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Department of Biochemical Engineering and Biotechnology

Indian Institute of Technology Delhi

Instructions for lab component of BBL132 for 2019 and 2020 batches

Mode of conduct: The practicals for BBL132 will be conducted from 2-5 pm (each working day of the week) in UG Lab, DBEB from Monday, 25th April 2022. For your convenience the schedule for the experiments is enclosed as Annexure 1. You will notice that we have experiments planned for 6 weeks. If possible we will accommodate the last two experiments in the 5-week semester that has been carved out. But for this, we seek your cooperation and willingness.

Your preparedness: It has been a while since the lectures for the course were held. You are requested to brush up the respective theory section before each practical. The handouts are available at: <http://privateweb.iitd.ac.in/~sundar/labhandouts/bbl132.html>

Evaluation: The total marks for evaluation is **50** of which 10 marks are for your lab record. The lab record will be checked once in the middle of the semester and then towards the end. For the remaining 40 marks you will have a quiz towards the end of the semester.

Basic Instructions:

*You are required to wear your lab coats during the entire duration that you are in the UG lab for the practicals.

*100% attendance is compulsory. *If due to any medical emergency you are forced to miss a class, we will try our best to accommodate you on any other day of the week for that particular set of experiments. But for this you need to reach out by email (shilpi@dbeb.iitd.ac.in) at the earliest (same week).*

*You should bring your updated lab records to every practical class.

Annexure 1

Schedule for experiments to be conducted in Lab Semester (2021-22), BBL132P

Week	Experiments
1 st week	To learn the principles of the microscope 1
	To prepare bacterial culture media for growth 2
2 nd week	To study morphology of bacterial organisms by simple staining and to perform Gram staining of the given bacterial cultures 3
	To study the morphotypic diversity in environmental samples 12
3 rd week	To determine total microbial population count by Neubar's Slide method (Direct microscopic count) 4
	To determine bacterial growth curve and to perform dilution plating experiment for enumeration 6
4 th week	To study the influence of different environmental factors (pH and temperature) on microbial growth 7
	To study the anti-microbial sensitivity of antibiotics 8
5 th week	Biochemical tests for identification of microorganisms 9
	To study the effect of ultraviolet radiation on bacterial growth 10
6 th week	To study bacterial spores by staining methods 5
	To screen colonies obtained through UV mutagenesis for presence of lactose non-utilizing mutants. 11

GENERAL FORMAT

PLEASE MENTION THE FOLLOWING ON THE LEFT HAND SIDE OF THE LAB RECORDS (BLANK SIDE)

- 1) AIM OF THE EXPERIMENT
- 2) DIAGRAMMATIC REPRESENTATION(S)
- 3) OBSERVATIONS
- 4) RESULTS

PLEASE MENTION THE FOLLOWING ON THE RIGHT HAND SIDE OF THE LAB RECORDS (RULED SIDE)

- 1) AIM OF THE EXPERIMENT
- 2) REQUIREMENTS
- 3) PRINCIPLES
- 4) PROCEDURE
- 5) PRECAUTIONS

- **I AM EXPLAINING IT BY TAKING AN EXAMPLE OF THE FIRST EXPERIMENT OF YOUR HANDOUTS
TO LEARN THE PRINCIPLES OF THE MICROSCOPES**

PLEASE MENTION THE FOLLOWING ON THE LEFT HAND SIDE

- 1) AIM OF THE EXPERIMENT : TO LEARN THE PRINCIPLES OF THE MICROSCOPE
- 2) DIAGRAMMATIC REPRESENTATION : PLEASE DRAW A DIAGRAM OF THE MICROSCOPE
- 3) OBSERVATIONS: OBJECT WAS OBSERVED AT 4X,10X,40X and 100X
- 4) RESULTS : AN INCREASED MAGNIFICATION WAS OBTAINED AS MAGNIFICATION POWER WAS INCREASED FROM 4 TO 100X

PLEASE MENTION THE FOLLOWING ON THE RIGHT HAND SIDE OF THE LAB RECORDS

- 5) AIM OF THE EXPERIMENT: TO LEARN THE PRINCIPLES OF THE MICROSCOPE**
- 6) REQUIREMENTS:MICROSCOPES,GLASS SLIDES,IMMERSION OIL**
- 7) PRINCIPLES: EXPLAIN IN BRIEF**
- 8) PROCEDURE :MENTIONED IN THE HANDOUTS**
- 9) PRECAUTIONS:MENTIONED OR AS EXPLAINED DURING CLASSES**

BBL132 – General Microbiology (Sem I)

List of practicals

- Expt 1. To learn the principles of the microscope
- Expt 2. To prepare bacterial culture media for growth
- Expt 3. To study morphology of bacterial organisms by simple staining and to perform Gram staining of the given bacterial cultures.
- Expt 4. To determine total microbial population count by Neubar's Slide method (Direct microscopic count)
- Expt 5. To study bacterial spores by staining methods.
- Expt. 6 To determine bacterial growth curve and to perform dilution plating experiments for enumeration.
- Expt. 7 To study the influence of different environmental factors (pH and temperature) on microbial growth.
- Expt. 8 To study the anti-microbial sensitivity of antibiotics.
- Expt. 9 Biochemical Tests for identification of microorganisms.
- Expt. 10 To study the effect of Ultraviolet radiation on bacterial growth.
- Expt. 11 To screen colonies obtained through UV mutagenesis for presence of lactose non-utilizing mutants.
- Expt 12 To study the morphotypic diversity in environmental samples

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EXPERIMENT # 1

AIM

To learn the principles of the microscope

LEARNING OBJECTIVES

- a) Learn the names of the major parts of the light microscope and how they function.
- b) Understand the importance of resolution and learn what affects the resolving power of a microscope.
- c) Learn how to check your microscope before you use it and before you put it away.
- d) Draw the microscope, label all of the parts. Briefly note various functions

What should you know by the end of this class?

1. Learn the principles of optical Microscopy and be able to identify each part of an optical microscope along with its function.
2. Understand the basic idea behind other types of microscopy (Dark field, DIC, Fluorescence, Confocal, Electron)
3. How to use a microscope (focusing, aperture adjustment etc.)
4. Precautions and instrument care

Using the Microscope

- a. **Lower** the Slide platform and place the slide in position using the **slide clips**.
- b. Use the **stage adjustment knobs** to position the slide such that the edge of a stain (where the dye seems to be brightest) is just above the **condenser** opening.
- c. Switch on the lamp and place it at an intermediate brightness. Also use the condenser slider to **full open position**.
- d. Position the 4X lens at the sampling position and lower it as much as possible **without** touching the slide, then while looking through the eyepiece, slowly raise the lens using the **coarse adjustment knob**. When you see something, use the fine adjustment knob to bring it into clear focus. The fine adjustment knob only works over a small range, so bring it as near as possible to focus using the coarse adjustment knob, then us the fine adjustment knob.
- e. Now that you have brought the right layer into focus, you can use the **stage adjustment knobs** to move the slide slowly and identify a region of good density of cells (At this stage, you cannot identify anything, but you can get identify the cells from the colour of the dye). If everything looks fine, then move on to the next step.
- f. **Without moving the slide (don't touch the stage adjustment knobs), use the coarse adjustment knob to lower the stage.** Put the 10X in the sampling position, and repeat the above procedure for focusing. Don't move the stage using the stage adjustment knobs until you have brought the slide into focus. After you have obtained the focus, then you can move the slide to find the best spot (most number of cells/best looking ones etc).
- g. Do the same with the 40X magnification.
- h. If required, then cover the slide with a cover slip and place a droplet or 2 of **oil** on the cover slip. Lower the 100X such that it is in contact with the oil and the **oil forms a layer between the objective lens and the coverslip**. This increases the resolution of the microscope allowing you to use the 100X. The same procedure for focusing may be followed as above, here the lower most position is when the lens just touches the cover slip.

Precautions

1. To move the microscope, hold it by the arm, don't ever hold it using the eyepiece or lens holder or the stage.
2. Switch off the lamp when not in use, since the lamps have a short lifetime.
3. Don't move the coarse adjustment knobs too fast, the lens might crash into the slide!!!
4. While changing the slide, always lower the stage.
5. Don't ever touch the lens bottom directly

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EXPERIMENT # 2

AIM

To prepare bacterial culture media for growth

BACKGROUND

Introduction

Microrganisms need nutrients, a source of energy and certain environmental conditions in order to grow and reproduce. In the environment, microbes have adapted to the habitats most suitable for their needs, in the laboratory, however, these requirements must be met by a culture medium. This is basically an aqueous solution to which all the necessary nutrients have been added. Depending on the type and combination of nutrients, different categories of media can be made.

Types of Media

1) Complex media / Non-synthetic media

Complex media are rich in nutrients, they contain water soluble extracts of plant or animal tissue (e.g., enzymatically digested animal proteins such as peptone and tryptone). Usually a sugar, often glucose is added to serve as the main carbon and energy source. The combination of extracts and sugar creates a medium which is rich in minerals and organic nutrients, but since the exact composition is unknown, the medium is called complex.

2) Defined media / Synthetic media

Defined media are media composed of pure ingredients in carefully measured concentrations dissolved in double distilled water i.e., the exact chemical composition of the medium is known. Typically, they contain a simple sugar as the carbon and energy source, an inorganic nitrogen source, various mineral salts and if necessary growth factors (purified amino acids, vitamins, purines and pyrimidines).

Selective/differential media are media based on either of the two categories above supplemented with growth-promoting or growth-inhibiting additives.

The mixture of necessary nutrients can be used as a liquid medium, or a solidifying agent can be added. "Agar agar" is a natural polysaccharide produced by marine algae and is the most commonly used solidifying agent added to media (end concentration usually 1.5 % w/v). If hydrolysis of the agar is suspected, a silica gel is used as a replacement solidifying agent. The most commonly used media for cultivation of bacteria is nutrient broth/ agar, for cultivation of fungi is potato dextrose agar and for yeast is Malt extract-peptone-dextrose agar.

PREPARATION OF MEDIA

Liquid media/broth: For propagation of large numbers of microbes, fermentation slides

Solid media : For propagation of large numbers of microbes, fermentation studies

Semi-solid media : For motility studies. More jelly like texture, less concentration of agar or gelatin

NUTRIENT BROTH / AGAR

Beef extract	3g
Peptone	5g
NaCl	5g
Distilled water	1L

MGYP BROTH / AGAR

Malt extract	3g
Yeast extract	5g
Glucose	10 g
Peptone	10 g
Distilled water	1L

For BROTH

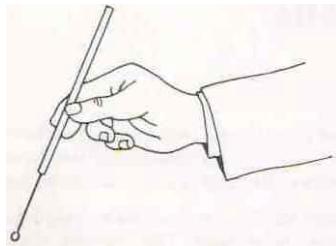
- Weigh the above ingredients, add it in an Erlenmeyer flask and dissolve in 800 ml of distilled water.
- Adjust pH 7.2.
- Make up the volume to 1 L.

For AGAR

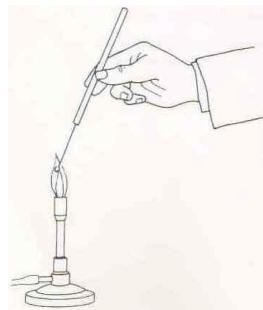
- To the above preparation add 2% w/v agar-agar.
- Plug the flask with cotton plug.
- Sterilize by autoclaving at 15 psi/ 121°C for 15 minutes.

ASEPTIC METHOD FOR TRANSFERRING CELLS

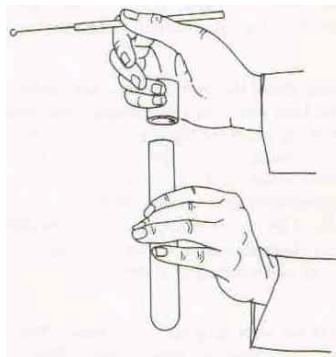
Grasping the inoculation loop



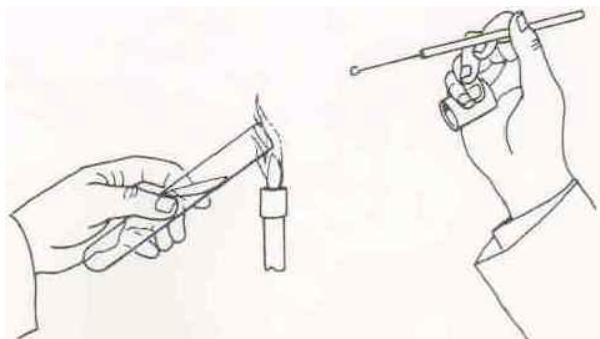
Holding the inoculating loop at a 60 degree angle in the hottest part of a Bunsen burner flame



Removing the plug or cap of the tube



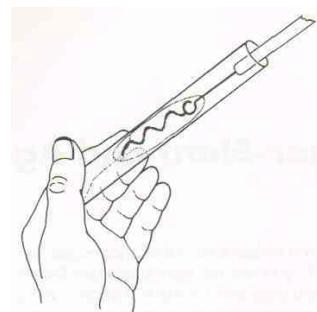
Flaming the tip of the tube



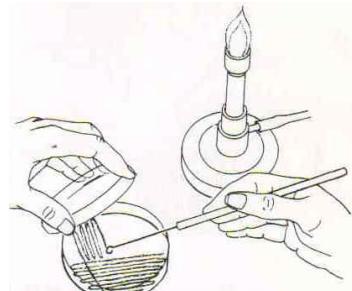
Removing a small amount of culture from the tube



Transfer of culture to Agar slants



Transfer of culture to Streak plates



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EXPERIMENT # 3

AIM

To study morphology of bacterial organisms by simple staining and to perform Gram staining of the given bacterial cultures.

BACKGROUND

Simple staining of bacterial organisms is very important starting point for identifying species. In this procedure, single stain is used to colour a bacterial organism. Most commonly used dye for simple staining is methylene blue, crystal violet, and basic fuchsin. These dyes contain colour-bearing ions (chromophores) and are positively charged (cationic). As bacteria are slightly negatively charged, they produce attraction with cationic chromophores of dyes called basic dyes.

Gram staining method is named after a Danish bacteriologist Hans Christian Gram (1882). It is one of the most important staining techniques in Microbiology and is usually the first step in identification of unknown culture. It is also used for the classification of bacteria into two general groups, namely, Gram positive and Gram negative.

MATERIALS REQUIRED

Microbial cultures, stain – methylene blue, basic fushein and crystal violet, ethanol, iodine, safarnin, distilled water, tissue paper, oil immersion, grease-free slides, microscope, etc

PROCEDURE FOR SIMPLE STAINING

- 1) Prepare a smear of culture on the microscopic slide and air dry.
- 2) Heat-fix cells by passing the slide rapidly across the flame (do not overheat as it may shrink the cells and distort the shape)
- 3) Flood the slide with methylene blue and allow it to react for 1 minute.
- 4) Tilt the slide and wash with distilled water to remove excess stain (be gentle).
- 5) Blot off excess water and allow it to dry completely.
- 6) Carefully position the oil immersion objective over the smear after adding immersion oil to the smear.

PROCEDURE FOR GRAM STAINING

- 1) Prepare a smear of culture on the microscopic slide and air dry.
- 2) Heat-fix cells by passing the slide rapidly across the flame (do not overheat as it may shrink the cells and distort the shape)
- 3) Flood the slide with crystal violet and allow it to react for 1 minute.
- 4) Tilt the slide and wash with distilled water to remove excess stain (be gentle).
- 5) Flood the slide with Gram's iodine and allow reacting for 1 minute.
- 6) Tilt the slide and wash with distilled water to remove excess stain.
- 7) Wash the smear with alcohol for 15-20 sec, by holding the slide in vertical position and observing the release of CV-I complex as blue drops. (This step is done till blue drops stop appearing).
- 8) Wash the smear with distilled water.
- 9) Flood the smear for one minute with safranin.
- 10) Tilt the slide and wash with distilled water to remove excess stain (be gentle).
- 11) Blot off excess water and allow it to dry completely.
- 12) Carefully position the oil immersion objective over the smear after adding immersion oil to the smear.

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EXPERIMENT # 12

AIM

To study the morphotypic diversity in environmental samples

BACKGROUND

Microbes grow on solid media as colonies. A colony is defined as a visible mass of microorganisms originating from a (single) mother cell, therefore a colony constitutes a clone of bacteria all genetically identical (except mutations that occur at low frequency). The number of cells within a colony can even reach a few billion. On a given medium, a colony's shape, colour, consistency, surface appearance and size - for a given incubation time - are often characteristic, and these features are often used in the identification of particular bacterial strains (Fig. 1).

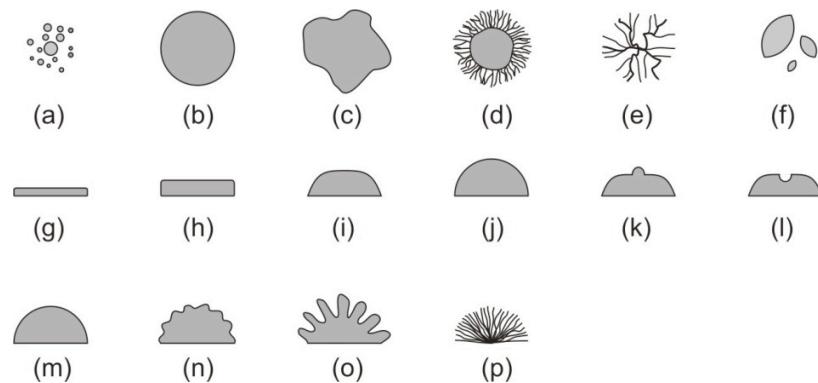


Fig. 1. Colony morphology of bacteria. Form: (a) punctiform (b) circular (c) irregular (d) filamentous (e) rhizoid (f) spindle. Elevation: (g) flat (h) raised (i) convex (j) pulvinate (k) umbonate (l) crateriform. Margin: (m) entire (n) undulate (o) lobate (p) filamentous.

Characteristics of cell morphology have great importance in the classification of bacteria using traditional taxonomical methods. Microorganisms cannot be identified solely by morphological characteristics, since bacterial cells can only be assigned to a limited number of categories

Bacteria are μm -sized organisms, where cell size is an important aspect of a thorough morphological characterization. The size and shape of the cells are usually determined following staining. The circumstances of culturing, the age of the culture and the physiological condition of bacterial cells can alter cell size and shape. According to their shape, bacteria can usually be identified as rods, cocci or spirals. An average rod-shaped bacterium is 2-5 μm long and 0.5-0.8 μm wide in diameter. The average diameter of a sphere-shaped bacterium is 0.8 μm . The size of some bacterial groups deviates from average values: spirochetes include some extremely thin (0.2 μm) bacteria, while there are some giants: *Thiomargarita namibiensis* (100-300 x 750 μm) and *Epulopiscium fishelsoni* (50 x 600 μm).

OBJECTIVE OF STUDY

Agar plates with colonies originating from an environmental sample

MATERIAL

Ruler, Magnifying glass, petridishes, saline, spreader, test tubes ,marker pen

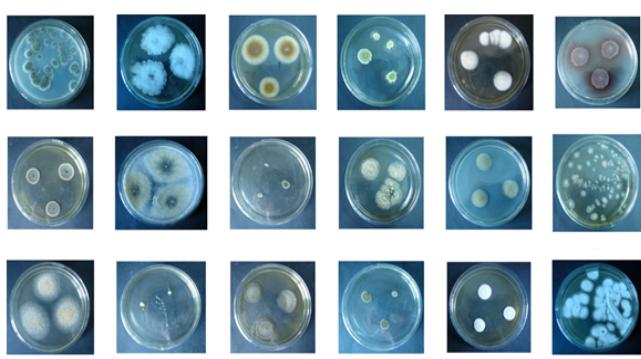
PROCEDURE

1. Collect various environmental samples like water, soil in a sterile container from different location near to your lab
2. Dilute samples obtained from soil to 10^{-6} to 10^{-8} and water to 10^{-2} in saline buffer and plate them on Nutrient agar by spread plate method
3. To know the morphotypic diversity in air simply expose your petriplates at different location like under laminar, outside laminar, inside lab or out side of lab in corridor
4. Incubate the plates for 24 to 48hrs at 28 °C and 37 °C
5. Select 5 different discrete colonies from the surface of a Petri plate and characterize them as follows:
 - size of the colony (diameter in mm),
 - shape or form of the colony (punctiform, circular, irregular, filamentous, rhizoid, spindle),
 - elevation of the colony (flat, convex, pulvinate, umbonate, crateriform),

- margin of the colony (entire, undulate, lobate, filamentous),
- pigmentation of the colony (diffusible water-soluble or water-insoluble pigments),
- surface of the colony (smooth, glistening, rough, dull, wrinkled),
- density of colony (transparent - clear, opaque, translucent - almost clear, but distorted vision-like looking through frosted glass, iridescent - changes colour in reflected light),
- consistency of colony by touching it with an inoculating loop (butyrous, viscid - sticks to loop, hard to get off, brittle - dry, breaks apart, mucoid),
- Presence or absence of diffusible pigment in the medium around the colony.

RESULTS

Results will be observed as mentioned in the pictures and will be recorded in tabular form



Precautions

1. Use sterile containers for collecting your samples
2. Make proper dilution for samples obtained from soil and water
3. Avoid any contact with cultures

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EXPERIMENT # 4

AIM

To determine total microbial population count by Neubar's Slide method (Direct microscopic count)

BACKGROUND

The number of cells in a microbial population can be measured by counting under microscope. Population of cells can be known by counting number of cells or weight of cell mass. There are several methods for counting microbial cells or estimating cell mass, suited to different organisms or different problems. Wolford stain distinguishes between dead and viable cells. Viable cells do not take up the stain and appear colorless while dead cells appear colored.

APPARATUS AND MATERIALS REQUIRED

Microbial culture (*Saccharomyces cerevisiae*), Wolford stain, Neubar's Slide, pipettes, coverslips, microcentrifuge tubes, microscope etc.

Details of Neubar's Slide: It is a thick glass slide (Fig. 1) on which there is an H-shaped trench enclosing two plateform A and B which is 1/10 mm deeper than rest of the slide.

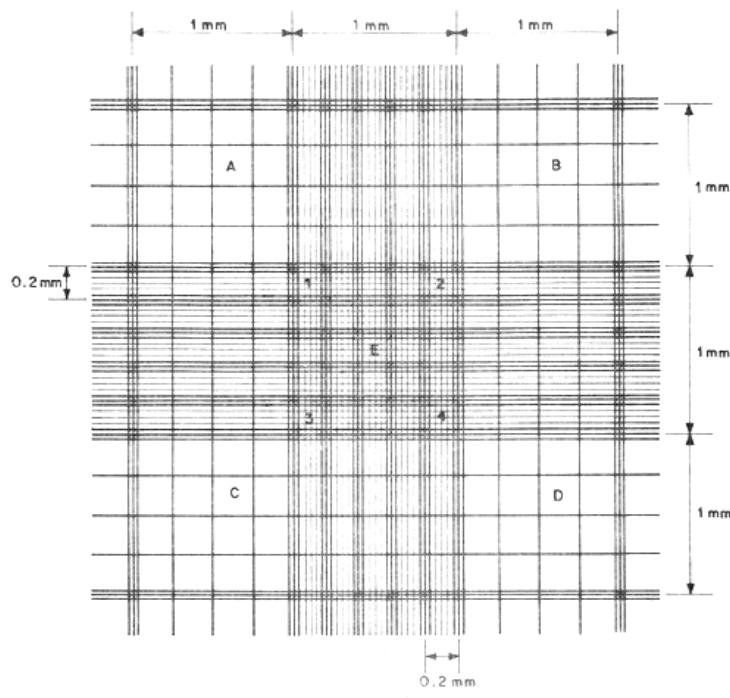


PROCEDURE

- 1) Take 1 ml of microbial culture after shaking in a test tube.

- 2) Add 1 ml of Wolford stain and mix properly
- 3) In 9 ml of distilled water, add 0.1 ml of stained microbial solution and make it upto 10 ml.
- 4) Place a cover glass on the Neubar's counting chamber; charge the solution of cells inside from the pipette. Wait for one minute so that the cells should settle down in chamber.

- 5) Count the number of microbial cells in the four corners squares 1, 2, 3, and 4 (Fig. 2) on Neubar Chamber under high power in Bright field microscope.
- 6) Calculate the total microbial cell count.



CALCULATION

Each corner square has a side of 1 mm and as the depth is 1/10 mm.

Volume of each square = 1/10 cubic mm

Therefore volume of 4 corner square = 4/10 cubic mm.

- a) Assume X be the number of microbial cells in four squares
- b) In 4/10 cubic mm area of slide, the number of microbial cells = N
- c) In 1 cubic mm of diluted mixture of cells = $\frac{N}{4} \times 10^3$

And as dilution of microbial culture is 1 x 200,

$$\text{Total population of microbial cells in undiluted culture 1 cubic mm} = \frac{N \times 10 \times 200}{4} = 500 \times N$$

DISADVANTAGES OF THE METHOD

1. Small cells are difficult to see under microscope and some cells may probably be missed.
2. Precision is difficult to achieve.
3. Unsuitable for low density culture.
4. It will be difficult to count highly motile cultures.

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EXPERIMENT # 6

AIM

To determine bacterial growth curve and to perform dilution plating experiments for enumeration.

BACKGROUND

Bacterial growth is the division of one bacterium into two identical daughter cells during a process called binary fission. Hence, doubling of the bacterial population occurs. Bacterial growth in a typical batch culture is divided into 4 phases: lag, log, stationary and death phases. Determination of the growth curve requires bacterial enumeration (cell counting) by direct and individual (microscopic), direct and bulk (biomass), indirect and individual (colony counting), or indirect and bulk (most probable number, turbidity, nutrient uptake) methods.

The simplest method to determine the growth curve is spectrophotometric. At 600 nm, the optical density (OD) is the measure of solution turbidity. Thus, the samples taken at different time intervals are analyzed by taking OD at 600 nm and plotted to give the growth curve. Death phase is not observed in such case because dead cells also contribute to absorbance.

Therefore, dilution plating is performed. In this method the culture is first serially diluted in normal saline and then plated on the appropriate medium (nutrient agar). Serial dilution involves adding 1 ml culture to the 9 ml normal saline to give 1/10 diluted culture. Now, 1 ml of 1/10 diluted culture is added to another 9 ml normal saline to give 1/100 diluted culture and so on.

After dilution, appropriate dilution is plated on the surface of nutrient agar. After the requisite incubation period, visible colonies appear on the plates which are counted to determine the number of viable cells originally present in the culture. Bacterial count here is described not in cells/ ml, but as colony forming units (CFU)/ml, because it may be a group of bacterium which has given rise to a colony.

$$\text{CFU/ ml} = \text{no. of colonies/volume plated} * \text{Dilution factor}$$

Dilution factor is the reciprocal of the dilution plated.

The colony count should be between 30-300. If it < 30, it is called ‘too less to count’ (TLTC) and if >300 it is called TNTC or ‘too numerous to count’.

APPARATUS AND MATERIALS REQUIRED

a) For turbidity measurements.

Bacterial culture (*Escherichia coli*)

Sterile nutrient broth.

Shaker incubator

Sterile eppendorfs

Laminar air flow

Cuvette

Spectrophotometer.

b) For dilution plating

Sterile 0.85 % NaCl solution (9 ml) in tubes.

Nutrient agar plates

Pipettes

Tips and spreader

Incubator (at 37°C)

Colony counter

PROCEDURE

- 1) Take 4 ml of given microbial culture and inoculate 100 ml of the sterile nutrient broth.
- 2) Harvest a 0 hour sample in sterile eppendorf and in a cuvette after proper shaking.
- 3) Sample taken in cuvette is measured for O.D at 600 nm.
- 4) Incubate the culture at 37°C with shaking at 250 rpm.
- 5) Harvest the culture in a manner similar to described in step 2 and perform step 3.
- 6) Plot the value of the O.D with time to get growth curve.
- 7) The samples harvested in sterile microfuge tubes are to be serially diluted and plate 100 µl of diluted culture on nutrient agar.
- 8) Incubate the plates at 37°C incubator.
- 9) After overnight incubation, count the colonies and calculate CFU/ ml and obtain CFU/ml v/s time plot to get the growth curve.

Precautions

1. Ensure adequate shaking before harvesting each sample and before taking O.D.
2. While doing dilution plating, vortex before taking samples for further dilution and for plating.
3. Perform serial dilution and plating under sterile conditions.

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EXPERIMENT # 7

AIM

To study the influence of different environmental factors (pH and temperature) on microbial growth.

BACKGROUND

The total environment must be sustained to achieve the desired growth of microorganisms. Factors such as temperature, hydrogen ion concentration of the organism's environment exerts the greatest influence on its growth. Microorganisms need optimum temperature and pH for their growth. If the pH and temperature are varied, then a marked effect on the growth can be observed. However, the optimum temperature varies from one organism to another. Some prefer neutral pH while others grow best under alkaline or acidic conditions because at their optimum temperature/pH, their enzymatic reactions proceed at maximum rates. The rates of reactions can slow down considerably or stop if the pH/temperature varies to a great extent.

PROCEDURE

(A). To study effect of temperature:

- 1) Inoculate four tubes of 10 ml nutrient broth with 100 μ l of given freshly grown microbial culture broth.
- 2) Incubate the tubes at different temperatures such as 4°C, 30°C, 37°C and 45°C for 24 h.
- 3) Read the optical density at 610 nm against medium as blank.
- 4) Plot optical density vs time at different temperatures and determine the optimum temperature for growth of the given microorganism.

(B). To study effect of pH:

- 1) Prepare five tubes of 10 ml nutrient broth and adjust the pH to 5, 6, 7, 8 and 9 separately.
- 2) Inoculate four tubes of 10 ml nutrient broth with 100 μ l of given freshly grown microbial culture broth and incubate at 30°C for 24h.
- 3) Read the optical density at 610 nm against medium as blank.
- 4) Plot optical density vs pH at different pH and determine the optimum pH for growth of the given microorganism.

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EXPERIMENT # 8

AIM

To study the anti-microbial sensitivity of antibiotics.

BACKGROUND

Different antibiotics are produced by different microorganisms (moulds and Streptomyces). Their mode of action on the microorganisms are different. Some antibiotics interfere with protein synthesis eg. Chloromycetin, kanamycin, tetracycline, etc. Others like penicillin and Ampicillin stop cell wall synthesis. Some affect DNA synthesis, eg. Novobiocin. Clinically different antibiotics are selected for different causative microorganisms of different diseases. Their selection depends upon antimicrobial sensitivity of antibiotics and their side effects.

After the causative organism is isolated, a broth culture is grown in a medium like Nutrient Broth and flooded on a suitable agar plate. After the surface is dry, the antibiotic discs are placed carefully and the plates are incubated for 18-20 hours. The zones of inhibition can be measured and the result is written as sensitive, moderately sensitive or resistant.

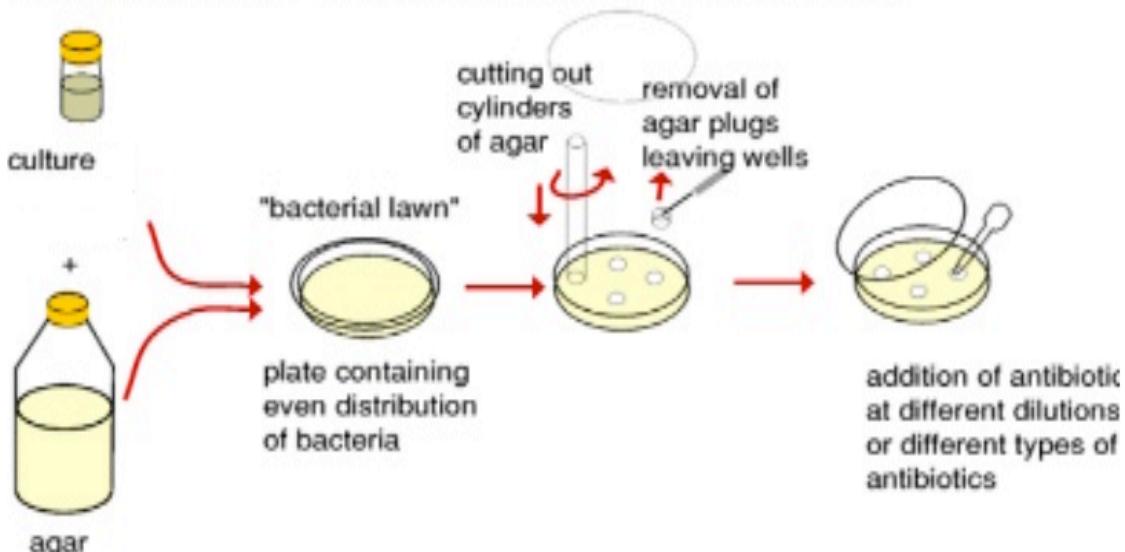
PROCEDURE

There are various methods for doing antimicrobial sensitivity, but the disc diffusion method is widely used

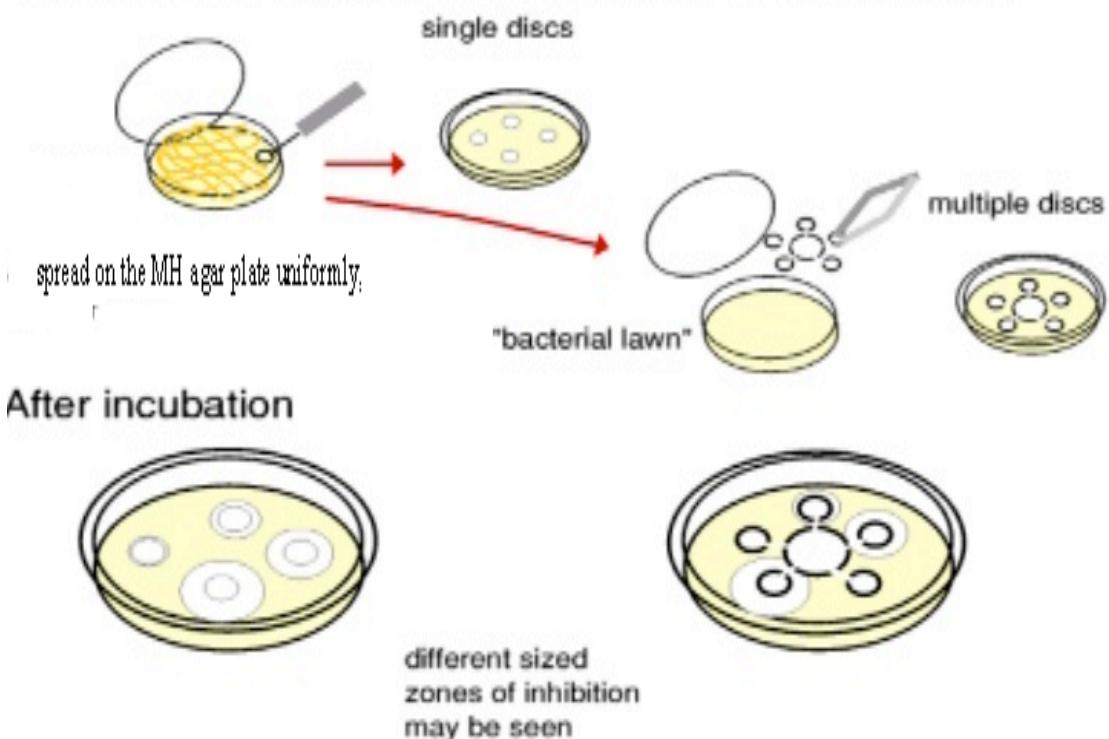
- 1) Take 5-10 similar clones of causative organisms/test culture and transfer to about 2 ml of Nutrient Broth in a test tube and incubate in a shaker at 30-37°C for 4-6 hours, until a very fine turbidity appears.
- 2) Take 100 µl(SECOND METHOD) of this culture and spread on the MH agar plate uniformly, using a glass rod and turntable and allow the surface to dry in Laminar Flow Hood for sometime (care must be taken that not a single drop of culture falls on the laminar flow bench).
- 3) Aseptically place the antibiotic disc, with a pair of forceps, onto the agar surface. The firm contact of these discs on the agar surface is necessary for uniform diffusion of antibiotic in the surrounding medium(Fig-1).
- 4) Write the codes of the antibiotics on the other side of the plate. Refer to Table 1 for the antibiotic codes.
- 5) Incubate the plates upside down in an incubator at 30-37°C for 18-20 hours.
- 6) Measure the zones of inhibition (clear zones) of each antibiotic with the help of a scale and express the results as sensitive, moderately sensitive or resistant(Pictures 1 and 2).

Antimicrobial sensitivity testing by different methods

Antibiotic assay by the zone of inhibition method



Alternative method using paper discs containing different antibiotics, or different concentrations of one antibiotic



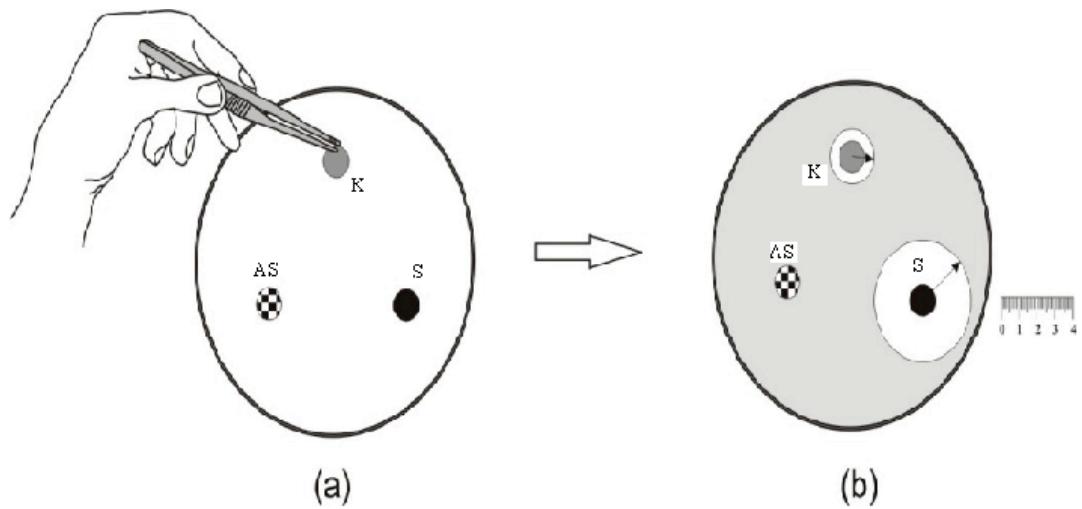
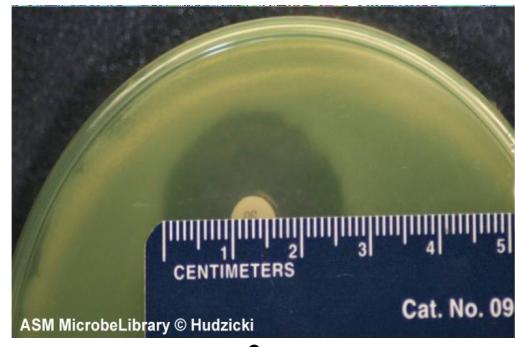
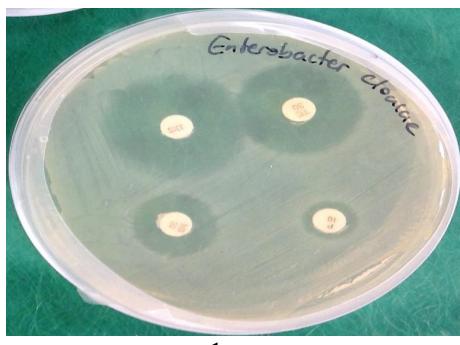


Fig-1

Table 1: Nomenclature for antibiotic discs to be used in diffusion sensitivity tests

Antibiotic	Symbol
Ampicillin	AS
Kanamycin	K
Streptomycin	S



PRECAUTIONS

1. Do not over incubate your test plates
2. Prepare bacterial lawn of appropriate dilution

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EXPERIMENT # 9

AIM

Biochemical tests for identification of microorganisms (Kit based)

BACKGROUND

All the metabolic activities in microorganisms are mediated by enzymes. The chemical end products of these enzymatic actions could be measured. By making a series of different biochemical and physiological tests, a pattern of activity can be established. On the basis of these, identification of different microorganisms can be made. You will be using performing some of the most common tests.

MATERIALS

(1) Two microbial cultures (*Escherichia coli*, *Pseudomonas* sp.) (2) Peptone broth with different sugars and phenol red (3) Simmons citrate agar slants (4) Kovacs reagent (5) Methyl red (6) Durham tubes (7) Triple sugar iron agar slants.

PROCEDURE

1. Acid and Gas production from Carbohydrates

The microbes breakdown common carbohydrates to produce various organic acids (like acetic and lactic acids) and gases (like carbon dioxide and hydrogen). The formation of acid can be detected by including a pH indicator in microbial growth media. The gas formation can be detected by placing an inverted Durham tube in the test tube.

- a) Prepare three tubes each of the following media: Glucose broth with phenol red and Durham tube, Lactose broth with phenol red and Durham tube.
- b) Inoculate the given strains of *E.coli* and others into each tube and incubate at 37 °C overnight. Include an uninoculated control for each set.
- c) After 24 hours, observe the cultures for acid and gas formation (Fig-1)

2. IMViC

2a) Indole production

Indole, a nitrogen-containing compound formed from the degradation of the amino acid tryptophan, produced only by certain microorganisms.

- a) Inoculate two tubes of 1% tryptone broth with *E.coli* and *Pseudomonas*.
- b) Incubate at 37 °C for 24 hours.
- c) To each tube, add 3 ml of Kovacs solution and mix well. Reddening of alcohol layer indicates the presence of indole (Fig-2)

2b) Methyl-Red/Voges-Proskauer (MR-VP) test

These tests are important in the identification of the gram-negative non-spore forming rods and some species of *Bacillus*. Instead of accumulating mostly acidic products from the fermentation of glucose, some bacteria convert the metabolic intermediate, pyruvic acid, to neutral products. When the Voges-Proskauer test is applied, acetyl methyl carbinol is an easily detected neutral product.

The methyl-red test detects organisms that do not convert acidic products to neutral products and thereby produce a final pH lower than that of organisms producing neutral products. Because of the lower pH, the MR indicator changes to red color, which is a positive test.

Voges-Proskauer (VP) test

- a) Inoculate a tube of MR-VP medium (5 ml) with *E.coli* and *Pseudomonas* and incubate for 48 hours at 37 °C.
- b) Test for acetyl methyl carbinol.
 - i. Decant about ¼ volume of culture into a clean test tube
 - ii. Add 0.5 ml (8-10 drops) of alpha naphthol solution (5% in alcohol)
 - iii. Add 0.5 ml of 40% KOH solution containing 0.3% creatine.
 - iv. Shake thoroughly and allow to stand for 5-30 minutes.
 - v. The appearance of a pink to red color indicates the presence of acetyl methyl carbinol. (Fig-2)

Methyl-Red (MR) test

- c) Test for acid production by adding few drops of an alcoholic solution of methyl red to the rest of the culture. A distinct red color is considered to be a positive test. Yellow is negative. (Fig-2)

2c) Citrate utilization

The ability to utilize citrate as a sole source of carbon and energy can be used to distinguish between certain gram-negative rods. Growth on Simmons citrate agar is a positive test for citrate utilization. Certain organisms that give a positive test increase the pH, changing the bromothymol blue indicator in the medium from green to blue.

- a) Inoculate slants of Simmons citrate agar with *E.coli* and Citrobacter strains.
- b) Incubate at 37 °C for 48 hours
- c) The presence of growth at the end of incubation is a positive test. (Fig-2)

3. Catalase activity

The catalase enzyme breaks down hydrogen peroxide to give free oxygen



The gas can be readily seen as a white froth when a few drops of 3% H_2O_2 is added to a microbial colony or to a broth culture.

- a) Add a few drops of 3% H_2O_2 to the culture growth and observe it closely for the appearance of bubbles of oxygen.
- b) Add a few drops of 3% H_2O_2 to broth culture and look for a streaming up of O_2 bubbles. (Fig-3)

RESULTS

Report your result as given below.

Acid and Gas production from Carbohydrates

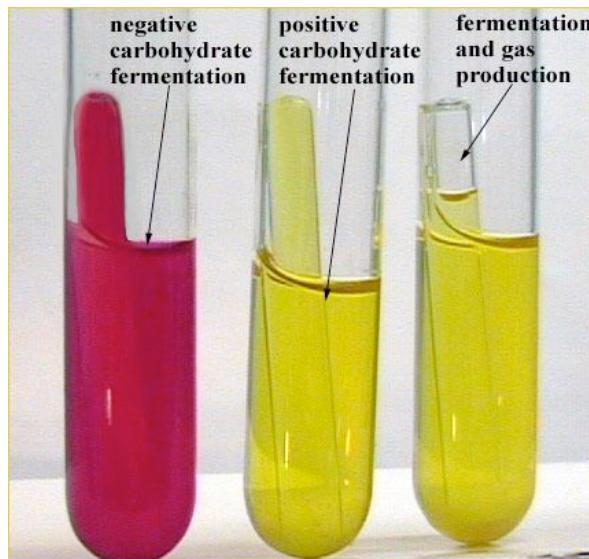


Fig-1

IMViC

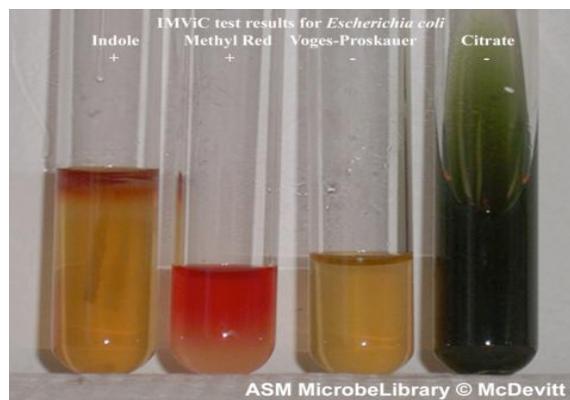


Fig-2

Catalase activity

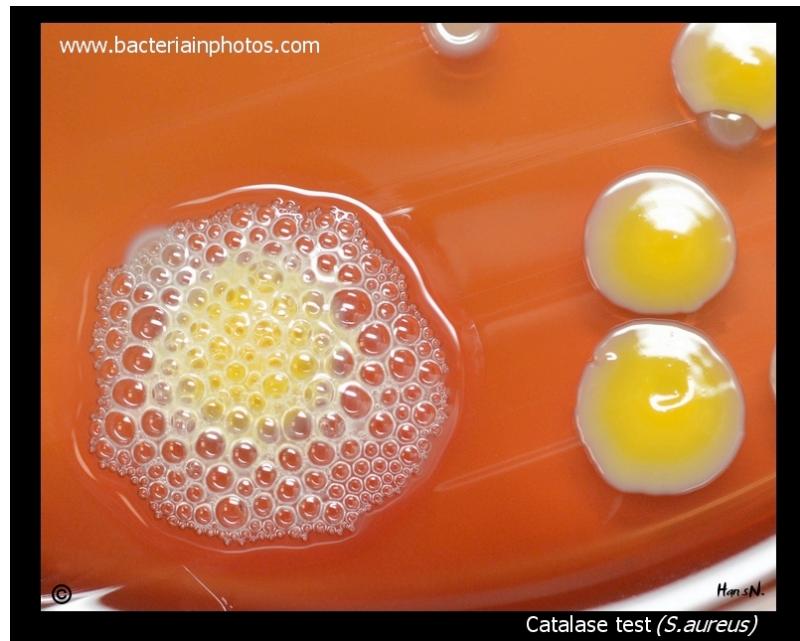


Fig-3

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EXPERIMENT # 10

AIM

To study the effect of ultraviolet radiation on bacterial growth.

BACKGROUND

In nature, the growth and survival of microbes are profoundly influenced by the physical factors in their environment. Ultraviolet radiation (100-400 nm) is of interest here because it is used in certain environments to kill microbes. However, very small dosage of UV light can make inheritable changes in the hereditary molecule, DNA. These slight changes in inheritable characteristics are often irreversible, but not lethal, to the cell. In this way, mutant off springs are produced. A mutant is a organism that has different genetic characteristics from its parent.

MATERIALS

(1) Broth culture of *Escherichia coli* (2) Nutrient agar plates (3) Ultraviolet lamp (4) Timer (5) Glass spreader (6) Sterile tubes.

PROCEDURE

1. Properly label the agar plates.
2. Inoculate each of the plate with 0.1 ml of the broth cultures; then spread the inoculum over the entire surface with a glass-spreading rod.
3. Place the plates under the UV lamp with lids removed and expose for different lengths of time (10-60 sec) at a distance of 2.4 inches above the plates.
4. Recover the plates with their lids and incubate inverted at 30°C for 24 hours.
5. Record the number of mutants survived at each exposure time.
6. Plot log CFU/ml v/s time of exposure to UV to get the kill curve.

PRECAUTION

Be careful not to look at the rays coming from the ultraviolet lamp.

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EXPERIMENT # 5

AIM

To study bacterial spores by staining methods.

BACKGROUND

Certain species of bacteria (Genera: *Bacillus*, *Clostridium*) in order to protect themselves from the vagary of adverse environment develops on its surface thick tough spore coat. **Endospores** are metabolically inactive, highly resistant structures produced by some bacteria as a defensive strategy against unfavorable environmental conditions like heat, short wave, radiation and toxic chemicals. Endospores can remain dormant for long periods of time. Spore coat is made up of protein dipicolinate. Simple staining will not stain endospores, however special stains and heating can dye the endospore coat. The endospore staining is a **differential staining method** which selectively stains bacterial endospores.

Schaeffer-Fulton Method - Malachite Green is a stain that is used to stain spores. It is triarylmethane dye with basic N-N- Dimethylaniline groups that interact with the thick & highly cross-linked surface of endospores.

MATERIALS REQUIRED

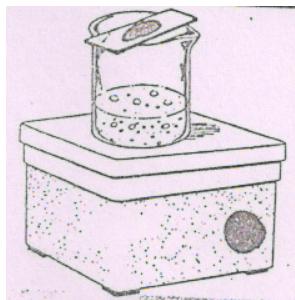
Culture : *Bacillus sp.*
Stains : 5% Malachite Green and Safranin
Apparatus : Slides, boiling water bath, pipettes, microscope, immersion oil.

PROCEDURE

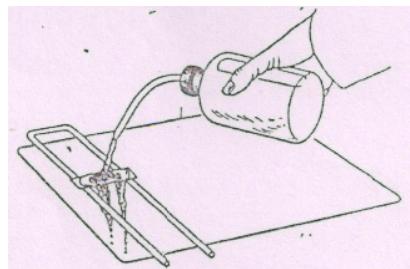
- 1) Prepare a thin bacterial smear on a clean grease free slide.
- 2) Air dry and heat-fix the smear.
- 3) Overlay the smear over a boiling water bath so that it is constantly exposed to steam.
- 4) Cover the smear with a sheet of blotting paper.
- 5) Flood the smear with malachite green stain for a period up to 20 min and place a cut piece of paper towel over the smear (Don't let the smear dry; keep adding stain to keep it wet).
- 6) Gently heat the under side of the slide with a burner by passing back and forth (Steam heating)
- 7) Keep the stain steaming for 15 min; add stain to keep the paper saturated.
- 8) Remove the paper towel and wash the slides with distilled water.

- 9) Counter stain with safranin for 1 min, wash and air dry.
- 10) Examine under oil-immersion objective. See spherical green endospores and pink vegetative portion of the cell (Sporangium).

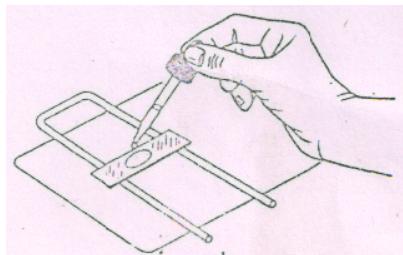
**1. Cover the smear with malachite green.
Steam over boiling water for 15 min.
Add additional stain if stain boils off.**



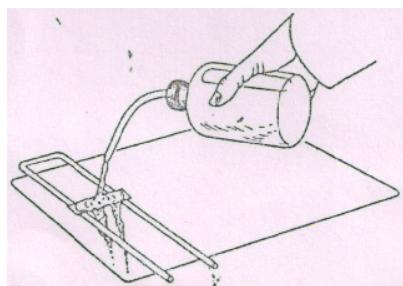
**2. After the slide has cooled sufficiently,
rinse with water for 30 seconds**



3. Counter stain with safranin



4. Rinse with water



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EXPERIMENT # 11

Aim

To screen colonies obtained through UV mutagenesis for presence of lactose non-utilizing mutants.

Background

A large variety of microbial mutants have been isolated and studied intensively by microbiologists. Some important categories of mutants include auxotrophic mutants, resistant mutants, metabolic mutants, cryptic mutants, antigenic mutants, conditional mutants etc. Lactose non-utilization is a metabolism-associated mutation.

Escherichia coli belong to the class enteribacteriaceae and are characterized as lactose fermenting organisms. Lactose non-fermenting *E. coli* are very important hosts for genetic manipulation and these mutants are called metabolic mutants. Certain differential media are their wherein lactose fermenting bacteria can be differentiated from non-lactose fermentors.

MacConkey agar is one such medium where lactose fermentors will form pink colonies whereas non lactose fermentors will form white colonies. Thus the mutant *E. coli* can easily be screened. It contains bile salts (to inhibit most Gram-positive bacteria, except *Enterococcus* and some species of *Staphylococcus*), crystal violet dye (which also inhibits certain Gram-positive bacteria), neutral red dye (which stains microbes fermenting lactose), lactose and peptone.



Fig 1: MacConkey Agar plate showing lactose fermenting and lactose non-fermenting bacteria.

Materials required

Mutant colonies of *E. coli* obtained through UV mutagenesis experiment, MacConkey agar plates, tooth picks, incubator etc.

Procedure

1. Assign a number (1-16) to the mutant colonies obtained in the previous experiments by labelling at the back of the plate.
2. Take a plate of Mac Conkey agar and at the back of the plate make 16 squares and label them as 1-16 and inoculate them with these mutants with a correspondingly assigned number using sterile tooth peaks (inoculate by making a small line streak not reaching out of the box boundary).
3. Incubate the plates at 37°C.
4. After overnight incubation, observe for the presence of white colonies.