East Point College of Pharmacy

East Point Campus, Jnana Prabha, Virgo Nagar Post
Bengaluru – 560049, Karnataka

Approved
by
Pharmacy Council of India, New Delhi

Affiliated
to
Rajiv Gandhi University of Health Sciences
Karnataka
Bengaluru – 560 041
India

LAB MANUAL

PHARMACEUTICAL MICROBIOLOGY
PHARM D
2nd Year

EAST POINT COLLEGE OF PHARMACY
East Point Campus, Jnana Prabha, Virgo Nagar Post,
Bengaluru – 560049, Karnataka

PROGRAM SPECIFIC OUTCOMES	
DOCTOR OF PHARMACY	
PSO1	Acquire a thorough foundational knowledge in pharmaceutical sciences, including pharmacology, pharmaceutics, pharmaceutical chemistry, pharmacognosy and pharmaceutical analysis to excel in further academic pursuits
PSO2	Acquire and apply the pharmacotherapeutic concepts for better patient care enhancing employability across various sectors including clinical research organizations, academic and hospitals
PSO3	Equip with entrepreneurial skills and knowledge of pharmacoepidemiological studies and regulatory aspects to initiate and run successful ventures in the healthcare sector

Course:	**Code: 2.2P** **Pharmaceutical Microbiology**
CO1	Able to identify specific organism by using morphological, cultural and biochemical test
CO2	Study and practically apply the importance of aseptic techniques while handling materials in microbiological laboratory
CO3	Know microorganism growth multiplication and their industrial usage
CO4	To learn about microbial sensitivity testing and minimum inhibitory concentration and viva voice

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Experiment No.1

Basic rules of a microbiology laboratory

AIM :- To study the basic rules of a microbiology laboratory.

A microbiology laboratory is a place for working with a variety of microorganisms. Since several culture medias are prepared and organic materials are present, the chances exist for the presence of high spectrum of microbial community. Secondly, while working with pure culture, one should always follow the microbiological rules so that neither the experiment should be unsuccessful nor any hazard may occur. There are some rules which must be observed for the successful completion of the laboratory exercise in pharmaceutical microbiology, personal and environmental safety and convenience for others working in the laboratory.

- Do not enter the laboratory without wearing apron and head cover.
- Check the following equipment required for practical before entering the laboratory:-

Record file	Note book
Clean apron and head cover	Match box/ lighter
Nichrome wire loop	Glass marker
Scales	Labeling paper/ stickers
Clean napkin	Weigh box
Microscope slides	Butter paper
Tissue paper	Microscope

- Upon entering the laboratory keep coats, books and other requirements at specified locations never on bench tops.
- Keep doors and windows closed during the laboratory session to prevent contamination from air currents.
- Put off electrical fans before starting experiment.
- At the beginning and end of each laboratory period, clean your working space with a disinfectant solution provided.

❖ Avoid following behavior in laboratory.
❖ Shaking hands
❖ Drinking or eating
❖ Laughing
❖ Biting nails
❖ Smoking
❖ Touching injured part with any laboratory chemical /surface

- ❖ Sucking culture/solution using pipette
- ❖ Touching or smelling smear culture
- ❖ Always use dustbin for throwing waste material and do not use wash basin or sink.
- ❖ Aseptic techniques must be vigorously followed at all times.
- ❖ Do not carry costly things likely to be lost in the laboratory.
- If there is gas leakage, light problem, injury or any other difficulty, immediately report to your laboratory technicians or teacher.
- Never remove media, equipment, especially bacterial cultures from the laboratory.
- Always sterilize inoculating loop or needle by holding it vertically on the flame, before and after it's use.
- Do not start your burner with adjacent burner or transfer fire by any means. Keep your burner off when it is not in use.
- You must know in advance about the exercise to be performed.
- Label all the plates, test tubes, media cultures properly before starting an exercise.
- Always clean microscope stage, eye piece and objective lenses, before and after use. All lenses must be wiped with the lens paper.
- As you perform the exercise, record your data or observations and make sketches and label them.
- On completion of laboratory period, place all glass wares and cultures in the disposal area.
- Clean your working place after completion of experiment.
- Wash your hand with liquid detergent and dry with towel up on entering and prior to leaving the laboratory.

Experiment No : 2

Study of compound Microscope

AIM: To study about the compound microscope.

A Microscope may be defined as an optical instrument , consisting of a lens or a combination of lenses , for making enlarged or magnified images of minute objects.

Depending on the number of lenses , microscopes are classifed into two types such as simple microscope and compound microscope and depending on number of eye piece , microscopes are classified into two types as monocular microscope (single eye piece) and binocular microscope (two eye piece). Microscope may be classified as light microscope and electron microscope , depending upon the source of illumination .

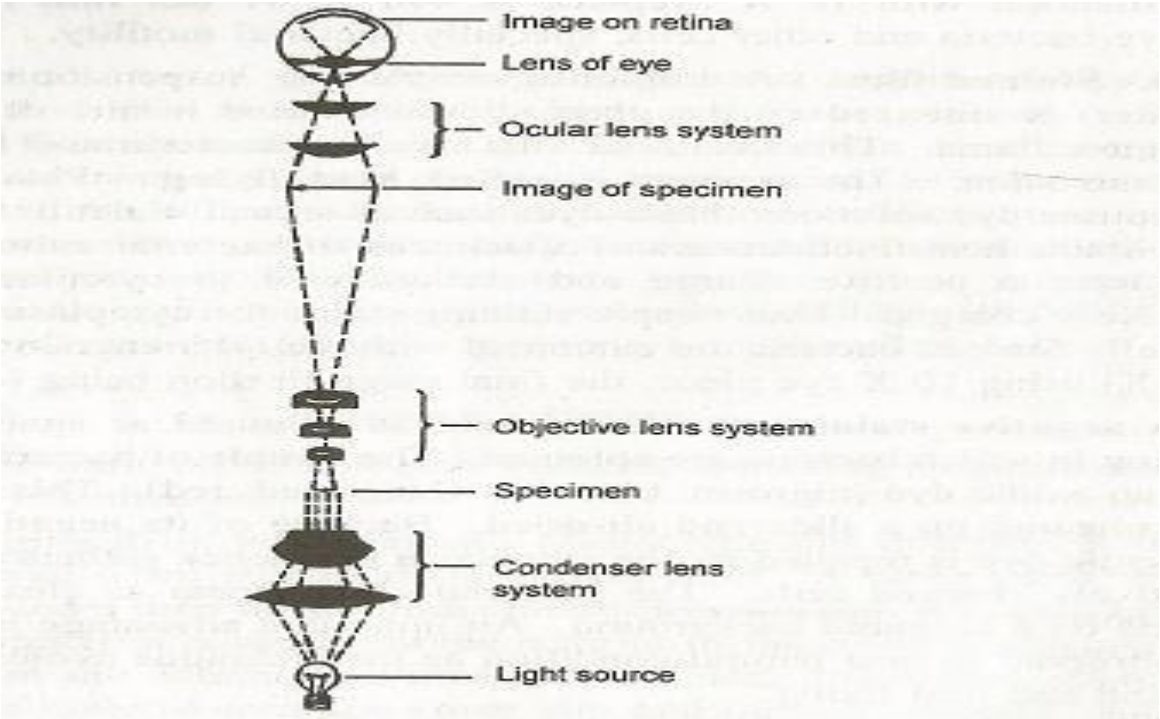

Fig. 3-2 Image formation in Compound microscope

1) Support system.
2) Illumination system.
3) Magnification system.

1) Support system:- It comprises of base, stage and body tube.
2) Illumination system:- It throws light on the object for proper viewing. It comprises of light source or mirror, iris diaphragm and condenser. The light source may be a concave or plain mirror or electrically illuminated by a tungsten filament lamp or a halogen lamp. Mirror and electric light source are generally interchangeable.
3) Magnification system:- It includes a set of lenses aligned in such a manner so that a magnified real image can be viewed. The objective is a set of lenses placed near the object, which can be observed through the eyepiece in a more magnified form.

Parts of compound microscope:-

1) Oculars:- A series of lenses (5x, 6x,10x, 15x) that magnify the object and corrects some of the defects of the objective. Huygenian , Ramsden and compensating oculars are commonly used in microscopy.
2) Objectives:- The objective is the most important lens on a microscope because it's properties make the final image. The objective lenses generally equipped with microscope are low power, high power and oil immersion lens having magnification of 10x, 40x, 45x and 100 x respectively. Functions of the objective lens are to gather the light rays coming from any point of the object and to unite the light of the image and magnify the image.
3) Condenser:- This component is found directly under the stage and contains two sets of lenses that collect and concentrate light passing upward from the light source into the lens system. There are several different types of condensers , depending upon the type of microscopy eg. Abbe condenser, variable focus condenser .
4) Iris Diaphragm:- It is equipped with a condenser . It controls the intensity of light entering the condenser. A lever is equipped with it to adjust the light intensity.
5) Illumination (light source) :- The light source is positioned in the base of the instrument. Some microscope are equipped with a built in light source to provide direct illumination. Others are provided with a mirror, with one side flat and the other concave. An external light source, such as a lamp , is placed in front of the mirror to direct the light upward into the lens system. The flat side of the mirror is used for artificial light and the concave side for sunlight.
6) Body tube:- Above the stage and attached to the arm of the microscope is the body tube. The upper end of the tube contains the ocular or eye piece lens. The power portion consists of a movable nose piece containing the objective lenses. It also provides sufficient space for image formation.
7) Revolving nose piece:- A base in which the objectives are fixed and it holds 2- 4 objectives and which can be revolved to align the required objective.
8) Focus adjustment knobs:- There are two focus adjustment knobs, a coarse adjustment and a fine adjustment . Coarse adjustment knob is used to bring the object into focus andfine adjustment knob is used for fine and clear focus of specimen.

9) Mechanical stage:- It is a platform on which the specimen to be viewed is placed. Some stages have clips to hold the glass slide. Others have a mechanical stage, which makes it possible to move the slide across the stage.

Light microscopy :- The basic microscopic system used in microbiology is the compound microscope (light microscope or bright field microscope), a two lens system with the objective lens nearer the objective & the ocular lens nearer the eye. Light passes through the objective lens, forming an inverted real image. This image serves as an object for the ocular lens which re-magnifies the image & forms the virtual image. The lens system of the eye perceives (understand) this image & captures it on the retina.

Usually there are three objective lenses in a light microscope, the low power lens (10 X), the high power lens (40X) & the oil immersion (100X) . The ocular lens further magnifies the real image. Thus a 10X ocular lens will show (10X10 =100X) or (40X10 =400X) or (100X10= 1000X) ie. 100 times, 400 times or 1000 times magnification. When 10x, 40X or 100 X objective lenses are employed . The oil (eg.Caedar wood oil) used in oil immersion microscopy provides a homogenous pathway for light from the slide to the objective & resolution of the object increases.

The resolving power (RP) or resolution of a lens system is calculated as

RP= λ/ 2xNA.

Where λ represents the wave length of light (usually set at 550 nm), and NA is the numerical aperture of the lens. NA refers to the size of the cone of light that will enter the objective & the medium in which the lens is suspended (usually air).

RP = 550nm/ 2x0.25 = 1100 nm= 1.1μm.

The RP of an oil immersion lens with a NA of 1.25 can be calculated as

RP=550nm/2x1.25 = 220 nm=0.22 μm.

Thus in the first case an object larger than 1.1 μm. & in the second case an object larger than 0.22 μm would be visualised.

Cedar wood oil and it's use in compound microscope:- Observation by compound microscope , using the 100 X objective requires cedar wood oil. It is popularly known as immersion oil and lens is known as oil immersion lens in microscopy. Immersion oil is colourless and has the refractive index similar to glass (1.55). The refractive index of air is lower than that of glass and as light rays pass from the glass slide into the air, they are bend or refracted so that they do not pass into the objective lens. This would cause a loss of light which would reduce the numerical aperture and diminish the resolving power of the objective lens. Loss of refracted light can be compensated by using cedar wood oil , which has the same refractive index as

glass, between the slide and the objective lens. In this way, decreased light refraction occurs and more light rays enter directly into the objective lens producing a vivid image with high resolution.

Use and care of microscope :-

Microscope is very expensive. Hence it must be observed the following regulations and procedure for handling and care of microscope.

1. Remove the microscope from the cabinet by grasping the microscope arm firmly with the right hand and the base with the left hand, carry close to the body and gently keep iton the laboratory table.
2. Place the microscope with it's arm facing the user approximately 15 cm from the edgeof the laboratory table.
3. Clean all lenses with dry, clean lens paper before and after use. Remove oily substances from glass parts by wiping with a lens paper moistened with xylol.
4. Set up the microscope so that light is properly reflected through the plate or mirror.
5. Place the slide with the object on the microscope stage and fix with the attached clips.
6. First focus the slide under low power objective (10X) and then focus under high power objective (40x or 45x) and oil immersion objective (100 x) if necessary.
7. While looking into the ocular lens, with the coarse adjustment knob slowly raise the stage (body tube with lens) until the specimen comes into focus . Adjust the specimen into sharp focus by using the fine adjustment knob.
8. Adjust the sub stage condenser and iris diaphragm to produce optimum illumination.
9. When using the oil immersion objective, swing the high power objective partially out of the way and place a drop of immersion oil (eg. liquid paraffin or cedar wood oil) on the area of the slide you are observing and bring the oil- immersion objective into position.

Experiment No :3

APPARATUS USED IN MICROBIOLOGY

AIM :- To study the various apparatus used in microbiology.

Culture Tubes:-

Usually in microbiology lab rimless test tubes are used so as to minimize the contamination . These are open ended small tubes cut from a piece of glass tubing and do not have a rim.

Uses:- They are used for growing microorganisms . A nutrient solution is provided in the tube where in the growth of microorganism occurs . The nutrient contains all substances required for growth.

Cultures :-

Common test tubes, which are used for chemical tests, may also used for culturing microorganisms. But some special tubes either of the round bases or flat base type may be obtained . These tubes are without any rim . Culture tubes having metal screw cap have more advantages than the conventional types.

There are three types of cultures .

1. Slant culture:- In slant culture , the culture is grown in the test tube on an inclined surface of solid medium. This provides more surface area and is suitable for aerobic organisms.
2. Stab culture :- In stab culture, the culture is grown in the test tube with medium solidified in upright position . The microorganisms are grown by stabbing them in side.
3. Broth culture :-In broth culture, the liquid culture is used for the growth of microorganisms .

Inoculation loop / needle:-

A measured length of Platinum wire, Nichrome,Tungsten or mixture wire is fused into a metal or glass rod at the end and bent suitable at the other end. The loop should be approximately 8 cmlong. In smaller loops the diameter is around 3.0 mm and large loop is 5.0 mm . It has a plastic handle. The needle have pointed end for stabbing.

Uses:

1. Platinum, Nichrome or Tungsten is used because they get heated to red hot fast and cool down fast.
2. The large loops are used to transfer quantities of inoculums and they may be used to transfer colonies from solid media and on solid surface.
3. It is also used to transfer culture to a slide.
4. The needles are used for stabbing and to transfer a small number of organisms.

Slide :-

A slide is made up of a glass and it is rectangular, plane in shape. Slides may be plane or cavity slide . A plane slide is simple with a smooth surface. Cavity slide has a cavity with a concave surface.

Uses :-

1. Plane slides are used to stain the cultures and observe under the microscope.
2. Cavity slides are used to study the motility of microorganism by hanging the culture in the concave surface of the slide in the form of a drop.

Bunsen Burner :-

It is one of the most important equipment used in the lab, which holds a rubber tube from below , this allows the gas to reach with a metallic body on the top of the burner.

Uses :-

1. Used for sterilization of loops .
2. It is used to heat the mouth of the test tube.
3. It is also used to get sterile and aseptic area.

Petri dishes :-

It contains two glass plates, having a base and a cover or lid . These are different in size but 10 to 15 cm is most suitable. These plates are designed in such a way that when the cover or lid is on the top of the base, exchange of air with the atmosphere can take place. It's quality must be such that it can withstand the vigorous nature of dry heat sterilization. Generally borosilicate glass petridishes are used.

Uses :

1. Provide large surface area for experiments like streaking, bacterial counting, studying colony counts and also assay of antibiotics.
2. We can measure zone of inhibition , sensitivity of microorganisms can also be studied.

Borer :-

Borer is made up of steel, iron or any other metal. It is used to make holes into culture medium. It is used at many places in micro biological works, mainly for sensitivity testing and antibiotic assay by cup plate method.

Durham's Tube :-

They are small tubes inserted in an inverted position at the bottom of test tube containing culture to detect the production of gas during the process of fermentation.

Uses :- Used to study biochemical reactions in an organism, especially for carbohydrate utilization test, where carbohydrate produce gas.

Autoclave :-

Culture media and other liquids when required must be sterilized by moist heat. For this purpose autoclave is used . Autoclave is an apparatus in which the steam is allowed to form in the inner cylinder by heating water. The steam pressure inside the cylinder increases with the time of heating. The rise in pressure is indicated by pressure gauge. The steam pressure increases the temperature inside to a desired level. The pressure most commonly allowed to develop inside the vessel is 15 pound per square inch (psi) in excess of atmospheric pressure, which is equivalent to a temperature of 121^0 C (250^0) F . Exposure of 15- 20 min is sufficient to sterilize any medium, provided the following points into consideration.

The load in autoclave should not exceed more than 3 liters; medium should not be more than 500ml in the container. This would provide proper circulation of steam inside the autoclave for satisfactory sterilization.

15 psi pressure at a temperature of 121^0 C must be maintained for 15 minutes.

The autoclave consists of inner cylinder, lid, safety valve, pressure gauge, exhaust valve and electric coil to heat the water. The lid is fastened to chamber by screw clamps and there is a perforated tray that covers the electric coil.

Uses

It is used to sterilize culture medium, glass apparatus, aqueous solutions and all those articles, which are not sensitive to moisture and temperature of 121^0C.

Pressure cooker :-

This is a common device for boiling water under pressure. It has a vertical metal chamber with a strong metal lid, which can be fastened down and sealed with rubber gasket. There is air and steam discharge valve, safety valve fitted on the lid. The water is heated with an electric coil

inside, which is covered with a perforated tray. There must be sufficient water inside the chamber. The auto clave is loaded (less than ¾ th) and lid is fastened down with the discharge open. There is weight , which is applied to increase the pressure. It has an automatic timer to adjust the time.

Hot air oven :-

This is electrically operated equipment used for heating dry glass ware and used for dry heat sterilization. It has a wide chamber into which the materials can be put. The walls of the oven are made of stain less steel or aluminum and devised to prevent the heat conduction from inside the chamber. A motor set at the bottom of oven forces the stream of hot air through the chamber, which raises the temperature inside and sterilize the contents. One and half hour exposure at the temperature 160^0C is sufficient to sterilize the glass ware. At this temperature all the living cells and viable spores are killed. The killing is due to the destructive oxidation of the cell contents. There is a thermostat inside the oven to regulate the temperature and there is a thermometer outside the chamber to note the temperature.

Uses :

1. This is used for the sterilization of petridishes , flasks, tubes, pipette etc. and other glass apparatus.
2. This is used for the sterilization of those articles, which cannot be sterilized by autoclave due to sensitivity towards moisture , eg, dry powders, oils, fats etc.

Refrigerator :-

It is an apparatus, which provides low temperature, required to preserve organisms. In the refrigerator, the temperature is maintained at 4^0C. The culture can be kept in the refrigerator , for a period of 2-3 weeks . At this temperature the microorganism metabolizes and multiply at a very slow rate, hence their growth is inhibited and preserve for longer period.

Uses:-

1. It helps to preserve microorganisms for 2-3 weeks.
2. Preserve the culture medium, antibiotics etc.

Incubator :-

It is electrically operated equipment designed to provide a controlled temperature for the growth and development of microorganisms in culture medium. Its construction and operation are more or less the same as those of hot air oven . Only the operational range of temperature is lower in an incubator, which generally lies between 5^0 -50^0 c. Incubator requires accurate temperature control , that can be maintained by a thermostat system. This consists of heat feed

back arrangement that is fitted by providing an extra thermostat to prevent any over shooting. There are two exhausts inside. There is thermometer outside to note the temperature . There are perforated trays to keep the articles . The chamber has two doors one of which is glass door to prevent the contamination .

Uses:- It is used to incubate or grow microorganisms by providing required temperature . Bacteria grow at 37^0C and Fungi at 25^0C.

Colony Counter:-

There are various types of devices to count the total viable cells in the culture in the form of colonies. The simplest is QUEBEC colony counter. In this petridishes having colonies of microorganisms developed on a solid medium is mounted on a plat form while the petridishesis illuminated from beneath , the visible colonies can be counted with the help of lens having magnification of 1.5x. Each colony can be touched by the electrode and recorded automatically in the counter. There is a counting area marked on the platform to help in counting.

Uses:- Counting colonies for viable count.

Electric loop sterilizer:-

It works on electricity and is a method of sterilizing the inoculation loop. There is a metal tube into which the inoculation loop is introduced and it gets heated to temperatures above 300^0c. Bythis method all kinds of organisms as well as spores get killed or destroyed. This method is used when burners are not required. Eg. The laminar air flow area.

Uses:- Sterilization of inoculation loop.

Inoculation chamber:-

This is a chamber, which is also called safety cabinet. Where the handling of pathogenic organisms is done. In this chamber the movement of air is restricted such that the organism do not contaminate the environment. The chamber is enclosed with an enclosure made of glass for viewing. There are two windows for introduction of hands for aseptic transfer and two doors for introduction of burners. There is a UV lamp that is switched on before and after the aseptic transfer.

Use:- Aseptic transfer.

Antibiotic Zone reader:-

This is an apparatus that is used to measure the diameter of zone of inhibition. Zone of inhibition is obtained when sensitivity of microorganisms against antibiotic is determined. The apparatus consist of a belt where a petridish can be placed and the belt is moved with the help of knob with scale. The petridish is illuminated from the bottom and the image of the zone is projected

on a mirror with the help of prism. By moving the petridish such that zone is visible through the mirror. The zone diameter can be accurately measured using the scale.

Uses:- Measurement of diameter of zone of inhibition.

Sterility testing apparatus:-

This is an apparatus used for sterility testing of large volume of fluids. It is a filtration unit consisting of 2-3 holding funnels with perforated plates to hold the membrane. There is a provision for application of vacuum. 2-3 fluids can be tested for sterility using this apparatus .The fluids are filtered using the filtration unit with membranes. The membranes are then added into culture media to test for the presence of viable organisms. If there is growth in the medium after incubation at 37^0c for seven days the fluid is not sterile otherwise it is sterile.

Laminar airflow unit:-

This is an aseptic technique that makes use of a laminar airflow unit. In this technique air of a closed room or cabinet is allowed to pass through a high efficiency particulate air (HEPA) filter pack and filtrate becomes free of all particles above 0.3μ (microns) dimensions. The technique involves sucking in of air of the room and blowing out the air through a filter withuniform velocity and in parallel flow line. Both horizontal and vertical laminar airflow systems are used in microbiological and pharmaceutical lab and in electronics and in aerospace industries. The advantage of this system is that the aseptic transfer can be done without using enclosed chamber and burners.

The laminar air flow unit consists of an area enclosed on all slides except one, HEPA filter, pre filter , UV light , Air blower , air sucking device, manometer and the working bench.

Uses:-

1. Transfer of culture.
2. Handling of lyophilized culture.
3. Sterility testing.
4. Filling of parenteral.
5. Other aseptic process.

Experiment No :4

STERILIZATION

AIM:- Sterilization of glass apparatus by heat sterilization.

Sterilization:- It is the process of freeing an article or material from all living microorganisms including spore forming bacteria, non- spore forming bacteria, fungus, protozoa, etc. The microorganisms are removed or killed from the articles.

The sterilization can be classified into the following four methods.

1. Heat sterilization.
2. Radiation sterilization.
3. Chemical sterilization.
4. Filtration sterilization.

Heat sterilization and radiation sterilization are the physical methods of sterilization and only filtration is the mechanical method of sterilization.

Heat steriliztion can be classified into :

Dry heat steriliztion	eg. Flaming , incineration, hot air oven.
Moist heat sterilization	eg. Autoclaving (a) simple (b) Steam jacketed.

Dry heat:

In this method articles are sterilized by using hot air oven. All the cellular contents of cells are oxidized and the bacteria are killed. For dry heat sterilizaion petridish, glass tubes, pipette etc are first dried and wrapped in a kraft paper and then exposed to hot air in electrical hot air oven at the temperature of 160^0 c for 1 ½ hrs. This time is sufficient to sterilize the glass ware. The temperature kills all living cells and viable spores due to destrructive oxidation of the cell contents.

- Precautions in using hot air oven:
  - Should not be overloaded.
  - There should be space between the articles.

- Materials should be wrapped in brown or kraft paper and paper should not touch the wall of oven as it is at a high temperature.
- Dimension of package should not be more than 4x4x12 inches.
- In case of powders or oil, the depth shouldn't be more than 2 inch in the tube.
- Before keeping the articles the hot air oven should be at operating temperature and then the time is set.

Moist heat:

In this method organism has to be in contact with moisture . Mechanism of killing of bacteria is coagulation of protein. This can be done in 3 ways.

a) At temperature $< 100^0$ C (pasteurization).
b) At temperature $= 100^0$C (Boiling)
c) At temperature $> 100^0$C (Autoclaving)

Pasteurization and boiling are not strictly sterilzaion process.Autoclaving is mostly used because it is less time consuming and also requires less temperature. ie. at 121^0 C for 15 minutes, culture media , aqueous solutions, clothes, rubber and other materials that would be destroyed by dry heating are sterilized by moist heating in autoclave.

- Precautions:
- The open end should be plugged with non adsorbent cotton.
- Should not be overloaded.
- Air must be displaced by steam.
- Time must be noted after applying pressure.

Advantages:

1. Moist heat is more efficient than dry heating because in dry air conduction is less rapid than in wet air.
2. Dry air requires longer time and death rate is less when compared to that of moist heating.
3. Moist heat kills all living cells by bringing about coagulation of structural proteins and essential enzymes at considerably lower temperature than in dry heating with out affecting the quality of the sterilization.
4. Superheated steam releases the latent heat (which represents approximately 80% of the total heat energy) on contact with a cooler surface, there by increasing peneration into the substances to be sterilized.

Plugging of test tubes:

For sterilization of test tube culture media , autoclave is one of the best methods. The culture tubes are plugged with non absorbent cotton wool before placing in the autoclave.Then it is kept in an autoclave for 15 minutes at 121^0c under a pressure of 15 psi. such plugging allows steam to flow in the medium. The plug should not be too light, which creats difficulties in opening while transferring or inoculating cultures.

Instructions:

- Before plugging the glass ware should be dry.

- When non absorbent cotton is not available, absobent cotton may be used but the plugging should be covered with a piece of kraft paper over the top and around the neck of the vessel to prevent the cotton from getting moist by the condensation of steam when sterilized in an autoclave.
- The plug should not be removed until required for the purpose of pouring of medium into tubesor flask or inoculating the medium with any culture only under aseptic conditions.

Plugging of pipette:

Graduated pipette which required for microbiological work need to be sterilized and hence plugging of cotton at the wide end is necessary to prevent entry of microorganisms into the mouth. A mouth piece may be an additional safety method. The plugged pipette is sterilized by wrapping it with a long thin piece of kraft paper with ends of kraft paper twisted and tied. They should not be unwrapped until use.

Wrapping and sterilization of the petri dish:

Before sterilization , clean and dry petri dishes should be wrapped individually or in groups of 4-5 with kraft paper and securely tied with a piece of string. This will prevent any contamination after sterilization. In this way these can be stored for a number of days and should not be unwrapped until use.

Wrapping and sterilization of the syringe:

The body, needle and piston are wrapped seperately and tied with a thread. They are also sterilized by hot air oven and before sterilization they should be neatly wrapped in kraft paper and then loaded into hot air oven and then temperature of 160^0 C for $1^{1/2}$ hrs is maintained to completely sterilize the syringe and all other precautions are taken while handling the hot air oven.

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CULTURE MEDIA INTRODUCTION

Microorganisms draw all the necessary nutrients for synthesis of their cell materials and for the generation of energy from the environment alone. These substances are termed nutrients. In nature microorganisms grow as a mixed population drawing their required nutrients from different sources available in nature. But when we want to grow them in lab we must provide these nutrientsin the culture medium in quantities appropriate to the specific requirements of the microorganisms. As microorganisms are very diverse in their nutritional requirment there are literally hundreds of different types of media. The major requirements are found to be water, oxygen, Carbon, Nitrogen, Phosphorous and sulfur. Water alone accounts for 80-90 % of the total weight and the next six elements accounts for about 95% of the cellular dry weight. The physiological functions of these elements accounts for about 95% of the cellular dry weight. The physiological functions of these elements are specific for construction of all cell materials. The minor nutrients are iron, copper, magnesium , sodium , calcium etc. Some microorganisms require certain more organic compounds as specific growth factors like amino acids, vitamins etc. for their metabolism. Therefore one must know the forms of life of certain microorganisms while preparing the medium for culturing and also what nutritional requirement theseorganisms prefer for their growth.

Culture medium is a mixture of nutrients used in the laboratory to support growth and multiplication of microorganisms. The survival and continuous growth of microorganisms depend on an adequate supply of nutrients and favourable growth envoronment. The nutrient on which microorganisms are grown in the laboratory is known as a culture medium and the growth itself is called a culture. Media are used for cultivation, isolation and seperation of microorganisms and sterility testing. The characteristics of an ideal culture media are:

1. It must give a satisfactory and rapid growth from a single inoculum.It should maintain sterility through out the experiment.
2. It should be reasonably cheap and easily reproducible.
3. It should maintain pH during storage and transport.

<u>General types of Media</u> :-

Media used for the growth of microrganisms are classified as follows.

I) On the basis of state:-
   a) Liquid media - eg. Nutrient broth.
   b) Semi solid media - eg. Nutrient broth + 0.5% agar.
   c) Solid media - eg. Nutrient agar.

II) Based on composition of media :-
   a) Simple media or basal media - eg. Peptone water, nutrient agar.
   b) Complex media - eg. Fluid thioglycollate media.
   c) Synthetic media or defined media - eg. Ashby's medium.
   d) Special media.
      i) Enriched media - eg. Blood agar, egg media , chocolate agar.
      ii) Enrichment media - Selenite – F broth, tetrathionate broth.
      iii) Seletive media - eg. Deoxycholate citrate medium which contains nutrient agar.

iv) Indicator media - eg. Wilson and Blair media for Salmonella typhi.
v) Hiss's serum - eg.sterile water with 25 % serum.
vi) Transport media - eg. Stuart's transport media , Amies transport media.
vii) Sugar media - eg. Peptone water+ 1% sugar.
viii) Storage media - eg. Robertson cooked meat media.

III) a) Aerobic culture media.
b) Anaerobic culture media
c) Facultaive culture media.

Peptone, Casein hydrolysate , meat extract, malt extract , blood , serum, agar , yeast extract and water are the common ingredients for different types of culture media. Culture media gives artificial environment stimulating natural conditions necessary for growth of bacteria. The basic requirement of culture media contains energy, carbon, and nitrogen sources, salts like sulphates, phosphates, chloride and carbonates of sodium , potassium, magnesium and Calcium. Culture media must provide satisfactory temperature and pH , and adequate oxidation- reduction potential.

Basal ingredients of culture media: Various ingredients , some of which are of plant origin and animal substances are used in natural media. Often some chemical substances are added to the natural media to fortify nutrition. In a synthetic medium ingredients are mainly chemical substances of known quantities. In special cases , when required to study metabolism and other biochemical factors, some growth factors such as vitamins , amino acids , hormones etc, are also added to the synthetic media.

1) Protein hydrolyzates:- A wide variety of sources are generally used to provide protein hydrolysis. Plant animal and milk proteins are also used. They are concentrated and hydrolyzed using heat with acid. Eg. Meat protein – peptone, milk protein – casein hydrolyzate. These are rich in nitrogen and carbohydrate.
2) Meat extract and inclusions: Meat or beef is extracted in water and concentrated into semi solid mass or powder. Meat extract and meat infusions for microbiological use should not contain any toxic substances and the contents of metal should be low. The extract should be free from fermentable carbohydrates. These substances are rich in minerls , organic micronutrients , protein derivatives , carbohydrates and vitamins.
3) Yeast extract: Yeast extract is prepared by autolysis or plasmolysis of cells of saccharomyces sp. by heating the cell in water at 50 -60^{0}C for 30 minutes and concentrate the extract. It is available in powder and paste form.
4) Agar:- Agar is a solidifying agent used for getting solid medium. These are rich in mineral and organic nutrients. It dissolves in water at 100^0 C and forms a gel at temperature below 45^{0}C. agar is a polysaccharide , which is extracted from several red sea weeds. It is a mixture of two polysaccharides agarose and agaropectin.
5) Gelatin:- Gelatin is prepared from bones, which is defatted and demineralized to yield a low ash bone matrix. This is also used for solidification.
6) Carbohydrates: various types of carbohydrates are used in media as energy sources for heterotropic microorganisms . The complex carbohydrates are polymers of the simple sugars or their derivatives and are present in plant cell in various forms.

Prepartion of culture media for microorganism involves the following steps.

- Ingredients in their required quantities are dissolved in appropriate volume of distillled water. The pH of the dissolved media is adjusted.
- The medium is dispensed into suitable containers whose mouths are then closed with cotton plug or metal caps.

- The medium is then sterilized by moist heat, generally by autoclaving . Some heat labile substances such as blood, sera , antibiotics and growth regulating substances that may be denatured by heating are added to the sterilized media under aseptic conditions . Heat labile substances are sterilizied by filteration . Blood should be warmed at 37^0c in water bath beforeadding to molten media at 45^0C.

- The pH of the media should be critically adjusted speciifc for the particular medium.

Experiment No :5
Nutrient broth

AIM :- Preparation and sterilization of Nutrient broth.

Requirements

Chemicals: Beef extract, peptone, sodium chloride, 1N HCL, 1N NaOH.

Apparatus : Conical flask, test tubes, measuring cylinder, glass rod , cotton.

Equipment's: Autoclave, oven, pH meter.

Principle:-

A broth media may be defined as a sterile liquid nutrient solution which does not have any solidifying agent. Broth cultures are ideal where growth of microbes are studied by turbidometric method. Nutrient broth is the general liquid media used for the cultivation of bacteria that contain beef extract and peptone.

Sl.No.	Nutrient Agar	Quantity required	Quantity used
1.	Beef extract	10 gm	
2.	Peptone	10 gm	
3.	Sodium chloride	5.0 gm	
4.	Distilled water to make	1000ml	

P^H - 7.0

Peptone- Peptone consists of water soluble products obtained from lean meat or other protein material such as heart muscle, casein, fibrin, or soya flour, usually by digestion with the proteolytic enzymes, pepsin, trypsin, or papain. The important constituents are peptones , proteoses, amino acids, a variety of inorganic salts including phosphate, potassium and magnesium. It also contains nicotic acid and riboflavin.

Beef extract:- Beef extract is an aqueous extract of lean beef tissue concentrated to a paste . It contains the water soluble substances of animal tissue which include carbohydrates , organic nitrogen compounds, water soluble vitamins and salts.

Yeast extract :- Yeast extract is prepared from washed cells of brew's or baker's yeast. Nutritionally yeast extract is the source of vitamin B, carbohydrates, minerals , amino acids and peptides. Yeast extract is used mainly as a comprehensive source of growth factors and may be substituted for meat extract or beef extract in culture media.

Water:- Tap water is suitable for culture media, if it has a low mineral content. But otherwise distilled water or deminerilized water may be used.

Procedure:-

- Put the weighed amount of beef extract , sodium chloride and peptone in required quantity of distilled water.
- Heat the mixture and agitate with glass rod to dissolve the ingredients.
- Add distilled water to make up the final volume.
- Adjust the pH of the medium to 7.0 by adding acid or alkali.
- Pour 10 ml of medium in each test tube or a conical flask may be used instead of test tube depending on the requirement.
- Apply cotton plugs to all test tubes or the conical flask.
- Sterilize in auto clave at 121^0C under 15 lbs pressure for 15 minutes.
- Allow the autoclave to cool.
- Remove the broth tubes or flask and store at room temperature for use.

Precautions.

Chemicals should be handled carefully and weighed on butter paper.

1. Check the sterility of media after incubation at 37^0C for 24 hours.
2. Cotton plugs are to be kept loose when autoclaving.
3. Prepare and store the medium in dust free environment.
4. Label the media with date and name.
5. Do not fill test tubes or flasks completely with the medium.

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Experiment No :6

Nutrient Agar

AIM :- To prepare and submit the sterilized nutrient agar.

Apparatus required:- Beaker, conical flask, pressure cooker, bunsen burner, petri dish, stirrer, culture tube and cotton.

Chemicals required :- Agar, Nacl, peptone, distilled water, beef extract.

Principle:- Nutrient agar is nutrient broth solidified by the addition of 1-2% agar. In addition to liquid media , solid and semi solid media are widely used for cultivation of bacteria. Solid media are useful for isolating bacteria or for determining the characteristics of colonies. Semi solid media are prepared with agar at concentration of 0.5 % or less and are useful for the cultivation of microaeroplilic bacteria and for the detection of bacterial motility.

Agar is a complex polysaccharide (carbohydrate) consisting of 3,6- anhydro- L- galactose and D-galactopyranose, produced from various red algae belonging to Gelidium, Gracilaria and Pterocladia. It melts at 95 to 100^0C and solidifies at 40- 45^0C. It does not provide any nutrition to the microorganisms and it acts only as solidifying agent. It is not metabolised by any pathogenic bacteria.

Solid media can be placed in test tubes, which are then allowed to cool and harden in slanted position , producing agar slants . The slant provides a growth surface and is an ideal way of maintaining cultures for study. Similar tubes are allowed to harden in the upright position designated as agar stabs. Agar stabs are used primarily for the study of the gaseous requirements of microorganisms. A deep or pour is liquified stabs usually contains 18 to 20 ml agar medium . The medium is liquified and held in a water bath at about 50 -55^0C. Cultures can be added to it and poured into petri plates producing agar plates, which provide large surface area for the isolation and study of microorganisms.

Sl.No.	Nutrient Agar	Quantity required	Quantity used
1.	Beef extract	10 gm	
2.	Peptone	10 gm	
3.	Sodium chloride	5.0 gm	
4.	Distilled water to make	1000ml	
5.	Agar	20 gm	

Procedure:-

1. Beef extract, Peptone and sodium chloride is dissolved in the appropriate volume of distilled water.
2. pH of the fluid medium is determined with a pH meter and adjusted to 7.0 by using 1 N NaOH.
3. Add agar powder (1.5%) and medium is heated to dissolve the agar to form a clear liquid.
4. The medium is dispensed into tubes (slants, stabs or deep) or flasks .
5. Plug the flasks and test tubes containing medium by using nonabsorbant cotton.
6. Sterilize the media at 121^0c, 15 lbs pressure for 15 minutes in an autoclave.
7. Allow the tubes to cool in a slanting position (for agar slants) and upright position (for stabs).
8. Allow the flasks to cool up to 50^0c and pour the medium quickly in to sterile petri plates under aseptic conditions.
9. Allow the medium to cool and to produce solid agar plates.

Precaution:-

1. Cotton plug are kept when autoclaving.
2. Store in a low temperature and adjust the free environment.

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Experiment No :7

PREPARATION OF MEDIA FOR FUNGI

AIM :- To prepare and submit media for fungi.

Chemicals required:- Glucose, Peptone, Agar, Distilled water.

Apparatus required :- Culture tubes, beaker, conical flask, auto clave, incubator, glass rod and cotton.

Susboraud's Agar	Quantity required	Quantity used
Glucose	20 gm	
Peptone	10 gm	
Distilled water to make	1000ml	
Agar	20 gm	

Procedure:-

1. Glucose and Peptone is added and dissolved in distilled water.
2. pH of the fluid medium is determined with a pH meter and adjusted to 5.4 by using 1N Hcl .
3. Add agar powder (1.5%) and medium is heated to dissolve the agar to form a clear liquid.
4. The medium is dispensed into tubes (slants, stabs or deep) or flasks .
5. Plug the flasks and test tubes containing medium by using nonabsorbant cotton.
6. Sterilize the media at 121^0c, 15 lbs pressure for 15 minutes in an autoclave.
7. Allow the tubes to cool in a slanting position (for agar slants) and upright position (for stabs).
8. Allow the flasks to cool up to 50^0c and pour the medium quickly in to sterile petri plates under aseptic conditions.
9. Allow the medium to cool and to produce solid agar plates.

Experiment No:8

Potato Dextrose Agar

AIM:- To prepare and submit potato dextrose Agar.

Apparatus required :- Beaker, conical flask, stirrer, aluminum foil, cotton.

Chemicals Required :- Potatoes, Dextrose, Agar, Distilled water.

Procedure:-

1. 20 gm of potato is skinned, sliced and boiled in 20 ml water.
2. They are mashed (crushed) by glass rod and filtered by muslin cloth to obtain potato extract.
3. It is then boiled with water to dissolve the contents & volume is made up with distilled water.
4. pH is adjusted to 7.0 and potato dextrose agar is sterilized by moist heat sterilization.

Sl.No.	Ingredients	Quantity required	Quantity used
1.	Peeled potato	250 gm	
2.	Glucose	20 gm	
3.	Agar	15gm	
4.	Distilled water	1000ml	

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BACTERIAL STAINING INTRODUCTION

Microorganisms are semitransparent , as the refractive index of microbes is approximately equal to the refractive index of the surrounding environment. Hence bacteria are not easily observed under the microscope in the unstained state .

Stains are used :-

1. To render microscopic and semitransparent objects visible.
2. To study the morphology (size, shape and arrangement of microorganisms).
3. To observe the presence of various internal and external structures (capsules, spore, flagella etc.)
4. To produce specific physical or chemical reactions.

The terms stains and dyes are generally used interchangeably by the biologist but they are not the same. A colouring agent that is to be used for general purposes is called a dye and used for biological is called a stain.

Stain is defined as an organic compound containing both chromophore and auxochrome groups linked to benzene ring. Chromophore group imparts colours to stain. Any substance which possesses only chromophore group is not a stain. To behave as stain, it must have an affinity to bind with cells or tissues. The group that imparts ability to binding tissue is auxochrome. The electrolyte dissociation of auxochrome group helps to bind the stain with the cell.

Stain:-

A) Acidic stain (anionic stain) :- A stain that possesses the negatively charged chromophore and therefore has a strong affinity for the positive constituents of the cell. Proteins, positively charged cellular components readily bind to and accept the colour of the negatively charged ,anionic chromogen of an acidic stain. Eg. sodium eosinate, picric acid, nigrosin, congo red , roseBengal etc.

B) Basic stain (cationic stain) has a strong affinity for the negative constituents of the cell. Nucleic acid, negatively charged cellular components readily bind to and accept the colour of the positively charged, cationic chromogen of a basic stain. Eg. methylene blue, safranin, crystal violet , malachite green etc.

A number of staining techniques are available for visualization , differentiation and separation of bacteria. A summary of commonly used staining techniques and their purposes is out lined.

All microbiological staining procedures require preparation of smears prior to the execution of any of the specific staining techniques listed above. The technique requires adequate care in it’s preparation.

1. Cleaning of glass slide :-
   Clean slides are required for preparation of microbial smears. Grease or oil from fingers on slides must be removed by washing the slides with soap solution. Slides are rinsed with water and followed by 95% alcohol. After cleaning, dry the slides and placed them on laboratory towels until ready for use.
2. Preparation of Smears:-
   A thin dried film of bacterial culture on glass slide prepared for staining is referred as smear. Good thin smear is prepared by avoiding thick and dense smears. Those made from broth culturesor cultures from a solid medium require variations in technique.

a) Broth cultures :- A target circle of approximately 1. 5 cm diameter is marked on the underside of a slide with a glass marking pencil. Aseptically transfer a loopful of bacterial suspension on the circled area and spread evenly on area of target circle with the help of nichrome wire loop. Allow the slide to air dry completely.

b) Cultures from a solid medium:- Microorganisms cultured in a solid medium produce thick, dense surface growth. These cultures must be diluted by placing a loopful of water on the glass slide . Transfer of cells from the culture requires the use of sterile inoculating needle. Only the tip of the needle should touch the culture to prevent the transfer of too many cells. The culture suspended in water drop on the glass slide and spread evenly on the area of target circle with the help of nichrome wire loop. The smear must be allowed to dry completely.

3) Fixation of smear:- Smear fixation causes bacteria to adhere to slide that they can be stained and observed . Generally, heat is used for fixation. In heat fixation, bacterial proteins are coagulated and fixed to the glass surface. Heat fixation is performed by the rapid passage of the air dried smear two or three times over the flame of the Bunsen burner.

Experiment No :9

SIMPLE STAINING

AIM :- To study the bacterial morphology by simple (monochrome) staining.

Requirements:-

Cultures :-

Stains:- Methylene blue or crystal violet or carbol fuchsin.

Apparatus:- Staining tray, glass slide, Bunsen burner, inoculating loop, lens paper, glass marker.

Equipment :- Microscope.

Principle :- The use of single stain to colour the bacteria is commonly called as monochrome staining (mono- single, chrome- colour).

The surface of the bacterial cell has acidic characteristics because of a large amount of carboxyl groups located on the cell surface due to acidic amino acids. There for when ionization of the carboxyl groups takes place it imparts negative charge to the cell surface.

ionisation

$COOH \rightarrow COO^{-} + H^{+}$

In nature, H^{+} is replaced by another positive charged ion . eg. Na^{+} or K^{+} and H^{+} bonds with oxygen to form water . Thus surface of an unstained bacterial cell is represented as shown below.

Basic dyes are commonly used for the monochrome staining . These dyes are available as a salt of acids eg. methylene blue chloride . When methylene blue rehydrates, it ionizes to form methylene blue and chloride ions. The positively charged ion have the colouring property .

On addition of methylene blue for staining , exchange of MB^{+} with Na^{+} on the bacterial cell surface takes place , resulting into ionic bond formation between MB^{+} and cell surface. Thus when colouring agent forms ionic bond with cell or cell components, it result into the staining of cell . The most commonly used basic stains are methylene blue (2- 3 minutes), crystal violet (1-2 minutes) and carbol fuchsin (15 to 30 seconds)

Procedure :-

1. Prepare separate bacterial smears of each microorganisms and fix it by heat.
2. Place a slide on the staining tray and flood the smear with one of the indicated stains , using the appropriate exposure time .
3. Pour off the staining solution and wash the slide in running tap water .
4. Dry the slide between blotting papers and examine the stained smear under microscope using oil immersion objective.

Composition of staining reagents:-

1. Loefflers methylene blue :-
   - Methylene blue - 0.2gm
   - Absolute alcohol -10ml
   - Distilled water - 90ml

   Procedure:- Dissolve in alcohol add water and filter.

2. Crystal violet :-
   - Solution A
     - Crystal violet -2gm
     - Ethyl alcohol (90%) -20 ml
   - Solution B
     - Ammonium oxalate -0.8 gm
     - Distilled water - 18ml

   Procedure:- Mix solutions A and B store for 24 hours.

3. Safranine :-
   - Safranine - 2.5 gm
   - Alcohol (90%) -10ml
   - Water -90 ml

   Procedure:- Dissolve in alcohol and water.

Procedure for staining:-

1. Clean the plane slide thoroughly with cleaning powder and wash with water. Heat the slide gently to destroy organisms adhering to it. Again wipe it with clean cotton piece.
2. Sterilize the inoculation loop, transfer a loop full of distilled water on to the slide.
3. Transfer a small quantity of colony on to the drop aseptically with the help of a loop and make a suspension of organism in water.
4. Spread the suspension with inoculation loop to make a smear.
5. Fix the smear by gentle warming by passing the slide over the burner.
6. Flood the smear with stain and allow it to stand for 2 minutes.
7. Wash the excess stain by exposing it to a thin stream of distilled water. Dry the slide in air after wiping with blotting paper.
8. Observe the slide under oil immersion lens using Cedar wood oil (100x).

Experiment No :10

Negative Staining

AIM : To carry out the negative staining of the given microorganisms.

Apparatus: Slides, acidic dye, bacterial culture, Inoculation loop, Bunsen burner, Cedar wood Oil, Microscope etc.

Discussion : The principle involved here is that when an acidic dye is added , the bacteria form a complex with the cations of the dye which are colourless and the anion of the acidic dye, which is coloured, gives a coloured background. Hence the bacteria remain colourless over a coloured background and can be observed under the microscope.

Bacteria + (Cations) + (anions) → (bacteria) (Cations) + anions

Colourless complex coloured

Negative staining has certain advantages over positive staining.

1. In Positive staining, fixation of the bacteria is done to the slide by slightly warming. This may change the shape of the bacteria, which is prevented in case of negative staining.
2. Slightly acidic bacteria, with basic dye when stained , do not get properly coloured or stained, as they are less acidic. Therefore for such organism negative staining can be done. Eg. lower class of bacteria like Spirochetes.Negative staining is also done for capsulated bacteria to study the capsule. Some of the acidicdyes are Nigrosin, Eosin, Indian ink etc.

The disadvantage of the negative staining is that when the smear is kept for a long period of time, the cells may shrink which is not seen in case of positive staining.

Procedure:

1. Take a clean slide and dry it.
2. Place a drop of the acidic dye at the end of the slide.
3. With the help of an inoculation loop following aseptic technique add a loop full of organism and mix it with the dye.
4. With the help of another clean slide spread the suspension by dragging it along the first.
5. Dry the smear in air, add a drop of Cedar wood oil and observe under the microscope using 100 X objective.

Experiment No :11

Gram's Staining

AIM :- To study the morphology of a given organism by Gram's staining method.

Requirements:- Microscope, plane slide, Bunsen burner , Inoculation loop, Staining material , culture tube containg organisms.

Discussion :- Gram's staining is an emperical method of differentiating bacterial species into two large groups (Gram – positive and Gram negative based on the chemical and physical properties of their cell walls. The gram staining is almost always the first step in the identification of bacterial organism. While Gram staining is a valuable diagnostic tool in both clinical and research settings , not all bacteriacan be definitively classified by this technique , thus forming Gram variable and Gram indeterminate groups as well.

Mechanism:-

Gram positive have a thick mesh like cell wall made of peptidoglycan (50 – 90% of cell wall), which stains purple while Gram negative bacteria have a thinner layer (10 % of cell wall), which stains pink. Gram negative bacteria also have an additional outer membrane which contains lipids , and is seperated from the cell wall by the periplasmic space. There are four basic steps of the gram stain , whichinclude applying a primary stain (crystal violet) to a heat fixed smear of a bacterial culture, followed by the addition of a trapping agent or mordant (Gram's iodine) rapid decolorization with alcohol or acetone, and counter staining with safranin.Crystal violet (CV) disssociates in aqueous solution into CV^+ and chloride (Cl^-) ions. These ions penetrate through the cell wall and cell membrane of both Gram+ ve and Gram – ve cells. The CV^+ ion interacts with negetively charged components of bacterial cells and stains the cells purple. Iodine (I^- or I_3^-) interacts with CV^+ and forms large complexes of crystal violet and iodine (CV-1) within the inner and outer layers of the cell. Iodine is often referred to as a mordent, but is a trapping agent that prevents the removal of the CV-I complex and therefore colour the cell.

When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram –ve cell will lose its outer lipopolysaccharide membrane and the inner peptidoglycan layer is left exposed. The CV-I complexes are washed from the Gram –ve cell along withthe outer membrane . In contrast , a Gram +ve cell becomes dehydrated from an ethanol treatment. The large CV-I complexes become trapped with in the Gram +ve cell due to the multilayered nature of it's peptidoglycan. The decolorization step is critical and must be timed correctly; the crystal violet stain will be removed from both Gram +ve and Gram -ve cells if the decolorizing agent is left on too long.

After decolorization , the Gram +ve cell remains purple and the Gram –ve cell loses it's purple colour. Counter stain , which is usually +vely charged safranin or basic fuchsin, is applied at last to give decolorized Gram –ve bacteria a pink or red colur.

Gram positives sometimes get converted to Gram –ve which may be due to

a) Removal of magnesium ribonucleic acid.
b) Rupture of the cell wall.

c) Treatment with lysozymes or antibiotics.
d) In old cultures.

Examples of Gram+ve bacteria	Gram-ve bacteria
Bacillus anthracis (Anthrax)	Bordetella pertussis (whooping cough).
Clostridium tetani (tetanus)	Escherichia coli (Urinary infections).
Clostridium botulinum (Botulism)	Haemophilus influenza.(Meningitis).
Coryne bacterium diphtheria(Diphtheria)	Klebsiella pneumonia.
Staphylo coccus aureus (Boils, carbuncles, sepitcemia).	Neisseria gonorrhoeae.
Strepto coccus pyrogenes (scarlet fever, rheumatic fever, septicaemia).	Salmonella typhi (Typhoid fever).
Strepto coccus pneumonia (Pneumonia)	Vibrio cholera (cholera).

Mycobacterium leprae (leprosy) & mycobacterium tuberculosis (tuberculosis) are gram variable ie.

Either +ve or –ve.

Primary stain :- Crystal violet (Crystal violet + Ammonium oxalate + distilled water)

Mordant :- Gram Iodine (1gm of Iodine + 2gms of KI+ 3gm. $NaHCO_3$ + distilled water)

Decolorizing agent :- alcohol

Secondary stain :- Safranine (2.5 gm of solid safranine+ alcohol+ distilled water)

Procedure:-

1. Clean a plane slide thoroughly with cleaning powder and then with 10- 12 drops of chromic acid . Warm it over the flame of burner to destroy organic adherents. Wipe with clean cotton.
2. Smear preparation: Aseptically transfer a loop full of distilled water on to the center of slide. Sterilize the loop and transfer a small quantity of the colony. Prepare a suspension by mixing . Spread to form a thin film. Fix the smear by gentle heating.
3. Cover the smear with crystal violet . Let stand for 20- 30 seconds.
4. Briefly wash off stain in a gentle stream of water.
5. Cover the film with gram's iodine and allow it to act for 1 minute (30- 60 seconds).
6. Rinse with water.
7. Decolorize with 95% alcohol or acetone for 10-20 seconds. This step is critical . Thick smears requires more time than thin ones. Decolorization has occurred when the solvent flows colorlessly from the slide.
8. By the end of 20 seconds rinse the slide with water.
9. Counter stain with safranine for 20- 30 seconds . Rinse with water and blot dry.
10. Examine under oil immersion objective.

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Experiment No :12

MOTILITY TESTING

AIM:- To carry out the motility studies of the given organism by Hanging drop method.

Requirement :- Microscope, inoculation loop, cavity slide, cover slip, bunsen burner, liquid paraffin, culture of organism (24 hrs).

Principle:-

Two general techniques are used to prepare specimen for light microscopic examination. They are.

1. Wet mount technique: The organisms are suspended in a liquid.
2. Hanging drop method: This will help in the observation of motility of an organism.

Motile strains of bacteria possess filamentous appendages known s flagella which effect screw like propulsive movements and act as organs of locomotion. A flagellum is a long , thin filament , twisted spirally in an open regular wave from it is about 0.02μm thick and is usually several times the length of a bacterial cell. According to the species there may be one to several flagella per cell in an elongated bacteria the arrangement may be peritrichous or lateral when they originate from sides or polar when they originate from both ends. They consist largely or completely of flagellin, a protein.

Wet preparation permits the exhibition in normal living conditions. A wet mount is made by placing a drop containing the organism on a slide, and is covered with a cover slip. To reduce the rate of evaporation and also to exclude the effect of air current the cover slip may be lined with petroleum jelly or similar material. The slide is used for observing the movement of bacteria.Alternatively a microbial suspension is placed on the cover slip. The slide with a concave depression lined with petroleum jelly is inverted over the drop to produce a hanging drop of the specimen.

Motility may be observed either microscopically or by observing the occurance of spreading growth in semi solid agar medium. On microscopic observation of wet fibers the motile bacteria are seen swimming in different directions across the field with a darting, wriggling, tumbling movement. True motility must be distinguished from a drifting movement of bacteria in a single direction due to the current in the liquid medium and also from Brownian movement, which is a rapid oscillation of each bacterium with in very limited area due to the bombardment by the water molecules.

Different types of movements of Protists: Basically four types of motility are seen in case of protists. They are:

1. Amoeboid movement :- The amoeba thrusts out a pseudopodium and moves by flowing into the projection. This type of movement is found in protozoan and is known as Amoeboid movement.
2. Gliding movement:- This type of motility is found in the blue green algae. It consist of slow, steady ‘to and fro’ movement as progressive gliding of algae cell in contact with a solid surface.
3. Rotatory movement:- The rotatory movement is seen in spirochetes . Where two axial filaments arising from opposite ends of cells meet to join at center of the cell these filaments appear structurally more complex than bacterial flagella.
4. Brownian movement:- This occurs in case of motile bacteria in which there will be rapid oscillation of each bacterium within the limited area. The bacteria also move because of the continuous ment

Function :- Though it is not exactly clear as what the bacteria desires for being motile, it may be beneficial in increasing the rate of uptake of nutrients solutes by continuously changing the environmental fluid in contact with the bacterial cell this movement ensures at least some cells of the strain to reach every locality suitable for colonization. Moreover it ensures migration of bacteria to favorable area from unfavorable area. Motile aerobes show movement to regions with higher oxygen concentration. Whereas anaerobes migrate away from such region. Other bacteria move towards regions with higher nutrient concentration. It may be supposed that the power of active location will assist pathogenic bacteria in penetration through mucous secretions and epithelial barrier and in spreading throughout the body fluid and tissue, although non motile pathogenic bacteria like Bruscella, Strepto cocci.

Procedure:-

1. Clean the slide and cover slip with cleaning powder. Then gently heat over a flame to destroy possible organic adherents. Then the slide is wiped with cotton. Apply grease or wax uniformly on the periphery of the cavity of the slide.
2. A drop of the culture broth is taken on the cover slip. Invert the cavity slide on the cover slip, invert back the slide so that the cover slip is on top of the slide to obtain a hanging drop.
3. Examine the drop under the high power objective of a microscope.

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PURE CULTURE TECHNIQUES INTRODUCTION

Microorganisms are isolated from various substrates or sources like soil, air, fresh water, sea water, food, body surfaces etc. Microbes in nature are found in mixed populations . In the laboratory , these populations can be separated into pure cultures . These pure cultures are suitable for the study of their morphological, cultural , biochemical , serological and genetic properties . In pure culture techniques, colonies are individual , isolated, visible masses and representing the multiplication of a single organisms . A culture that contains only kind of microorganisms is called a pure culture. A culture which contains more than one kind of microorganism is called a mixed culture.

The technique to obtain pure cultures may be classified as follows .

A) Common methods:
   1) Streak plate method.
   2) Pour plate method (loop dilution and serial dilution)
   3) Spread plate method.
B) Special methods:
   1) Single cell isolation (capillary pipette method and micromanipulator method).
   2) Enrichment method
   3) Selective media method
   4) Differential media method

Pure cultures are mainly studied by following tests such as colony characteristic, morphology, biochemical tests, staining reactions, immunological reactions etc. Most microbiological laboratories usually maintain a large collection of pure cultures as well as subcultures of authentic species purchased from various culture collection centres.eg. National collection of industrial bacteria (NCIB), Nationalchemical laboratory (NCL) , India etc.

Experiment No :13

STREAK PLATE METHOD

AIM:- Isolation of pure cultures by using streak plate

method.Requirements:-

Cultures:-

Chemicals:- Peptone, , beef/ yeast extract, NaCl, agar, alcohol.

Apparatus:- Petri plate, conical flask, beaker, test tube, nichrome wire loop, glass marking pencil.

Equipment:- Auto clave, hot air oven, balance, pH meter.

Principle:-

Inoculation of microbial culture to the surface of the sterile agar plate and spreading it by an inoculation wire loop is called streak plate method. Streak plate techniques is used for the cultivation, isolation and separation of microorganisms from mixed populations. Streak plate technique is used to produce well separated colonies of bacteria from mixed suspension and the size, shape, colour and other physical characteristics of isolated colonies are studied.

Procedure:-

1. Prepare 30ml of nutrient medium and sterile by 121^0C, 15 lbs for 15 minutes.
2. Transfer the sterile medium (about 15 to 20ml) into a Petri dish carefully. Rotate the petri plate to allow for uniform distribution of the medium under strict asepticconditions and allow to solidify.
3. Take a small amount of bacterial inoculum by nichrome wire loop from agar slants or agar plates. Carefully lift the lid and streak the culture on the agar medium .
4. Streak the culture as a first zone by using inoculum and then flame the wire loop to destroy the culture present on wire loop.

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Experiment No :14

POUR PLATE TECHNIQUE

AIM:- Isolation of cultures by pour plate .

Requirements:-

Culture:-
Chemicals:- Peptone, beef/yeast extract, Nacl, agar, alcohol.
Apparatus :- conical flask, test tube (big size), petri plate, nichrome wire loop, beaker, test tube rack.
Equipment :- Autoclave, hot air oven, pH meter, balance, water bath, thermo meter.

Principle:-

In the pour plate method the mixed culture is diluted directly in tubes of liquid (cooled) agar medium. The medium is maintained in a liquid state at a temperature of 42 to 45 ^{0} c allow proper distribution of the inoculum. The medium is then poured into petri plates and allowed to solidify. Incubate all plates under suitable conditions for the growth of microorganisms. A series of agar plates showing decreasing number of colonies resulting from the dilution technique. In this technique proper distribution of colonies are observed. If this technique is performed by serial dilution (by using pipette) method.

The pour plate technique has a number of disadvantages.Some of the microorganisms are trapped beneath the surface of the medium when it is in the liquid form. Hence surface and subsurface colonies are developed. It is very difficult to remove or transfer the subsurface colonies to fresh media.

1) Microorganisms used for isolation must be able to withstand temporary exposure to the 42to 45^{0}C temperature of the liquid agar medium. Hence pour plate method is unsuitable for isolation of psychrophilic microorganisms.

Procedure:-

1. Prepare nutrient agar medium and transfer 15 to 20 ml medium in each test tubes.
2. Sterilize all test tubes in autoclave at 121^0, 15lbs for 15 minutes.
3. Simultaneously sterilize three petri plates in a hot air oven and mark by using glass marking Pencil A, B, and C.
4. Cool all test tubes to 42 to 45^0 C temperature.
5. Transfer one loop full of the culture in first test tube(A). Gently mix the culture by rolling the tube between the palms.
6. Transfer one loop full of the culture from first tube (A) to second tube (B) and mix as first inoculated tube.

Experiment No: 15

COLONY CHARACTER

AIM- To study the colony characteristics of bacteria in agar plates.Apparatus

Requirement: - Petri dish, 50ml capacity conical flask, Bunsen burner.

Principle:- Microorganisms are exhibiting differences in the microscopic appearance of their growth on different types of media. These differences are called as cultural characteristics.

Characteristics such as shape, size, pigmentation, form, elevation, growth pattern etc. provides useful information for identification of microorganisms. They are determined by inoculation of microorganisms on nutrient agar slants, plates, in nutrient gelatin.

Colonies on agar plate:-

The well isolated colonies on nutrient agar plates are studied as follows.

a) Size:- Colonies range in size from extremely small (pin point) , measuring a fraction of a mm in diameter to large colonies measuring 5 to 10mm in diameter. Depending on size of colonies, they are classified as pinpoint, small, moderate and large.
b) Form:- The total appearance of the colony is called form. The shape of the colony is described as follows.
    1. Circular- like circles 2. Irregular- no definite shape 3. Rhizoid – root like spreading growth.
c) Margin or edge :- The appearance of the outer edge of the colony is described as
    1. Entire- Sharply defined, smooth, even margin 2. Lobate- marked , lobed margin.3. undulate- wavy margin 4.Serrate- tooth like appearance 5. Filamentous- thread like appearance.
d) Pigmentation or chromogenesis:- Many microorganisms produce intracellular water insoluble pigments and these pigments impart various colouration to the colonies eg. Staphylo coccus aureus (gold).

Some microorganisms produce water soluble pigments that diffuse into the surrounding agar and stain it. Eg. Pseudomonas aeruginosa forms a blue water soluble pigment called pyocyanin. Some water soluble pigments are fluorescent that colonies glows white or blue –green when exposed to ultra violet light. Pseudomonas aeruginosa produces pyoverdin fluorescent pigment.

e) Elevation :- The surface view of the colony is called elevation. The following types of elevationare seen in bacterial colonies.
    1. Flat- same level of the medium 2. Raised – slightly above the level of the medium.

    3. Convex- raised above the medium in a convex shape.

    4. Umbonate- raised above the medium with elevated convex in central region.

f) Texture :- colonies may have varied surfaces, depending on the microorganisms.

1. Smooth , shiny or glistening.

2. Rough- dull granular.

3. Mucoid or gummy.

4. Wrinkled or folded surface.

g) Optical characteristics:- Optical characteristics may be evaluated on the basis of the amount of light transmitted through the growth.

1. Opaque - No light transmission.
2. Translucent- Partial transmission of light.
3. Transparent- Full transmission of light.

A) Growth on Agar slant:-

Cultural characteristics of bacteria on agar slants. Pigmentation, optical characteristics and texture of cultures are studied on agar slants as per agar plates.

a) Amount of growth :- The amount of growth is designated as none, slight, moderate or large.
b) Form:- The appearance of the single line streak of growth on the agar slants is designated as follows.
   1. Filiform : continuous and thread like growth.
   2. Echinulate : continuous with pointed outgrowths.
   3. Beaded : Non confluent like beads.
   4. Effuse : Thin, spread over the surface.
   5. Arborescent : branched, tree like growth.

B) Rhizoid: Root like growth.Growth on nutrient gelatin stabs:
The solid medium may be liquefied by the enzymatic action of gelatinase released from microorganisms. Liquefaction occurs in a variety of patters in gelatin stab.

a) Crateriform : Saucer shaper liquefied area.
b) Napiform : Bulbous shaped area at surface.
c) Infundibuiform : Funnel shaped
d) Saccate : Elongated.
e) Stratiform : Complete liquefaction of the upper half of the medium.

C) Growth in nutrient broth:
In liquid medium such as nutrient broth, growth characteristics of microorganisms are evaluated as follows.

a) Amount of growth:- The amount of growth is designated as none, slight , moderate and large.
b) Distribution of growth:- the following types of growth distribution or accumulation are observed.

1) Uniform fine turbidity - Finely dispersed growth through out the media.
2) Flocculant - Flaky aggregates dispersed through out the media.
3) Pellicle - Tick, pad like growth on surface of the broth.
4) Sediment -Granular, flaky or flocculant growth at the bottom of broth.

Procedure:-

1. Prepare 30 ml of the nutrient agar medium and keep it for sterilization in an autoclave.
2. Then prepare a suspension of mud in water and add 1ml of this mud water into the sterilized conical flask containing 30 ml of nutrient agar medium. Mud water should only be added till the content of the conical flask is 50ml.
3. Pour the contents of conical flask into the sterilized petridish in an aseptic area between the two burners.
4. Keep the petri dish for incubation for 24 hours.
5. Observe it after 24 hours and take the observation.

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Experiment No:16

VIABLE COUNT

AIM :- To find the number of viable bacteria present in the given sample of water.

Requirements:- Sterile petridish , syringe , test tube , burner , culture .

Discussion :- Viable counting is done to find out the purity of milk , water, soil etc. The basic principle of this method is that viable spores of cells grow multiply if they are provided with normal growth condition and that each organism grow and multiply ultimately forming a visible mass of organisms called colony. Each organism grows into a separate colony each with characteristic type of growth. Observation of their specific growth characteristics often provides useful information for identification. Each organism grows into a separate colony. The number of colonies present in an agar plate therefore indicates the number of organisms present in the original sample.

To achieve the objective , a measured quantity of diluted sample must be taken. The sample is diluted so that the number of organisms (colony forming units) on each plate falls in the range of 30 -300. It helps to achieve accuracy and reduces the interference of one colony with the other. The total number of viable organisms is reported in terms of colony forming units.

There are many disadvantages for the plate count technique like:

- The only bacteria that will be counted are those which can grow on the medium used and under the conditions of incubation provided.
- One colony may have developed from more than one organism.
- All the organisms may not grow in the nutrient agar medium.
- Spores may not have germinated because we observe after 24 hours of incubation only.
- Since the sample is added around 40- 50 ^{0}C. Organisms sensitive, to this temperature may die.
- The development of one colony from one cell can occur when the bacterial suspension is homogenous and no aggregate of cells are present. But if the cells have the tendency to aggregate like cocci in cluster (staphylo coccus), cocci in chains (strepto coccus) cocci in pairs (diplo coccus).
- The resulting counts will be lower than the number of individual cells.
- For this reason the counts are often reported as colony forming units per ml rather than number of bacteria per ml.

Advantages:

- The plate count technique is used routinely and gives satisfactory results for the estimation of bacteria.
- It is easy to perform and can be adapted for the measurement of population of any magnitude.
- It has the advantage of sensitivity , since very small number of organisms can be counted.

Procedure :

1. Prepare 30 ml of nutrient agar medium and sterilize in a pressure cooker.
2. Then take 1ml of tap water (for dilution) dilute with 9ml of sterile water and from this take 1ml of water.
3. Add 1ml of the diluted sample into a conical flask containing 30ml of sterile nutrient agar cooled to 50^0C.
4. Pour the contents of conical flask into a previously sterilized petri dish in an aseptic area.
5. Keep this petri dish for incubation for 24 hours.
6. Observe it after 24 hours and report the observation.

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Experiment No:-17

Biochemical Reactions : Indole Production.

AIM: To carry out Indole production test.

Apparatus: Test tube, Inoculation loop, Bunsen burner.

Principle:

Certain bacteria which posses enzyme tryptophanase degrade amino acid tryptophan to Indole, pyruvic acid and ammonia. Indole production is detected by inoculating the test organism in peptone water and incubating at 37^0 C for 48- 96 hrs. The kovac's reagent is added and gently shaken. A red colour in the alcohol layer indicates a positive reaction.

Composition of Kovac's reagent:-

p-dimethyl amino benzaldehyde		- 10 g
Amyl or Isoamyl alcohol		-150ml
Con. Hcl		-50ml
Peptone water medium (1% w/v)		
Peptone		- 10 gm
Distilled water	qs to	- 1000ml

Procedure:

1. Inoculate a loop full of microorganism under test to a test tube containing peptone water medium.
2. Disperse the inoculums uniformly by shaking gently.
3. Incubate at 37^0 C for 48- 96 hrs.
4. After incubation, add 0.5ml of Kovac's reagent and shake gently.
5. A red colour in the alcohol layer indicates positive reaction.

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Experiment No: 18

Biochemical Reactions: Methyl Red Test.

AIM: To carry out methyl red test.

Apparatus : Test tube, Inoculation loop, Bunsen burner.

Principle:

This test detects the production of sufficient acid by fermentation of glucose so that pH of the medium falls and it is maintained below pH 4.5.

Composition of Glucose phosphate medium:

Peptone	- 10 gm
Dipotassium hydrogen phosphate (k_2HPO_4)	- 5gm
Glucose	- 5gm
Distilled water	- 5 gm

pH 7.2± 0.2 at 25^0 C

Procedure:

1. Inoculate the test organism in glucose phosphate broth and incubate at 37^0c for 2-5 days.
2. Add 5 drops of 0.04% solution of methyl red , mix well and read the result immediately.
3. Positive tests are bright red (indicating a low pH) and negetaive ones are yellow.
4. If the test is negative after 2 days repeat after 5 days.

Experiment No: 19

Biochemical Reactions: Voges - Proskauer (VP) Test

AIM: To carry out Voges - Proskauer test for Acetoin Production.

Apparatus: Test tube, Inoculation loop, Bunsen burner.

Principle:

Many bacteria ferment carbohydrates with the production of acetyl methyl carbinol (acetoin). In the presence of KOH and atmospheric oxygen, acetoin is converted to dicetyl , and α- naphthol, present in the test , serves as a catalyst to form a red complex. This test is usually done in conjugation with methyl red test. An organism of the Enterobacteriaceae is usually either methyl red positive and VP negative or methyl red negative and VP positive.

Procedure:

1. Inoculate test organism in glucose phosphate broth and incubate at 37^0C for 48 hours.
2. After incubation add 1ml of KOH and 3ml of 5% α- naphthol solution in absolute ethanol.
3. A positive reaction is indicated by the development of pink colour in 2-5 minutes and Crimson in 30 minutes.

Experiment No: 20

Biochemical Reactions: Citrate Utilization Test.

AIM: To carry out citrate utilization test.

Apparatus : Test tube, Petri dish, Inoculation loop, Bunsen burner.

Principle:

This test is used to study the ability of an organism to utilize citrate as a sole source of carbon for growth. Liquid (koser's) and (Simmon's) media containing culture as a so le source of carbon can be used. The ability of an organism to utilize citrate as a sole source of carbon is detected by the production of turbidity (due to growth) in liquid medium. Solid medium also contains bromothymol Blue as an indicator, therefore , on the solid medium the appearance of growth and blue colour is positive; and original green colour and no growth is negative. The blue colour is due to the alkaline pH that results from the utilization of citrate. It turns the indicator in the medium from green to blue. It is important to keep the inoculum light, since dead organism can be source of carbon, producing a false result.

Procedure:

1. Prepare Koser's liquid and Simmon's solid media separately.
2. Inoculate the broth medium with the organism under test. Keep the inoculums light.
3. Incubate at 37^0C for 24- 48 hrs.
4. Turbidity in Koser's medium and change in colour from green to blue, and growth in Simmon's medium indicate positive reaction.

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Experiment No: 21

Biochemical Reactions: Carbohydrate Utilization.

AIM: To carry out carbohydrate utilization.

Apparatus : Test tube, Petri dish, Inoculation loop, Bunsen burner.

Principle:

Bacteria derive energy by fermentation. This involves breaking down of a complex substance into simple ones by the action of enzymes present in bacteria. These enzymes differ from species to species. By the enzymatic activity bacteria can break down various sugars, and carbohydrates , proteins and fat. The end products of fermentation are fairly constant. Therefore these reactions help in the identification of pathogenic organisms. By a process of fermentation the carbohydrates (sugar, hexahydric alcohols, polysaccharides) are split up into acid or simpler substances like CO_2, H_2O etc. For routine work, sugar tubes are used to detect fermentation .These sugar tubes may contain monosaccharides , disaccharides, polysaccharides or alcohol like Mannitol , dulcitol etc. The sugar tubes contain a particular fermentable sugar in peptone water which favors the growth of the organism and Andrade's neutral red indicator indicates acid production, a small inverted tube called Durham's tube for showing production of gas. With the production of acid , the pH of the medium is reduced to pH 6.2 the indicator in the solution shows a pink colour. The production of gas is detected by the presence of air bubbles in the Durham's tube. The acids produced vary from a complex acid like formic acid/ lactic acid and the later in their turn are split up into the CO_2 and H_2O. Certain bacteria can however produce only acid and no gas.

Substrates are used in the sugar tubes in the proportion of 0.5- 1%.

Procedure:

1. Prepare the medium for glucose , sucrose and lactose formation.Dissolve all ingredients properly and indicator should be added ie. bromo cresol purple.
2. Transfer these media into each test tube and label them . keep them for sterilization after putting the Durham's tube in such a way that it is completely filled with the medium without air bubbles.
3. Wrap them in a craft paper and keep for sterilization in an autoclave.
4. After the sterilization is complete bring the test tube to the aseptic area and put a loop full of bacteria into it when temperature is slightly low.
5. Keep them for inoculation and observe the result after 24 hrs.
6. If the colour changes from blue to yellow then the organism has utilized the glucose, sucrose and lactose present by conversion of the sugar provided in the medium to acidic metabolites. The production of gas by the organism by utilization of the given sugar is indicated by the presence of gas in the Durham's tube.
7. A blank is also performed with out the addition of organism to it .

Glucose broth

Sl.No.	Name of Chemicals	Quantity required	Quantity used
1	Peptone	5 gm	
2	Beef extract	5 gm	
3	Water	1 ltr	
4	Glucose	5 gm	
5	Bromocresol purple	QS	

Lactose broth

Sl.No.	Name of Chemicals	Quantity required	Quantity used
1	Peptone	5 gm	
2	Beef extract	3 gm	
3	Water	1 ltr	
4	Lactose	10 gm	
5	Bromocresol purple	QS	

Sucrose broth

Sl.No.	Name of Chemicals	Quantity required	Quantity used
1	Peptone	5 gm	
2	Sodium chloride	5 gm	
3	Water	1 ltr	
4	Sucrose	5 gm	
5	Bromocresol purple	QS	

Experiment No: 22

SENSITIVITY TESTING

AIM :- To carry out the test for sensitivity of bacteria to antibiotics by diffusion method.

Discussion :- Most antibacterial drugs used today are antibiotics and the most important commonly used antibiotics are penicillin, ampicillin, streptomycin, tetracycline, chloramphenicol, Chloromycetin, gentamycin etc. Many antibiotics inhibit the growth of a wide range of bacteria and they are usually called as broad spectrum antibiotics. Eg. Tetracycline. Others have more restricted antibacterial spectrum eg. Penicillin. Streptomycin is more active against gram –ve and acid fast bacteria (Mycobacterium tuberculosis). One of the most characteristic structures in bacteria is the cell wall which is quite different from those of higher organisms. It is probable that many antibiotics , owe their specific anti microbial activity to an inhibition of bacterial cell wall synthesis.

Principle :-

These tests are carried out by the same methods as used in other forms of microbiological assay. A concentration gradient of the antibiotics is prepared in a medium fully adequate to support growth. Growth occurs in the medium where concentration of antibiotic is below the inhibitory level and ceases where this level is adequate for inhibition thus allowing assessment of the susceptibility of microorganisms to the antibiotics under study. An antibiotic is put in the cup made in the agar medium from where it diffuses through the agar medium to form a diffusion gradient to which the microorganism is susceptible. The size of the zone of inhibition depends up on the factors that influencethe diffusion of the substances in the gel. By measuring the zone of inhibition it can be known which organism are more sensitive to the presence of a particular antibiotic.

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Measurement of anti- microbial activities.

Determination of anti- microbial activity can be carried out by two methods. Diffusion / Dilution using an appropriate standard organism and known standard for comparison , these methods can be employed either to detect the anti- microbial property of antibiotics in the samples or to determine the sensitivity of the organism.

1. Dilution method:- Serially diluted amounts of anti- microbial substances is incorporated into liquid or solid bacteriologic medium and incubated. The result is taken as the amount of anti- microbial substance required to inhibit the growth of the tested bacteria.
2. Diffusion method:- A filter paper disc or porous cup containing measured quantities of drug is

placed as a solid media that has been heavily seeded with the tested organism. After incubation, the diameter of the clear zone of inhibition surrounding the deposit of drug is taken as a measure of the inhibitory power of the drug against the particular test organism.

While determining bacterial sensitivity by diffusion method in laboratories, we use filter paper impregnated with antibiotics. A concentration gradient of antibiotics is produced in the medium by diffusion from disc , but as diffusion is a continuous process, concentration gradient is never stable for a long time, some stabilization can be achieved by refrigerating the seeded agar plate with the anti-microbial agent for 2-3 hours before incubating it.

Procedure:

1. Prepare about 50ml of nutrient agar medium and sterilize by autoclaving.
2. Suspension of the organism is made using 0.9% of NaCl (Saline). The inoculums density should be - at 600 nm the absorbance is 0.1 , which is approximately equal to 10^6 cells/ml.
3. This is aseptically transferred into a sterilized petridish and allowed to solidify.
4. Add 0.1ml of inoculums to the solid agar plate and spread it over the entire surface with a Sterile spreader.
5. Decant the excess liquid.
6. Bore 2 wells on the agar surface with a sterile borer which is previously sterilized by dipping in alcohol and flaming.
7. Add 0.1ml of different antibiotics solution to the 2 wells with a micropipette.
8. Close the petri plate and keep for incubation at 37^0C for 24 hours.

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Experiment No:23

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

AIM:- To determine minimum inhibitory concentration for the given antibiotics.

Discussion :- Minimum inhibitory concentration of an antibiotic is the least concentration at which the anti-microbial, agent is active against the given organism. The antibiotic which is to be administered should have low concentration because at higher concentration the antibiotics produces side effects, hence for an antibiotic to be effective it should be active at low concentration. The minimum inhibitory concentration indicates whether the organism is sensitive or resistant to that antibiotic at a particular dose.

Dilution method :-

There are various method to determine the minimum inhibitory concentration of the antibiotic of which dilution method is one . In this method the antibiotic is first diluted to various concentration of the antibiotic medium, equal volumes of media with different concentration of the antibiotic are taken in culture tubes and equal amount of organism is added. It is important that the amount of the organism is kept constant because if the amount changes then the activity of the antibiotic is also changed. Then these tubes are incubated and the concentration of antibiotic in the tube showing no growth is taken as the MIC.

Diffusion Method:-

This method was standarddized by Kerby and Boyes and hence it is called Kerby boyes cup plate method. In this method medium is taken along with the organism in a petridish and to this antibiotic is added by means of Whatman filter paper. This is cut into discs and it is considered that 100 discs will absorb 1ml of the antibiotic and the concentration of antibiotic as well as amount of organisms is also kept constant. MIC is calculated from the zones of inhibtion.

Minimum inhibitiory concentration is an absolute value which is not based up on comparison with a standard reference preparation as in the case of antibiotic assay and certain disinfectant tests.

Procedure:-

1. Prepare antibiotic solutions of different concentration of antibiotics
   a. 2ml → 2000 mg(stock solution)
   b. 1ml of antibiotic + 1ml medium
   c. 1ml of medium + 1ml of above mixture.
   d. 1ml of medium + 1ml of above mixture.
   e. 1ml of medium + 1ml of above mixture.
   f. 1ml of medium + 1ml of above mixture.
   g. 1ml of medium + 1ml of above mixture.
   h. 1ml of medium + 1ml of above mixture.
   i. 1ml of medium + 1ml of above mixture.
   j. Blank - only 1ml medium.
2. Prepare the Muller Tinton medium and keep it for sterilization.
3. Keep the medium seperately and test seperately for sterilization.
4. Keep 100ml of water for sterilization to prepare the antibiotics.

5. After the sterilization is complete bring the medium in an aseptic area wait till it gets cooled.
6. After the temperature of the medium falls , pour about 10 ml into the starter to prepare 50 % bacterial suspension.
7. Then take 2ml of the antibiotic in test tube no.1 and from this take 1ml into tube no.2 with sterile syringe.
8. Into same test tube no.2 add 1ml of medium so now the total volume of test tube no.2.is 2ml.
9. From this 2ml, take 1ml in test tube no.3. and also add 1ml medium to test tube no.2. Then again take 1ml in test tube no.3. Continue the same till test tube no.9. Each time adding 1ml offresh medium to each of the tubes before the next transfer.
10. In test tube no.10. Take only the medium which serves as blank. ie. Only medium+ 1ml of organism- no antibiotics is added to this.
11. The voulme of all the test tube should be 2ml only after the addition of 1ml of organism.
12. Incubate for 24hrs and take the reading after the period of 24 hrs.
13. Calculate the MIC (MIC- It is the minimum concentration of antibiotic that will inhibit the growth of microorganism).
14. The turbidity of the test tube is compared with that of the blank.

Experiment No: 24

ANTIBIOTIC ASSAY

AIM :- To carry out the assay of the given antibiotic by cup plate

method.Requirements:- Petridish, Borer, Culture of organism.

Discussion:-

Biological assay are the methods used to study the potency of the preparation by means of it's effects on living systems , when the potency cannot be determined by physical or chemical methods.

A typical biological assay involves substances like vitamins, antibiotics , fungicides or other drugs etc. applied to the animal tissues or bacterial culture. The intensity of the stimulus applied to the subject is known as the dose . The dose is measured by weight / volume concentration of the preparation. The observed effects on the subject are known as the response.

Antibiotic assay:-

Microbial growth inhibition under standard conditions may be utilized for demonstrating therapeutic effects of the antibiotics. Any change in the antibiotic molecule will result in change of their growth or inhibition effect. The potency of an antibiotic sample is assayed by comparing the degree of microbiological growth inhibition of susceptible microorganisms by demonstrating the same degree of inhibition.

Methods of assay are:

1. Chemical or physical method.
2. Biological method.

Chemical method:- When an antibiotic exists in pure form it's potency expressed in mg of pure chemical per mg of the sample. This method is the chemical assay of the antibiotics. Different chemicals give different colour. They are generally more accurate but they may be less sensitive.

Biological method: Here the potency is expressed in terms of mg or undetermined by comparing the amount of killing or bacteriostasis of a tested organism caused by the substance with that caused by the different substance under rapidly controlled conditions. The inhibition of microbial growth under standard conditions is utalized in demonstrating the therapeutics effects of antibioics . Any change in antibiotic molecule which may not be detected by chemical methods will be revealed by a reduction or increase in Antimicorbial activity and hence the microbial assay are very useful for resolving the doubts regarding the production of the antibiotic having little or no activity.

Procedure:-

1. Prepare nutrient agar medium, take about 35ml of the medium in conical flask plug it and keep for sterilization.
2. Take the sterilized medium to an aseptic area prepare a suspension of the organism with 0.9% NaCl. Transfer about 0.1ml of this suspension into the conical flask at 50^0C and shake.
3. In between the two burners transfer seeded medium into a sterile petri plate allow it to solidify for 15 minutes.
4. With the help of sterile borer make five bores label center as unknown (U) and remaining

four as std. Antibiotics (S).

5. Make dilution of known concentration for standards and transfer into the bores with the help of a micro plate.
6. Prepare a solution of known concentration of the sample and transfer to the central bore.
7. Keep in refrigerator for 2-3 hours for diffusion and incubate for 24 hrs at 37^0C.

Experiment No:25

STERILITY TESTING FOR WATER FOR INJECTION

AIM:- To carry out sterility testing for water for injection.

Requirements:- Test tube, conical flask, inoculating loop, syringe, needle, water for injection, fluid thioglycolate medium and soyabean casein digest medium.

Discussion:-

Sterility test is applied to products intended to be sterile to check that the products are free from detectable contamination of both bacteria and fungi. Virus, pyrogens and toxins cannot be tested for because they are not detectable under the conditions of the experiment. The test is based on the principle that if viable organism is present and provided with all necessary growth conditions, then it will grow and show growth in the form of turbidity in the fluid medium. If no turbidity is present, then the product is sterile. To obtain a conclusion, a proper medium is required for growth which serves as a nutrient for bacteria.

Media:- The media used must be capable of supporting the growth of even small numbers of microorganism. Generally two media are preferred.

1. Fluid thioglycollate medium:- Also called as bacterial medium. It supports the growth of anaerobic organism.
2. Soybean casein digest medium:- Also called as fungal medium. It supports the growth of aerobic bacteria and fungi.

Time and temperature:- The incubation time and temperature are important since the temperature mustbe suitable for growth and the time should be long enough for visible growth. Bacteria , spores and fungi will show growth within 7-14 days.

Controls :- Two types of controls used are positive and negative control.

Positive controls:- Intended to ensure that the sterility test system ie. Medium+ Sample+ Inhibitor, will support the growth of microorganism and is likely to detect all potentially viable contaminants.(Eg. Inoculate the tube with known organism and incubate it. Add *Clostridium tetani* sporogenes (anaerobic), Staphylococcus aureus (aerobic). Here the media are incubated with a known organism and

incubated. The organism generally used are *Clostridium tetani* sporogenes (anaerobic), Staphhylococcus aureus (aerobic) and Candida albicans (fungi).

Negative control:- Ensure the sterility of all materials (media and apparatus) used in the test, here in one test tube only media is incubated without the sample.

Environmental Control:-

1. To indicate that the environment during transfer is sterile (Aseptic precautions) validation of sterility testing is always done in laminar air flow.

2. Person performing the sterility testing must be highly skilled and familiar with aseptic technique.

3. Area where the sterility testing is taking place should have no UV light or disinfectant sprays (which might destroy organisms).

Other consideration:-

1. Some parental sample contains preservative, which may inhibit growth of organisms. For this , some modifications are made before performing the sterility test . Dilution with water is carried out for alcohol containing preparations and for other preservative containing preparations. Coloured preparations membrane filtration is carried out.
2. For soluble powders, powder is added into the media and incubated for 7 days.
3. For insoluble powders , powder is added into the media and incubated for 3 days on the 4^{th} day the supernatant is removed and added to fresh media and incubated for 11 days.
4. Sulfa drugs:- In case of sulfa drugs sterility testing should be done in the presence of para-amino benzoic acid.
5. Ophthalmic ointments : If such ointments contain paraffin base, the paraffin is dissolved using isopropyl myristate and tested for sterility by membrane filtration technique.
6. In ointments with bases other than paraffin 100gm of ointment is shaken with 100ml of water, the water is used s sample.
7. Sterility testing is a destructive method as the sample while being tested is destroyed.

The I.P. gives the standards for taking standard representative out of the batch.

Statistical method modification : Do the sterility testing. If no turbidity is observed, the sample is said to be sterile, if turbidity is observed then the sterility testing is repeated second time. Look for growthin both tubes. If the organisms present is the same, the test fails to be sterile. If both the organisms are different then the sterility testing is carried out for the 3^{rd} time. If test passes, conclude that the test passes. If there is turbidity present, the sample fails the test for sterility.

Methods for modification:-

By dilution: eg. Alcohol is diluted with sterile water to 1% concentration.

By Inactivation:-eg. Antibiotic penicillin is inactivated by enzyme penicillinase.

By membrane filtration:-

Eg. 1) When preservatives are added pass the sample through membrane filter and wash with peptone water, cut the membrane into two parts. Add one to each media. This method may remove the organism that is present.

2) If product is coloured (dye) turbidity can not be seen, so here membrane filtration method can be applied.

3) Where volume is larger , the sample taken may or may not contain organism , so here again membrane filtration is applicable. Neutralization of acidic or basic substances.

Sterile water for Injection :- Sterile water for injection is water for injection distributed in suitable container of glass or any other material, sealed and sterilized by heat under conditions that ensure that the water for injection is maintained sterile and permits the withdrawal of the nominal volume.

Procedure:-

1. Prepare fluid thioglycollate medium and soyabean casein digest medium.
2. Transfer about 10 ml of fluid thioglycollate medium in three labeled test tubes (one for sample, one for positive control and one for – ve control.
3. Plug the test tube and wrap in a brown paper and tie it.
4. Transfer about 10ml of soybean casein digest medium in three labeled test tubes (one for sample, one for positive control and one for negative control .
5. Plug the test tube wrap it and tie it.
6. Keep all six test tubes for sterilization in an autoclave.
7. Place the two negative control test tubes in the incubator.
8. Add the organism Staphylo coccus aureus in the two positive control tubes aseptically.
9. Note down the label of the ampoule of sterile water for injection I.P. Cut open the ampoule . With the help of a sterile syringe and needle, transfer 1ml of the sample into the sample test tubes with the different media aseptically.
10. Keep the test tube for incubation for 7 days at temperatures of about 20- 25^0C.
11. Observe the presence or absence of turbidity after 7 days. If the turbidity is present in the sample tubes the sample is not sterile and if the turbidity is absent the sample is sterile provided , the negative controls shows no growth at all and the positive controls show growth. Otherwise the test is invalid and needs to be repeated.

Vision and Mission of the Institution

Vision

The East Point College of Pharmacy aspires to be a globally acclaimed institution, **recognized** for **excellence in** pharmaceutical education, research and nurturing students for **holistic development.**

Mission

M1 Create pharmacy graduates through **quality education**

M2 Promote innovation, **creativity**, and excellence **in teaching**, learning, and **research**

M3 **Inspire** integrity, teamwork, critical thinking**, personal** development, and ethics in **students** and lay **the** foundation for lifelong learning

M4 **Serve** the **healthcare, technological, scientific**, and **economic** needs of then **society**.