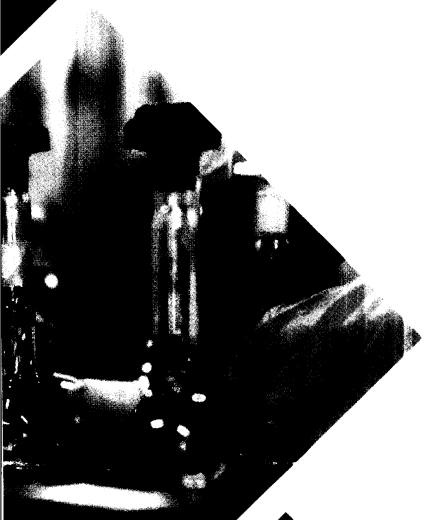

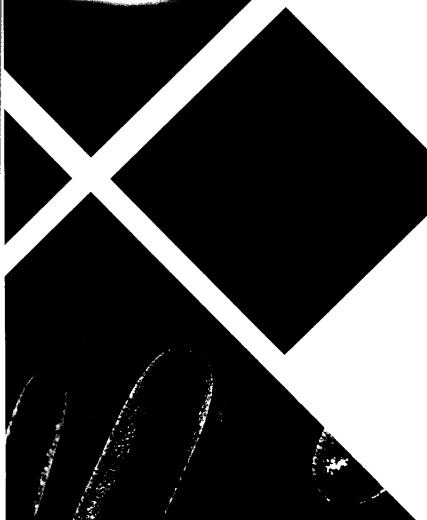

PRACTICAL MANUAL

GENERAL MICROBIOLOGY

B. Tech. (Food Technology)

Semester : I

Course No. : FMS -111

Course credits : 3(2+1)

Department of Food Microbiology and Safety
College of Food Technology

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Certificate

This is to certify that Shri/ku._____

Reg. No. _____ has completed the practical of Course No. FMS-111(General Microbiology) as per the syllabus for B. Tech. (Food Tech.) first year I semester as prescribed by MCAER, Pune.

Date: / /

Course Teacher

INDEX

Microscopy

Objective: To study and understand the various parts of a compound microscope and their functions. Examine the pond water under compound microscope.

Theory

The science of microscopy rests on the resolving power. A power to distinguish objects lying very close together. The resolving power of a human eye is 0.1 mm. Objects closer than this can't be distinguished. They appear in blood or invisible using the magnifying power of lense. Microscope is designed to increase the resolving power and the magnified view of the object.

Definition

Wavelength: Is the distance between two successive crest of through and is represented by λ.

Magnification: Is the degree of enlargement of an object.

Magnification of a Compound microscope = Magnification of the objective X Magnification of eye piece

Numerical aperture

It is the measure of resolving power of an objective. It can be expressed as the ratio of the diameter of objective lense to its focal length.

$$NA = \frac{\text{Diameter of the objective lense}}{\text{Focal length of objective lense}}$$

or $NA = \mu \sin \theta$

Where μ = Refractive index of medium

Air $\mu = 1$

Cedar wood immersion oil $\mu = 1.514$

Resolving power

The resolving power of a lens is its ability to separate two adjacent points (elements) in the structure of an object that are close together.

$$\text{Resolving power} = \frac{\lambda}{2 \times NP} \quad \text{i.e.} \frac{\text{Wave length of light}}{2 \times \text{numerical aperture}}$$

As we decreases the wavelength of light, the more closer elements are seen clearly

Example -		
	UV light wavelength	2000-4000 A
	Ordinary light	4000-7000 A
	Electron	0.5 nm or 5A

The use of wavelength in optical microscope is limited.

Materials

a) A compound microscope
b) Electric lamp
c) Pond water
d) Slides and cover slips

Compound microscope

Objective: In compound microscope consists two lenses (magnifying) one is eyepiece and other is objective. An object is placed beyond the focal length of an objective, it gives magnified inverted real image. A compound microscope is built up around the basel foot limb which stands out from base.

Parts of a compound microscope

1) Mechanical parts : It includes;

1) Base
2) Pillar
3) Arm
4) Mechanical stage
5) Coarse adjustment
6) Fine adjustment
7) Iris diaphragm m. (condenser)

2) Optical parts: It includes;

Magnifying parts –

1) Ocular (10 X)
2) Objectives
Low power 10X
High power 40 X
Medium power 20X
Oil immersion 1000X

b) Illuminating parts

1) Lamp 2) Mirror 3) Condenser

How to use microscope

I) Illumination
i) Rack up fully the sub stage condenser.
ii) Place the lamp 8-10" away from the mirror.
iii) In case of built-in lamp arrangement (as in binocular microscope). Check that lamp filament entered exactly with the lamp condenser lens.
II) a) Switch on the lamp. Open the iris diaphragon fully, Adjust the lamp so that bright beam of light shines on the center of place side of the mirror.
b) In case of built-in lamps, it is important to ensure full resistance is befores witching on the lamp and

then for illumination the resistance should be increased or decreased as desired. Before switching off the lamp dim the bulb to the full amount. These precautions will improve the fire of the bulb.

III) The amount of light vary with the type of objective used. This is because the size of cone of light passing in to microscope differ with each objective. Since the working distance decreases with increase in the magnification of the objective lens. With the increasing magnification a larger cone of light must enter the objective lens. This can be regulated by iris diaphragm. When the low power and high power objections are used, do not open the iris diaphragm fully, with the oil immersion objective the working distance is very small and therefore open the iris diaphragm more fully.

Steps to see an objective under a microscope

When one wish to examine a specimen, prepare a thin layer on a clean no greasy slide. Before we start examining the specimens following precautions should be taken.

a) Clean the objectives and ocular lenses free from dust and immersion oil.

b) Place the plane side of mirror in position.

c) Rack the sub stage condenser until its top surface is 1-2 mm below the slide.

After illuminating, following steps to be taken.

1) Place the slide on stage, specimen should face the objective lens. Centre the slide as accurately as possible over the hole in the center of the stage.
2) Focus the specimen with low power objective, using the course adjustment. Lower the body tube by means of coarse adjustment until the objective is about 1/4 from slide.
3) Look through the eyepiece and slowly raise the objective with coarse adjustment until the specimen is in focus. With the fine adjustment bring the specimen to sharp focus.
4) After we examine the specimen with low power objective, it is shifted to high power objective by rotating revolving nose piece until the objective clicks into the place.
5) Look through the eye piece and focus. Never attempt to bring the specimen into focus by lowering the body tube while looking through the eye piece. Always watch the objective lens from the side while lowering it and then focus by raising the body too slowly, when the specimen is in focus adjust the mirror and iris to obtain a clear image.
6) Raise the condenser so that it's upper surface in practically leveled with stage.
7) Open the iris diaphragm fully.
8) Raise the body tube and rotate the revolving nose piece until the oil immersion objectives clicks into position. Put a drop of immersion oil on the portion of the slide directly below the objective. Carefully lower the objective by watching from the side until it touches the oil. Never allow the objective to touch the slide. Slowly focus upward with fine adjustment by looking through the ocular.

Under compound microscope we have seen the following microorganisms in pond water

1) Protozoa: These are unicellular microorganisms, which are motile. They were moving very fastly.
2) Spirogyra: They were seen under microscope, spiral in shape. Ribbon like chloroplast bands. They were green fresh water algae.

Microscope are divided into two categories

Light microscopes – (Optical) it includes

a) Bright field microscope b) Dark field microscope c) Ultraviolet microscope d) Fluorescence e) Phase-contract microscope.

Electron microscope: In this a beam of electrons in place of light waves to product the magnified image is used.

Dark Field Microscope

The effect produced by the dark field technique is a black back ground against which objects are brilliantly illuminated. This is accomplished by equipping the light microscope with a special kind of condenser that directs the light path from the source of illumination. If the specimen is completely transparent and homogeneous, the light directed through the condenser does not enter the objective and the entire field of view is dark. If, however, the transparent medium contains objects that differs from it in refractive index, there will be scattering of light by reflection and refraction. The scattered light will enter the objective and thus the object will appeared bright, otherwise dark microscopic field. This is particularly helpful for examination of unstained microorganisms suspended in fluid and hanging drop preparations.

Ultraviolet Microscope

Ultraviolet microscope permits greater resolution and hence greater magnification than conventional light microscope. This is because UV light has shorter wave length (2000 - 400 A versus 4000-7000 A) than visible light. The principal advantage is that specific or different component of specimen are specific in their absorption. The characteristic ultraviolet absorption of certain substances permits their localization, distinction and measurement in a suitable specimen. Ultraviolet microscope differs from the conventional light microscope in having optics which transmit or reflect in the region of practical interest, 230 through 350 um. Since the UV rations are invisible image are made visible by recording on a photographic emulsion, by use of an image converter tube, or by display on a television screen.

To use UV ration require 1) Filters 2) Quartz lenses, we cannot use it directly because, it causes injury to eyes.

Phase Contrast Microscope

It is extremely valuable for studying living cells and has wide application in biological studies. It employees controlled illumination of the specimen, accomplished by special phase contrast objectives and a condenser assembly attached to a conventional light microscope. Light passing from one material through another of a slightly different density will be bent or refracted from its original path. In this microscope two modification are;

1) Introduced phase shift element.

2) Annular ring diaphragm just below the condenser. By phase contrast microscope we can see even the hyaline (colorless) bacteria and also it can be measured for their size and shape.

Electron Microscope

Electron microscope has advantage of tremendous magnification, as the resolving power is hundred times that of light microscope. This is only because of extremely short wave length of electron beam. In this the use of waves of electrons and magnetic fields to produce the image, where as the light microscope uses waves of light and lenses. It produce useful magnifications of about 200000. For electron microscope the specimen to be examined is prepared as an extremely thin dry film an screen. It is introduced in between the magnetic condenser and magnetic objectives. The magnified image may be viewed on a florescent screen through an air tight window. In this electrons are produced by electron gun tungsten filament of 32-150 kilovolts and passes through vacuum. Such high magnification is used to study the sub cellular substances of viruses and bacteria.

Experiment No. 2

Micrometry

Object: To determine the size of the micro-organisms.

Theory

The dimension of the cell is not a very reliable clue for identification of the culture unless it is mean of large number of cells, a minimum of hundred. Moreover the microorganisms growing in rich nutrient medium may be found to have large dimensions as compared to those growing in moderate or poor nutrient media. In other words the sine of the microorganisms to determine by various external factors. But a rough identification can be done, if the average sine of the culture is known.

Materials

1) Compound microscope
2) Stage micrometer
3) Ocular micrometer
4) Culture of yeasts and mold
5) Loop

Procedure

Setting up of the compound microscope properly keeping in view the precautions involved in its handling and avoiding them to minimum account then proceed as follows;

A) Standardization of ocular micrometer: With an ocular micrometer in eye piece and the stage micrometer on the stage focus with the low power and noted the number of divisions of the stage micrometer, covered by 10 divisions of ocular micrometer. Repeat the standardization by using high power calculated the number of that corresponds the division of ocular micrometer.

a) Standardization of ocular micrometer

2 mm stage micrometer is divided into 200 parts

Hence 1 division of stage micrometer = $\frac{2}{200} \times \frac{2}{200} = 10u$

To divisions of ocular micrometer coincides with 3.5 divisions of stage micrometer.

Hence 1 division of ocular micrometer = 0.35 division of stage micrometer or 3.5 μ

Hence 1 division of stage micrometer =

B) Measurement of the organism

Prepared a cell suspension of the given culture i.e. yeast and mold in distilled water, one or two loops of the suspensions were transferred to a clean slide. It is coved with clean glass cover. The slide is kept on the stage of microscope. Using high power objective the size of the yeast and mold is measured in terms of micrometer units.

Result: 1) Size of yeast = _____ µm

2) Sine of mold = _____µm

Experiment No. 3

Cleaning and sterilization of glassware

Object: To describe the procedure for cleaning of glassware used in microbiology laboratory.

Materials

Flasks, pipettes, beaker, conical flasks, test tubes bottle and petri plates.

Procedure

General condition

Use the flasks, pipettes within 3 days of sterilization.

For Volumetric Flasks, beaker, conical flasks, test tubes bottle

Drain out solution from glassware completely and wash these with potable water to remove the solution. Scrub the interior and exterior surfaces of the glassware with 2% soap solution or 0.5% labolene using a long handle brush. Drain out the soap solution and wash the glassware thoroughly with potable water to remove the detergent completely and then rinse with purified water. Dry the glassware in oven at 60 to 65^{0}C. Plug the mouth of the dried flasks, test tubes with non-absorbant cotton plug and wrap with cello fen/butter paper. Affix indicator if required. Autoclave the glassware at 121^{0}C, 15 lbs pressure for validated cycle time.

For Pipettes and burettes

Immediately after use dip the pipettes/burettes in freshly prepared 2% liquid soap or 0.5% labolene solution. Flush each pipettes/burettes with jet of potable water. Remove all traces of liquid soap by flushing jet of potable water thoroughly. Rinse the pipettes/burettes with purified water and distilled water and dry in oven at 60 to 65^{0}C. Plug the mouth of the dried pipettes/burettes with non-absorbent cotton plug. Wrap with cello fen/butter paper, affix indicator if required and autoclave at 121^{0}C, 15 lbs pressure for validated cycle time. Affix labels having date of sterilization and use before.

For Petri Plates

Remove agar from the petri plates with a blunt knife. Collect the agar in a stainless steel container and autoclave at 121^{0}C, 15 lbs pressure for 30 min. Immediately dispose the medium by burning in the specified place or drain it in ETP. Dip the plate in a washtub filled with disinfectant solution. Rinse the plates with tap water and dip them in a wash tub containing 2% soap solution or 0.5% labolene. Scrub each plate with a brush or scrubber. Wash each plate thoroughly with tap water to remove soap with purified water. Finally rinse with purified water. Keep the plates for drying in oven at

60 - 65°C. After drying pack 5 - 10 Petri dishes in bundle and wrap with cello fen/butter paper. Affix indicator if required and autoclave at 121°C, 15 lbs pressure for validated cycle time. Affix labels having date of sterilization and use before.

Precaution

While handling used Petri Plates, Pipettes and Flasks wear disposable latex rubber hand gloves. All contaminated glass wares should be sterilized at 121°C for validated cycle time.

Frequency

After every use of glassware.

Results_____

Experiment No. 4

Acquainting with equipments used in microbiology

Object: To acquaint with equipments used in microbiology

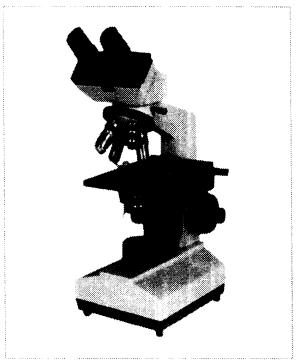
Theory

The goal of the laboratory is to expose students to the wide variety of life in the microbial world. Although the study of microbiology includes bacteria, viruses, algae and protozoa.

Materials

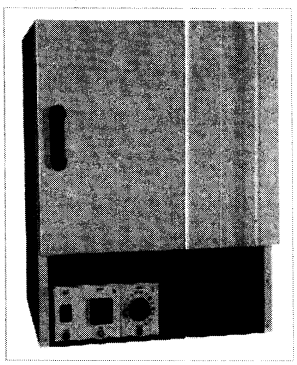
1. Microscope 2. Autoclave 3. Incubator 4. Laminar flow Hood
5. B.O.D. Incubator 6. Digital Colony Counter 7. Hot Water Bath 8. Turbidity Meter

Procedure

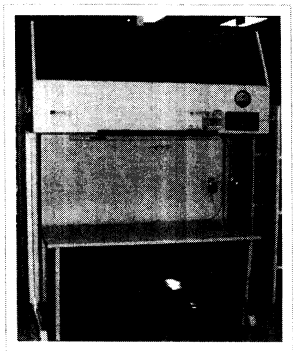


Microscope

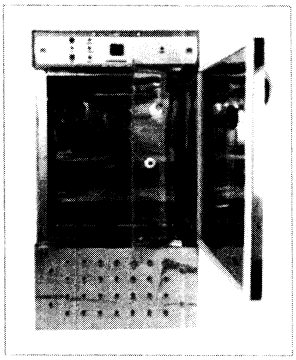
Autoclave



Incubator

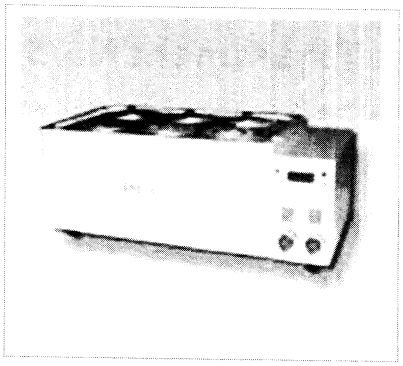


Laminar Flow Hood

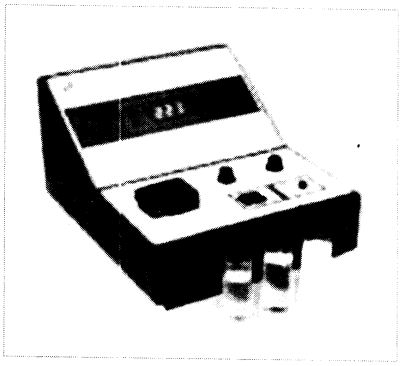


B.O.D. Incubator

Digital Colony Counter



Hot Water Bath



Turbidity Meter

Results: _____

Experiment No. 5

Preparation of nutrient agar media

Object: To prepare of nutrient agar media.

Theory

Bacteriological media come on a wide range of types. Nutrient Agar is a **complex**medium because it contains ingredients with contain unknown amounts or types of nutrients.Nutrient agar contains beef extract (0.3%), peptone (0.5%) and agar (1.5%) in water. Beef extract is the commercially prepared dehydrated form of autolysed beef and is supplied in theform of a paste. Peptone is casein (milk protein) that has been digested with the enzyme pepsin.Peptone is dehydrated and supplied as a powder. Peptone and beef extract contain a mixture ofamino acids and peptides. Beef extract also contains water soluble digest products of all othermacromolecules (nucleic acids, fats, polysaccharides) as well as vitamins and trace minerals.There are many media ingredients which are complex i.e. yeast extract, tryptone, and others. The advantage of complex media is that they support the growth of a wide range of microbes. Agar is purified from red algae in which it is an accessory polysaccharide (polygalacturonic acid) of their cell walls. Agar is added to microbiological media only as a**solidification agent**. Agar for most purposes has no nutrient value. Agar is an excellent solidification agent because it dissolves at near boiling but solidifies at 45^{0}C. Thus, one canprepare molten (liquid) agar at 45^{0}C, mix cells with it and then allow it to solidify thereby trappingliving cells. Below 45^{0}C agar is a solid and remains so as the temperature is raised melting onlywhen >95^{0}C is obtained.

Materials

1. Electronic or beam balances
2. Weigh boats, tongue depressors
3. Tripods, asbestos wire-gauze, asbestos gloves
4. 10 mL non-sterile pipettes
5. pH paper or pH meter with standard buffers
6. 413x100 mm screw capped culture tubes
7. Graduated Cylinder, 250 mL
8. 2500mL Erlenmeyer Flasks
9. Beef Extract, Peptone, Agar
10. 3 N HCl, 3 N KOH
11. 16 x 150 mm screw cap culture tubes
12. Non-absorbent cotton and gauze to make cotton stoppers

Nutrient Agar

Beef extract: 0.3%
Peptone: 0.5%
Agar: 1.5%

Procedure

For preparation of 200mL of nutrient agar. Weigh out beef extract, first tare a tonguedepressor, then dip it into the beef extract and weigh. Adjust the amount of beef extract until the correct amount is obtained. Be sure to be careful not to get beef extract on to the balance. Weigh out enough beef extract to get a 0.3% solution. Place the tongue depressorinto the flask, beef extract side down. Tare a weigh boat and weigh out enough peptone and add that to the flask. Add 200 mL of distilled water and swirl to dissolve the peptone and beef extract. Check thepH, it should be 7.Tare a weigh boat and weigh out enough agar and add that to the flask. With a Bunsen burner, tripods, asbestos wire-gauze, heat the medium to boiling to dissolve the agar. While the agar is still warm, but not hot, pipette 3 mL each into 413x100 mm screw capculture tubes.Label the flask and tubes.After preparation of medium, Place media in the autoclave. Autoclave the medium for 20 min. The media will be saved and used.

Precaution

1. Keep the rotating the flasks to prevent the agar from cooking onto the bottom of the flask.
2) Watch out: Boiling agar can froth and boil out all over the lab bench. As soon as it begins to boil take it off the heat and put it on to the bench. Allow it to cool a few min.

Observations

Results: _____

Experiment No. 6

Inoculation of Culture Media

Object: Inoculation of pure culture media.

Theory

Since most of the bacteriological work is done with the pure cultures. The importance of the techniques used in inoculating need not be stressed. In nature microorganisms as a mixed population of many widely different types. For studying a particular organism it is necessary to isolate and grow in the laboratory conditions. To grow a single species (pure culture) in laboratory, it is necessary to sterile the culture medium suitable for its growth. This sterile media should be inoculated with a pure culture of microorganisms without outside contamination.

Material

Pure cultures of the following;

1) Bacillus substilis
2) Staphylococcus aureus
3) Arthrobacter globiformis
4) Sarcina lutea
5) Sterilized nutrient agar
6) Sterilized nutrient broth tubes
7) Inoculating needle

Procedure

From the sterilized nutrient agar, four test tubes are taken and two of the nutrient broth. The slants are made by keeping the nutrient agar tubes in warm bath and cooled it at slanting position. It is increase the surface area of medium.

Preparation of subcultures of *bacillus subtilis* slant to slant

The given test tube of pure culture of *B. substilis* and the one to be inoculated were hold in left hand palm in the horizontal position near to the flame. Inoculating loop was heated to red hot by flaming to sterilize it. The needle was held between two fingers of right hand and the cotton piug of tubes with the same hand is removed clear to the flame. The mouth of both the tubes flamed immediately. The heated (red hot) loop is cooled in the tube was to be inoculated and gently small portion of culture was taken in loop and transformed to inoculating tube by moving loop gently on slant

agar surface. Taking care not to gauge the agar. The mouth of the tubes flamed and plugged immediately. The loop was again heated to red hot to destroy the inoculums before taking to table. The inoculated tubes were labeled with the names of the organisms (*B. substilis*) and the date of inoculation.

A sub-culture of *S. aureus* was also prepared by the same way slant to slant. A slant broth culture of *Arthrobacter globiformis* and *Sarcina lutea* were prepared.

Results: _____

Experiment No. 7

Preparation of Staining Material

Object: To prepare different dyes, for the staining microorganisms, to observe them.

Theory

Microorganisms are observed by two ways.

1) Living unstained. 2) Observing dead cells stained with dyes.

But living are colorless and with insufficient contrast in presence of water. Some are by line, so the stains are also used for examining the internal structure of bacterial cells. Thus the advantages of staining;

1) Provide contrast between microorganisms and their back ground.
2) Permit study of internal structures.
3) Enables to use higher magnificence.

Stains are of two types

1) Negative and 2) Positive stain.

Negative strains are very thick in consistency and so can't enter inside the cell. While positive enter in the cell.

Stains

A. Simple stains

1) Basic dyes
2) Acidic dyes
3) In different dyes

B. Differential stains

1) Gram stain
2) Acid fast stain

C. Structural stains

1) Fuelgen (for nuclear material)
2) Endospore stain
3) Cell wall stain
4) Capsule stain
5) Flagella stain

D. Micro-chemical tests for reserve materials

1) Glycogen
2) Lipid
3) Volutin.

Composition of some stains

1) Safranin: 2.5% solution in 95% ethyl alcohol 100mL. Distilled water 100mL.
2) Malachite green: Solution
   Malachite green - 5g
   Distilled water - 100 mL
3) Loeffler's methylene blue
   Solution A – Methylene blue 0.3 g + ethyl alcohol 95% 30mL
   Solution B –KOH 0.01 g + distilled water 100mL.
   Mix the solution A and B
4) Hucker's crystal violet (gram stain)
   Crystal violet - 2 g
   Solution A – Ethanol 95% 20mL
   Solution B – Ammonium oxalate - 0.8 g
   Distilled water - 80 mL
5) Lugol's Iodine KI – 2 g
   Iodine - 1 g
   First dissolve KI in small amount of water and then
   Iodine – Add 300mL of water
6) Ziell's Carbol fuschin

Solution A – Basic fuschin - 0.3g
E. alcohol - 10mL
Solution B – Phenol - 5g
Distilled water - 95mL
Mix the A and B solutions.

7) Leifsons solution

Tannic acid - 10g
NaCl - 5g
Basic fuschin - 4g
Mix and ground to a fine powder. Before using the stain it is dissolved in 33mL of 95% alcohol and pH is adjusted to 5 and stored at 35°C temperature.

8) Borex methylene blue
   Methylene blue - 2g
   Borax - 5g

Add to the warm water at least 60°C with continuously stirring.
Preparation of Carbol fuschin

Material

1) Basic fuschin 2) Alcohol 3) Phenol

Procedure

Preparation of

1) Solution A – Weighed out 0.9g of Basic fuschin and added to 30mL of alcohol.
2) Solution B –Phenol solution is weighed 15g and added to 285mL of water.

Carbolfuschin solution was prepared by mixing solution A and Solution B and kept in bottle and labeled.

Results: _____

Monochrome Staining

Object: To study about various cultures by monochrome staining.

Theory

The numbers of dyes are available and having two groups, i.e. chromophore group and auxo chrome group. Chromophore group gives rise color to the compound and auxo chrome group imparts salt forming properly and helps in the fixation of dyes. The practical classification of dyes based upon the chemical behavior of dyes in acidic, basic and neutral. These may be thought as salts of two kinds.

(i) Those in which the color bearing ions (chromophore) is the anion e.g. Na-Eosinate and

(ii) Those in which the chromophore is cation e.g. methylene blue chloride. The first and second are acidic and basic dyes respectively and can be react with salt and acid to form dye salt.

Basic stain acts as cation replacing similarly charged ions from the cell membrane so they reach with negatively charged ions of cell membrane and dye the membrane.

Nature of staining: Ziehl's Carbolfuschin is best as it stained the cells out line bright and contrasting with less time.Certain dyes have the property of staining cell wall increase the contrast in the microscope. This makes it possible to observe the morphology (shape, size etc.) of microorganisms.

Material

A) Stains

Ziehl's Carbolfuchsin, Loeffler's methylene blue, Safranin.

B) Cultures

Bacillus substilis
Arthrobacterg lobiformis
Staphylococcus aureus
Sarcina lutea

Procedure

The cleaned slides are taken and dried then with the dry cloth. By means of sterile wire loop (heated to red hot) transferred a loopful of culture to the control of slide. This drop was spread in the form of thin film on the slide and allow to air dry and fixed the smear and then passed through the low flame. Smear is covered with several drops of dyes and allowed to remain for following intervals;

1) Carbolfuchsin or Safranin - 1 min
2) Methylene blue - 3-5 min

The slide is washed with gentle stream of water and dried with filter paper and rapidly passing over flame. Slide is examined under oil immersion objective and observed the shape and side.

Precaution

The slide must be free from grease, by rapidly passing over flame, cleaned slides should be handled by grasping only at their dyes, to prevent the re-deposition of grease, by fingers.

Results: _____

Gram Staining

Objective: To study the gram positive and gram negative bacteria and to become familiar with the technique involved in a differential staining.

Theory

Differential staining consists use of more than one dye, to stain the microorganisms and are divided into Gram positive and Gram negative. This difference in staining due to variation in cell wall. The bacteria which retain crystal violet color (Gram positive) have special complex of Mg ribonucleic acid, protein, carbohydrate form insoluble compound with dye and iodine. Hence the dye can't come out through small pores of cell wall. It can be proved by treating gram positive bacteria with ribonuclease enzyme which degrades ribonucleic acid into small fractions making possible that substance to corne out of cell pores and thus behavior of bacteria changes to gram negative by losing dye retention power. In Gram positive bacteria cell wall is compared of polysaccharide. They contain very less or 2% lipid layer (lipopolysaccharide) while in case of Gram negative it is thick and it is removed by dissolving in alcohol, so the only Gram positive retain the color, while in Gram negative removal of lipoproly saccharide layer increases the permeability of cell wall and loses the color due to washing of alcohol. Iodine acts as a mordant in staining procedure. It reacts, with primary stain i.e. crystal violet and form the (CV+I) complex, which helps in fixing the primary dye to the bacterial cell. Age of the culture generally 12 - 24h are taken, more than 24h culture, the Gram positive bacterial tends to become Gram negative. pH of the medium (In acid medium Gram positive bacteria tends to become Gram negative). Certain bacteria require 5% blood serum in medium if it is not present in the medium, the Gram positive bacteria tends to become Gram negative. Techniques gives the method for differentiating in to Gram positive and negative which are fundamentally different Yeasts and actinomycetes are characterized if stained by this stain giving Gram positive and Gram negative reactions.

Materials

A) Cultures (nutrient broth 24h)

1) Escherichia coli

2) Sarcina lutea

3) Arthrobacter globiformis

4) Staphylococcus aureus

B) Staining reagents

1)	Crystal violet	Primary dye
2)	Lugol's iodine	Mordant
3)	Safranin	Counter stain
4)	Alcohol (95%)	

C) Slides, loop needle, flame etc.

Procedure

Smear of provided cultures numbered and saliva prepared separately by putting two loops of suspension each on cleaned glass slides, marked the slides with number, Flooded the mass with crystal violet and allowed to remain it for 1 min. Washed with gentle stream of water, dried and again flooded with lugol's iodine solution and allow it for a minute and excess iodine is removed by gentle stream of water for decolourization, washed the slides with the help of 95% alcohol for 10-20 sec and rinsed with water. Allow to dry and counter stained with Safranin for a minute. Again rinse with gentle stream of water and dried over hot air. The slides are examined under oil immersion.

Results	**Number of slide**
1) Staphylococcus aureus Gram positive	1
2) Arthrobacter globiformis Gram negative	2
3) Sarcina lutea Gram positive	3
4) Escherichia coli Gram negative	4

Negative or Indirect Staining

Objective: To introduce a rapid method for demonstrating microorganisms against a dark background and for use in measuring the size of bacteria because the cells are not destroyed as they may be when the usual staining procedures are employed.

Theory

Negative staining gives more accurate view of bacterial cell with only air drying because of this we can measure size and observe shape. On the other hand in positive stain by use of heat smear is fixed which changes shape and size of microorganisms (bacteria).

Material

Cultures of *B. subtilis, S. aureus, Sarcina lutea, Arthrobacter globiformis,* Dorner's nigrosin solution (staining reagent), Clean slides.

Procedure

Placed two loopful of bacterial suspension on the slide and 1 loopful of the nigrosin solution. It is mixed thoroughly. Material is spread on oil, to form a thin film. Air dried. Slide is examined under oil immersion objective.

Results: _____.

Experiment No. 11

Capsule Staining

Object: To demonstrate the presence of gum like material surrounding certain bacteria This substanc is called a capsule when it envelops individual cells.

Theory

Capsule conform the property of virulence as in case of *Diplococcus pneumoniae*. In the industries like paper mills, capsulated bacteria are responsible for development of slim. Slime to mode of polysaccharide and proteins. These are various types as dextrin – *Leuconostoc mesenteroides* which clogs the pipe in sugar refineness, dextrin, cellulose, glucosamine. Capsule of *B. anthracin* to polypeptide made up of dightanic acid. The capsule material is antigenic in nature as it promotes synthesis of antibiotics in our body. These capsular forming bacteria adhers to tooth and causes tooth decay. The organisms like *Coxilla burnetii* is resistance due to capsule, so the (in pasteurization) temperature used in 62°C/30 min. in folder method and 71.7°C/15 sec in HTST method was raised to 62.8°C/30 min, as in case of milk, it carries through milk and causes Q-fever.

Material

Culture –*Alcaligenes viscosus* (milla-48 h)
Staining reagents
a) (Anthony's) crystal violet
(95% dry content)- 1g
b) Distilled water-100mL
b) Copper sulphate solution – 20%
Copper sulphate (CP) - 200g
Distilled water- 1000mL
Clean slides
Blotting paper etc.

Procedure

Prepared a smear of *Alcalginesvi scosus* bacterial cultural on a clean slide and air dried. This is stained by crystal violet for 2 min. Washed the slide with $CuSo_4$ solution (20%) and dried by blotting. Examined the prepared slide under oil immersion objective, best result found all edge of smear.

Result: The capsule appeared unstained while the organisms stained with deep purple and appeared within colorless hallow (capsule) the background is colored purple.

Flagella Staining

Object: To stain flagella of cell.

Theory

Bacteria have two types of locomotory organs and that are flagella and pili. Here we are studying about flagella staining. Flagella are a thin, hair like structure made up protein called as flagellin. It size ranges from 20 - 200 μ in length. Flagella is one of the most important locomotory organ. It is mainly made up of three parts;

1) Basal body 2) Filament 3) Hook

Flagella are generally present in rod shape bacteria and very few cocci shape bacteria posses flagella. As flagella are very thin and hair like they cannot be easily observed under microscope. So a special technique is design to increase thickness of flagella as well as stain it. Due to these technique we can observe structure of flagella easily under microscope.

Materials

1. Flagellated cell culture slant
2. Leifson's stain
3. 1 % Methylene blue
4. Distilled water

Procedure

First of all take two hours old flagellated cell culture slant and add two to three drops of sterile distill water in the slant with the help of sterile pipette. Note that the distill water is added slowly without disturbing the growth of cells. After addition of distill water incubated the slant for 20 min. Then take a drop of suspension from the slant and place the drop on a clean slide which is kept in slanting position. The drop should flow slowly from one end of slide to other end to avoid folding of flagella on cell. Allow smear to air dry here we don't use heat fixation treatment. After air drying the slide is flooded with Leifson's stain till a thin film of shinny surface appear. After this give a gentle stream of water wash treatment to a slide. Now treat the slide with 1% methylene blue treatment for 1 min. Give the slide water wash treatment, air dry and observe under oil immersion lens.

Mechanism

First of all in this procedure thickness of flagella is increase so it can be visible. The Leifson's stain is made up of tannic acid, basic fuschin stain prepared in alcohol base. When we treat Leifson's stain with cell the tannic acid get attach to the flagella and alcohol get evaporated.

After evaporation of alcohol the thickness of flagella is increased due to deposition of tannic acid. Whereas basic fuschin stain the flagella. After Leifson's stain treatment cells are treated with methylene blue stain. This methylene blue stains the cell.

Result: After observation under microscope we can observe that flagella appear red in colour and bacterial cell appear blue in colour.

Endospore Staining

Object: A special staining procedure must be used to stain the endospore properly

Theory

The production of bacterial endospore is not a method or mode of reproduction but it is the mechanism to tide over unfavorable conditions, as the spores are highly resistant cell to any drastic change in their environment, as compare to vegetative cells from which they are produced. The case in yeast and fungi is different, the spores are produced for the perpetuation. Each spore is capable of giving rise to one or more cells of hyphae and so the spores of yeast and fungi are essentially reproduction. Fungi can produce innumerable spores, whereas bacteria can enclose one or two spores. The position (location) of endospores in cell may be central, terminal or sub terminal and is not same for all the species. The diameter of the endospore may be smaller or lager than that of vegetative cell. The spore may be oval, spherical or cylindrical in shape. The spore is much more resistance than vegetative cell, most of the activities are stopped, but TCA cycle is continued spore spare dormant. Even after processing and taking precaution, if the canned foods undergo spoilage, can attributed the presence of spores and thus this is the in direct method to detect the presence of spores by means other than staining. The spore forming bacteria (spore) are resistance to heat, can be survive at pasteurization and sterilization temperature. So they can bring about the spoilage of food, if not properly pasteurized or sterilized. Ex. *B.Stearo thermophiles* which causes the flat sour in canned foods. Moreover they are also known to produce deadly toxins. Ex. *Clostridium, Cl. tetani* – lockjaw. Botulinun in food.

Material and reagents

I) Culture of *Bacillus subtilis* (24h. old)

II) Staining reagents

a) Zichh's Carbolfuschin
b) Dorner'snigrosin
c) Malachite green
d) Safranin

III) Distilled water, clean and dried slides, flame, loop etc.

Procedure

There are two different methods for spore stain, i.e.

i) Dorner's method ii) Wirtz method

i) Dorner's method

Two to three drops of *Bacillus subtilis* culture suspension is taken in a clean and dry test tube. Added equal quantity of Carbol fuschin (2-3 drops).This mixture is allowed to stand in boiling water for 10 min. On the slide, mixed the one loopful the stained preparation with one loopful of the nigrosin solution. This is made to thin smear and dried immediately, without forming the cracks. This slide was examined under oil immersion objective.

Result: Endospore is of brilliant red colored and background is grayish colored.

ii) Wirtz method

A smear of *B.subtilis* is prepared and heat fixed. The slide was neatly flooded with malachite green and kept over a streaming water bath for 5 min. Care is taken that the stain does not dry up. This is done by repeatedly adding malachite green stain. The calcium dipicdivate wall of the spore which is refractive takes up the stain. Excess stain is removed by means of gently flooding of water. A counter stain a safranin is added (30 sec). The vegetative body which does not take the malachite green has taken the counter stain Safranin and appears pink. The excess counter stain is removed by the gentle stream of water and allow to air dry. The slide is examined under oil immersion objective.

Results: Body of cell – pink colored and endospore is of bright green colored.

Pure Culture Technique

Objectives: To isolate pure cultures from a mixture of bacteria in a suspension by serial dilution (pour-plate method) and to study the morphology of deep and surface colonies.

Theory

Preliminary

The desk was made clean and top was washed with disinfectant before pouring plates to avoid contamination and other necessary thing were kept ready.

Culture: Growth of the organism is called culture.

Pure culture: The growth of one kind of organism is called pure culture

Isolation: Separation of one kind of organism from mixture for study of culture is known as isolation.

Material

1) Suspension of mixed bacteria
2) Nutrient agar for plating
3) Sterile petri dishes (6 pairs)
4) Saline solution ^{0}(867%) Nacl-10mL/tube and 6 No. of tubes
5) Pipette graduated 1mL-6

Procedure

For obtaining pure cultures there are two methods.

1. Pour plate method (loop dilution)

This method was investigated by Joseph Listor. Six test tube containing each 9mL 9% saline solution was taken and was labeled as 1,2,3,4,5,6. 1mL of bacterial suspension from mixture was taken with the help of graduated pipette and transferred it to 1st test tube. Subsequently by the second sterile pipette, after mixing thoroughly in first test tube, 1mL was transferred to second, and from second to third is repeated upto the 6 test tubes. These are known as dilution blanks, Take the sterilized agar and pour 15mL. In each petri dish and agitate it to obtain uniform surface cooled it but do not allow to solidify. Three petri dishes were taken after adding this 15mL. And added 1mL. Bacterial mixture suspension from 5 and 6th tube and plate named as 5 and 6th kept one as controlled. The care was taken the temp erature of petridishes was moderate and process was carried aseptically. After solidification invert the petridishes and allow to incubate at 37^{0}C.

2. Streak plate method

Nutrient agar is boiled in hot water till it becomes liquid, poured 15mL. Nutrient agar in each petri dish, total three and allows them to cool and marked them as 1 2 3. The solution from the 6th tube prepared by above serial dilution technique used for the cultivation.A loopful of culture from the 6th test tube was taken, raised the lid of petri dish and streak the media with loop from top to bottom in zigzag manner closed the lid inverted the petridish and with the same loop streaked with second and third petri dishes. Inverted the petri dishes and incubated at 37^{0}C for 24-48h.

Examination of bacterial growth

1. The colonies were examined on next period, first with necked eye and then with low power objective, without removing the covers of the petri dish.
2. Selected two representative colonies from poured plates and following observations were made. From each colony smear is prepared for gram staining.

Stock cultures: From the well isolated, selected colonies, inoculated in to other sterile slant prepared test tubes, each into separate test tubes and grow them at 37^{0}C, (i.e. kept for incubation at 37^{0}C)

Results: 1. Colonies in the 6th Petri dish were well isolated then 5th , in pour plate method.

2. In streak method colonies in the 3 plate are very rare and in 2nd are rare then that of 1st plate.

Macroscopic observation

Parameters	**Type I**	**Type II**
Shape	Round	Irregular
Size (dia in mm)	0.25 mm	0.4 mm
Surface topography	Smooth	Concentrically rigid
Surface elevation	Pulvinate	Umbonate
Colour	Yellow	Pinkish
Optical characteristic	Opaque glistening	Opaque dull
Consistency	Butyrous	Brittle

Microscopic characteristics

Margin, (edge)	**Entire**	**Slightly lobate**
Gram reaction	Gram positive cocci irregular bunches	Gram negative, rod irregular
Internal structure	Curveled	Curveled

Identification procedure of unknown bacteria

Object: To identify unknown bacteria

Theory

Identification of unknown bacteria is one of the major responsibilities of the microbiologists. Samples of blood, tissue, food, water and cosmetics are examined daily in laboratories throughout the world for the presence of contaminating microorganisms.

In addition, industrial organizations are constantly screening materials to isolate new antibiotic producing microbes or microbes that will increase the yield of marketable products, such as vitamins, solvents and enzymes. Once isolated, these unknown microbes must be identified and classified.

The science of classification is called taxonomy and deals with the separation of living organisms into interrelated groups. Bergey's Manual of Determinative Bacteriology, 8th edition, is the official taxonomic key containing the orders, families, genera and species of all known classified bacteria.

With the fundamental knowledge in staining methods, isolation techniques, bacterial nutrition, biochemical activities and growth characteristics of bacteria, it becomes easier for identification of any unknown bacteria.

Characteristics of few bacteria have been given in Table 1. Other bacteria can be identified in a similar way based on the observations and results obtained following the experimental procedures.

Table 1: The identifying characteristics of few bacteria

Organism	Gram Stain	Agar siant cultural characteristics	Litmus milk reaction	carbohydrate fermentation			H2S production	Nitrate reduction	Indole production	MR reaction	VP roaction	Citrate utilisatuion	Urease activity	Catalase activity	Oxidas activity	Gelatin liquetaction	Starch hydrolysis	Lipid hydrolysis
				Lactose	Dextrose	Sucrose												
Eschenchia coli	Rod -	While, moist, glistening growth	Acid,curd ± gas ±, reduction ±	AG	AG	A ±	-	+	+	+	-	+	-	+	-	-	-	-
Enterobacter aerogenes	Rod -	Thick, white, glistening growth	Acid	AG	AG	AG ±	-	+	-	-	+	+	-	+	-	-	-	-
Klebsiella pneumoniae	Rod -	Slimy, while, somewhat-transtucent raised growth	Acid, gas curd ±,	AG	AG	AG	-	+	-	-	±	+	+	+	-	-	-	-
Shigella dysenteriae	Rod -	Thin, even, grayish growth	Alkaline	-	A	A ±	-	+	+	+	-	-	-	+	-	-	-	-
Saimonella typhimurium	Rod -	Thin, even, grayish growth	Alkaline	-	AG ±	A ±	+	+	-	+	-	+	-	+	-	-	-	-
Proteus vulgaris	Rod -	Thin, even, grayish growth	Alkaline	-	AG	AG ±	+	+	+	+	-	-	-	+	+	+	-	-
Pseudomonas aeruginosa	Rod -	Abundant, thin white growth, with medium turning green	Rapid peptoni-sation	-	-	-	-	+	-	-	-	+	-	+	+	+ Repid	-	-
Alcaligens taecalis	Rod -	Thin, white, spreading viscous growth	Alkaline	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-
Streptococcus aureus	Coccus +	Abundant opaque, golden growth	Acid, reduction ±,	A	A	A	-	+	-	+	±	-	-	+	-	+	-	+
Streptococcus lactis	Coccus +	Thin, evem, growth	Acid, rapid reduction with curd,	A	A	A	-	-	-	+	-	-	-	-	-	-	-	-
Micrococcus luteus	Coccus +	Soft, smooth, yellow growth	Alkaline	-	-	-	-	±	-	-	-	-	+	+	-	+ Show	-	-
Corynebacte-rium xerosis	Rod +	Graysih granular, limited growth	Alkaline	-	A ±	A ±	-	-	-	-	-	-	-	+	-	-	-	-
Bacillus cerous	Rod +	Abundant, opaque, white waxy growth	Peptoni-sation	-	A	A	-	+	-	-	±	-	-	+	-	+ Repid	+	+

Materials

Slides, cavity slides, petri dishes, conical flasks, cotton plugs, inoculating loop, autoclave, bunsen burner, laminar flow chamber, dispose jar, incubator, nutrient broth, nutrient agar, gram staining reagents, media and reagents for biochemical tests, compound microscope, isolated colonies or pure cultures of bacteria.

Procedure

1. Gram staining of the unknown bacteria is performed. Besides gram staining, its morphology and arrangement is also recorded. Motility test of the bacteria is determined by hanging drop preparation (Figure 2).

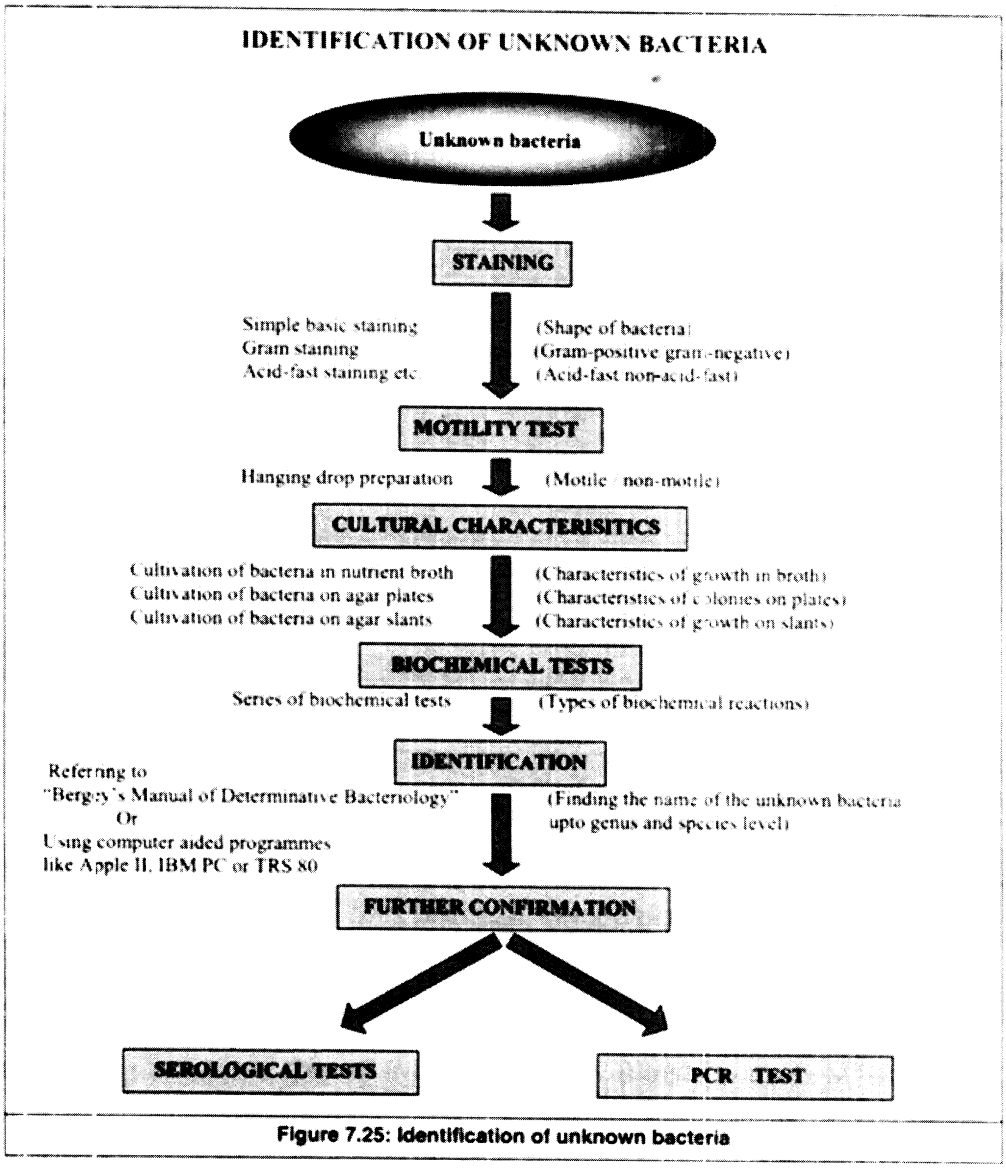


Figure 7.25: Identification of unknown bacteria

2. Using sterile inoculating technique, the bacteria is inoculated into two nutrient agar slants, a nutrient broth tube and to a nutrient agar plate by means of streak inoculation. Following incubation, one slant culture is used to determine the cultural characteristics of the unknown bacteria. The second is used as a stock sub-culture, should it be necessary to repeat any of the tests. Growth characteristics are also observed in the broth tube and colony characteristics on the plate.
3. Exercising care in sterile technique, so as not to contaminate cultures and thereby obtain spurious results, the bacteria is inoculated into respective media to carry out the different biochemical tests.
4. The inoculated media are incubated at the required temperatures for the required lengths of time.

Observations

1. In gram staining, besides gram reaction, the morphology and arrangement of the bacteria are also recorded.
2. The cultural characteristics of the bacteria in nutrient broth, on agar slant and on nutrient agar plate are noted.
3. Results of the biochemical reactions are recorded.

Results: _____ .

Measuring the microbial number

Object: To enumerate the microbial number in a given sample.

Theory

The number of cells in a population is measured most directly by counting under the microscope, a method is called the direct microscopic count. The haemocytometer is like a slide with 2 notches and elevate centre. It has (centre) some fixed scale and there are horizontal and vertical lines engraved on it. When a drop of culture put on the centre on which cover slip is put we can calculate the number of the cells, in a square under microscope. The number of cells for a certain area gives number of cell per mL. This is the direct count. Viable count can be taken by serial dilution followed by pour platting method, using nutrient agar medium, incubation and counting colonies.

Material

1) Yeast extract
2) Nutrient agar
3) Petri dishes
4) Saline blank (0.9%)
5) Haemocytometer
6) Sample culture-yeast

Procedure

Direct count method

This small size of most microbes and their large densities make it necessary to use special chambers such as the haemocytometer chambers to count the number of cells in a sample. Haemocytometer was cleaned and a drop of culture from a known concentration was put in the centre with sterile wire loop, avoiding the overflow, and covered with cover slip. This was observed under high power objective of microscope. Firs the cells in the 80 small squares of first row was counted and cells in the 3rd row, 40 squares of its (3rd row) 2nd 3rd row in small squares was counted and the cells in the 5th row in 80 small squares was calculated. The total area of all 400 small squares is 1x1 mm. and the height of the drop kept under the cover slip (thin film) is 0.1 mm. So the volume covered by 400 squares (small) is (1x1x1) cub mm = 0.1.0 mm. Above count was for 200 small layers, is doubled and calculated for 400 squares, i.e. for 0.1 mm vol. and thereby for per mL. Viable count is done frequently in both research and applied microbiology. The plate count is very sensitive because in principle any viable cell, when place on an appropriate medium will give rise to colony. Different organisms often produces colonies of different shape, size texture and colour, so several kinds of organisms can be

counted in the mixture. The given sample was serially diluted as 10^1, 10^2, 10^3, 10^4. One ml. of cultural solution was taken from 3rd and 4th and poured in petri dishes, counting melted (40^0C) cooled nutrient agar and shake well and incubated at room temperature for four days and counted the colonies.

Result: For a given yeast sample, microscopic count = _____ cells/mL.
Viable count 1mL. of sample = _____ cells/mL.

Factors influencing growth i.e. pH and temperature

Object: To measure the growth of bacteria at different temperature and pH.

Theory

pH: pH requirement for bacterial growth is specific, as the all bacterial activities are enzymatic in nature and these enzymes are sensitive to pH.

Ex: pH 6.5-7 common for all bacterium.

pH 9 (alkaline) *vibrio.*

pH 12 *Agrobacterium* (soil bacterium).

Temperature requirement: Bacterial growth is also affected by temperature. Because all bacterial activities are enzymatic in nature, and these enzymes are sensitive to temperature (only active at certain temperature). So different bacteria requires different minimum, optimum and maximum temperature.

Bacteria	**Temperature**		
	Minimum (^{0}C)	**Optimum (^{0}C)**	**Maximum (^{0}C)**
Psychrophils	0	4-15	30
Mesophils	5-25	25-40	30-50
Thermophiles	25-45	45-55 (60)	60-90

Procedure

For testing pH requirement triplicate (three) test tubes of pH 3, 5, 7, 9 and 11 nutrient broth 10 mL in each test tube was filled and sterilized in autoclave. To test the temperature requirement line (g) test tubes was filled with nutrient broth pH 7, each with 10 mL and sterilized in autoclave. Culture was made from previous pure culture into liquid form by adding nutrient broth (sterilized) pH 7 and shake. For studying pH requirement (effect) the cultures are made by adding a drop of inoculum in duplicates of each pH test tubes and simultaneously blank was also carried out and incubated at 37°C for 24 h.

pH					
	3	5	7	9	11
Culture tube	2	2	2	2	2
Blank	1	1	1	1	1
Total	3	3	3	3	3

For testing temperature requirement cultures was inoculated in the nutrient broth 8 ubes by adding one drop of culture and incubated at different temperatures as follows for 24 h.

Temperature	Tubes	
4^0C	2	
25^0C	2	Culture tubes
37^0C	2	
55^0C	2	
37^0C	1	Blank

Results:

Temperature

4^0C	No growth
25^0C	Slow growth
37^0C	Good growth
55^0C	No growth

pH

3	No growth
5	Not good growth
7	Good growth
9	Not good growth
11	No growth