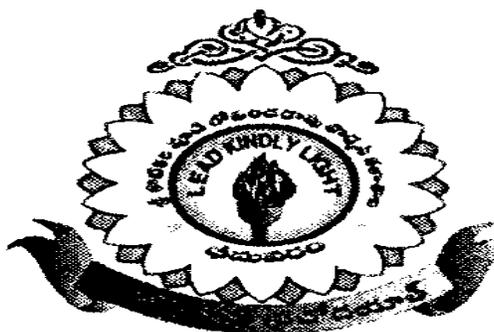


K.G.R.L.COLLEGE OF PHARMACY
DIRUSUMARRU ROAD, BHIMAVARAM – 534 201



LAB MANUAL

SUBJECT: Pharmaceutical Microbiology

Year: II/II B.Pharm-I Sem

PREPARED BY

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About K C O P



To be an educational Institute of par excellence and produce competent pharmacy professionals to serve the community through research and the ever-increasing needs of Industry.



1. Imparting quality education and innovative research for various career opportunities.
2. Creating conducive academic environment to produce competent pharmacy professionals.
3. Indoctrination of students adorned with high human values and makes them aware of their responsibility as health care professionals.

Program Educational Objectives

PEO 1: To produce graduates with sound theoretical knowledge and technical skills required for their career opportunities in various domains.

PEO 2: To incite the students towards research and to address the challenges with their innovative contributions for the benefit of the mankind.

PEO 3: To instill the essence of professionalism, ethical commitment to become a health care professional with sound integrity and adherence to the core human values in the service of the society.



PROGRAM OUTCOMES

Pharmacy Knowledge: Possess knowledge and comprehension of the core and basic knowledge associated with the profession of pharmacy, including biomedical sciences; pharmaceutical sciences; behavioral, social, and administrative pharmacy sciences; and manufacturing practices.

Planning Abilities: Demonstrate effective planning abilities including time management, resource management, delegation skills and organizational skills. Develop and implement plans and organize work to meet deadlines.

Problem analysis: Utilize the principles of scientific enquiry, thinking analytically, clearly and critically, while solving problems and making decisions during daily practice. Find, analyze, evaluate and apply information systematically and shall make defensible decisions.

Modern tool usage: Learn, select, and apply appropriate methods and procedures, resources, and modern pharmacy-related computing tools with an understanding of the limitations.

Leadership skills: Understand and consider the human reaction to change, motivation issues, leadership and team-building when planning changes required for fulfillment of practice, professional and societal responsibilities. Assume participatory roles as responsible citizens or leadership roles when appropriate to facilitate improvement in health and well-being.

Professional Identity: Understand, analyze and communicate the value of their professional roles in society (e.g. health care professionals, promoters of health, educators, managers, employers, employees).

Pharmaceutical Ethics: Honour personal values and apply ethical principles in professional and social contexts. Demonstrate behavior that recognizes cultural and personal variability in values, communication and lifestyles. Use ethical frameworks; apply ethical

principles while making decisions and take responsibility for the outcomes associated with the decisions.

Communication: Communicate effectively with the pharmacy community and with society at large, such as, being able to comprehend and write effective reports, make effective presentations and documentation, and give and receive clear instructions.

The Pharmacist and society: Apply reasoning informed by the contextual knowledge to assess societal, health, safety and legal issues and the consequent responsibilities relevant to the professional pharmacy practice.

Environment and sustainability: Understand the impact of the professional pharmacy solutions in societal and environmental contexts, and demonstrate the knowledge of, and need for sustainable development.

Life-long learning: Recognize the need for and have the preparation and ability to engage in independent and life-long learning in the broadest context of technological change. Self-assess and use feedback effectively from others to identify learning needs and to satisfy these needs on an ongoing basis.

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Rules and Precautions for The Microbiological Laboratory

- Always wear apron before entering laboratory.
- Clean the work bench before and after the laboratory work.
- Never eat or drink in the laboratory.
- Long hair should be tied back to minimize the contamination of culture and fire hazards.
- Sterilize all the equipment before use.
- All cultures and prepared solutions should be properly labeled or marked.
- Before discarding any culture, the organism must be killed.
- Put off the burners when not in use.
- Broth cultures should never be pipette out.
- Label all plates and tube cultures properly before starting the experiment.
- Open the mouth of the culture tubes and petri plates near the flame.
- Aseptic conditions should be strictly followed at all the times.
- It is advisable to wear gloves when work with chemicals, hazardous to eye and skin.
- Protect yourself from exposure of eyes and skin to UV light by wearing goggles and clothing respectively.

EXPERIMENT-01

INTRODUCTION AND STUDY OF DIFFERENT EQUIPMENTS AND PROCESSING

Aim: To study different equipment and processing used in Pharmaceutical microbiology lab

BOD INCUBATOR:

It is often called low temperature incubators are one of the most important lab equipment in many research centers, hospitals and other pharmaceutical labs. BOD incubators is the most versatile and reliable low temperature incubator which is designed to maintain at 20°C , necessary for biological oxygen demand/ biochemical oxygen demand determination. BOD incubators provide controlled temperature conditions accelerated tests and exposures.



Applications:

- It measuring waste loadings to treatment plants and in evaluating the BOD removal efficiency of such treatment systems.
- It measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic materials and the oxygen used to oxidized in organic materials such as sulfides and ferrous iron.

ASEPTIC HOOD

The air of laboratory is full of microorganism. Air borne microorganism may contaminate sterile medium while handling pure culture during inoculation. Thus, an inoculation procedure is usually carried out under a hood or special cabinet. To have aseptic environment, two types of the devices are placed in practice – laminar air flow aseptic cabinets.

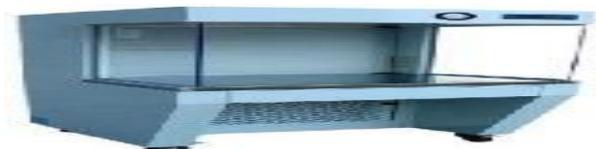


The inoculation hood is used to create bacteria, fungal free atmosphere in the chamber with ultra violet germicidal light and it is used in biological culture studies.

LAMINAR AIR FLOW UNIT:

All the microbiological operations are to be carried out under aseptic conditions to prevent contamination. Similarly when we handle hazardous and pathogenic organisms, precautions should be taken. Laminar air flow cabinets provide aseptic working environment and also prevents spillover of pathogenic organisms. . There is a special filter system of high efficiency particulate air filter (HEPA) which can remove particles as small as 0.3mm. The laminar air flow is based on flow of air currents of uniform velocity along parallel flow lines which helps in transferring microbial cultures in aseptic condition. It is passed through the filters will not allow any kind of microbe to enter into the system.

Inside the chamber fluorescent tube and U.V tube is fitted. Due to the uniform velocity and flow of air current all microbial operations like transfer of cultures, pouring of media, plating etc. can be carried without any contamination. Both horizontal and vertical laminar air flow cabinets are



available.

OPERATION:

Open the front door of the laminar air flow cabinet and clean the platform with any disinfectant (preferably alcohol) with the help of smooth cloth.

Switch on the U.V light and continue for 3 min so as to decontaminate the chamber.

Switch off the U.V light and start work on platform.

All operations inside the cabinet should be carried out in flame zones of burner or spirit lamp.

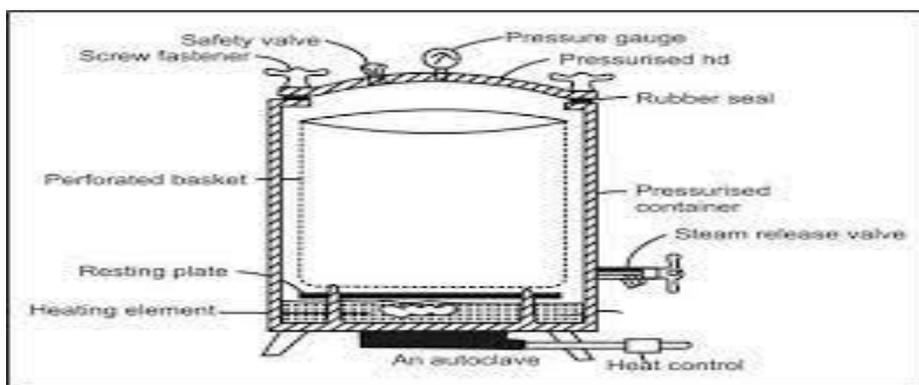
PRECAUTIONS:

- Don't get exposed to U.V light, wear black goggles.
- Leave the shoes before entering to operate the apparatus.
- Wash hands with detergent or soap before starting the work.
- After finishing the work clean the platform with surgical spirit, switch on UV light and keep for 15 min before final switch off the cabinet

AUTOCLAVE:

Autoclave is a common and the most essential instrument in every microbiology laboratory. It is used for sterilization of media (solid and liquid), heat stable liquids, heat resistant instruments, glass wares and rubber products. The autoclave is based on principle that saturated steam heats an object many times more efficiently than hot air at the same temperature. The increased pressure results in the elevation of boiling point of water and produces steam with the high temperature. However, it is important to note that it is not the pressure that kills the organisms but the high temperature of the steam. The boiling point of water at 15 lbs. pressure is 121⁰C. Most of the organisms are killed at 121⁰C (15 psi) in 15 minutes.

An autoclave is a double walled metallic vessel. The body is usually made up of steel. Both vertical and horizontal types of autoclaves are available, but for routine laboratory use vertical types are commonly used. Fully open able lid is provided with a pressure gauge for recording pressure, steam clock (exhaust valve) for air exhaustion, a safety valve to avoid explosions.



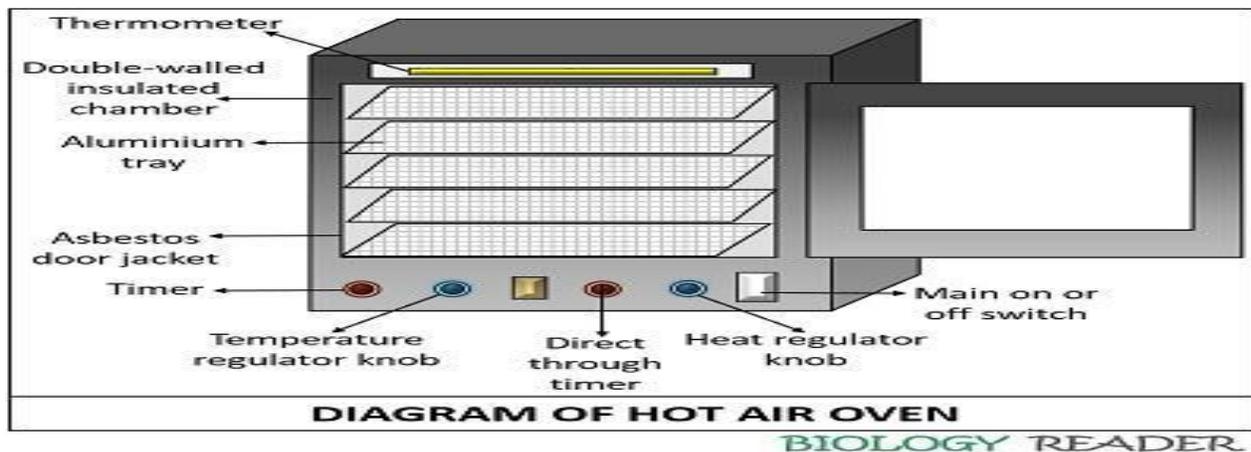
PRECAUTIONS:

- Water level inside the autoclave should be well above the heating mantle.
- Tighten the screw fasteners equally on all sides.
- The required 15 psi pressure must be maintained constantly for the required period of time.
- Over sterilization likely to change the composition of the medium.
- Agar may also lose the jellifying (solidifying) property.
- Do not open the lid until the pressure gauge shows 0 (The zero)

HOT AIR OVEN:

It is another common instrument of microbiology laboratory and it is used for sterilization of glass ware. It is based on the principle where sterilization is accomplished by dry heat or hot air. Hot air oven is most widely used instrument for sterilization of glass ware; dry heat is most reliable method for sterilization. Material that may be damaged by heat can be sterilized to protein denaturation and oxidative damage, toxic effect of elevated levels of electrolytes in a completely moisture free atmosphere. Bacterial spores are more resistant to heat. So they are killed with oxidation for coagulation. Flaming, incineration and hot air oven methods are based on dry heat principle. Hot air is bad conductor of heat and its penetrating power is low. The oven is usually heated electrically with heating elements in the wall of chamber it should not be overloaded and material should be arranged in such a way to allow free circulation of air between the objects. Glass ware should be preferably dried before placing in the oven. Test tube and flasks should be wrapped in foil. The oven must be allowed to cool slowly for about 2 hours before the door is opened because the glass ware may break due to sudden un even cooling. A holding period of 160⁰C for 2 hours is used to sterilize the glass ware like Petri plates, test tubes, pipettes, metal instruments like forceps, spatula, glass syringes and some pharmaceutical products like dusting powders, liquid paraffin, mineral oils and grease, rubber materials except silica rubber can with stand high temperatures can also be sterilized by using hot air oven. Cutting instruments such as those used in ophthalmic surgeries should be sterilized for 2 hrs. at 150⁰C. Oils, dusting powders require 150⁰C for 1 hr. for sterilization.

S.No	Temperature (⁰C)	Sterilization time (minutes)
1.	120	480
2.	140	180
3.	150	150
4.	160	120
5.	170	60
6.	180	20



DEEP FREEZERS FOR MEDICAL LABORATORIES

Deep freezers are the testing equipment that are used in scientific laboratories to preserve and store medical equipment, blood samples, medicines and injections, etc for a long period of time. There are numerous types of deep freezers such as blood bank refrigerators, freezer drier, ultra-low deep freezer. These devices are available in different sizes and shapes sometimes it is designed with compact designs and sometimes with regular designs. The specifications and functions of the instruments varies as per the requirements of the test application.



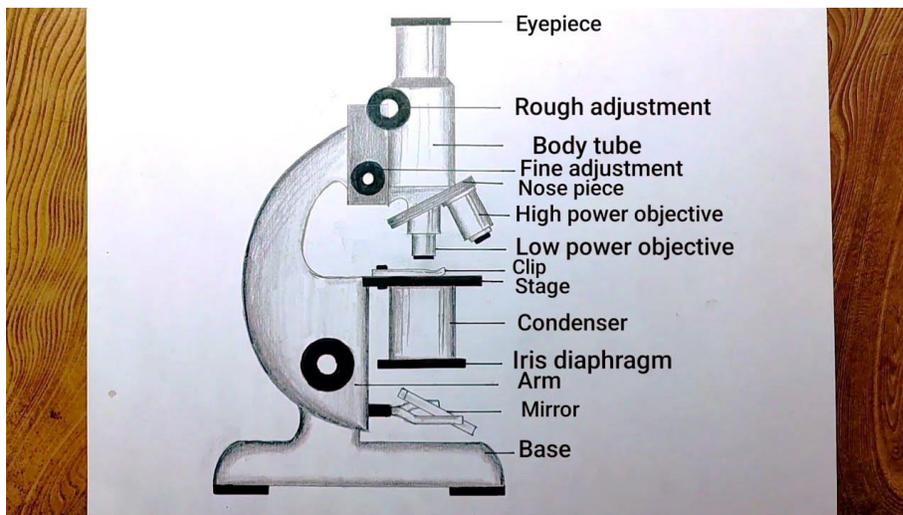
MICROSCOPE:

Construction and Working:

Microscope is defined as an optical instrument comprising of lens or combination of lenses.

It enables to magnify image. Microscope is of 2 types

- Simple Microscope
- Compound Microscope



SIMPLE MICROSCOPE: It is also called as dissecting microscope it consists of one set of lenses and gives lower magnification. It mainly helps to relieve the morphological characters of the compound.

COMPOUND MICROSCOPE: It consists of two sets of lenses 1. Eye piece 2.Objective.

Compound microscope mainly consists of 3 major systems

Support System

Illumination System

Magnification System

Support System: It consists of base, stage, body tube, mechanical stage inclination joint.

Illumination System: It consists of light source iris diaphragm and condenser; light source may be plane or concave mirror. Electrically illuminated by a tungsten filament lamp or halogen lamp.

Magnification System: This includes the set of lenses aligned such a manner that a magnified real image can be viewed. The object is a set of lenses placed near the object. It partially magnifies the object which can be observed through eye piece in a more magnified form.

Base: It forms the foundation of microscope.

Stage: It holds the object or slide.

Body Tube: It holds the object at the bottom and eyepieces at top.

Mechanical Stage: For planar, forward, back ward, left and right movement of object with the help of knob.

Inclination Joint: This help to incline the microscope to avoid the strain on neck and back.

Eye piece: It is used to observe the magnified real image.

Draw Tube: It is used to fit eye piece inside.

Resolving Nose piece: It holds 2, 3 or 4 objectives which can be resolved the aligned the required objective.

Objective: It produces the first magnification showing real inverted image it may be 6, 10, 40, 45, 100x.

Mirror or Light Source: It reflects the light through the sub stage condenser and stage aperture.

Iris Diaphragm: Light illuminating object may be adjusted with this it may be opened or closed to diminish the light falling on the condenser and hence making the object more or less bright.

Condenser: It allows the parallel beam of light to pass through the stage aperture.

Knob: It is used for vertical movement of condenser and adjustment of knob. In case of compound microscope knob. It is of 2 types

Course adjustment knob

Fine adjustment knob

Course adjustment knob: It is used to focus on object

Fine adjustment knob: It helps to focus the specimen reveal the fine characteristics features.

Magnification System:

The objective lens is placed closed to the object to be viewed and the ocular lens is placed closed to the eye. The primary enlargement of the object is produced by the objective

lens. The image produced this is transmitted to ocular lens where final enlargement occurs. Therefore the magnifying capabilities of compound microscope are the product of magnification of objective and ocular lenses. For example using an objective lens of 40X ocular lens of 10X total magnification is 400X this magnification of microscope depends on following factors.

Optical tube length, focal length of objective, magnification of eye piece
Primary magnification = optical length / focal length of objective.

The optical tube length of microscope is the d/w upper focal plane of objective and the lower focal plane of eyepiece or where the primary image is formed the image produced by objective is real inverted image close of focal plane of eye piece. The eye piece is used in the compound microscope or simple magnifiers image produced by the objective. The image produced by the eyepiece is virtual inverted magnified image. It is fixed inside the draw it is used to observe more magnified real image. Eyepiece of 5x, 10x and 15x are available.

RESOLVING POWER OF OBJECTIVE:

An important property of microscope is its resolving power it is defined as its ability to distinguish 2 points that are closer together as distinct and separate. The greater the resolving power greater is efficiency of microscope it depend on the wave length of light optical property that is numerical aperture. This relationship can be expressed as

Resolving power = λ / NA where λ is wave length of light NA is numerical aperture of objective lens.

WORKING DISTANCE:

It is the distance b/w the objective and the object. It decreases with increase in magnification.

FOCUSING:

Focusing an objective is viewing through the eye piece that is adjusting of working distance this is done with coarse and fine adjustment of knob. Coarse adjustment knob is used to bring the object bin field of view fine adjustment knob is related to get the short image.

PRECAUTIONS:

- Observe the slide with both eyes open to have less strain on eyes.
- Always focus your slide slowly and carefully and with low power objective
- .Keep the microscope and its parts clean and hands with care

DIGITAL COLONY COUNTER:



The most time consuming part of carrying out a microbial count is the actual counting process on petri-dishes. Digital Colony Counter is designed for quick and accurate counting of bacterial and mould colonies in petri dishes. Feature packed and easy to use, this is an indispensable bench top tool for the busy microbiologist.

It is designed for rapid and accurate counting of bacterial and mould colonies. Simply place the petri dish on the illuminated pad and touch the dish with the pen provided to mark each colony in turn. This causes a count to be registered on the digital display and an audible tone confirms each count made. Marking the dish with the pen avoids missing colonies or double counting. The digital count on the display can be reset manually any time by pressing reset key provided. Optimum viewing of colonies is aided by peripheral glare free illumination graticuled disc provided in the instrument. The magnifier magnifies the colony area. This is an indispensable tool for the microbiologist.

Specifications

Model	361	362	363
Display	3 Digit, 7-Seg. LED	4 Digit, 7-Seg. LED	6 Digit, 7-Seg. LED
Max. Counts	999	9999	999999

Dish Size	110 mm
Magnification	x 1.7 Surface Magnification
Power	230 V \pm 10% AC, 50 Hz,
Dimensions	225 x 250 x 280 mm (L x B x H) (Approx.)
Weight	3 Kg (Approx.)
Accessories	Marking Pen, Magnifying Lens, Lens Holder, Operation Manual and Dust Cover

CYCLOMIXERS / VORTEX MIXERS:

SALIENT FEATURES

- Designed for mixing liquids in Schools, Laboratories & Factories
- Touch/ Continuous Operation mode Selection through bi-directional Switch
- Speed Regulation through knob provided on the control panel
- Interchangeable mixing heads for use with variety of tubes
- Supplied with all interchangeable mixing heads

Operation

- Connect the Cyclo mixer plug to the main power supply socket and switch on the main supply.
- Press the button up to start the Cyclo mixer.
- Regulate the speed of Cyclo mixer by rotating knob in clockwise direction to achieve the vortex formation speed.
- Hold the test tube containing the sample on the holder of Cyclo mixer.

- The Cyclo mixer can start directly by pressing the button in down direction.
- Hold the test tube containing the sample on the holder of Cyclo mixer and start the mixing by pressing the tube on the holder of Cyclo mixer.
- Wait till there is vortex formation or specified time.
- Remove the test tube after vortex formation from the vortex mixer.
- Turn the control knob anti-clockwise direction to stop the holder from further rotations.
- Switch off the Cyclo mixture by pressing the button in middle.
- Switch off the main supply of the Cyclo mixer.

Cleaning

- Insure that the power supply to the instrument is switched off and main cord is removed from the supply.
- Clean the Cyclo mixture with a clean lint free cloth every day.
- Clean the Cyclo mixture with lint free cloth along with disinfectant.

SAFETY & PRECAUTIONS:

Before cleaning or any maintenance remove the power supply cord of the Cyclo mixer from the main supply.



DIGITAL ANTIBIOTIC ZONE READER:

Features

- Accurate method for determining the strength of antibiotic materials.
- Electronic 'zero' adjustment eliminate tedious zero setting on the scale.
- LED illumination for better visibility of zone.
- Fine focus adjustment for clear image on the viewing Glass.
- IQ /OQ Documentation.
- No effect of ambient light on image.
- No parallax so no human error in reading.
- Wide range of measuring diameters from 0 to 80.0 mm.



- EI Digital Antibiotic Zone Reader provides the user, distance in mm with accurate and fast method for determining the strength of antibiotic material. It functions by measuring the diameter from 0 to 80.0 mm of an inhibition zone in a petri-dish.
- Wells are created on a petri-dish, prepared with agar – agar solution and inoculated with bacteria. After incubation, the bacterial growth cover the dish except for a circular inhibited zone around each disc, it being a function of the strength of the antibiotic.
- The zone reader accurately measures the diameter of the inhibited zone to 0.1 mm within range of 0 to 80.0 mm diameter. Light is passed through the transparent and semitransparent portions of the agar from a source at base towards up. It then passed to a reflecting mirror supported by an arm above the unit, which reflects the light to glass prism mounted at the front of the unit and magnified image of the zone of inhibition is clearly visible on the prism.

Specifications

Zone Diameter Range	0 to 80.0 mm
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Accuracy	0.1 mm
Resolution	0.1 mm
Illumination Lamp/LED	12 V DC
Display	4 digit seven segment LED display
Power Supply	230V±10% AC, 50Hz
Dimensions	12'' X 14'' X 17'' (L X W X H)
Weight	Net 10 Kgs. (Approx.)

EXPERIMENT-02

STERILISATION OF GLASS WARE PREPARATION & STERILISATION OF MEDIA

AIM: To sterilize glassware, prepare media and sterilize the media prepared.

REQUIREMENTS: agar, peptone, beef extract or meat extract, NaCl, distilled water, Test tubes, measuring cylinder, conical flask, nonabsorbent cotton, pH meter, autoclave, Hot air oven.

PRINCIPLE:

Sterilization of equipment and materials is carried by:

Wire loop: Heat to redness in Bunsen burner flame.

Empty glassware: pipettes and Petri Dishes: hot air oven at 160 °C for 2 hours, allowing additional time for items to come to temperature.

Culture media and solutions: Autoclave/pressure cooker.

Glass spreaders and metal forceps: Flaming in alcohol (70 % IDA).

Culture medium is a nutrient preparation which provides a balanced mixture of required nutrients that will permit good growth of microbes or other cells. Isolation, growth and, maintenance of microbes necessitates the preparation of suitable medium. Although all organisms need sources of energy, carbon, nitrogen, phosphorous, sulphur and various minerals, the composition of the medium will depend on the species to be isolated and cultured. Culture media are based on their components function and use. Depending on physical state; media are classified into 3 types.

SOLID MEDIA:

Solid media are needed for surface cultivation of microbes. Agar is widely used as a solidifying agent as it dissolves in boiling water and solidifies at about 40-42⁰ C. Agar is a sulphated polymer composed of D-glucose, 3, 6-anhydro levo-galactose, D-galacturonic acid. It is prepared

by addition of 105% agar to the corresponding liquid or broth medium. It is also an excellent hardening agent because most organisms cannot degraded it

SEMI SOLID MEDIUM:

It is prepared by the addition of 0.1-0.4% agar to the liquid medium. These media are used for demonstrating the motilities of the bacteria.

LIQUID OR BROTH MEDIUM:

It is prepared without adding solidifying agent. These media are ideal for physiological and biochemical studies.

Nutrient agar and nutrient broth are routinely used in the laboratory hence they are referred as general purpose media. These media are essential in the isolation and identification of microbes. These media are frequently used in water and food analysis, industrial microbiology and also to find out antibiotic sensitivity.

PROCEDURE:

COMPOSITION OF NUTRIENT AGAR

It is a solid medium and its composition is

- Agar-15 G
- Peptone-5G
- Beef extract-3G
- NaCl-5G
- Distilled water-1000ml

PREPARATION OF NUTRIENT AGAR

- Dissolve required quantity of given chemicals in 1000ml distilled water.
- Find out the pH of the solution by pH meter/pH paper.
- Adjust pH (if required) by NaOH and HCl solution drop by drop to pH 7.0.
- Now, put a cotton plug in the mouth of the conical flask and cover it with the aluminum foil.
- Sterilize the medium in autoclave for 15-20 minutes at a pressure of 15 lbs and temperature of 121⁰c.

PREPARATION OF AGAR SLANT

After allowing the tubes containing agar medium to cool down to 45-60⁰C keep them in slanting position by resting the plugged ends over a glass rod on the table and leave the test tube until the medium is cooled to room temperature.

COMPOSITION OF NUTRIENT BROTH

It is liquid medium and its composition is

- Peptone-5 G
- Beef extract-3 G
- NaCl-5 G
- Distilled water-1000ml

PREPARATION OF NUTRIENT BROTH:

- All steps are similar to the preparation of nutrient agar except the addition of agar.
- All ingredients are weighed and dissolved in water. Agar is not added in broth preparation and the sterilized liquid medium is directly distributed in to flask and culture tubes.

REPORT:

Experiment No -3

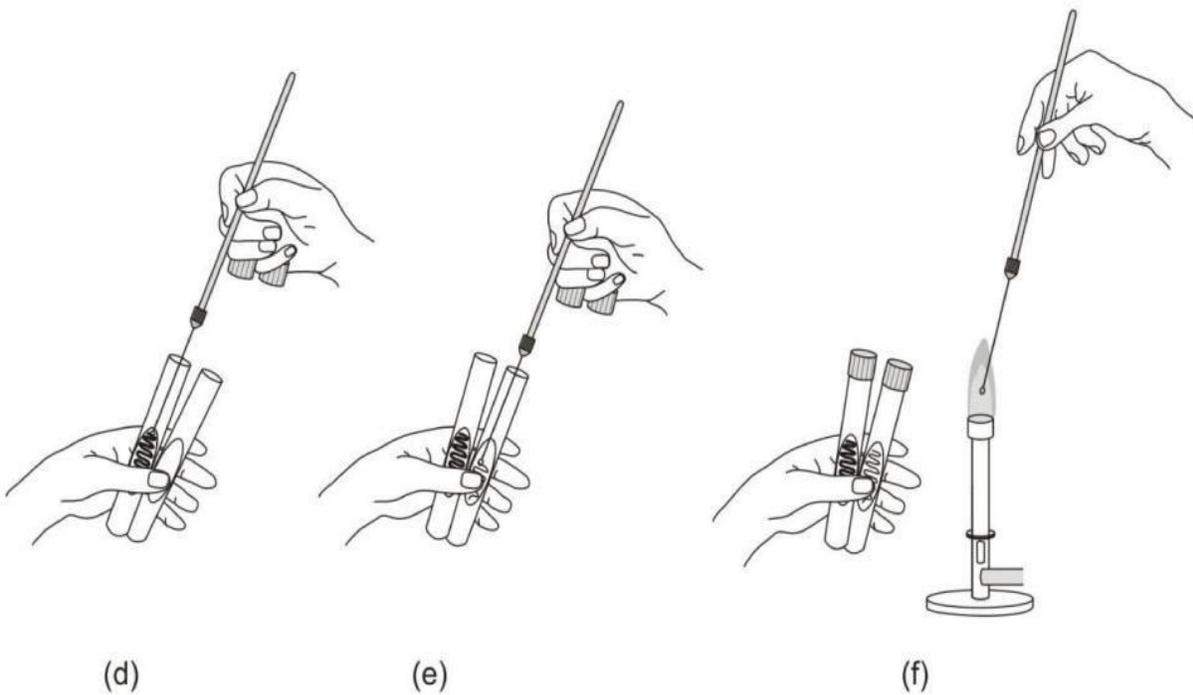
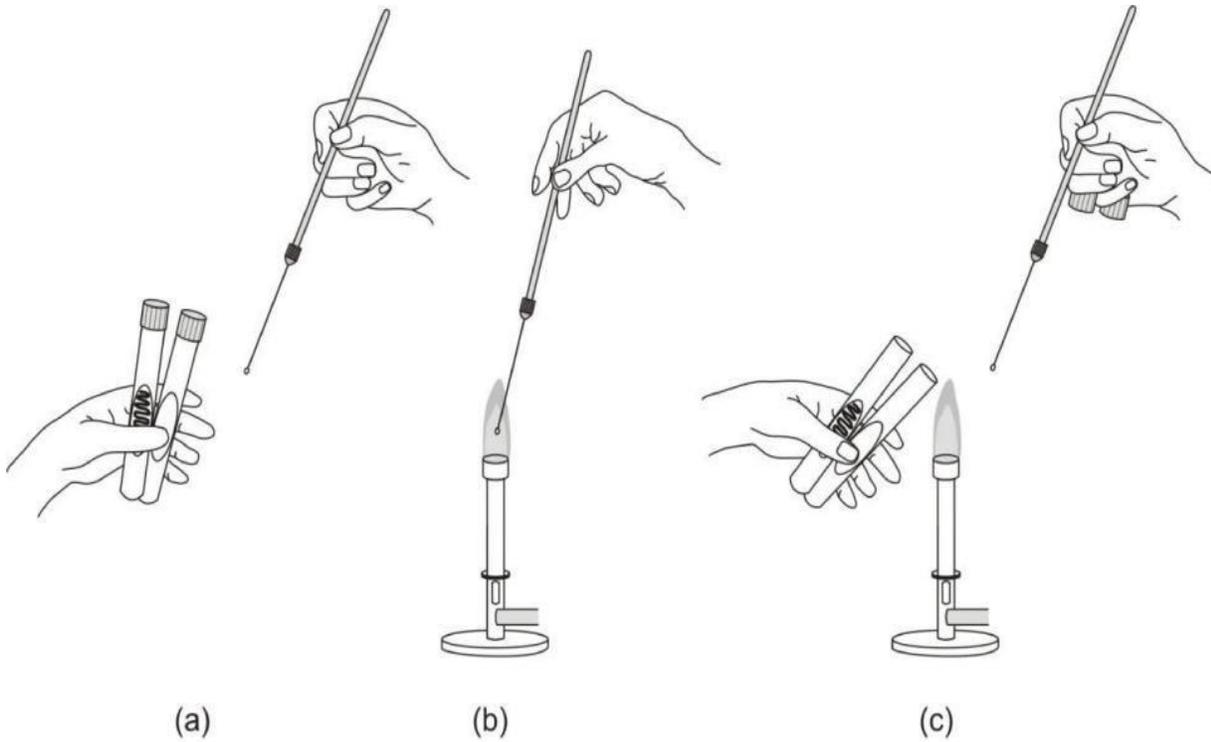
SUB CULTURING OF BACTERIA & FUNGUS

Aim: To carryout sub-culturing of bacteria and fungus

After incubation has been completed in streak plate , pour plate or spread plate techniques and appearance of the discrete, well separated colonies has been examined, the next step is to subculture some of the cells from one of the colonies to separate agar plates or nutrient agar slants with a sterilized needle or loop for further examination and use. Each of these new cultures represents the growth of a single species called a pure culture or stock culture. Sub culturing term is the term used to describe the procedure of transferring o microorganism from their parent growth source to a fresh one or from one medium to another medium.

REQUIREMENTS:

Mixed nutrient agar streaks, pour plate and spread preparations of may references bacteria
Nutrient agar slants or nutrient agar plates
Inoculating loop Wax marking pencil Microscope



PROCEDURE:

- With a wax pencil label the nutrient agar slants and agar plates as bacteria A & B.
- sterilize the inoculating loop by holding it in the hottest portion of the Bunsen burner flame
- Flame until entire wire become red hot
- Allow the loop to cool for a few seconds or cool it by dipping In a fresh agar plate
- Touch the tip of the loop to the surface of a selected discrete colony or the agar streakplate or the pour plate
- Remove the plug of the agar slants, grasp the plug with thee little finger of the left hand and pass the neck tube rapidly over the Bunsen burner flame. Inset the loop into the subculture tube rapidly over the Bunsen burner flame. Inset the loop into the subculture tube and inoculate it lightly over the hardened surface in a straight or zig zag line and recap tube.
- Reflame the inoculating loop/ needle to destroy existing organism
- Incubate the culture for 48-72hrs.

Observations:

After incubation, observe the slants or plates for the growth of pure colonies.

Experiment – 04

PREPARATION OF NUTRIENT SLAB AND SLANT

Aim: To Prepare Nutrient slab and agar slant

The sterilized agar medium in test tube is kept in an inclined position while hot making a slope of 20° angle or less. The tubes are allowed to cool in that position to make slants. The sloped surface provides more surface area for the growth of the inoculated organism. Which is easily inoculated with loop or needle, for each stab culture a straight needle is used and stabbed down to the bottom of the agar medium called “butt”

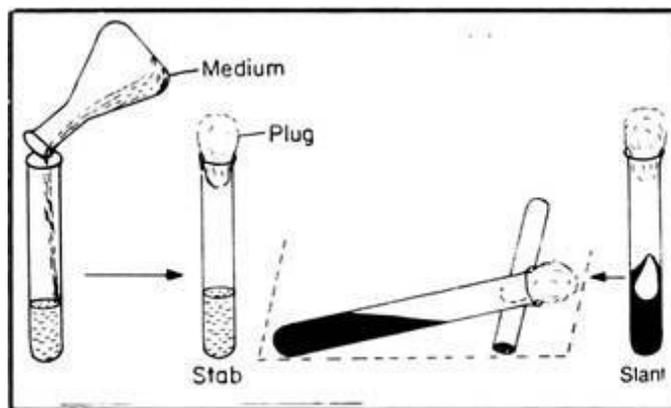


Fig. 2.2 Preparation of stab and slant.

Preparation of Culture Plates Procedure:

- The hot sterilized medium in test tubes is allowed to cool to 55°C and poured into sterilized petri dishes under aseptic conditions.
- The medium is allowed to solidify in flat position and the plates are left overnight at room temperature in an inverted position. This prevents the condensation on the lid and keeps the plates clear for viewing.
- The plates are stored in refrigerator or in a cool place in inverted position, avoiding drying of the medium by keeping them in plastic bags.

Observations:

Experiment – 05

STAINING METHODS – SIMPLE STAINING

Aim: To identify the given bacterial culture by simple staining

Simple Staining methods:

Normally there two staining procedures for light microscopy

Simple attaining

Differential staining

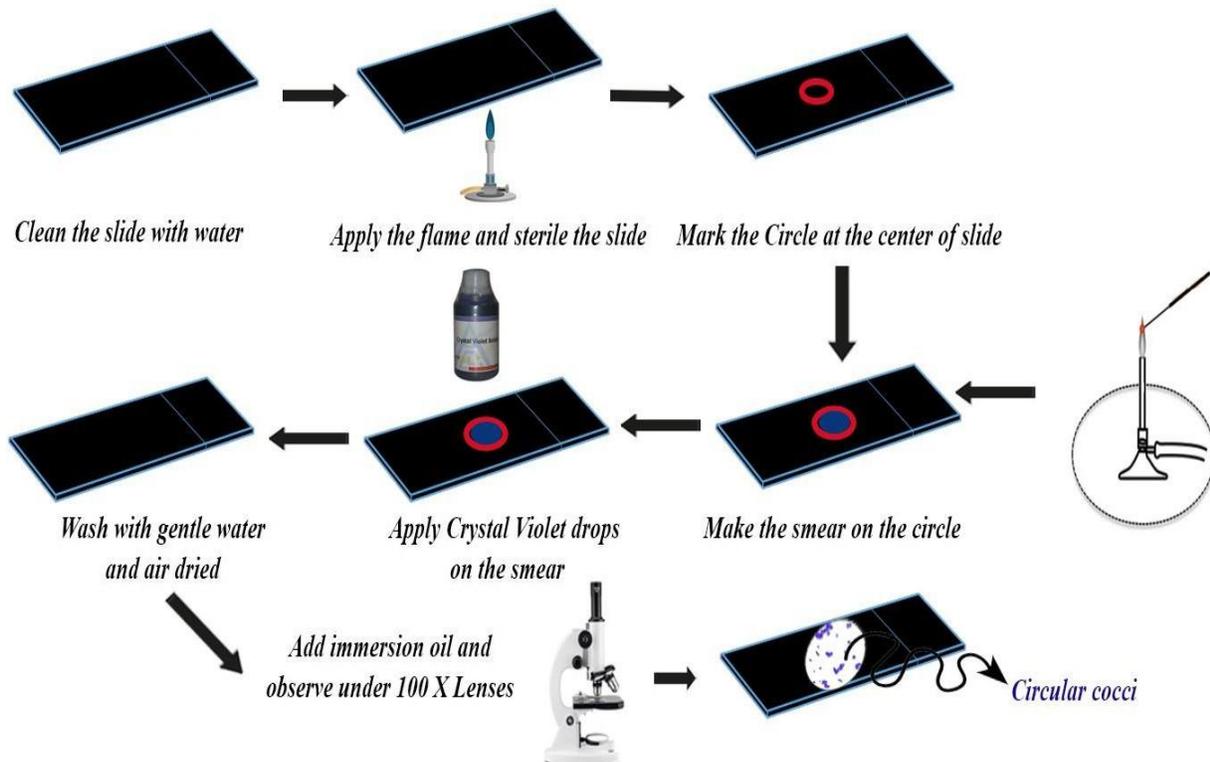
- **Simple staining:** In positive, the stain is basic having positive charge and attaches to the surface of the object that is negatively charged
- **Negative staining:** in this procedure, more than one staining reagents are used and specific objects exhibit different staining reactions which are readily distinguishable. Two most widely differential procedures are gram staining an acid fast staining.

SIMPLE STAINING

Principle :

simple staining involves single dye or staining reagents. The purpose of staining is to demonstrate cell size, shape and arrangement of bacterial cells. Since bacterial cells usually have a negative charge on their surface, they are most readily colored by basic stains. These compounds will either give up, which is attracted to the negatively charged cell surface.

Reagents: Loeffler's methylene blue solution



Sequential Diagrammatic steps of Simple Staining

Procedure:

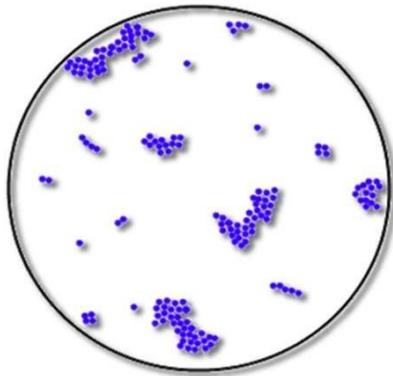
- prepare a smear of a given culture or materials by spreading a thin film on a clean glass slide
- Dry it by waving in air and then heat fix by passing the slides 2 to 3 times through the flame with the smeared slide facing upwards
- Stain the smear by flooding it with one of the staining solutions and allowing it to remain covered with the stain for the time designated below.

Methylene blue – 1 min

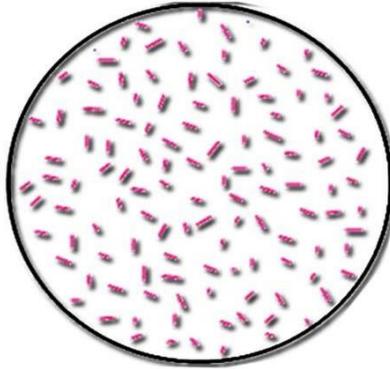
Crystal violet- 30 sec

Carbol fuchsin – 20 sec

- Wash the slide gently with running water to remove the stain Air dry the slide or blot with blotting paper
- Apply oil directly to the smear, and focus the smear first under low power objective and then under oil immersion objectives.



Spherical shape Cocci



Rod shape - Bacilli

INTERPRETATION:

Bacilli and diplobacilli: Rod-shaped bacteria, purple

Spirilla: spiral-shaped bacteria, purple

Cocci: spherical-shaped, bacteria, purple

OBSERVATIONS AND RESULTS: Cells stain uniformly with sample staining. Bacterial cells stained with different staining solutions take the following colours methylene blue- blue, crystal violet-violet, safranin- pink.

REPORT:

Experiment – 06

STAINING METHODS –GRAM STAINING

Aim: To identify the given bacterial culture gram staining method

INTRODUCTION:

The technique was developed by a Danish physician Dr. Hanes Christian gram. This is useful differential staining procedure in bacteriology which besides determining gross morphology differentiates bacteria into two major distinct groups:

Gram positive bacteria Gram negative bacteria

The differentiation assists in determining subsequent biochemical tests and the respective media for their culture in laboratory

The technique involve 6 basic steps

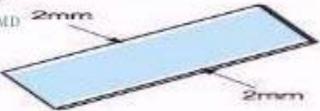
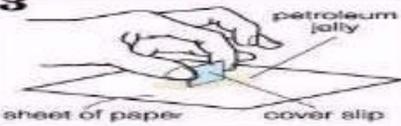
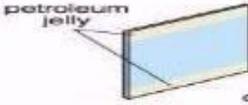
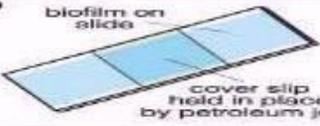
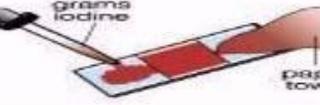
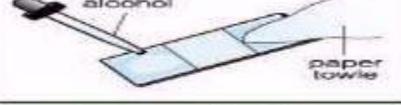
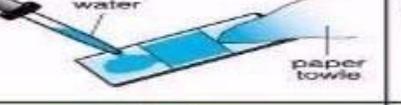
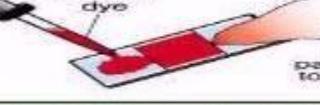
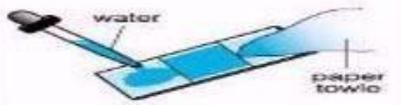
1. Smear preparation
2. Heat fixation
3. Staining with crystal violet
4. Use of iodine/lugol's soln
5. Treatment with acetone alcohol mix
6. Use of safranin

Principle:

The peculiar response towards the staining is related to physical and chemical difference in the cell walls of the two groups of bacteria. In gram negative bacteria, the cell wall is thin, multi layered containing high lipid contents which are readily dissolved by alcohol, resulting in pore formation in the cell wall facilitating the leakage of the crystal violet iodine complex and resulting in discolouration of gram negative bacteria which takes safranin and appears red.

On the other hand cell walls of gram positive bacteria are thick composed mainly proteins and cross linked mucopeptides. On application of decolorizing agent, dehydration is caused resulting in closure of pores of the cell wall thereby retaining the CV-I complex and do appear blue or purple.

18

GRAM STAINING				
1		2		
Wipe bottom of biofilm slide clean		Clean top edges of slide about 2mm		
3		4		
Build up a ridge of petroleum jelly on the top and bottom of a cover slip		Cover slip with petroleum jelly		
5			6	
Biofilm on slide with cover slip		Add crystal violet-wait 30 sec.		
7			8	
Wash with water		Add Grams iodine -wait 1.5 min.		
9			10	
Decolorize with alcohol		Wash with water		
11			12	
Stain with Safranin dye-wait 30 sec.		Wash with water		
13				
Examine under oil immersion through the cover slip				

Procedure:

- Make smears of the given culture on a clean glass slide
- Air dry the smear and heat fix it
- Cover the smear completely with crystal violet stain and leave the stain on the slide for one min.
- Wash the slide gently with distilled water or tap water
- Flood the smear with gram iodine solution and wait for one min
- Wash with tap water gently and drain carefully
- Add ethyl alcohol or alcohol acetone (1:1) solution drop by drop until the smear becomes free from any colorization
- Wash the slide gently under running tap water and drain
- Now counter stain with safranin and wait for 30 sec
- Wash again and blot dry with blotting paper or simply air dry the slide and observe under oil immersion objectives.

RESULT:

Bacteria that appear blue/ violet/ purple are assigned as gram positive bacteria
Bacteria that appear red/ pink are assigned as gram negative bacteria

Experiment – 07

STAINING METHODS –ACID FAST STAINING

Aim: To identify the given bacterial culture acid fast staining method

INTRODUCTION:

The technique was developed by Paul Ehrlich (1882) and was modified later by Ziehl-Neelsen and therefore also known as Ziehl-Neelsen staining. This is a differential staining used to identify mainly the members of Mycobacterium tuberculosis and leprae. These organisms are difficult to stain by ordinary staining methods due to the presence of high lipid content in their cell wall. Bacteria are classified as

ACID FAST: if they retain the primary stain after the application of strong acid and appear red.

NON ACID FAST: if they do not retain the primary stain and are counterstained by methylene blue.

Reagents: Carbol fuchsin solution,

Acid alcohol solution (3% HCl in alcohol)

Alternatively: sulphuric acid solution (20-25% v/v in water)

Methylene blue counter stain (0.3% w/v aq)

Procedure:

- Prepare a smear of purulent portion of the specimen on a clean glass slide
- Air dry and heat fix the smear
- Flood the smear with freshly filtered carbol fuchsin. Heat gently until steam rises
- Continue to heat for 5 min so that steam is seen but without boiling. Do not allow the slide to dry and add more stain from time to time to prevent this drying
- Cool and wash the stain of the slide with water.
- Cover the slide with acid alcohol solution for 3 min. wash with running water and drain. Repeat the decolorization process until the smear becomes faint pink in color.
- Cover the slide with methylene blue stain and leave it for 2 min

- Wash with tap water , blot dry air the slide under the oil immersion object

Results: Acid fast organism will appear bright red on a blue background while non acid fast organism will appear dark blue in color.

EXPERIMENT-8

ISOLATION OF PURE CULTURE BY MULTIPLE STREAK AND SPREAD OTHER TECHNIQUES

AIM: Isolation of pure culture by the use of plate, streak plate and spread plate techniques.

Introduction

All inanimate surfaces contain microorganisms, which makes them common sources of potential contamination in scientific settings. The outcome of an experiment depends on the scientist's capacity to sterilise tools and work surfaces as well as to keep sterile instruments and solutions away from non-sterile surfaces.

Several plating techniques are often employed in laboratories to isolate, multiply, or count microorganisms including bacteria and phage. All five techniques use aseptic technique, or steps used to keep experimental materials sterile. Procedures described include.

- **streak-plating bacterial cultures to isolate single colonies**
- **pour-plating**
- **spread plating to enumerate viable bacterial colonies.**
- **Isolation of fungi from the given soil sample**

Principle

It is a typical technique to identify and isolate a specific bacterial colony from a bacterial swarm. In the streak plate approach, the number of bacterial colonies is greater at the beginning of the streak and gradually decreases towards the end. Each colony is regarded as a pure colony, which aids in separating it from other colonies.

Requirements

Bacterial culture: Bacillus subtilis and Staphylococcus aureus 24-hour bacterial culture.

Instruments: sterile Petri plates, a nichrome wire loop for inoculation, a burner, and a marking pen.

The medium is nutrient agar.

Equipment: an incubator, a hot air oven, an autoclave, and a colony counter.

1.Streak Plate Method:

- The Culture techniques are commonly used in the laboratory for various purposes for which they are intended.
- To show how the bacteria's cultural traits are expressed.
- To separate the distinct colonies of bacteria from the specimen that contains several microorganisms.
- To assess a bacterium's sensitivity to drugs, antibiotics, or test chemicals, as well as its resistance to them.
- To ensure that the bacteria grows sufficiently for certain biochemical and other tests.
- To calculate the number of bacteria in the specimen that are still alive.
- To keep the stock cultures alive.
- To convey biological materials or store specimens temporarily
- Standard Procedure
- The bottom of the petri dish has the label. The name of the organism and the date are often on labels.
- Use the heat of the Bunsen burner to sterilise the nichrome wire loop.
- Use a sterile nichrome wire loop to collect a sample of the bacterial culture after opening the bacterial culture tube.
- Spread bacterial culture on the nutrient agar plate. To remove the designated quadrants, the agar plate cover must be opened between the Bunsen burners.
- The entire procedure is carried out in a laminar airflow cabinet under highly aseptic conditions.

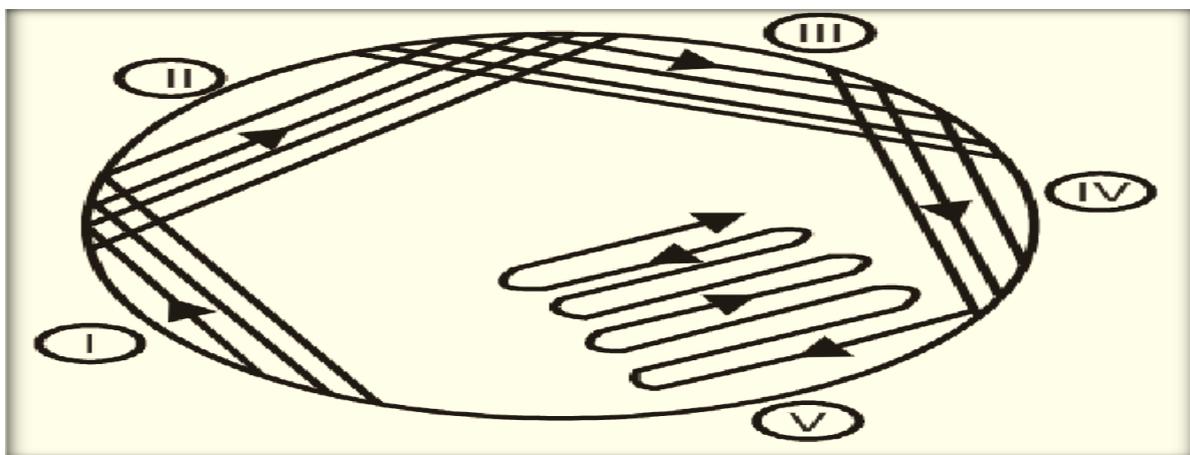
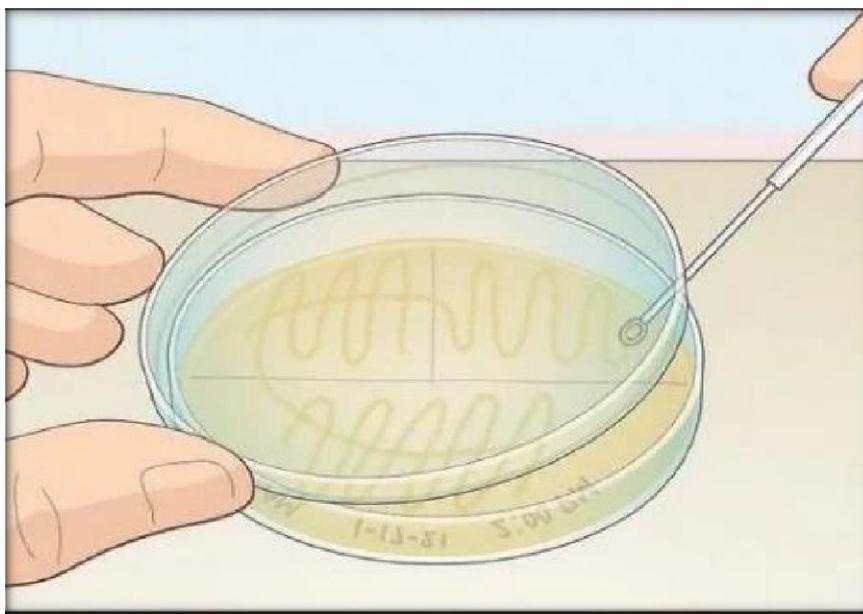


Fig 7.1: Streak Plate

Three Sector streak (t streak)

- Put the nichrome wire loop over Bunsen burners flame to sterilise it.
- Cool the loop of wire in-between the Bunsen burners.
- Submerge the wire loop in the broth culture that contains the assortment of bacteria.
- Streak the bacterial suspension in a zigzag pattern to create T-shaped streaks on the nutrient agar plate using the nichrome wire loop.
- After incubating the plate for 24 hours, the third sector will contain solitary colonies. The first sector will have the strongest growth, with the second sector experiencing less of it.

Fig :Three Sector streak (t streak)



Four – Quadrant streak:

- Put the Nichrome wire loop over a Bunsen burner flame to sterilise it.
- Cool the loop of wire that runs between the burners.
- Use a marker to write the petri dish's name and draw a four-quadrant grid on the bottom of the dish.
- Set a test tube containing a bacterial culture on fire.
- Insert the culture tube with the nichrome wire loop.
- Spread the bacterial suspension across the plate's four quadrants, between the two burners.
- Incubate the plate for 24 hours at 37 °C.

Observation: Examine the growth of isolated colonies on the surface of the nutrient agar plate.

Result: Few numbers of isolated colonies appear along with the points of the streak.

Advantages:

- Distinct separate colonies are obtained by the streak plate method.
- It is a simple method for the isolation of microorganisms.
- Commonly used for isolation of colonies from pharmaceutical products.

Disadvantage:

- Before isolation, there was a higher possibility of contamination.
- This approach only works in terms of quality.
- The colony count is not relevant in other quadrants since only isolation is acquired in the fourth quadrant.

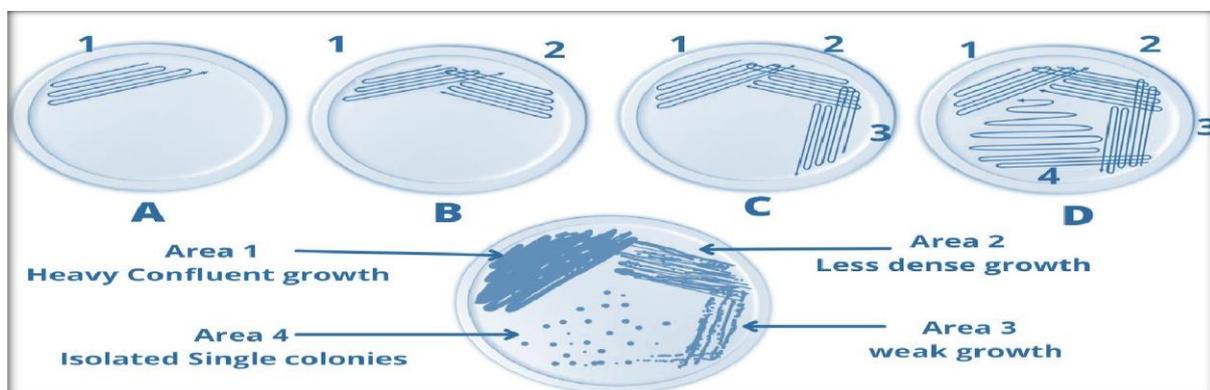


Fig techniques of Streak plate method

2. Pour plate method:

Introduction

For obligate and anaerobic microorganisms, the pour plate method is a typical plating technique. By serially diluting microbial colonies and then counting the colony forming units (CFUs), this approach is used to isolate the colonies. In this procedure, the liquid sample is added to the petri dish before the agar medium has a chance to set.

Colonies develop within and outside of the medium after solidification. Confluent colonies, however, are developing within the medium; the colonies on the surface are counted as viable.

Principle:

The pour plate method involves using a sterile pipette to transfer a predetermined volume of inoculum, typically 1 ml, from a broth or sample into the centre of a sterile Petri dish. After that, 15mL of the molten, cooled agar is added and well mixed in the Petri plate holding the inoculum. The plate is turned over and incubated at 37°C for 24- 48 hours following the agar's solidification.

Both on the surface and within the medium, microorganisms will proliferate. Most colonies that develop beneath the medium are typically tiny and may even be confluent; the few that develop on the agar surface are similar in size and resemble those on a streak plate. Each colony, big and little, is meticulously tallied (using a magnifying colony counter if necessary). The number of microorganisms present in the test sample is determined using the formula:

$$CFU/ml = \frac{\text{Total number of colonies obtained} \times \text{dilution factor}}{\dots \text{Volume of specimen used (aliquot)}}$$

Requirements:

- Test sample
- Nutrient agar or plate count agar (PCA)
- A 45 °C hot water bath
- Sanitised Petri plates
- Flame
- A colony counter with a microscope
- Test tubes 16*150 mm with sterile caps
- Pipettes in a range of sizes, such as 0.1, 1.0, and 2.0 mL

Procedure:

- Sterilise every piece of equipment, flask, and medium needed for the streaking process.
- To reduce infection, sanitise the space where you operate.
- Carefully assemble the Bunsen burner at your workspace.
- Prior to handling any microbiological solution, wash your hands with an antiseptic solution.
- Write down all pertinent details on the petri dish, including your name, the date, the medium you used, and the culture that is being injected.
- Since the sample is already liquid, produce successive dilutions to reduce the concentration of microbial colonies, which vary from 20 to 300 CFU per millilitre. Dilutions up to 10⁻¹⁰ can be made.
- To inoculate, remove the Petri dish covers and add 1 millilitre of the diluted sample within. 15 to 18 ml of the molten agar should be poured onto the sample once it has been slightly heated. Agar shouldn't be too hot or too cold, so keep that in mind. Put the dish's cover on and give it a steady stir.
- Gently combining the diluted material with the agar medium and then pouring it onto

the petri dish is another way for inoculation.

- Permit the plate to set.
- Flip the dish over and let it sit for 24 to 48 hours at the ideal temperature, which is often 37°C.

Result Interpretation

After 24 to 48 hours of incubation, count all colonies. A magnifying colony counter may assist in the counting of tiny, embedded colonies.

Applications of the Pour Plate Method

- It is used by scientists to obtain microbial growth curves and in the calculation of the concentration of cells in a particular sample.
- It is also used to check the effect of various growth factors and environmental factors on the growth rate of the bacteria.
- It is used to separate pure cultures from mixed cultures.
- It is used to identify and count viable fungi and bacteria (calculate CFU per ml) from liquid samples.
- Utilized to create growth curves to study biochemical and microbial metabolic processes and the effect of environmental conditions on the growth of microbial species.

Advantages of the Pour Plate Method

- It is useful for counting viable colonies.
- It can detect very low loads of bacterial counts as well.
- It does not require previously solidified agar plates.
- It can also be used for clinical and environmental samples.

Precautions

- The protocol must be followed in all aseptic conditions, particularly in Laminar air

Flow (Safety Cabinet) to prevent contamination.

- Make sure you accurately measure the amount when preparing the serial dilutions from the sample.
- Use sterile pipettes each time to prevent any mistakes or contamination that may occur.
- Determine the exact amount of dilute specimen prior to injecting it onto Plates of Solidified Media Plates.
- Spread the specimen uniformly on the Media plate until you get clear and healthy colonies.

Limitations

- The organisms that are heat sensitive are susceptible to being affected by molten media between 40-45degC.
- Colonies could be smaller than those in spreading or streaking, which can increase the probability of ignoring them.
- Obligate aerobes could have trouble growing towards the lower portion of the plate.
- Some don't even grow.
- It is time-consuming since it requires dissolving these solids, a series of diluting, and then melting the media at a particular temperature 42 to 45degc.
- Semisolid or solid samples should be suspended prior to the inoculation. It can be very difficult to do this to inoculate if the sample isn't easily dissolved.
- It is necessary to perform regular dilutions of the sample or else many colonies will form that aren't able to be counted and recognized as distinct.
- It takes time for the growth of the organisms and the creation of colonies.

3.Spread plate method.

Introduction:

A mixed sample is spread out across the surface of an agar plate, and a method called the spread plate is used to separate the bacteria from the material. To separate and isolate each bacterial colony, samples are diluted. In enrichment, selection, and screening experiments, the spread plate is frequently employed.

Principle

One of the popular culture techniques for isolating microorganisms, particularly bacteria, in the lab is the spread plate culture method. Using a sterile L-shaped glass rod (Spreader), a serially diluted specimen containing two or more bacteria is applied in a thin layer to the solidified agar media plates while the media plate is being spun on a turntable.

Requirements:

Nutritional agar,
beaker, a busen burner,
an L-shaped bent glass rod and a wax marking pencil,
alcohol with a 95% purity

Bacterial Culture: Bacillus subtilis, Chromobacterium indica, and Staphylococcus album, magnifying colony counter, an autoclave, a hot air oven, and an incubator.

Procedure:

- Set up the nutritional agar plate and label it with the appropriate bacteria, such as Staphylococcus aureus, Staphylococcus album, and Bacillus subtilis.
- Transfer a loopful of the bacterial culture aseptically from the medium in the appropriate Petri plate.
- Place the bent section of the glass rod in a beaker filled with 95% alcohol to sterilise it.

- Next, slide the beaker over the flame of a Bunsen burner.
- Let the rod cool for 15 to 30 seconds.
- Tilt the plate's cover, gently press a sterile rod on the agar surface, and disperse the bacterial suspension evenly.
- Use an alcohol bath to sterilise the bent rod, then reflare it over a Bunsen burner.
- Inoculate the remaining two plates with the bacterial sample by using the same procedure.
- For 24 to 48 hours, incubate all plates at 25°C while they are inverted.

Observation: Few colonies may be separate while the rest of the colonies are maybe in a bunch. Record the result of colonies their form, elevation, pigment formation by colonies, and their size.

Results: Isolated colonies have appeared on the surface of the nutrient agar plate.

Advantages

- It is a simple, easy, and quick method of culturing microorganisms.
- A very low microbial load can be detected.
- The colony morphology of a microorganism can be studied by this method.
- It is a qualitative and quantitative isolation approach that makes it easy to isolate and count.
- It is used in preparing and maintaining stock culture.
- It is the most appropriate method for culturing the aerobic microorganism.

Disadvantages

- The spread plate method allows the growth of other microbes along with desired microbes.
- The spread plate method allows the growth of obligate anaerobic microorganisms.

- Accidental contamination and hence the growth of undesired microbes may be possible.

Limitations:

- Additional tools, such as a spreader, are needed.
- The technique is a little complicated since the material must be in liquid or suspension form and must be serially diluted.
- Before inoculation, solid or semisolid materials must be suspended. If the sample is not readily soluble, it is quite challenging.
- Anaerobes and microaerophiles can't grow there in sufficient numbers.
- If the sample's microbial burden is too high, it is inappropriate. In order to lower the microbial burden at 20–300 CFU/mL, the sample must be serially diluted. We might even need to do pilot tests to obtain this dilution range.

Applications:

- Used to isolate bacteria and fungi from a given sample.
- Used in antimicrobial sensitivity testing, and enrichment and screening experiments.
- Used to calculate the number of viable microorganisms i.e., calculate CFU/mL in a sample.
- Used in food industries, pharmaceutical industries, soil studies, etc.
- Used to mass culture the stock culture or fresh specimen
- Used in clinical laboratories to inoculate the clinical specimens.
- Used to study growth curves, metabolic activities, and biochemical features of microorganisms,
- Used in separating pure culture from a mixed culture.

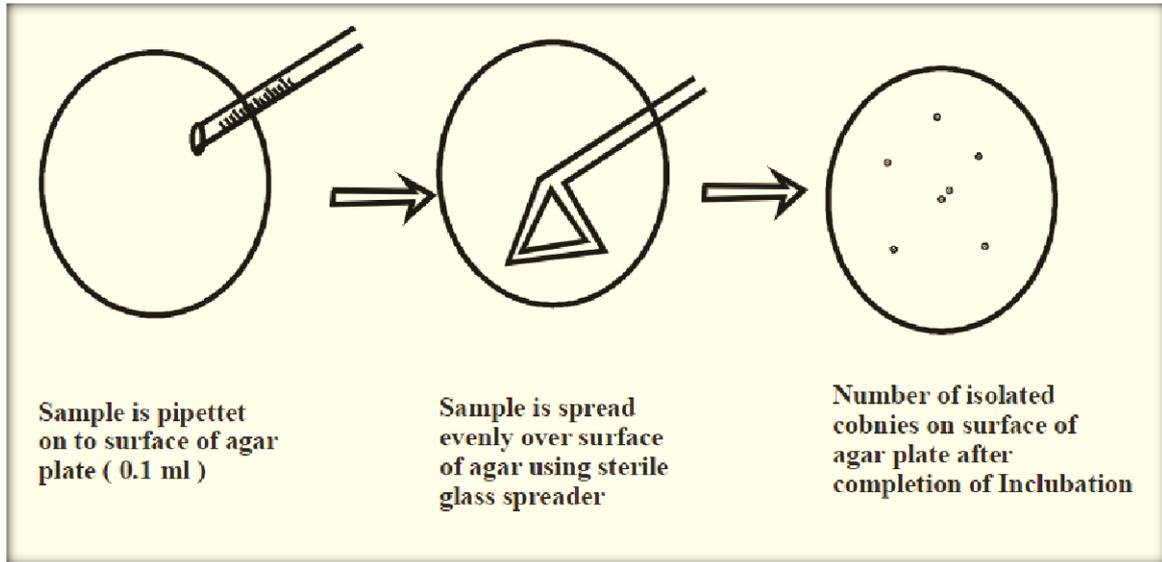


Fig 7.4 Spread Plate Method

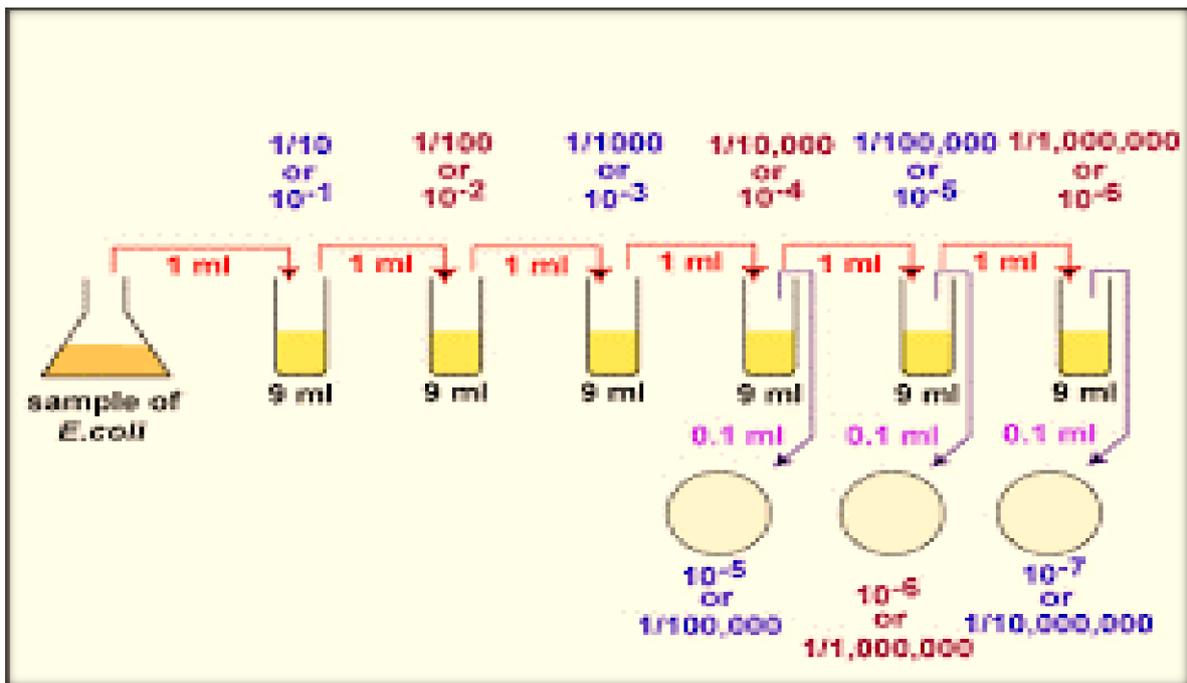


Fig 7.5 Serial dilution of sample

EXPERIMENT-9

MICROBIAL ASSAY OF PENICILLIN (CUP PLATE METHOD)

AIM: To determine the potency of given sample of penicillin and to construct a standard graph.

REQUIREMENTS: Sterile petri plates, sterile pipettes, test tubes, boiling tubes and nutrient agar.

COMPOSITION:

Meat extract – 0.3%, peptone – 0.5%, Agar – 2%, Sodium chloride – 0.5%, distilled water up to 100 ml.

TEST ORGANISM: Staphylococcus aureus.

PRINCIPLE:

The inhibition of microbial growth under the standard condition may be utilized for demonstrating the therapeutic efficiency of an antibiotic. Any subsequent change in the antibiotic molecule which may not be detected by chemical methods can be revealed by microbial activity. Here, microbial assays are very useful to know the possible loss of antibiotic activity. There are also routinely employed to determine the potency of all antibiotic preparations at various stages of development from their crude forms to finished product. The microbial assay is based upon the comparison of inhibition of bacterial growth by measured amount of antibiotic to be examined with that produced by the known concentration of standard preparation of the antibiotic having known activity.

PROCEDURE:

For the microbial assay of penicillin, cup plate method is employed. The melted agar medium which is previously inoculated with the sensitive organism is poured into a petridish of uniform depth of 3.4mm. 2 or 3 plates are prepared for each concentration of penicillin upon solidification of medium, 4 cavities are made at equal distances with a cup borer aseptically.

Penicillin stock solution of 100ug/ml was prepared by dissolving 20mg of penicillin in 33.4ml of sterile water. From the stock solution, different concentrations of 5ug/ml, 10ug/ml, 15ug/ml, 20ug/ml, 25ug/ml of penicillin were prepared. They are designated as S1, S 2, S 3, S 4 and S5 respectively. S3 concentration is taken as reference concentration of penicillin (suppose S1) and reference concentration (S3) are poured aseptically in alternate petridish in cups.

The same is followed for other concentrations replacing with S2, S4 and S5. In each plate 2 cups are filled with one concentration and two cups with another concentration (reference).

The petri plates are kept for some time at room temperature to allow the antibiotic to diffuse in agar medium. Then the plates are incubated at 37°C for 18hrs. After incubation, the diameter of zone of inhibition is measured.

Standard graph is obtained by taking log concentration on x-axis and zone of inhibition on y-axis.

REPORT: the concentration of unknown antibiotic sample was found to be.....

EXPERIMENT-10

MICROBIAL ASSAY OF ANTIBIOTIC (DISC PLATE METHOD)

AIM: To determine microbial assay of penicillin by disc plate method.

APPARATUS: Sterile petriplates, petri dishes, forceps, boiling tubes and nutrient agar medium.

TEST ORGANISM: Staphylococcus aureus.

PRINCIPLE: The inhibition of microbial growth under the standard condition may be utilized for demonstrating the therapeutic efficiency of an antibiotic. Any subsequent change in the antibiotic molecule which may not be detected by chemical methods can be revealed by microbial activity. Here, microbial assays are very useful to know the possible loss of antibiotic activity

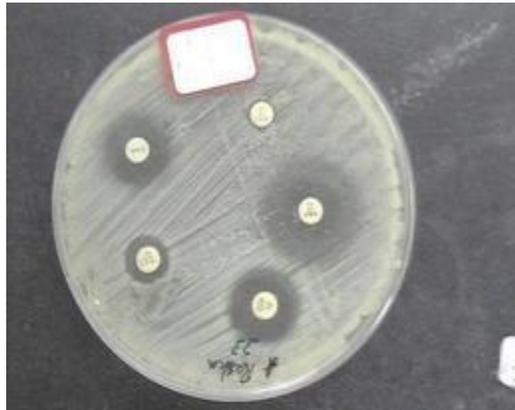
There are also routinely employed to determine the potency of all antibiotic preparations at various stages of development from their crude forms to finished product. The microbial assay is based upon the Comparison of inhibition of bacterial growth by measured amount of antibiotic to be examined with that produced by the known concentration of standard preparation of the antibiotic having known activity.

PROCEDURE:

The required materials were weighed and nutrient agar medium was prepared. It was sterilized by autoclaving at 121°C at 15lb pressure per inch for 15-20 min. after sterilization it was inoculated with Staphylococcus aureus and then the medium was transferred into petridish which was sterilized.

Different concentrations of penicillin (5, 10, 15, 20, 25 ug/ml) were prepared. Take the sterilized paper discs and dip in different concentrations of penicillin with forceps. In each plate, 2 paper discs were placed with one concentration and 2 paper discs with reference concentrations. Then the petriplates were kept aside for 45min for diffusion of antibiotic into agar. Then they are kept for incubation at 37°C for 18-24 hrs in an inverted position.

After incubation, the zone of inhibition was measured and standard graph was plotted on graph by taking log concentration on x-axis and inhibition zone on y-axis.



OBSERVATION:

REPORT: The concentration of unknown antibiotic sample was found to be...

EXPERIMENT-11

MOTILITY DETERMINATION BY HANGING DROP METHOD

AIM: To observe the motility of bacteria in a given bacterial culture by hanging drop method.

REQUIREMENTS: Sprit lamp, inoculation needle, cavity slides, cover slip, Vaseline, young bacterial broth culture, microscope with oil immersion object.

PRINCIPLE: The inhibition of microbial growth under the standard condition may be utilized for demonstrating the therapeutic efficiency of an antibiotic. Any subsequent change in the antibiotic molecule which may not be detected by chemical methods can be revealed by microbial activity. Here, microbial assays are very useful to know the possible loss of antibiotic activity

There are also routinely employed to determine the potency of all antibiotic preparations at various stages of development from their crude forms to finished product. The microbial assay is based upon the Comparison of inhibition of bacterial growth by measured amount of antibiotic to be examined with that produced by the known concentration of standard preparation of the antibiotic having known activity.

PROCEDURE:

- Take a clean glass cavity slide and apply Vaseline around the cavity with the help of needle.
- Using the sterile inoculation loop, place a drop of culture in the centre of cover-slip.
- Invert the cavity slide gently over the cover-slip and see that the drop of culture is in the centre.
- Press down on the edges of cover-slip and see that the Vaseline makes a seal. See that the drop does not touch the slide.
- Quickly and carefully turn the slide right side up, so that the cover-slip is on the upper side and the drop is suspended from cover-slip.

OBSERVATION: Motility is much observed along the edges of the drop.

REPORT:

EXPERIMENT-12

BACTERIOLOGICAL ANALYSIS OF WATER

AIM: To determine the microbiological water Quality.

THEORY:

Natural water supplies such as river, lakes and streams contain sufficient nutrients to support growth of various microorganisms. Microorganisms enter in the water by domestic waste. Hence it is necessary to test the water quality. Water quality can be tested by the presence of some indicator organism. The most frequently used indicator organism is the coliform bacterium, E.Coli. But its presence does not prove the presence of pathogenic bacteria. But this establishes the possibility of the presence of such pathogenic bacteria. Thus presence of coliform bacteria in water is regarded as warning signal.

REQUIREMENT:

Nutrient agar medium, Water sample, Sterilized petriplate, conical flask, Glass spreader.

PROCEDURE:

Put 0.1ml of water sample on the nutrient agar medium.

Spread it with the help of spreader.

Now, incubate the plates at 37⁰C for 18-24hrs.

Count the colonies appeared.

OBSERVATION:

Bacterial colonies appear on the surface of the medium. Count the colonies by Colony counter and record the average number of colonies of all plates.

No. of bacteria/100ml= No. of colonies x 1000

RESULT:

EXPERIMENT-13

BIOCHEMICAL TEST

IMVIC tests includes four tests i.e., Indole test, Methyl red, Voges-Proskauer and citrate tests. These tests are meant to differentiate the Gram-negative intestinal bacilli (enteric bacilli)

AIM: To study the characterization of microbes through biochemical reactions.

PRINCIPLE: Some bacteria oxidise tryptophan resulting in the formation of indole, pyruvic acid and ammonia. Indole thus formed reacts with Kovac's reagent (Para dimethyl amino benzaldehyde) resulting in the formation of cherry red coloured complex.

Tryptophan → Indole + Pyruvic acid + ammonia

Indole → P-dimethylamino benzaldehyde → Quinoidal red-violet complex

MATERIALS REQUIRED:

Nutrient broth culture of Escherichia coli, Proteus vulgaris, Enterobacter aerogenes: SIM agar deep tubes, Kovac's reagent, bunsen burner, inoculating needle, marking pencil.

PROCEDURE:

Inoculate each experimental organism in to its appropriately labeled deep tube by stab inoculation. Maintain one as control (Uninoculated)

Incubate the tubes at 37⁰c for 24 hours

After incubation add 10 drops of Kovac's reagent to all deep tube cultures and agitate the cultures gently.

OBSERVATION AND RESULTS:

Development of cherry red colour on the top layer of the tube is positive for indole test, while absence of such red colouration is negative. For E.coli this is positive, while for E.aerogenes it is negative.

REPORT:

EXPERIMENT-14

IMVIC TESTS

AIM: To study the characterization of microbes through biochemical reactions.

MATERIALS REQUIRED: Nutrient broth, culture of E.coli, Enterobacter aerogenes. MR-VP broth tubes, methyl red, pH indicator, Barrit's reagent

PRINCIPLE:

Methyl red and voges- proskauer tests are performed simultaneously on the same medium and they are used to distinguish bacteria that produce large amount of acid and those that produce neutral product acetone as the end product. Opposite results are usually obtained for the Methyl red and voges- proskauer test. If organisms produce large amount of organic acids from the glucose, the medium turns acidic and methyl red remains red, while other organisms which do not produce acids, the pH of medium will remain above 6.0 and methyl red turns yellow.

MR-VP test is of value in the separation of E.coli, Enterobacter aerogenes which are identical except these characters. This test is also useful as an indicator of sanitary quality of water.

PROCEDURE:

- Take 5 ml of MR-VP broth in each tube and sterilize at 15 lbs pressure for 15 minutes.
- Inoculate two MR-VP tubes with E.coli and another two with Enterobacter aerogenes
- and incubate at 35⁰c for 48 hours
- Uninoculated tube serves as control.
- At the end of incubation period, add 1-2 drops of methyl red and 2-3 drops of reagent II to each tube and shake well after removing the caps so as to expose optimum amount of oxygen.
- Allow the reaction to complete for 15-30 minutes
- Observe the colour changes in microbial culture broth.

OBSERVATIONS AND RESULTS:

Methyl red remains as red in E.coli inoculated broth is a positive for MR test, while Enterobacter aerogenes methyl red gets decolorized and turn yellow indicating negative for MR test. Appearance of crimson to ruby pink colour is a positive for VP test in E.coli culture broth, while no colour change is indicative of negative in Enterobacter aerogenes.

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