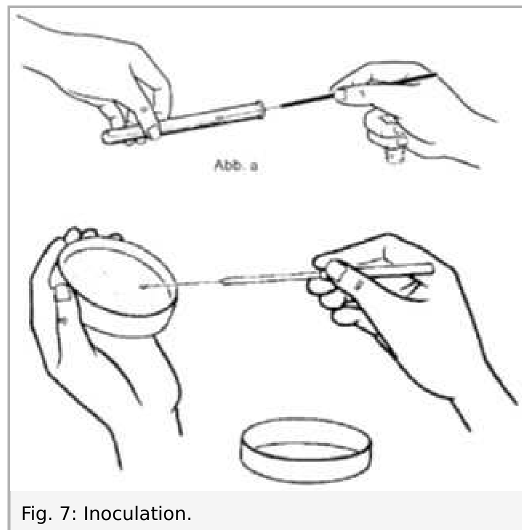


Fig. 7: Inoculation.

On all types of nutrient media, the inoculation material is usually spread out in a zigzag pattern (Fig. 8) in order to make optimum use of the available nutrient surface.
After the inoculation, flame the inoculation loop immediately in the flame of a gas burner until it is red hot in order to clean and sterilise it. Flame the grip as far as it came into contact with the inoculation material.

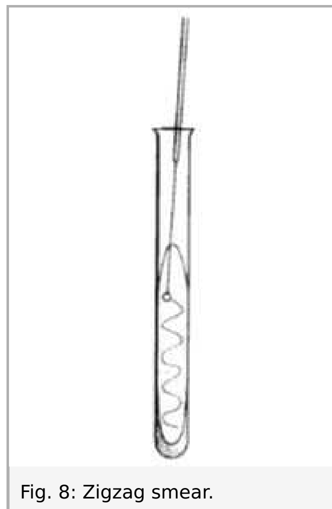


Fig. 8: Zigzag smear.