LABORATORY TECHNIQUES IN

MICROBIOLOGY & BIOTECHNOLOGY

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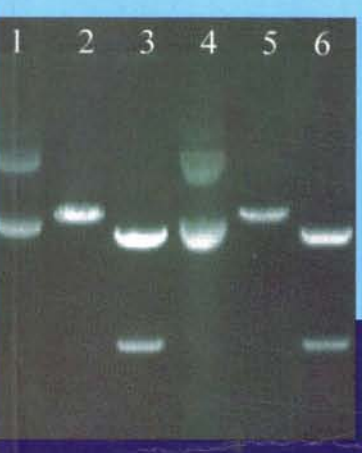

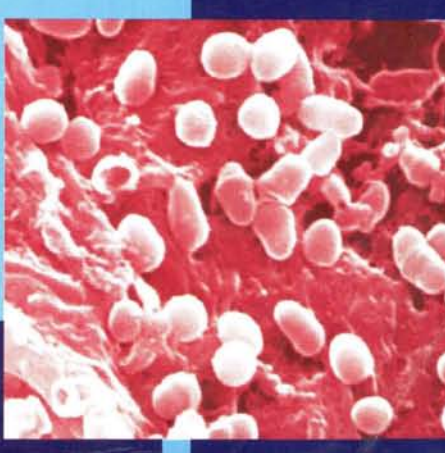

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Preface

The present manual comes from the teachers who have more than twenty-five years experience of teaching and research in microbiology and biotechnology to undergraduate and postgraduate classes. The manual has been written for University undergraduates (Medical and Engineering streams) having interest in Microbiology and Biotechnology. Students joining these disciplines are primarily the science students who may have only a brief exposure to microbiology being taught as small component along with biology. Much emphasis is on the exercises in botany and zoology and that through models. Some students from non-medical stream who even lack the basics of biology or have forgotten what they have learnt up to matriculation join B.E Biotechnology and Food Processing Technology courses. Students often lack the knowledge about the handling of microbes despite the fact they have the information about their potential as foes and friends. Hence it becomes obligatory to impart them good training comprising basics of biosciences, microbiology and biotechnology at least at the university or college level to comprehend the concept of sterile techniques, cross contamination, decontamination in laboratory, aseptic transfer techniques form one culture medium to another especially during cultivation and examination of microbial and cellular forms.

Although there has been a sea change in the research activities with the advent of, molecular biology and biotechnology but it is not possible to make progress unless one is conversant with the microbiological techniques pertaining to handling of microbes which has not changed over years. In recent years, very few books have been published pertaining to basic techniques, which have been neglected because of hype created with the emergence of relatively newer branches like biotechnology that also require one to efficient and proficient in handling of microbes to be a good biotechnologist.

We have the pleasure in bringing about the manual of techniques in microbiology and biotechnology that would be highly useful for the students of biosciences, microbiology and biotechnology. The manual has been divided in six sections pertaining to exercises in GENERAL MICROBIOLOGY, APPLIED MICROBIOLOGY, BACTERIAL PHYSIOLOGY, MEDICAL MICROBIOLOGY, IMMUNOLOGY, BACTERIAL GENETICS AND BIOTECHNOLOGY. The experiments have been given in chronological orders and most of these can be performed with materials available in most biological sciences laboratories. For some of the experiments even alternate procedures have been suggested. The experiments covered in this manual are easy to perform, and have been used over the years in our laboratory for imparting practical training regularly to undergraduates and postgraduate students and summer trainees. In the beginning of each experiment, a brief introduction of the exercise familiarizes the students about the nature of experiment and its objective. It is followed by a detailed stepwise protocol. Exercises have been explained in easy and explicit language. Suggestions from readers and users are welcome for its improvement in future (Email: ramptiwari@yahoo.com).

Authors

Introduction

Microbiology and biotechnology are not mere facts, terms, and concepts. In the present era of biological research, these two applied subjects are making significant contributions towards scientific knowledge and solving human problems. In order to be an accomplished biotechnologist, a thorough knowledge of Microbiology is essential.

Microorganisms were probably the first living forms to appear on the earth. Microorganisms are omnipresent in biosphere. Most microorganisms are free living and perform useful activities. Biosphere in general comprises of two categories: animate or living and inanimate or non-living beings. Biology is the science that deals with the living organisms. The branch of biology that deals with microscopic forms of living organisms (microbes) is termed as the "microbiology".

The emergence and development of microbiology has been highly erratic in the beginning. Credit for introduction to microbial world goes to Antony van Leeuwenhoek and to vistas of microbial activities to Louis Pasteur and Robert Koch. This period is known as the "Golden Era" of microbiology because of the significant discoveries on various aspects of microbiology: microbial fermentations, discovery of various disease causing agents, microbes cultivations, differences in metabolic activities, development of attenuated strains and their use as immunoprophylactic agents.

Microbial kingdom includes the study of bacteria, rickettsiae, viruses, fungi, algae and protozoa. Microbiology is further subdivided into bacteriology dealing with bacteria and rickettsiae, virology the study of viruses, mycology study of fungi and protozoology deals with protozoa. Microbiology considers the microscopic forms of life as to their occurrence in nature, their reproduction and physiology, their harmful and beneficial relationship with other living things and their significance in science and industry. Microbiologists study bacteria in many ways. The methods used to study bacteria and other microbes include direct microscopic examination, cultivation, biochemical tests, animal inoculation, serological reactivity and recent molecular biological techniques. Currently because of their high versatility and rapid turn over microorganisms are used extensively in genetic engineering and biotechnological processes and new processes are being developed to produce variety of enzymes of industrial importance, vaccines, insecticides, pharmaceuticals and other biological products of interest to mankind, animals and plants.

Laboratory Safety Rules

1. Always, mop the bench with disinfectant such as 2% phenol or polysan before and after use.
2. Always, wash your hands thoroughly with soap and water at the beginning and at the end of each laboratory period.
3. Keep windows and doors closed to reduce air borne contamination.
4. Be systematic and logical. Keep a faithful record of all the experiments and observations. Update it regularly and submit it for evaluation at the end of each exercise.
5. Always, wear overcoat/apron while working in laboratory. It should be washed at least once a week. Keep the hairs and loose garments under check.
6. Always, wear gloves if there are cuts on hands or working with hazardous chemicals.
7. Be familiar about the working of instrument prior to handling it independently. Keep all the laboratory equipments at their respective places after use.
8. Do not remove any culture or any other article from the laboratory.
9. In case of any accident or spillage of culture and stain, immediately report to the instructor or laboratory in-charge for its proper care and disposal.
10. Always, discard the disposables and used cotton, matchstick, paper pieces etc. to trash cans and never into the sink.
11. Always keep disinfectant solution jar on the working bench for disposal of refuse.
12. Do not bring and consume eatables in the laboratory.
13. Dispose of infectious material, cultures and contaminated material carefully in container of disinfectant and check its disinfecting activity regularly.
14. Dispose of all cultures after autoclaving and used pipettes should be placed in disinfectant after use.
15. Always flame the inoculating loops before and after use. Similarly sterilize the necks of all tubes and flasks by passing it through the burner flame before and after each operation.
16. Arrange the cultures and bench equipment at respective place on the desk. Do not allow unused articles to accumulate in your work area.
17. Always, follow the instructions sincerely.
18. Work either using laminar flow chamber or light the burner at least five min prior to making any inoculations and work near the burner.
19. Avoid horseplay and vocalization in the laboratory

Unit one

Observations and study of structure of microbes

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Exercise 1: Demonstration of omnipresence of microbes

Microorganisms are present everywhere in the universe. They are part and parcel of our life. These are **omnipresent** i.e. present in air we breathe, food we eat and water we drink as well as on our body surface. Some of these comprise the resident flora of skin and mucosal surfaces of our body that are in contact with the atmosphere. Distribution and composition of microflora at particular niches depend on the prevailing environmental conditions and availability of nutrients. It is very difficult to dislodge them from these places. They colonize quickly if removed deliberately using cleansing devices, e.g. washing of hands with simple water or soap. However, scrubbing does decrease their number temporarily. It took very long to prove the existence of these microscopic forms until the developments of microscope and culturing techniques of these organisms. Presence of these organisms can be demonstrated by fingerprinting experiment. Each organism transferred to nutrient medium grows and produces a visible growth called colony representing the progeny of single organism. Number of such colonies appearing on nutrient medium on incubation decreases as the same finger is touched again and again at different places subsequently.

Requirements

a. Nutrient agar plates
b. Soap or any other detergent, alcohol
c. Incubator.

Procedure

1. Take a nutrient agar plate. Remove its lid near the Bunsen burner flame. Now, touch the agar surface at 5-6 places with the forefinger.
2. Repeat the same protocol on second plate with soap or alcohol washed hands.
3. Incubate both the plates in inverted position in the incubator at 37°C.
4. Next day observe both the plates and note the size and number of colonies in each fingerprint impression.
5. Study the colonial morphology of different types of colonies in term of size, shape, color, consistency, translucency, elevation etc. Keep these plates to be used to study the morphology of organisms in next period.

Questions

1. What do you mean by a colony?
2. What is the source of organisms found in air?
3. Why are the plates incubated in inverted position?
4. How did the invention of agar helped in development of microbiology?
5. Name the procedures used in the study of bacteria.

Exercise 2: Microscopy: Study, use and care of microscope

Microscope is an important tool for the microbiologist as the microorganisms are invisible to naked eye unless magnified. Antony van Leeuwenhoek called as "the father of microbiology" depicted the drawings of major forms of microorganisms while looking through a magnifying glass mounted on mechanical device for observing microbes. It was called a **simple microscope**. It contains a biconvex lens whose movement could be mechanically regulated.

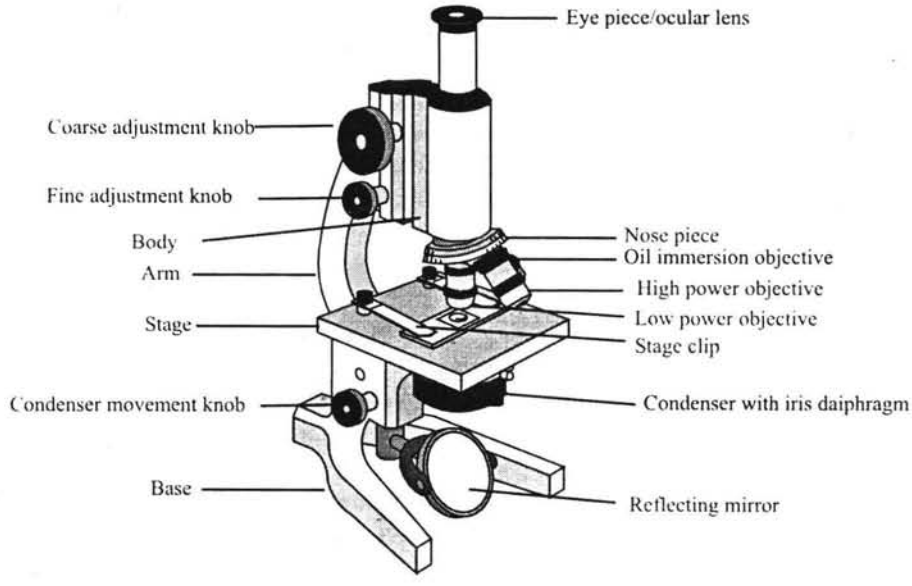
Bright field microscope, a compound microscope, incorporates two or more lens system. The arrangement increases the net magnification of the object many folds depending upon the magnification of two lenses. Zacharias Jansen introduced microscope currently used in most laboratories. Quality of microscope depends on its resolution or resolving power of the objective lens. Microscope comprises two main parts, the supporting stand and the optical system.

The supporting stand includes: (1) a **base** to hold the microscope in its position, (2) **an arm** to support the optical system and house the fine adjustment, (3) a **stage** or **platform** on which the object to be examined is placed. It is usually equipped with mechanical device that holds the glass slide firmly and on which the object is mounted so that it can be moved from place to place by setscrews. (4) **Condenser and mirror** is fitted beneath the stage. Condenser and mirror focus the light through a central opening in the stage on the object to be examined.

The optical system consists of a body tube, which supports ocular lens (eye piece) at top and a set of objective lenses attached to revolving nose piece at the other end. Optical system is connected to the arm of the supporting stand by an intermediate slide, which moves up and down on the arm in response to movement of fine adjustment. The intermediate slide contains the rack and pinion for the coarse adjustment, which acts directly on the tube of the optical system. **Iris diaphragm** controls the intensity of light entering the object through condenser. **Ocular lens** (5x, 10x or 15x) is placed at the upper end of the tube. Monocular microscope has one-lens while the binocular has two lenses. **Set of objective lenses** (10x, 40x and 100x an oil immersion objective): Above the stage on other end of mechanical tube is a revolving nosepiece holding three/four objectives. These primary lenses magnify the specimen. The objective can be moved away or closer to the object through **coarse** (low power objective) and **fine adjustment knobs** (for high power and oil immersion objective) for focusing the image. **Reflecting mirror/ light source:** Reflecting mirror has two planes, one concave for incandescent or artificial light and other plane for daylight.

Magnification of microscope is the multiplication product of the powers of objective and ocular lenses. Magnification of an objective is usually designated by its equivalent focal length (the focal distance/ working distance of a lens). 16 mm objective magnifies 10 times; 4 mm objective magnifies 40-45 times and most 1.8 mm objectives 97 times.

A **phase contrast microscope** converts slight differences in refractive index and density into easily detectable variations in light intensity and is an excellent device to observe living cells, as there are little differences in contrast between the cells and water. **Microscope condenser** has an annular stop and an opaque disk with a thin transparent ring which produces a hollow cone of light so that background formed by non-deviated light is bright while the unstained object appears dark as the light passing through cone/cell. This type of microscope is highly useful for the detection of bacterial components such as endospores and inclusion bodies containing polyβ hydroxybutyrate, polyphosphates, sulfur or other substances.



Compound light microscope

Fluorescence microscope : Fluorescence is the property of emitting rays having wavelength different from that of incident rays. Object becomes luminous against dark background. Fluorescent materials are generally of two kinds: one present naturally in cells and the other include the induced one by staining the object with fluorescent dyes or flurochromes. In fluorescent microscope, an object emits light when examined under UV rays. Radiations exciting the luminosity do not contribute to image formation. Such objects absorb radiant energy and release trapped energy when excited as visible light quickly to return to more stable state. Two kinds of filters are used for filtering harmful rays. **Excitation filters**, which transmit only the rays of visible range while blocking UV radiations the illuminating beam only excluding the radiations, and **Barrier filters** prevent passing of excited radiations in microscope to protect eyes from UV rays. The technique has become very important in medical microbiology, microbial ecology and study of bacterial pathogenesis. The objects are identified after staining with fluorescent dyes or flurochromes or specifically labeled fluorescent antibodies using immunofluorescence procedures.

Handling and use of microscope

1. Always, carry the microscope with both hands, one beneath and other on the arm.
2. Always focus the object by moving the *Objective* away from the glass slide. Avoid focusing downwards.
3. With coarse adjustment knob move the Objective to be used until it nearly touches the surface upper surface of the mounted specimen. Then focus by moving the coarse knob

upwards until the object comes into view. Complete the focusing with Fine adjustment knob.

4. Adjust the mirror and light while using low power Objective to give adequate illumination.
5. While observing unstained objects, the *Iris diaphragm* should be barely open to achieve good contrast. Iris diaphragm is fully open with higher magnification and while viewing stained objects.
6. Always, clean the lenses before and after use with lens paper. Do not touch the lenses with hands. Leave *Objectives* with lowest power in working position.
7. Keep the microscope covered when not in use.
8. Observe the slides with both eyes open. Do not incline the microscope. Adjust the stool to use the instrument comfortably.
9. Avoid direct sunlight. North light is advantageous.
10. To achieve a good contrast adjust the *Iris diaphragm*. Usually the Iris diaphragm is open to the minimal level while examining the unstained objects and it is fully open when stained specimens are examined. Observe the change in specimen when the Iris diaphragm is opened or closed for obtaining good contrast.
11. Place a drop of immersion oil on the illuminated area of slide and shift to 100X objective. Lower the *Objective* slowly viewing from sides until the lens contacts the oil drop and then the surface of slide.
12. Prior you place the microscope in wooden box at the conclusion of each laboratory period turn the nosepiece until the low power objective is in place and lower to the maximum until it reaches stop.

Questions

1. Which objective focuses closest to the object?
2. What controls the light entering the *Ocular lens*?
3. What is false and useful magnification?
4. What is field of vision?
5. How can you enhance the resolving power of microscope?
6. What would occur if you use water instead of immersion oil?
7. Examine a drop of urine sediment under microscope and make sketch showing different objects seen.
8. What is "working distance" in regard to compound microscope?
9. How would you distinguish dust particle and microorganisms?

Exercise 3: Examination of microorganisms in live preparations.

i. **Hay infusion examination.**
ii. **Examination of protozoa**
iii. **Hanging drop technique**
iv. **Motility in semi solid agar.**

Microorganisms can be divided into two broad groups based on their cell structure: the prokaryotes and eucaryotes. The former lack nucleus and several other membranes bound organelles include bacteria and blue-green algae or the cyanobacteria. Fungi, algae, protozoa and the multicellular parasites are some examples of eucaryotes. Staining procedures used for examining microorganisms are usually harsh and often distort the natural structure, shape and formations. Bacteria can be observed under a microscope in unstained or stained preparation. Even the motility in bacteria can be observed in live preparations using wet preparations. A wet mount is the fast way to observe bacteria. In hanging drop technique the application of petroleum jelly seal around the cavity reduces the evaporation of suspended fluid drop. Making it possible to observe larger microbes and motile organisms more easily because of greater depth provided by the hanging drop. Motility of bacteria can also detected by observing the growth pattern of culture in semisolid or motility agar inoculated with a straight wire on overnight incubation.

Requirements

a. Hay infusion or pond water
b. Microscopic slide
c. Cavity slide, cover slip petroleum jelly, matchstick
d. Inoculating wire
e. Motility agar or Hugh Leifson medium in sugar tubes
f. Bacterial culture: *Proteus vulgaris* broth culture (8-16 h old).

Procedure

(i) Hay infusion or pond water examination

1. Take a drop of hay infusion or pond water on clean grease free glass slide.
2. Place a cover slip on the drop from the edge to avoid air bubbles entrapment.
3. Focus the slide under microscope, with condenser lowered to maximum and iris opening narrowed down. Examine the slide under microscope initially under 10x objective and then shift to 40x objective.
4. Record the observation regarding various forms of organisms seen differing in cell structure, motility, size, shape, color etc and illustrate these diagrammatically.

(ii) Examination of protozoa

The protozoa can be seen more distinctly in unstained condition as compared to bacteria under bright field microscope. The protozoa are larger as compared to bacteria. In some, even the internal structures can easily be observed. Morphology of protozoa is usually diagnostic in many cases. The group display different types of reproduction activities rather than merely dividing by binary fission seen like bacteria.

Requirements

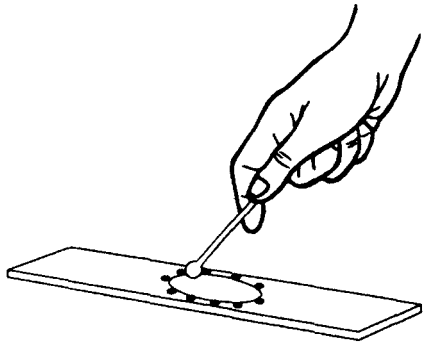
a. Hay infusion
b. 3% gelatin solution
c. Plastic dropping pipette

Procedure

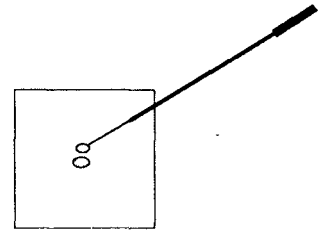
1. Allow the melted gelatin solution to cool to room temperature
2. Mix one drop of gelatin and one drop of hay infusion on the microscopic slide.
3. Place cover slip and examine under microscope.
4. Note down the morphology observed under different objectives (10x, 40x and 100x) and sketch the observations.

(iii) Hanging drop technique

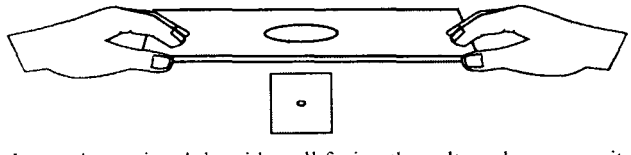
1. Take a clean cavity slide. Apply petroleum jelly around the well with matchstick.
2. Pick up a cover slip and place it on filter paper. Transfer a loopful culture in the center of cover slip.
3. Now, carefully put the slide over the cover slip so that drop is in the center of cavity. Carefully invert the slide so that drop hangs in the well.
4. Examine the slide under low power objective. Locate the edge of the drop by moving the slide with pinion arrangement and position the slide so that the edge of the drop crosses the center of the field.
5. Reduce the light adjusting iris diaphragm and condenser and focus. Observe the different sizes, shapes, and types of movement.



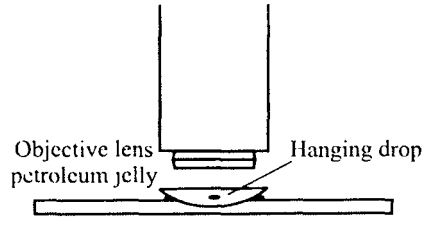
(I) Apply petroleum jelly around the well



(II) Place a drop of culture in the center of cover slip



(III) Invert the cavity slide with well facing the culture drop on cavity slide and gently press it and turn up slide



(IV) Observe under microscope

Hanging drop technique

(iv) Motility in semisolid agar

1. Aseptically inoculate the sterile semi solid medium in tube by stabbing with a straight inoculating needle.
2. Incubate the inoculated tubes at 37°C for overnight.
3. Next day examine the semi solid agar tubes for bacterial growth pattern of culture.
4. The culture is non motile if the growth is restricted to the inoculated path and motile if the tube shows turbidity diffusing along the streak or throughout the medium.

Questions

1. Why are microorganisms hard to see in wet preparations?
2. Why the oil immersion lens is not used in examination of hanging drop procedure?
3. Why did you use young cultures for observing bacterial movement?
4. What is the motility component in bacteria?
5. Why do we add gelatin prior to observing under microscope?
6. What would be the effect of culture shaking on bacterial motility when examined in hanging drop?
7. Enlist the similarities and differences between protozoa and algae.
8. What is the purpose of a hanging-drop preparation?

Exercise 4: Examination of blue green algae (BGA)

Blue green algae are found throughout world in fresh water and saltwater and in moist microaerophilic habitats. These are abundant in fresh water near the surface than deep in water or in saline habitats. Accumulation of water for prolonged periods under sunny habitats is usually good for algal growth as theses conditions favor the development of blue green algae (BGA), which have become dormant during dry seasons. Red sea has been so named due to the abundance of red BGA. Most algae are forage plants or planktons for fishes and other sea animals. However the presence of BGA in drinking water is discouraged as they impart disagreeable smell, odors and taste and some may cause bloom. Some are even rich source of vitamins and minerals.

Requirements

a. Prepared slides of mixed BGA
b. Water sample from shady pond

Procedure

1. Compare the morphology of algal cells on the reference slides.
2. Sketch and label the different parts of the cells seen on each slide.
3. Place a drop of pond water on the slide and cover it with the cover slip.
4. Examine care fully and try to identify the type of algae present and note down their movement. Sketch the different types of cells seen and the movements.

Questions

1. What is the economic importance of BGA?
2. What are planktons?
3. What disease or nuisance does BGA cause to mankind?
4. Compare and contrast the properties of BGA and bacteria.

Exercise 5 : Preparation of bacterial smear and simple staining

Microorganisms are small, transparent and motile in fluids hence it is very difficult to observe under microscope unless these are immobilized (fixed) and stained with a suitable stain. Placing and spreading of sample on microscopic slide is called a **smear**. The air-dried smear is fixed to glass to avoid it being washed when treated with liquid stain.

Fixation is accomplished by passing the smear through the top part of Bunsen burner flame 2-4 times taking care that glass slide is quite hot but bearable to the reverse of your hand skin. Alternatively the smear can be chemically fixed by covering the smear with 95% methanol for 1-5 min. **Fixation** denatures the bacterial enzymes and prevents autolysis and ensures bacterial adherence to microscopic slide. Soon after fixing, slide can be stained.

In bacteriology, three classes of stains are used: simple stain, differential stain and special stain or structural stain. Simple stain is usually an aqueous or alcoholic solution of dye applied 1-2 min to the smear and then washed off. Most common simple stains are Loeffler's alkaline methylene blue, methylene blue, safranine, carbol fuchsin and gentian violet. Most bacteria stain easily and quickly with simple stains while the capsules and spores remain unstained.

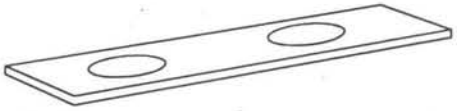
Most stains used in microbiology laboratory are the aniline dyes. The ion imparting color to the dye is called chromophore. The dyes are usually salts, a few are acids or bases composed of colored ions. If chromophore is a positive ion like methylene blue, it is called a basic stain and if negative, it is known as acidic stain.

Methylene blue chloride $\longrightarrow$ methylene blue (chromophore) + chloride

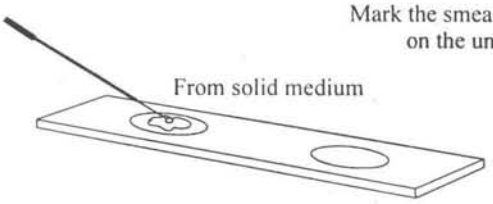
Staining procedure that uses only one stain is called simple staining. Simple stain that stains bacteria is direct stain and which does not stain bacteria but stains background is called negative staining. Simple staining is easy, cost effective and very useful in studying the morphology, size and arrangement of microorganisms.

Requirements

a. Stains: Methylene blue, safranine, malachite green or diluted carbol fuchsin solution.
b. Bacterial cultures: *Staphylococcus epidermidis* slant and *Bacillus megaterium* and *Escherichia coli* broth.

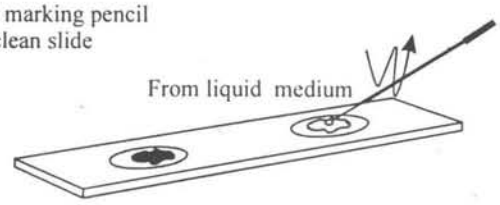


Mark the smear areas with a marking pencil on the underside of a clean slide



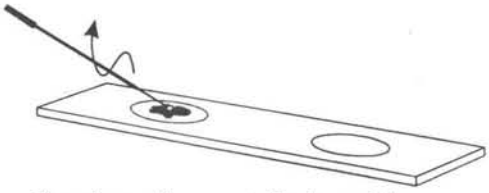
From solid medium

Place one loopful of water on the slide

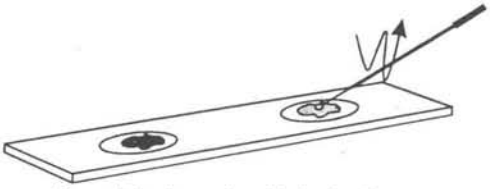


From liquid medium

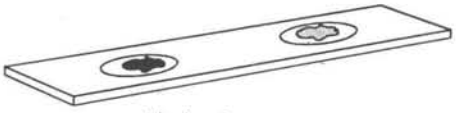
Place a loopful of culture on the slide with sterile loop



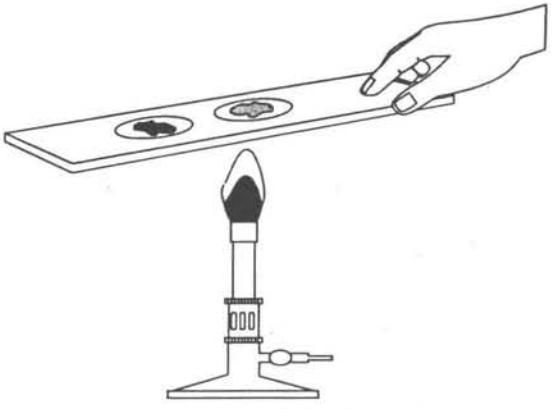
Transfer small amount of culture with a sterile loop. Mix with water and spread in the marked area



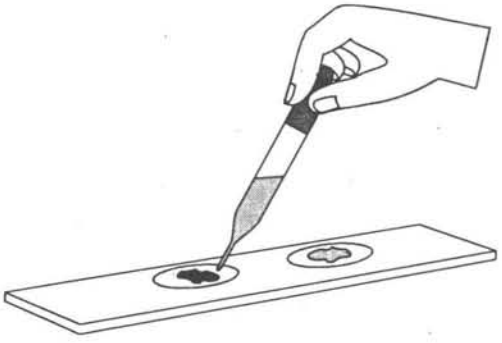
Spread the bacteria with in the ring



Air dry the smears



Pass the slide through the flame of a burner, two or three times



Cover the smears with 95% methyl alcohol for one minute, than let the smears air dry

Smear making and fixation of bacterial culture for staining

Procedure

1. Clean microscopic glass slides with cleanser, rinse with water and air dry. Hold the slides from edge and mark two circles on slide (underneath) in the center with a marker. Label each circle.
2. Place one loopful of sterile distilled water in the center of a circle for bacterial culture on slant with inoculating loop. Loop must be sterilized before and after use by making it red hot in Bunsen burner flame.
3. Allow the loop to cool. Now transfer small amount of culture from solid medium to distilled water drop and loopful culture from broth to another circle and spread them evenly as thin smear.
4. Allow the smears to air dry at room temperature. Fix the smear by passing slides through burner flame 2-4 times or covering it with methanol for 5 min. and air-dry before staining.
5. Place the slide on staining rack and cover the smears with any one stain and let it stain for 30-60 seconds. Then drain off the stain and wash the slides under running tap water or water from wash bottle.
6. Gently blot dry the smear with absorbent/ filter paper and air dry.
7. Place a drop of immersion oil on each smear, examine the slides under oil immersion objective, and record your observations.

Questions

1. Which culture shows rods and which appeared coccus to you?
2. What would happen if you apply too much heat while heat fixing the smear?
3. What are the advantages of simple stain?
4. Why is it necessary to cool the loop prior to picking the culture?
5. Does heat fixation kill all the microorganisms?
6. What is fixation? What is the alternate method to heat fixation of bacteria?
7. What are three classes of stains used in bacteriology?
8. Why is it customary to flame the inoculating needle, flask or tube mouth immediately prior to and after inoculation?

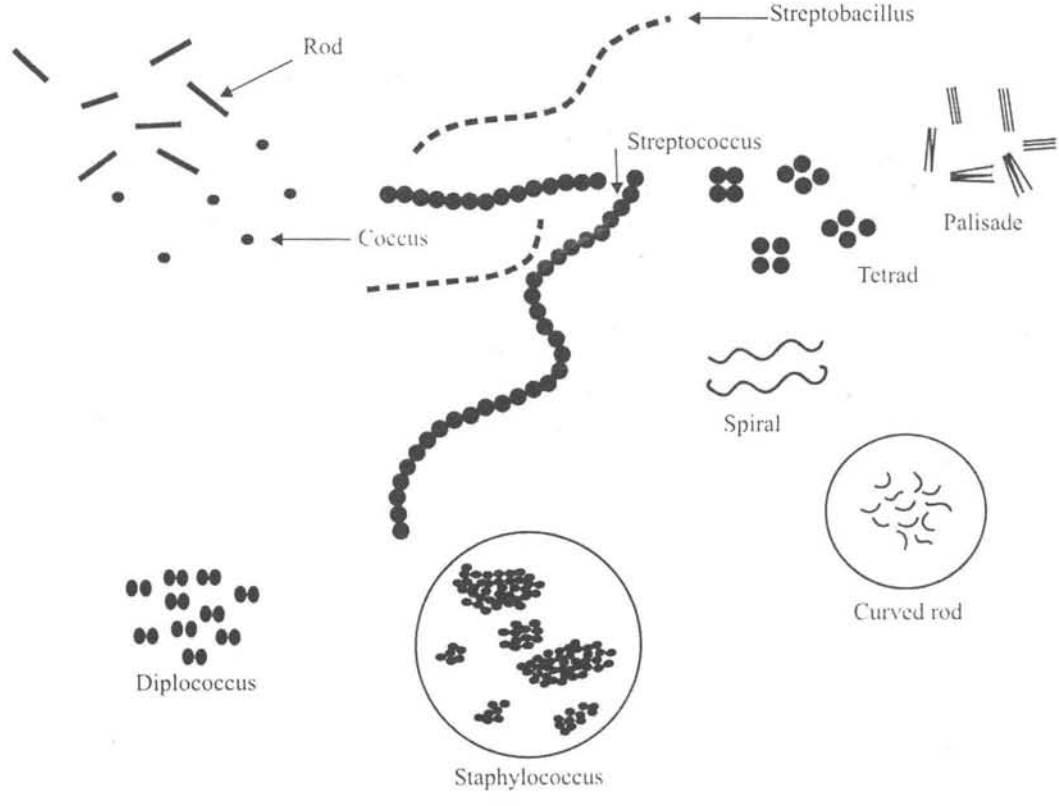
Exercise 6: Study of morphology of bacteria

In nature, the microorganisms exist in different sizes, shapes and morphology. These characteristics are inherent properties of each genus and are highly useful in differentiation and identification of strains. It is also useful for taxonomical classification. Broadly, the bacteria are of three shapes: small rod (straight or curved), spherical (coccus) and spiral. Microorganisms are grouped based on arrangement of cells. Some bacteria after binary fission do not separate and remain attached, giving rise to specific cell arrangements depending on the planes of cell division. Some strains change their form after being cultured *in-vitro*. Organisms showing such variations are called pleomorphic organisms and the phenomenon pleomorphism. The following arrangements are usually observed in cocci and rods:

Cocci: *Staphylococci* (organisms arranged in bunches), *streptococci* (cocci in chains), tetrads (a packet of four cells), *diplococci* (cocci in pairs) and as coccus (as individual spherical cell)

Rods: May exist as singles, in pairs (diplobacilli), chains (streptobacillus), palisade arrangement rods arranged like a pile of coins called slipping (*Mycobacterium* sp.) and may show Chinese letter arrangement when the cells after division do not fall apart and remain attached at different angles. This is known as snapping (*Corynebacterium* sp.).

Spirals: (*Spirochete, Spirillum* and *Vibrio*), no specific arrangement.



Morphological arrangement in bacteria

Requirements

a. Bacterial cultures: *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Bacillus subtilis, Sarcina lutea, Treponema pallidum, and Vibrio cholerae.*
b. Inoculating needle, glass slides, microscope.
c. Stains: Methylene blue, crystal violet

Procedure

1. Prepare the smear for each strain on separate slide.
2. Air-dry and heat-fix the smears. Simple stain each slide using methylene blue or crystal violet just for 30 sec. Make use of negative staining in case of *Treponema pallidum.*
3. Examine under oil immersion objective and record the observation with respect to shape, size and arrangement of different cultures.

Questions

1. Why do the microorganisms exist in different morphological forms?
2. What is the basis of palisade and Chinese letter morphological arrangement in *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae?*
3. What causes the microorganisms to exist in different morphological forms?
4. Diagrammatically represent the morphological arrangement of following cultures: *Escherichia coli, Vibrio cholerae, Sarcina lutea* and *Streptococcus pneumoniae.*
5. Comment on the possible morphological arrangement of organisms:
   a. Bacteria divide in one plane but do not separate after division
   b. Bacteria divide in one plane but fall apart after division
   c. Bacteria divide in two planes and do not separate
   d. Bacteria divide in more than two planes and do not separate
6. Do all bacteria stain with simple stains?

Exercise 7: Observation of capsule and bacteria using negative staining

Negative stain does not stain bacteria but imparts color to the background. The bacteria and capsule appear colorless against colored background. While looking for capsule if culture is treated with crystal violet and negative stain mixture, the **capsule** appears unstained sandwiched between colored bacterial cell and background. In negative staining, smears are not heat fixed; hence, no bacterial cells distortion. This staining is highly useful where other techniques do not clearly indicate cell morphology or size.

In most strains capsule is present as slime layer exterior to cell wall. Capsule bearing organisms are hygroscopic and often produce smooth colonies on solid medium. Serum treated capsules visualized using negative staining appear bigger. This is called "Quellung reaction".

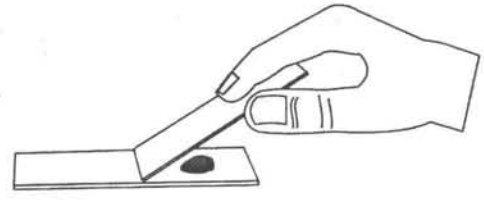
Requirements

a. Stain: Nigrosine and Crystal violet
b. Bacterial cultures: *Klebsiella pneumoniae, Bacillus subtilis and Staphylococcus albus*

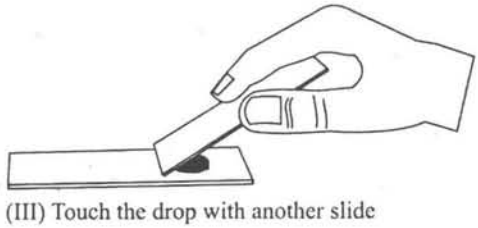
Procedure

1. Place a small drop of nigrosine at the end of clean grease free slide.
2. Transfer small amount of culture to the drop and emulsify. Addition of one drop of crystal violet prior to emulsification is good for examining capsular bacteria.
3. Using the end edge of another slide spread the drop to produce a smear of varying thickness (opaque black to gray). The angle of spreading slide determines the thickness of smear. Let the smear air-dry. Do not heat fix.
4. Examine the slides under oil immersion objective and record the observations.

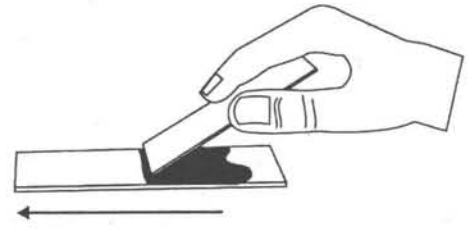
(I) Mix a drop of dye and culture on the slide



(II) With another slide at angle spread the drop as below



(III) Touch the drop with another slide



(IV) Push the top slide to the left along the entire surface of the bottom slide

(V) Let the smear air dry

Negative Staining

Questions

1. What is the mechanism of negative staining?
2. Can other dye be used instead of nigrosine?
3. Can this technique be used for demonstration of spores?
4. What is negative or relief staining?
5. How does the addition of crystal violet prior to emulsification help in differentiating capsular bacteria?

Exercise 8 : Differential staining of bacteria (Gram staining)

Gram stain is a differential stain as it differentiates bacteria in two groups (**Gram positive** and **Gram negative**). Hans Christian Gram introduced this technique in 1884. **Gram** stain is highly useful for identification of microorganisms as the bacteria stain differently because of chemical and physical differences in their cell walls. Crystal violet-iodine complex (CVI) formed because of crystal violet and iodine penetrating the bacterial cell does not leach out on treatment with alcohol because of **thick** peptidoglycan layer in Gram-positive bacteria. Alcohol dissolves the outer lipopolysaccharide layer; consequently, the CVI complex leaches out through **thin** layer of peptidoglycan in Gram-negative bacteria.

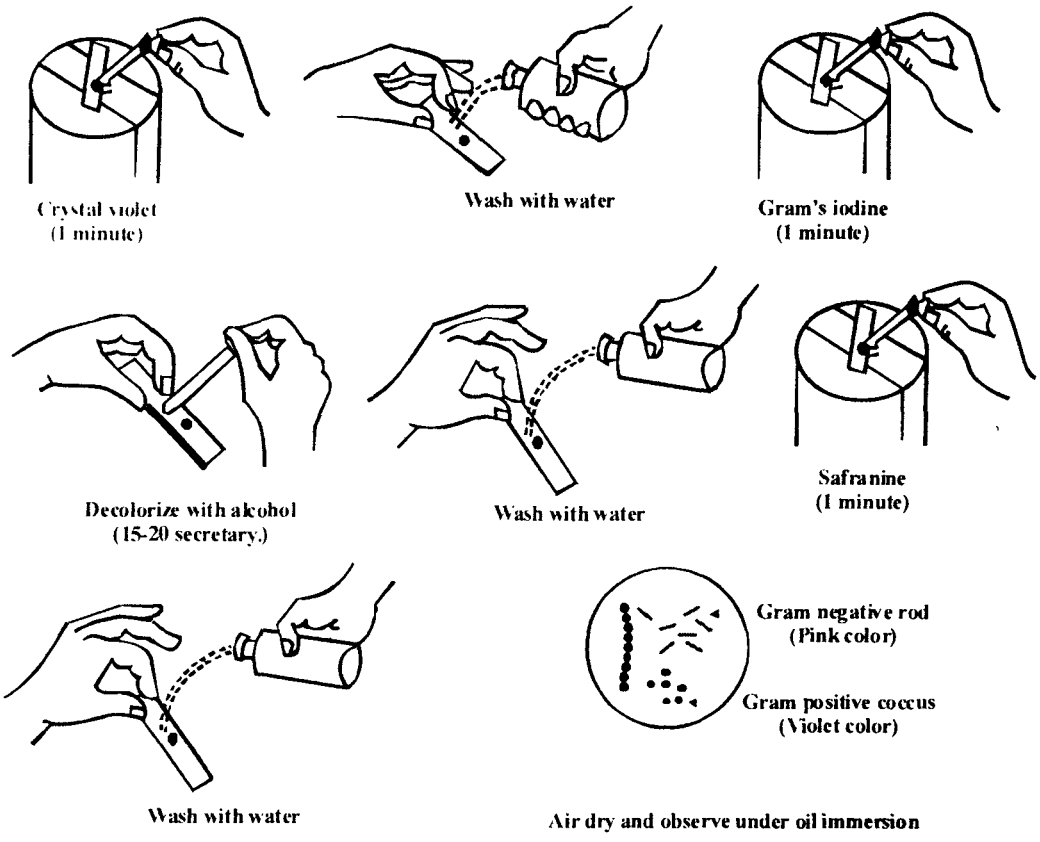
Gram stain is more consistent with young cultures (8-16 h old). Old cultures may show variation in **Gram** staining. The technique makes use of: **Primary stain** (crystal violet) that stains all bacteria purple, **Mordant** (iodine) combines with crystal violet to form a CVI complex, **Decolorizing agent** (95% alcohol or acetone) washes out the CVI complex from some bacteria, and **Counter stain or secondary stain** (safranine) to stain the decolorized bacteria. Gram staining gives information about its morphology, arrangement and occasionally spore, capsules or granules in addition to gram reaction of organism. It is also the first step in the identification of microorganisms.

Requirement

a. Gram stain set:
   Crystal violet
   Gram's iodine
   95% ethyl alcohol or acetone
   Safranine
b. Bacterial cultures: *Staphylococcus aureus*, *Escherichia coli* and *Bacillus megaterium*

Procedure

1. Prepare and heat fix smears on clean glass slides as explained earlier and mark circle around each smear on reverse side of slide with a marker.
2. Place the slide on the staining rack and cover the smear with crystal violet and leave it for one min. Wash the slide with tap water gently.
3. Cover the smear with Gram's iodine for one min.
4. Wash off the iodine by tilting the slide and pour water over the smear.
5. Decolorize with ethyl alcohol or acetone for about 10-15 sec. or until no large amount of purple color wash out. However, do not over or under decolorize. Immediately wash with water.
6. Cover the smear with safranine for 30 seconds. Wash with water. Blot dry, examine under oil immersion objective, and record the observations indicating the Gram character and morphological arrangement of the microorganisms in each culture.



Gram staining

Precautions

The factors that may affect the reliability of Gram staining are:

a. Gram-positive bacteria staining gram-negative : if the culture is old more than 24 h.
b. Over decolorizing is another most common error.
c. Heat fixation is the most important. If the smear is over heated it may char the organisms or create artifacts, which will adsorb and retain crystal violet that may be mistaken as Gram-positive organisms.

Questions

1. What would be the Gram's reaction for human cells?
2. Why do the old Gram-positive cultures stain Gram negative?
3. What do you conclude if you see both rods and cocci in Gram stained field?
4. What do you conclude if you find red and purple cocci in pure culture?
5. Name two each Gram's positive rods and cocci that grow in chains.

Exercise 9: Demonstration of pleomorphism in microorganisms

Morphological changes that usually occur during bacterial growth not only alter the normal growth cycle but also the cell shape and size of the cells in population. Pleomorphism is also observed within population growing in natural habitats. Number of rod shaped pathogens produce coccoid forms in the late phases of growth. Changes in morphological characteristics may result in increase or decrease in cell size, changes in cell opacity, cellular refractive index and resulting in aggregation of cells and sometime giving rise to cells of different shapes and sizes both. Pleomorphism can best be explained by examining the organisms growing in nodules. *Rhizobium* sp. cell population present in young (pink color) and old (brownish color) nodules show great variability, the cells may be thin or plump short rods, elongated, star shaped, y shaped, rods and coccoid forms. All these forms cultured on yeast extract mannitol agar produce uniform gram-negative rod shaped organisms. During fermentation some of the organisms produce filamentous growth because of slow diffusion of nutrients.

Requirements

a. Root nodules
b. Alcohol
c. Teasing needles, forceps
d. Gram stain set
e. Plating medium: yeast extract mannitol agar plates

Procedure

1. Select out the young pink colored nodules from the leguminous plant. Initially wash with water to remove dirt and soil.
2. Then give 2-3 washing with 70% alcohol and then wash it with sterile distilled water. Place the nodules in sterile petri plate.
3. Either crush the nodules by placing a nodule between two sterile slides or tease the nodules with the help of teasing needles.
4. Make a smear from the teased material on the clean glass slide. Let the smear air dry and Gram stain the slide.
5. Examine the slide under oil immersion objective and note down and sketch the different shapes of organisms seen.
6. Take a loopful of the crushed nodule and streak it on the plating medium. Incubate the plate for 24-48 h at 37°C.
7. Observe the type of growth appearing on the plate. Make a smear from the colony and examine it after gram staining. Record the observations.

Questions

1. What are the differences you observed in Gram stains of slides prepared from nodules and from colony?
2. How many types of colony did you observe on plating medium?
3. Why the alterations in cell size and shape was observed in nodules only?

Exercise 10: Examination of spirochetes

Spirochetes are minute spiral bacteria that are associated with number of disease in humans and even live as well as resident flora in the crevices of teeth. Most are anaerobes or extremely microaerophilic and can grow only under highly reduced conditions. The important genera that cause diseases in humans and animals include *Treponema pallidum, Borrellia recurrentis, B.burgdorferi, Leptospira interogans* etc. In contrast to other bacteria, which possess flagella and exhibit active motility, the spirochetes exhibit flexing and corkscrew like motion as flagella are encased in membrane that encloses the organism. Spirochetes vary in size, shape and number of spirals. These are stained with difficulty with ordinary staining methods. Special staining techniques make use of mordant like silver salts so that these are visible under microscope after staining.

Requirements

Fontana' method

a. Fixative (1 ml acetic acid, formalin 2 ml /100 ml distilled water)
b. Ammonical silver nitrate stain (10% ammonia to 0.5% silver nitrate in distilled water until precipitate form and redissolve. Now add more Ag NO_3 solution drop wise until precipitates returns and does not dissolve)
c. Mordant (phenol 1g and tannic acid 5g dissolved in 100 ml distilled water)

Becker's method

a. Fixative and mordant same as above.
b. Stain: Basic fuchsin saturated alcoholic solution 45 ml mixed with 18 ml Shunk's mordant (spirit 18 ml and aniline oil 4 ml) and volume made to 100 ml with distilled water.

Procedure

Fontana's method

1. Scrap material from the teeth using sterile needle. Transfer the material to slide and spread this on to the slide to prepare a thin smear.
2. Air dry. Fix the smear by giving three successive treatments with fixative 30 seconds each.
3. Decant off the fixative and add absolute alcohol for 3 min to wash off the fixative. Drain off the excess of alcohol and burn off the remaining by passing through burner flame until the film is dry.
4. Cover the smear with mordant for 30 seconds and heat the mordant on slide to steaming.
5. Wash the slide well with distilled water and air dry. Treat the slide with steaming ammonical silver nitrate stain for 30 seconds until the smear turns brown in color.
6. Wash the slide with distilled water.
7. Air dry and mount the slide in Canada balsam as the oil immersion may cause the film to fade. Observe the slides using oil immersion objective. The spirochetes are stained brownish black against the brownish yellow background.

Becker's method

1. Make the film and air dry. Cover the film for 3 min with fixative.
2. Wash in water for 30 seconds. Treat the slide with mordant for 3-5 min.

3. Again wash in water and stain the slide for 5 min. Wash the slide well and drain dry.
4. Observe the slides under oil immersion objective

Questions

1. Why is the immersion oil not added directly upon the stained smear?
2. What are the functions of a fixative?
3. Can spirochetes be stained with Gram's staining?

Exercise11: Slide culture technique for fungi (microscopic examination)

The fungus may be sub cultured on an agar block held between cover slip and a slide. This enables the study of various stages of fungal growth. This technique is highly useful for observing the dimorphic fungus and for study of morphologic characteristics of growing molds without disturbing the arrangement of spores and conidiophores.

Requirement

a Test fungus culture
b. Sterile Sabouraud's agar medium or Czapek Dox agar plate.
c. Inoculating needle, cover slip, forceps, alcohol

Procedure

1. Cut the agar in petri plate into square blocks (1sq cm) with sterile blade.
2. Place an agar square on sterile slide, inoculate a needle tip of culture into the mid point of each block and cover the block fully with flame sterilized cover slip from the surface.
3. Place the preparation in a covered jar or petri plate containing a layer of blotting paper soaked in 20% glycerol so that fungus can grow in well-aerated humid atmosphere without drying.
4. Incubate at room temperature or 28°C. Examine the slide daily after removing from the jar without disturbing the cover slip microscopically and record the observations.

Questions

1. What is advantage of slide culture over the plate culture?
2. Can this technique be used for examining bacteria as well?
3. Why this technique is most suited for fungal identification?

Exercise 12: Lacto phenol cotton blue staining for fungi

Nutritionally all fungi are heterotrophic, eucaryotic microorganisms that grow as saprophyte on variety of substrates particularly under moist conditions. Mycologists have classified true fungi classified into four classes; Phycomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes based on sexual modes of reproduction. **Phycomycetes** the water and bread molds produce reproductive spores that are external and uncovered. **Ascomycetes** yeast and molds bear sexual spores called ascospores that remain encased in a sac like structure called an ascus. **Basidiomycetes** produce basidiospores budding from basidia. Examples include the fleshy fungi; mushrooms and puffballs **Deuteromycetes** do not bear sexual spores hence are also called the **fungi imperfecti**.

Fungus can be seen growing on bread and spoiled citrus fruits producing white cottony, green, brown, orange, red or black growth. Some of these are cultured specifically as edible mushrooms, producing several industrial products of human interest including food, medicines and beverages. Fungi decompose dead plants and animal tissues and contribute to the fertility of soil. Some fungi are even harmful found associated with superficial and systemic infections. Some even produce potent carcinogens and other toxins. In contrast to bacteria the molds can be seen easily with naked eye. The filaments that comprise the mycelium are the intertwining **hyphae**. Mycelium growing on surface is called vegetative mycelium and that rises upward is referred as aerial mycelium. Specialized hyphae on aerial mycelium give rise to spores the reproductive elements of molds.

Lacto phenol cotton blue stain is used for making semi permanent fungal slides. An inoculum from fungus culture is teased with help of teasing needle directly in lacto phenol cotton blue stain and examined under microscope. The stain imparts blue color to cytoplasm against light blue background. Against which the walls of hyphae can be visualized easily. This stain comprises three different components that perform different important functions. Phenol present in stain is fungicidal. Lactic acid acts as clearing agent and the cotton blue stains the cytoplasm blue. Glycerin present is good for preparing semi permanent slides that may be sealed with nail polish. It can be replaced with polyvinyl alcohol or Canada balsam for permanent mounts.

Requirements

a. Fungus culture.
b. Lacto phenol cotton blue stain.
c. Glass slides
d. Teasing needle, burner and microscope

Procedure

1. Place a drop of stain on clean microscopic slide and transfer an inoculum from fungus culture representing all fungal structures.
2. Separate out fungal inoculum with teasing needle while mixing it with stain.
3. Place cover slip avoiding any air entrapment and examine under microscope.
4. For making semi permanent mount seal the cover slip margins with nail polish and let it dry for 30 min. Excess stain if any may be removed with alcohol prior to applying nail polish.

5. Sketch the different structures seen and describe the morphology of each and identify the fungus based on these characteristics.

Questions

1. What are sporangium, stroma, conodiophore and what are their functions?
2. How the edible and poisonous mushrooms can be distinguished?
3. Enlist the contrasting features of fungi from bacteria and algae.

Exercise 13: Acid fast staining

It is also a differential staining. In 1882, Paul Ehrlich discovered that in contrast to most bacteria *Mycobacterium tuberculosis* did not stain readily with primary stain but once stained, did not lose the stain even after washing with acid alcohol mixture. Hence, they are called "**acid fast**" bacteria. The technique is diagnostically important in identification of acid-fast *Mycobacterium* species and *Nocardia* species. Acid-fast organisms contain mycolic acid that renders the cell wall impermeable to most stains and detergents. Therefore these organisms remain alive in clinical specimens treated with 4% NaOH. This feature is exploited in inactivation of non acid-fast organisms in clinical samples for culturing acid-fast bacilli.

Currently **Ziehl-Neelsen** and **Kinyon** procedures are the most widely used acid-fast stains. In Ziehl-Neelsen procedure, the smear is flooded with hot carbolfuchsin or the stain is heated on the slide from underneath to facilitate stain penetration into bacteria. Heating is avoided in Kinyon modified cold stain procedure. Higher concentration of phenol and carbol fuchsin in stain facilitates the penetration of stain. Stained smears are washed with acid alcohol mixture that decolorizes non acid-fast bacteria. Methylene blue is used as counter stain for staining non acid-fast organisms. Carbol fuchsin has more affinity for lipids than acid alcohol hence remains bound to cell wall of acid-fast bacteria when washed with acid alcohol.

Requirements

Ziehl- Neelsen Carbol fuchsin method

a. Ziehl- Neelsen Carbolfuchsin stain
b. Acid alcohol or 20 % H_2SO_4
c. Counter stain: Methylene blue or malachite green

Acridine orange method

a. Acridine orange stain

Rhodamine-auramine method

a. Rhodamine-auramine stain
b. Decolorizer
c. Counter stain 0.5% $KMnO_4$
d. Bacterial cultures: *Mycobacterium phlei* and *Escherichia coli*

Procedure

Ziehl- Neelsen Carbol fuchsin method

1. Prepare and heat fix the smears of both the cultures on clean grease free slides.
2. Cover the smears with boiled carbolfuchsin and leave it for 5-10min.
3. Gently wash with water and then with decolorizer (acid alcohol) for 1 min. or until no more color comes out. Wash the slides with water.
4. Counter stain for1 min. with methylene blue or malachite green.
5. Wash with water and blot dry and examine the slides under oil immersion objective and record the observations.

Acridine orange method

1. Fix the slide in methanol or with heat.
2. Flood the slide with acridine orange stain. Do not let the stain dry and allow the stain to act for 3 min.
3. Rinse the slide with tap water. Keep the slide upright to drain water and air dry.

4. Examine the slide under UV light. Bacteria will fluoresce bright red-orange, leukocyte pale apple green against a green fluorescence or dark background. The nuclei may also fluoresce.

Rhodamine-auramine method

1. Heat fix the slides. Cover the slides with rhodamine-auramine stain. Allow the stain to remain on slide for 15min do not allow stain to dry on slide.
2. Rinse the slides with distilled water and shake off excess liquid.
3. Destain with decolorizer for 2-3 min. slide will appear pink.
4. Rinse thoroughly with distilled water and shake off excess water.
5. Counter stain for 2-3 min. do not allow the slides to dry.
6. Rinse with water and air dry.
7. Examine under UV source. AFB appears yellow orange against green background. For quick examination, slides can be screened initially under 40x and then confirmed under oil immersion objective.

Questions

1. What are diseases diagnosed with acid-fast procedure?
2. What is the arrangement of acid-fast bacilli in the smear?
3. Why are the clinical specimens suspected to contain *mycobacteria* digested with sodium hydroxide prior to staining and culture?
4. What is the concentration of H_2S0_4 as decolorizer while looking for acid-fast *Nocardia*, *Mycobacterium leprae* and *Mycobacterium bovis*?

Exercise 14 : Staining of bacterial spores

Out of the ten genera that form endospores, two genera *Bacillus* and *Clostridium* are the most common. The spores of bacteria do not stain as easily as vegetative cells. With ordinary stain, spores remain unstained or slightly tinged with stain. Endospores are metabolically inactive and are resistant to heat, chemicals and harsh environmental conditions. Spores contain dipicolinic acid which complexes with calcium ions and thus imparts heat resistance to the spores. The cell wall disintegrates soon after endospores formation. Spore staining procedures make use of strong stain such as carbol fuchsin and prolonged contact with stain or the stain is poured on the smear and heated underneath. Spores after staining resist decolorisation. The information regarding spores (shape, diameter and position of endospores) is very useful for taxonomy.

Requirements

ZNCF method

a. Ziehl-Neelsen Carbolfuchsin stain
b. Malachite green and methylene blue as counter stain

Darner's method

a. Nigrosine
b. ZNCF stain

Wirtz-Conklin method

a. Malachite green (0.5% aqueous)
b. Safranine or Mercurochrome (0.5% aqueous).
c. Bacterial culture: *Bacillus megaterium, B.subtilis* and *B.subtilis* (16-72h old)

Procedure

ZNCF method

1. Make smears from each culture on clean glass slide air dry and heat fix.
2. Boil malachite green in a test tube and pour it over the smears for 5-10 min. Alternatively place the slide on the boiling water beaker, pour malachite green onto the smear, and let it remain for 5 min.
3. Wash the stained smear thoroughly with distilled water.
4. Counter stain, the slides for 30 seconds with safranine.
5. Wash with distilled water. Blot-dry the smear and examine under oil immersion objective. Record the observations.
6. Repeat the spore staining using ZNCF instead of malachite green and malachite green as counter stain in lieu of safranine. Note the color of spore and cell in each.

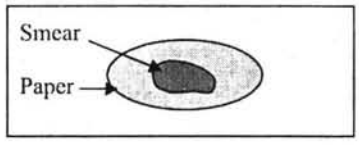
Darner's method

1. Make smears from each culture on clean glass slide air dry and heat fix.
2. Boil ZNCF stain in a test tube and pour it over the smear and let stain for 5 min.
3. Wash gently with water and air dry. Add a drop of nigrosine and spread it on the slide with another slide. Air-dry and observe under oil immersion objective.
4. Spores are stained red and cells appear colorless against black background.

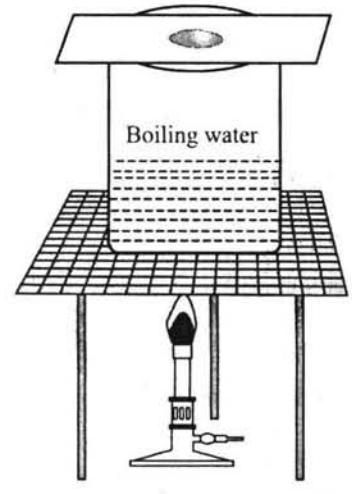
Wirtz-Conklin method

1. Make smears from each culture on clean glass slide air dry and heat fix.

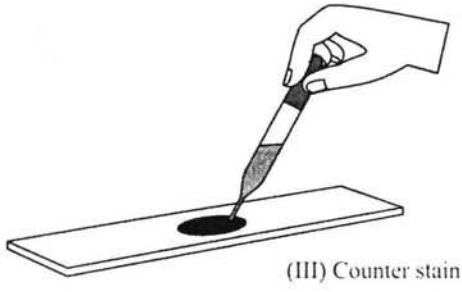
2. Boil malachite green in a test tube and pour it on the smear for 5-10min. Alternatively put the slide on a beaker containing boiling water and stain for 10 min with malachite green.
3. Wash with water and counter stain with either safranine or mercurochrome for 30 seconds.
4. Wash with water, air dry and observe under oil immersion objective.
5. Spores appear green in red stained cells.



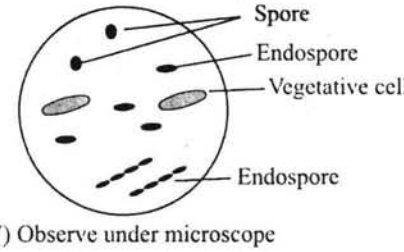
(I) make a smear, air dry and cover with filter paper



(II) Add ZNCF stain and heat the slide



(III) Counter stain



(IV) Observe under microscope

Spore staining technique

Questions

1. How do you account for the differences observed in 24 and 72 h old *B.subtilis* culture?
2. What prevents the cell from appearing green in the finished endospores stain?
3. Name the diseases caused by spore forming Gram-positive bacteria.

Exercise 15 : Capsule staining

Capsule is a gelatinous and slimy extra cellular material formed by bacteria, which remains adhered to and covers the cell as a layer. This is called a **capsule** when it is thick and regular, round or oval in shape and **slime layer** when it is irregular and loosely bound to bacterium. Ability to form capsule is inherent but the thickness depends on cultural conditions. Majority of the capsules are water soluble, uncharged polysaccharides hence do not imbibe simple stain. Some capsules are protein in nature as in *Bacillus anthracis*.

Capsular organisms usually make the broth viscous and stringy and the colonies produced on solid media are generally moist, glistening, mucoid and sticky. These are antiphagocytic in nature and play an important role in the virulence. Some capsule producing organisms are: *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Haemophillus influenzae* etc. Capsule bearing strains produce smooth colonies. Rough strains of *Streptococcus pneumoniae* (lack capsule) are avirulent. Capsule producing organisms are also troublesome for sugar and paper industries resulting in clogging of pipes and pores in paper. These are also useful as blood extender and in molecular sieve chromatography.

Requirements

a. Bacterial culture: *Klebsiella pneumoniae*, *Alcaligenes viscolactis*, *Staphylococcus aureus*
b. Capsule stain:

Anthony's method/ Hiss method

a. Anthony's crystal violet (0.2% aqueous solution)
b. 20% $CuSO_4$
c. Inactivated serum or skimmed milk.

Maneval's method

a. Congo red (1% aqueous solution)
b. Maneval's stain

Howie and Kirk Patrick method

a. Eosin stain (Eosin 10% aqueous-20 ml)
b. Inactivated serum-5ml
c. Zeihl-Neelsen Carbol fuchsin (1:5 diluted)

Procedure

Anthony's method

1. Prepare thin smear of culture with a loopful of skimmed milk on clean glass slide. Air-dry the smear. Do not heat fix.
2. Cover the smear with 1% crystal violet for 1min.
3. Drain off crystal violet by pouring 20%$CuSO_4$ on tilted slide. Let copper sulphate remain for 30 sec. Drain off copper sulphate and air-dry the slide.
4. Examine the slides under microscope. Capsules appear light blue and cell dark blue or purple against faint blue background.

Hiss Method

1. Mix a loopful of culture with a drop of serum on a glass slide and spread it into a thin smear. Allow the smear to air dry and gently heat fix.

2. Cover the smear with 1% crystal violet and steam the preparation for 1min and rinse with 20% CuSO4.
3. Air dry and observe under microscope. Capsules appear faint blue halos around dark blue to purple cells.

Maneval's method

1. Prepare a thick smear in a loopful of congo red stain. Spread it evenly with another slide. Let the smear air-dry.
2. Fix the smear with acid alcohol for 15 seconds.
3. Wash with distilled water and cover the smear with acid fuchsin for 1min.
4. Wash with water, blot dry, and examine under oil immersion objective.
5. The bacteria will stain red and capsules will be colorless against a dark blue background.

Howie and Kirk Patrick method

1. Mix a loopful of culture with one drop of Ziehl Neelsen carbol fuchsin (1:5 diluted) and let it react for 30 seconds.
2. Now, add eosin solution, mix and leave it for 1min. and then spread the mixture with another glass slide.
3. Let the smear air-dry. Examine under oil immersion objective and record your observations.
4. Capsules appear colorless as halo around red cells in a red background.

Questions

1. How does the capsule contribute to organism's virulence?
2. What is the nature of capsule?
3. Name any five diseases along with the etiological agent caused by capsular organisms.
4. How do the capsule bearing organisms appear on solid media?
5. What will happen to milk or sugar solution if it is contaminated with capsulated bacteria?

Exercise 16 : Demonstration of bacterial cell wall

Bacterial cell wall encasing the bacterial cytoplasm is rigid in nature with little plasticity. Besides protecting the bacterial internal structure, it assigns cell shape, size and integrity to bacteria. Even rod shaped bacteria deprived of cell wall often assume spherical shapes in isotonic solutions. Bacteria devoid of complete cell wall are called **protoplasts** and the bacteria with incomplete cell wall are known as **spheroplasts**. Though the bacterial cell wall structure varies from one cell to another but in general the basic structure is made up of **peptidoglycan.** Cell wall is thinner in Gram-negative bacteria as compared to Gram-positive bacteria. It is not visible in bacteria stained with simple stain as cell wall is very thin and is not within the resolving power of ordinary microscope. Therefore, cell wall demonstration technique makes use of mordant like tannic acid that makes the cell wall thicker thus making it visible after staining under microscope.

Requirements

a. Bacterial cultures: *Bacillus megaterium*, *Staphylococcus aureus* and *Proteus vulgaris*

Rainbow method

(i) Bouin's fixative (ii) 0.2% crystal violet in ethanol (iii) 1% congo red.

Ringer's method

(i) Bouin's fixative (ii) 10% tannic acid
(iii) 0.5% crystal violet in ethanol (iv) 0.5% congo red.

Cetylpyridinium chloride method

(i) 0.34% cetylpyridinium chloride (ii) Saturated congo red solution
(iii) Loeffler methylene blue.

Procedure

Rainbow method

1. Prepare the smear and air dry. Cover it with Bouin's fixative for 30 min.
2. Drain off the fixative by tilting the slide and add tannic acid and let remain for 30 min.
3. Wash gently with water and stain with crystal violet for 5-10 seconds.
4. Wash with water, blot dry, and examine under oil immersion objective. Record the observations.
5. Cell wall appears as violet colored around light blue colored cytoplasm.

Ringer's method

1. Prepare the smear on clean grease free slide and air dry.
2. Cover the smear with Bouin's fixative for 30 min. for fixation of smear.
3. Pour off the fixative and cover it with tannic acid for another 30min.
4. Drain off the tannic acid and stain with crystal violet for 1-2 min.
5. Wash of the stain and treat the smear for 2-3 min with congo red.
6. Decant off congo red, blot dry the smear and wash with distilled water.
7. Air-dry and examine under oil immersion objective.
8. Cell is stained violet in contrast to pinkish cytoplasm.

Cetylpyridinium chloride method

1. Prepare the smear on clean grease free slide and air dry.

2. Add three drops of cetylpyridinium chloride and one drop of congo red to the smear and mix the drops well with inoculating needle taking care not to scratch the smear. Let it stain for 5 min.
3. Rinse the smear with tap water and blot dry or air dry.
4. Stain the smear with methylene blue for 10 sec. Rinse off the dye with water.
5. Air dry and examine under oil immersion objective and record the observations.

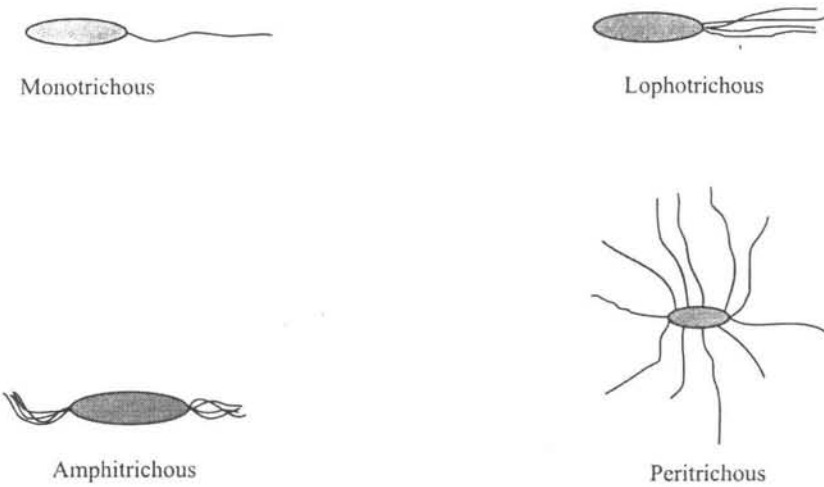
Questions

1. What is the color of cell cytoplasm?
2. What is the function of tannic acid in cell wall staining?
3. List the differences in cell wall of Gram positive and Gram-negative bacteria.
4. Why the cell wall is not stained with ordinary staining?
5. What are the differences in bacterial, fungal and plant cell walls?

Exercise 17 : Demonstration of flagella in bacteria

Flagella are fine thread like appendages arising from cytoplasm of motile bacteria. Most motile bacteria possess flagella but other forms of motility are also seen in bacteria. *Myxobacteria* exhibit gliding motion and spirochetes exhibit screw like motion using axial filament. Flagella are protein in nature and project out from cell wall. They are very fragile and break on mere shaking, heating and on treating with acid or detergent.

Flagella are not visible with light microscope being very thin much below the resolving power of bright field microscope. Hence, special staining methods are employed to increase the thickness of the flagella by depositing coats of mordant that increases their diameter. Presence and location of flagella is also helpful in the identification and classification of bacteria. Based on flagellation the bacteria have been grouped as : **Peritrichous:** flagella all around the surface; **Amphitrichous:** two or more flagella on both the ends; **Lophotrichous:** a tuft of flagella at one end and **Monotrichous:** single flagellum at one end.



Flagellar arrangement in bacteria

Requirements

a. Bacterial culture: *Proteus vulgaris* (8-16 h old), *Pseudomonas aeruginosa* (8 h old), and *Escherichia coli* (8-16 h old).
d. Stain: Ziehl-Neelsen carbol fuchsin
e. Mordant (tannic acid 10% in 5% NaCl solution)

Procedure

1. Take a clean grease free slide and pass it through Bunsen burner blue flame.
2. Add 2-3 ml sterile saline to the slant and keep it for an hour. Now with sterile pipette or sterile loop transfer a drop of culture on one end of the slide, tilt the slide, and allow the drop to trickle down slowly.
3. Air-dry the film. Do not heat fix.
4. Cover the smear with mordant for 10-30 min. Then rinse it gently with water.
5. Now, add stain over the smear and let it remain for 5-15 min. Rinse off the stain with water, air dry and observe under microscope using oil immersion objective. Record your observations.

Questions

1. What is need of a mordant in case of flagella staining procedure?
2. What are the different types of flagellation patterns encountered in bacteria?
3. What is the nature of flagella?
4. What is the difference between flagella and fimbriae?
5. Name and explain other methods for checking bacterial motility.
6. Where do flagella originate in bacterial cell?

Exercise 18 : Demonstration of metachromatic granules in bacteria

Some organisms contain intensely stained bodies exhibiting different chromatic behavior. These bodies are named metachromatic granules. These are the storehouses of energy in the form of ribitol phosphates. The mere presence of these granules in throat smear indicates *Corynebacterium diphtheriae* infection, an etiological agent of diphtheria. Non-pathogenic diphtheroid strains are devoid of these granules. *Corynebacterium diphtheriae* young culture grown on Loeffler' serum slope are rich in volutin granules.

Requirements

a. Bacterial cultures: *Corynebacterium diphtheriae, Bacillus subtilis, and Staphylococcus* aureus

Albert method

(i) Albert stain and
(ii) Albert's iodine

Modified Neisser method

(i) Neisser methylene blue
(ii) Iodine solution: for modified Neisser method - Mix 20 g iodine in100 ml 1 N NaOH and make the volume to 1litre with distilled water.
(iii) Neutral red solution: for modified Neisser method - Mix 1 g neutral red and 2 ml 1% glacial acetic acid in 1000 ml distilled water.

Loeffler method

Loeffler's methylene blue stain

Procedure

Albert method

1. Prepare bacterial smear on clean grease free slide, air dry and heat fix it.
2. Stain it with Albert stain for 5 min.
3. Wash the stain with running tap water. Add Albert's iodine for 1min.
4. Wash with water, blot dry, and examine under oil immersion objective. Record the observations.
5. Volutin granules are stained dark green to bluish in contrast to light green cell cytoplasm.

Modified Neisser method

1. Prepare bacterial smear on clean grease free slide, air dry and heat fix it.
2. Stain it with Neisser methylene blue stain for 3 min.
3. Wash off the stain with iodine solution and let it remain on slide for 1 min.
4. Wash with distilled water and counter stain with neutral red solution for 3 min.
5. Wash with distilled water. Blot dry and observe under oil immersion objective.
6. Organisms are stained pink and the granules blue in color.

Loeffler method

1. Prepare bacterial smear on clean grease free slide, air dry and heat fix it.
2. Stain it with Loeffler's methylene blue stain for 5 min.
3. Wash off the stain with distilled water. Blot dry.
4. Observe under oil immersion objective. Granules appear dark blue in light blue stained cells.

Questions

1. What is the composition and function of volutin granules?
2. Why are these called metachromatic granules?
3. What is the significance of volutin granules?
4. Do the bacteria possess any other storage granule other than metachromatic granules? If yes, name the granules.

Exercise 19: Demonstration of fat storage globules in bacteria

Some organisms if grown in nutrient media rich in fat content, store fat in the form of fat globules or lipid granules as reserve material for use in adversity or during starvation. These granules comprise of polyhydroxy butyric acid (PHBA). On staining with **lipophilic dyes** such as Sudan black, these granules appear as dark black bodies in cytoplasm, which takes the color of counter stain. However, if these cells are treated with alcohol or any other organic solvent before staining, these granules disappear. Organisms are grown in glycerol medium (nutrient agar supplemented with 5% glycerol) for making organisms rich in fat granules.

Requirements

a. Bacterial cultures: *Bacillus megaterium* (24-48 h old slants) grown in nutrient agar and nutrient agar supplemented with 5% glycerol
b. *Saccharomyces cerevisiae* on yeast extract potato dextrose agar (YPDA).
c. Stains:
   Sudan black B: 0.3% in 70% ethanol. Store the solution in stopper bottle.
   Safranine: 0.5% in distilled water.
   Ziehl Nelson carbol fuchsin (ZNCF)
   Xylene

Procedure

Wet preparation

1. Put a drop of Sudan black B on a clean glass slide.
2. Transfer a loopful culture to this drop and mix well.
3. Place a cover slip over the mixture and observe under oil immersion objective.
4. Fat granules appear as blue-black bodies.

Fixed preparations

1. Prepare bacterial smear on clean grease free slide, air dry and heat fix it.
2. Cover the smear with Sudan black B and stain for 10 min. do not let the stain dry on the slide.
3. Drain off the stain. Blot dry. Tilt the slide, pour xylene drop wise on top and let it trickle until no more color elutes out.
4. Blot dry and counter stain with safranine or1: 5 diluted ZNCF for 30 seconds. Do not over stain.
5. Rinse with distilled water, blot dry and examine under oil immersion objective.
6. Fat granules appear blue-black in red colored cells.

Questions

1. What is the function of fat granules?
2. What is the nature of fat granules?
3. Where the fat globules are synthesized?
4. Differentiate fat granules from metachromatic granules.

Exercise 20: Nucleic acid staining in bacteria

In a typical eucaryotic cell, the nucleus is encased in a thin nuclear membrane and positioned centrally inside the cytoplasm. In contrast, the prokaryotes, including bacteria, lack a well-demarcated nucleus. Hence the term nuclear material is used instead of nucleus. The nuclear material being rich in chromatin has great affinity for coal tar dyes that stain it intensely. It is the hub of all inheritable properties and phenotypic activities of bacterial cell. Nuclear stains color the whole bacterial cell cytoplasm suggesting it being distributed in the whole cytoplasm though at specific points it may be more localized. In most stained preparations, the basophilic nature of bacterial cytoplasm masks the chromatin material staining.

Requirements

Bacterial cultures: B*acillus subtilis*, *Staphylococcus aureus* and *E.coli*.

Feulgen's method

a. Bouin's fixative
b. 1N HCl
c. Schiff's fuchsin sulphate (Schiff's base)

Giemsa's method

a. Bouin's fixative
b. 1N HCl
c. Giemsa stain

Procedure

Feulgen's method

1. Prepare bacterial smear on clean grease free slide and air dry.
2. Cover the smear with Bouins fixative for 30 min.
3. Keep the slide on a beaker containing boiling water and add a few drops of 1N HCl. Let it remain for 10 min.
4. Wash it with Schiff's reagent for 20 min.
5. Wash with water.
6. Immerse the slides in sodium bisulphate solution for 10 min.
7. Wash with water. Air dry, examine under oil immersion objective, and record the observation with illustration.
8. Nuclear material appears pinkish in a colorless cytoplasm.

Giemsa's method

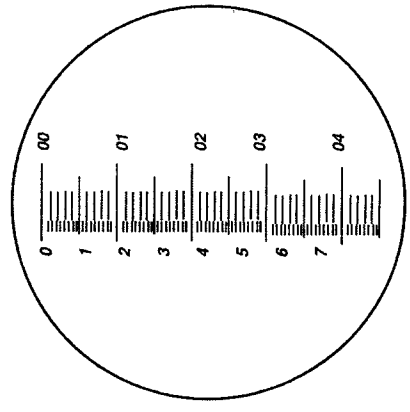
1. Prepare bacterial smear on clean glass slide and air dry.
2. Cover the smear with Bouins fixative for 30 min.
3. Keep the slide on a beaker containing boiling water and add a few drops of 1N HCl. Let it remain for 10 min
4. Rinse with water and stain with Giemsa's stain for 1-2 min.
5. Rinse with water and air dry. Examine under oil immersion objective and record the observation with illustration.
6. Nuclear material appears pinkish in a colorless cytoplasm.

Questions

1. What is the chemical nature of nuclear material?
2. What is the function of nucleic acid in bacterial cell?
3. What is the location of nuclear material in bacterial cell?

Exercise 21: Determination of size of bacteria

The size of microscopic objects including bacteria is expressed in **microns** (10^{-6}m) or nanometers (10^{-9}m). Such measurements are done with the ocular micrometer and a stage micrometer (for calibrating ocular micrometer). Ocular micrometer is placed in the ocular region of the eyepiece. The ruled divisions superimposing specific distance on stage micrometer are counted. By determining the number of divisions of the ocular micrometer that superimpose a known distance marked on the stage micrometer, one is able to calculate precisely the distance of each division on ocular micrometer. After calibration, the ocular micrometer can be used for determining the size of various microscopic objects. The size of bacteria is normally determined in viable stained state (intra-vital staining).



Calibration of ocular micrometer

Requirements

a. Bacterial culture: *S.aureus*, *Bacillus subtilis*, *E.coli*, *Salmonella typhi*, *Klebsiella pneumoniae*
b. Ocular micrometer and stage micrometer
c. Intra-vital stain (crystal violet 1: 120000).

Procedure

1. Remove the ocular lens and insert the ocular micrometer in ocular tube and replace the ocular lens and mount the eyepiece into the optical tube.
2. Mount the stage micrometer on the microscope stage.
3. Center the scale of the stage micrometer (with low power objective in position) while observing through eyepiece.
4. Bring oil immersion objective into position for observation.
5. Rotate the ocular micrometer containing eye piece so that the lines on it superimpose upon the stage micrometer divisions. Now make the lines of two micrometers coincide at one end.
6. Count the number of ocular micrometer divisions coinciding with stage micrometer exactly. Each stage micrometer corresponds to 10 microns.
7. Replace the stage micrometer with bacterial smears and count the number of divisions in the ocular scale that cover the bacterium.

8. Focus under oil immersion objective and record the observations for calculating the size of bacteria. Measure the size of 5-6 different cells to find the average size of bacterial cell.

Questions

1. What is the average size of *S.aureus* and *E.coli*?
2. Why do we use highly diluted crystal violet?

Exercise 22: Differentiation between live and dead microorganisms

Distinction between live and dead cells is possible because of differential staining behavior of these cells. The technique exploits the changes occurring in dead cells that stain differently. This technique is used for finding morphological index (MI) to find the ratio of live and dead *Mycobacterium leprae* in acid-fast stained smear. Trypan blue **dye exclusion** phenomenon is used for finding the percentage of live cells in eucaryotic cell suspensions.

Requirements

a. *Bacillus megaterium* old culture.
b. Loeffler's methylene blue solution
c. Dilute carbol fuchsin solution

Procedure

1. Make a thin smear of culture on the clean glass slide.
2. Heat fix the smear and treat the smear for 10 min. with methylene blue stain.
3. Wash the slide under running tap water and stain the smear with carbol fuchsin for a short while (5 sec) and wash immediately with water.
4. Blot dry and examine under oil immersion objective. Live vegetative cells appear purple and dead are stained red or pink. Live spores take up faint pink color and dead take up blue color.

Questions

1. What is the concentration of methylene blue or carbol fuchsin?
2. What is intravital stain?
3. What is morphological index (MI)?
4. Explain the use of MI in study of drug response against *M.leprae*.

Unit two

Microbial physiology : growth and metabolism

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Exercise 23: Preparation of nutrient media

Nutrient medium is a cocktail of chemicals and substrates that fulfills the growth requirements of organism being cultured. Culture media are divided into two broad groups: **solid** and **liquid** (broth) media. Solid media have a solidifying agent usually an agar and are referred to as **slopes**, **slants** or **plates**. They are used for many purposes e.g. isolation, identification, characterization and study of physiological characteristics.

Microorganisms differ widely in their nutritional requirements. Based on their requirements for growth, microorganisms have been categorized in two major groups: **fastidious** and **non-fastidious**. The former do not grow on ordinary media and require additional growth factors in the medium and the latter can grow well on ordinary medium such as nutrient broth and nutrient agar.

In general, the nutrient medium provides carbon, nitrogen, minerals and other growth factors for the growth of microorganisms. Substances like sugars, proteins, fatty acids, lipid, serum, blood, detergents, antibiotics etc. are supplemented to basal medium to meet the exacting growth requirements of a particular group of organisms and discouraging the growth of unwarranted organisms. Adding agar at 1.5-2.0% level can solidify liquid medium. **Agar** is derived from seaweeds. Agar melts at 95-98°C and solidifies around 45°C. The nutrient medium is freed of all kind of viable organisms (sterilized) prior to its usage. The medium preparation is accomplished in three steps: (a) weighing and dissolving the ingredients, (b) dispensing in suitable container, plugging and (c) sterilization.

Chemically the medium can be grouped into two main categories: **synthetic or chemically defined medium** and **the complex medium.** Chemically defined medium is the nutrient medium in which the kind and concentration of each constituent is known e.g. minimal medium and the biological assay medium. These media are useful in microbiological assays of microbial growth factors, assay of antibiotics, vitamins, amino acids and other products of microbial origin. In complex medium the medium components and their chemical composition is grossly known e.g. nutrient broth contains beef extract and peptones which provide vitamins, minerals and amino acids.

Based on their functional usages the media are classified as:

1. **Enriched medium:** Such medium supports the growth of vast majority of organisms being rich in nutrients. Nutrient media are enriched by adding blood, hemolysed blood, serum, and ascitic fluid as additional supplement to the basal medium such as nutrient agar. Examples include blood agar, hemolysed blood agar, chocolate blood agar, Loeffler's serum slope etc.
2. **Enrichment medium:** The medium composition is altered by adding chemicals to favor the survival or growth of a particular group of organisms and inhibiting the growth of others. Enrichment media are useful in selectively isolation of pathogens or isolation of organisms with specifically defined characteristic which are present in small numbers along with large population of resident flora Examples: alkaline peptone water (pH 8.5-9.0) is used for enrichment of *Vibrio*, Selenite F medium and Tetra Thionate Broth (TTB) for enrichment of *Salmonella* and *Shigella* in stool samples wherein their numbers is highly diluted as compared to *E.coli*.
3. **Differential medium:** This is a solid medium. Organisms inoculated on differential medium produce different types of colonies. Some bacteria cultured on blood agar lyse

the red blood cells and produce a hemolytic zone around the colonies while the others never do so as they do not produce enzyme needed to lyse red blood cells and hence always produce non-hemolytic colonies. Similarly, bacteria growing on MacConkey's agar are referred to as lactose fermenters (LF) that utilize lactose and produce red colonies and the non-lactose fermenters (NLF) give rise to colorless or light brownish colonies.

4. **Selective medium:** Media that encourages the growth of a particular kind of organism and retard the growth of other organisms are called selective media. It contains besides carbon source the chemicals that prevent the growth of unwanted bacteria with no effect on desired organism e.g. crystal violet blood agar is inhibitory for *Staphylococcus* sp but has no effect on the growth of *Streptococcus pyogenes.* Blood potassium tellurite agar (BPTA) is used for isolation of *Corynebacterium diphtheriae* and salt mannitol agar (SMA) and Baird-Parker medium selectively favor the growth of *Staphylococcus aureus.* Brilliant green agar (BGA) and Bismuth sulfite agar (BSA) are suitable for the isolation of typhoid bacilli from feces.

5. **Selective/Differential medium:** Some culture media are both selective and differential. These are particularly helpful in differentiation of enteropathogens. Medium like MacConkey's agar contains bile salt and crystal violet to inhibit the Gram positives and lactose to differentiate Gram negatives into LF and NLF.

6. **Biochemical medium:** Bacteria derive energy for growth and other metabolites utilizing variety of carbon and nitrogenous sources through oxidation and fermentation. Studies of such activities are possible with biochemical media only. Carbon, nitrogen sources are supplemented as exclusive nutrient sources to basal medium (like peptone water)) e.g. sugars at 0.5-1% levels are added to peptone water along with an indicator which imparts different color to the medium at acidic and alkaline pH. A combination of four tests called, IMViC test (Indole, Methyl Red, Voges Praskuer, Citrate utilization) is very important for grouping of organisms belonging to family *Enterobacteriaceae.*

7. **Assay medium:** Such types of media are used to study either stimulation or inhibition of growth in response to substance (vitamins/ antibiotics) present in sample. The degree of inhibition/stimulation is proportional to the amount of drug, antibiotic or vitamins etc. present in the growth medium. Microbiological assays of antibiotics are generally recommended for assaying pharmaceutical products, animal feed and other materials.

Requirements

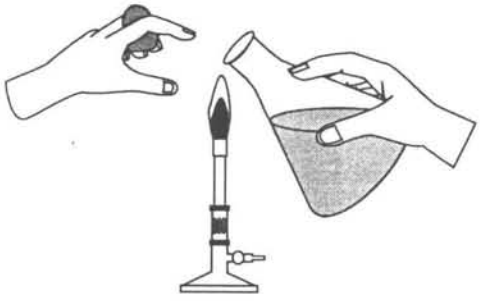
a. Beef extract
b. Peptone
c. Agar
d. Sodium chloride, flasks, cotton, measuring cylinder, beaker, test tubes and magnetic stirrer.

Procedure

Nutrient broth

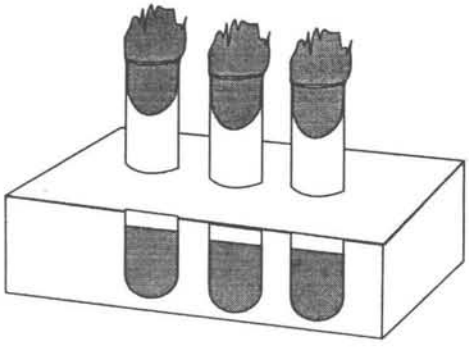
1. Weigh beef extract-3 g, peptone-5 g and sodium chloride-5 g. Dissolve these in distilled water on a magnetic stirrer and make the volume to 1litre.

2. Adjust its pH 7.2 with 1N NaOH using pH meter and dispense the medium (150 ml /250 ml Erlenmeyer flask).
3. Dispense 5 ml per ten ml tube in ten tubes.
4. Plug the flasks and tubes with cotton plug as instructed. Autoclave the dispensed medium at 121°C (15lbs/sq. inch) for 20 min.

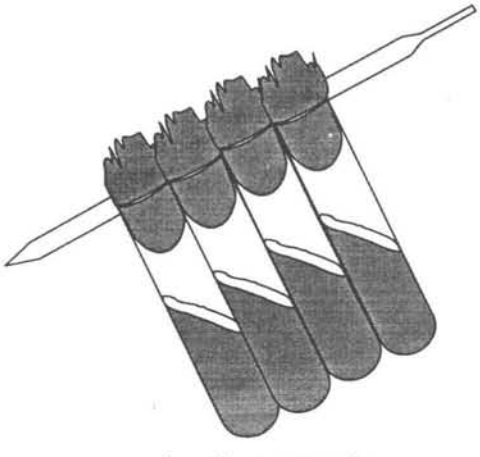


Flame the neck of flask

Pour the medium in petri plate



Preparation of stabs



Agar slants perparation

Preparation of agar plate, stabs and slants

Nutrient agar

1. Take two flasks containing nutrient broth (150 ml/250 ml flask).
2. Add 3 g agar powder to each flask. Shake the contents thoroughly.
3. Keep one flask on hot plate and let the agar melt. Now from this flask dispense about 5 ml each to screw capped tubes for making stabs and slants.

4. Plug the flasks with cotton plug.
5. Sterilize the flasks and tubes at 121°C (15 lbs/sq. inch) for 20 min in an autoclave.

Slants, stabs and nutrient agar plates

1. At the end of autoclaving, take out the flasks and tubes and l cool to 55-65°C.
2. Arrange sterile petri plates on the bench. Hold the nutrient agar flask with left hand; unplug the flask by holding the cotton plug in-between fingers of reversed right hand near the flame.
3. Transfer the flask to right hand and flame the mouth of flask. Open the petri plate with left hand and pour about 20 ml melted nutrient agar to each plate and immediately replace the lid. Let the agar solidify. These **nutrient agar plates** should be used after surface drying.
4. Arrange five tubes containing autoclaved nutrient agar in a test tube rack. Let the agar solidify. These **agar stabs** are useful for storage of cultures.
5. Take the remaining tubes containing nutrient agar and keep these in slanting position by resting these against the glass rod or pipette. Leave the tubes in this position until agar solidifies. **Agar slants** are also used for culture maintenance.
6. Always flame the mouths of container after removal and prior replacement of cap or cotton plug.

Questions

1. Why is it necessary to flame the container mouth prior to and after inoculation?
2. Enlist the user organism (s) against each of the following enriched selective growth medium:

   Neomycin blood agar ----------
   Wilson and Blair medium ----------
   Alkaline peptone water ----------
   Loeffler's serum slope ----------
3. What is indicator medium?
4. Name any three seaweeds as source of agar.
5. Why is the petri plate inverted for incubation?
6. Which is the correct way to stack petri plates on bench?

Exercise 24: Aseptic transfer of culture

In the laboratory, it is necessary to culture bacteria for characterization and study of its metabolic activities. Therefore, transfer of microorganisms is made from one growth medium to another for its propagation and maintenance. These transfers or inoculations must always be carried out avoiding the entry of unwanted microbes. This is called aseptic transfer technique.

Prior to making any transfer, growth medium is ascertained to be free from all kinds of living microbes. Sterility is accomplished using suitable method of sterilization depending upon the nature of medium. Preferably, one should use pre-incubated media and these should never be opened prior to use. Sterilized media are often stored in cold room or refrigerator at 4 ℃.

Broth cultures in tubes, agar slants and stabs are easy to carry. Agar stabs are used for maintenance of cultures for routine use. Semisolid agar containing 0.3-0.5% agar instead of 1.5-2% agar in tubes is used for determining motility in bacteria. It can also be used to study oxidation and fermentation of sugars if it contains utilizable sugar and a suitable indicator. In microbiological laboratory, cultures are usually transferred using inoculating loop/needle (straight wire). Inoculating loop is used for surface inoculation. Straight wire is used for stab culture alone or stab and surface inoculation.

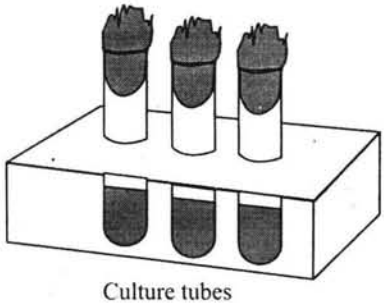
Requirements

a. Nutrient broth tubes
b. Nutrient agar slants
c. Hugh-Leifson medium tubes
d. Triple Sugar Iron tube (TSI)
e. Inoculating loop, inoculating needle and
f. Gram stain set

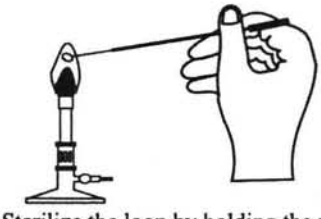
Procedure

1. Hold the inoculating needle in right hand and the **broth culture** in left hand.
2. Sterilize the inoculating loop and take off the cotton plug or cap, holding it with little finger twist the cap to unscrew it or loosen the cotton plug. Gently pull off the plug or cap while it is grasped with little finger.
3. Hold the broth culture tube at angle and flame the mouth of tube.
4. Introduce the sterilized loop into the tube, dip the loop into culture, obtain a loopful culture, and with draw the loop from tube. Holding the loop still in hand, flame the mouth of tube and replace the cap or cotton plug by turning the tube into the cap. Place the tube in the test tube rack.
5. Remove the cap and flame the mouth of **nutrient broth** tube to be inoculated following aseptic conditions as described while withdrawing inoculum from broth culture. Dip the inoculating loop into sterile both and then withdraw it from the tube. Flame the mouth of tube and replace the cap or cotton plug. Transfer the inoculum and reflame the loop to red hot and cool prior to making next transfer or placing loop in stand.
6. Same procedure is followed for transfer of culture to **nutrient agar slant** except that loop holding the culture is rubbed or moved gently across the agar surface from bottom of slant to top, taking care not to injure the agar. Withdraw the inoculating loop, flame the mouth of agar slant, replace the cap and finally sterilize the loop again prior to making another transfer or placing it on the rack.

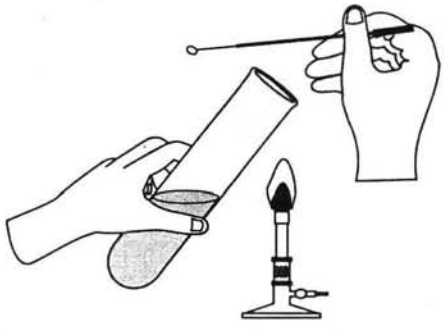
7. Inoculate **nutrient agar stab, semisolid agar medium** or **TSI tube** with inoculating needle deep by inserting needle straight down in the middle of tube and then pull out through the same path and inoculate the slant of TSI by moving the needle gently across the surface of agar following aseptic conditions as described above.
8. Incubate the inoculated media at 37°C for 24 h. Record the appearance of each culture and consult the teacher for interpretation of the patterns of growth in each medium.



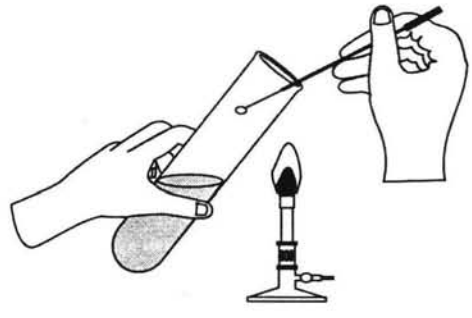
Culture tubes



Sterilize the loop by holding the wire in the flame until it is red hot

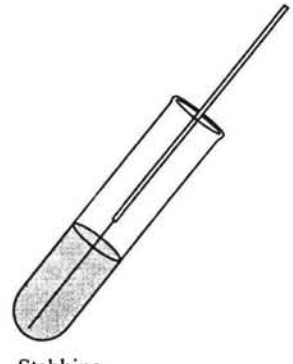


Briefly heat the mouth of the tube in the flame before inserting the loop for an inoculum



Get a loopful of culture, heat the mouth of the tube, and replace the cotton plug

Inoculating slant



Stabbing

Inoculating procedure

Precautions

1. Always, flame the mouth of container soon after it is opened and prior to replacing the cotton plug or cap.
2. Always, sterilize the inoculating loop or needle before and after each transfer.
3. The nutrient media stored in cold must be equilibrated to room temperature and agar plates must be surface dried prior to use.
4. While transferring organisms, first inoculate the growth media and in the last transfer to microscopic slide if handling clinical samples.
5. Handle one sample at a time to avoid any mix up or cross-contamination.

Questions

1. What is the primary use of slants and stabs?
2. Why is aseptic transfer so important?
3. Can you demonstrate motility in bacteria by any other method?
4. How do you determine the organism's motility in semisolid agar?
5. Why the agar plate with medium splashed between top and bottom lids should not be used?
6. Why is the mouth of flask or test tube flamed while making transfer of cultures?

Exercise 25: Isolation of pure culture of bacteria

Small size, similarity in morphological characteristics and staining reactions of most microbes make it difficult to identify organisms exclusively based on by microscopic observations. One of the methods is to culture the microorganisms on artificial medium and observe the growth pattern and colonial characteristics. Cultural methods not only help in identification but also are useful in isolation and determination of kind and load of microorganisms present in foods and clinical specimens.

Practically one may find hundreds of colonies of organisms growing on culture media on plating of sample showing few or no organism in smear staining. Isolation of organisms in pure culture has been a stumbling block in the development of microbiology, which was resolved by Robert Koch. Culturing of organisms encompasses the knowledge of sterilization and nutritional requirements of the organisms to be isolated, techniques for inoculation and transfer under aseptic conditions and incubation. Pure culture represents the progeny of a single species only.

Pure cultures can be obtained using dilution methods: **pour plate, spread plate or streak plate** methods. In **streak plate** method, mixed sample is streaked many times with inoculating loop over the surface of solid culture medium. Spread plate and pour plate are quantitative that determines even the number of bacteria in sample. In spread plate, a known amount of diluted sample is spread over the surface of nutrient medium with the help of a spreader while in pour plate, diluted sample is mixed under aseptic conditions with melted nutrient medium in sterile petri plates. At the end of incubation bacterial growth is visible as surface colonies (in case of spread plate technique) and surface/embedded colonies (in case of pour plate technique).

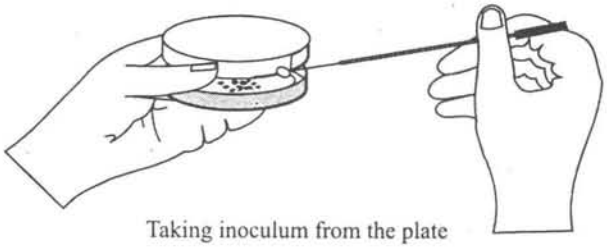
Requirements

Nutrient agar plate or any other growth medium plate, nutrient broth, sterile petri plates, sterile pipettes, dilution blanks, spreader, vortex mixer, burner and bacterial culture or the specimen.

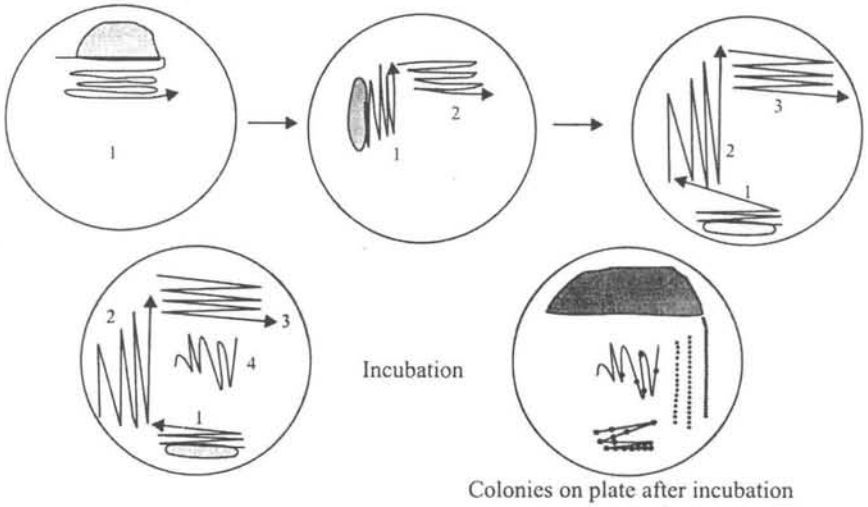
Procedure

Streak plate method

1. Flame the inoculating loop to red-hot, allow it to cool near burner in air.
2. Hold the culture in left hand near the flame. Remove the cotton plug or unscrew the tube with right hand and flame the mouth of tube for few seconds. Aseptically withdraw a loopful culture with needle.
3. Place the inoculum on the agar plate at least 1 cm away from sides. Spread it in two to three square cm areas.
4. With sterile cool needle streak or spread the culture from one corner to another and rotating the plate by 90° after streaking 4-5 times in one direction without overlapping previous streak as demonstrated by the instructor or shown in picture as below:



Taking inoculum from the plate



Colonies on plate after incubation

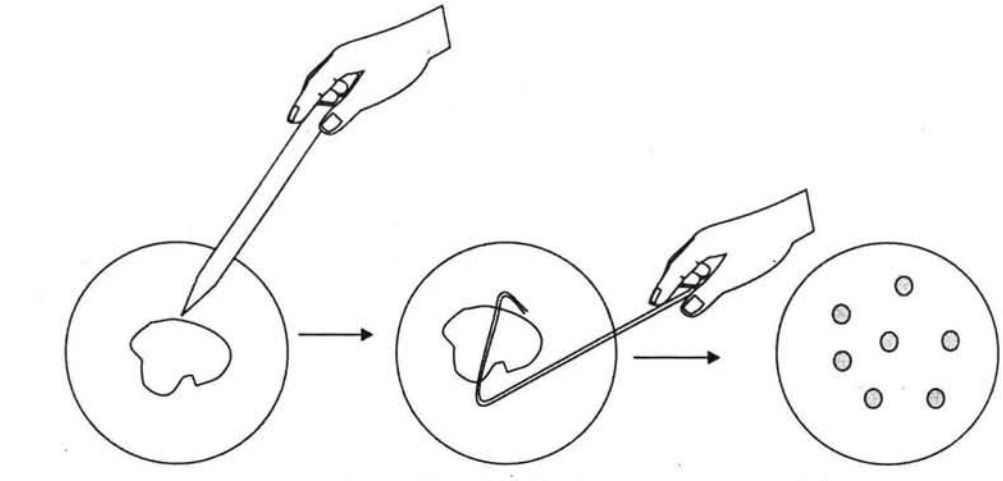
Streak plate technique

Pour plate method

1. Melt the nutrient agar and cool and place it in water bath set at 50°C.
2. Label the sterile petri plates and dilution blanks. Serially dilute the mixed culture in dilution blanks and from each dilution transfer 1 ml to respectively labeled petri plate using separate sterile pipette.
3. Pour 20-25 ml cooled nutrient agar to each plate and mix the sample with agar by rotating the petri plates on the bench. Let the agar solidify.
4. Incubate the plates in inverted position in the incubator at 37°C.

Spread plate method

1. Transfer 0.1 ml of the diluted culture as above to labeled surface dried nutrient agar plates.
2. Spread the culture on agar surface with spreader.
3. Invert the plates and keep in incubator for 16-18hr.
4. Next day observe the plates and study the colonial characteristics of isolated colonies.



Spread plate technique

Questions

1. What is colony-forming unit (CFU)?
2. Why is 37°C selected as temperature of incubation?
3. Describe the differences in size and shape of surface and submerged colonies.
4. Why do the colonies appearing on primary, secondary and tertiary streak area differ in number and size?
5. What is the range of colonies you count in pour plating and why?

Exercise 26: Isolation of bacteriophages from sewage

Viruses are ultramicroscopic, obligate intracellular parasites that cannot be seen with light microscope. They possess nucleic acid genome either as DNA or RNA which is encased in protein coat. Viruses are host specific and survive outside host as non-living inert bodies. Viruses that infect bacteria are called bacteriophages. Viruses lack the energy generating system but can effectively make use of host cell metabolic activities for their growth and replication. Coliphages are present in sewage (10^5-10^7 per litre) wherever the coliforms are present in plenty. Coliphages in sewage can be assayed by mixing sewage and log phase *E.coli* culture in top agar that is overlaid onto nutrient agar and incubated. Bacteria produce a confluent lawn except in the clear areas called plaques where the bacteriophages have killed the bacterial population. Hence presence of coliphages can be potential environmental indicator of sewage contamination, determining efficiency of water and waste treatment processes indicating the survival of enteric viruses and bacteria in the environment.

Requirements

a. Sewage sample
b. *E.coli* broth culture 3-5 h old
c. Soft agar (top agar- nutrient agar with 0.7% agar)
d. Nutrient agar plates
e. Sterile 1ml pipettes,
f. 9.0 ml buffered saline blanks
g. Water bath set at 50°C and incubator at 37°C.

Procedure

1. Dilute the sewage sample 1:10 and 1:100 in buffered saline blank.
2. Cool under running tap water four tubes containing sterile soft agar (3 ml/tube) to 50°C. Label them as 1,2,3 and 4.
3. Aseptically add 1 ml each of undiluted sewage and *E.coli* young culture to tube 1 and mix the tube contents thoroughly. Pour it immediately onto surface of dried nutrient agar and let it spread uniformly over the entire surface by rotating the petri plate.
4. Repeat the experiment adding 1ml diluted sewage (1:10 and 1:100) to tube 2 and 3 and mixing it with 1ml bacterial culture. Treat control tube 4 similarly but add buffered saline in lieu of sewage sample.
5. Let the agar solidify. Invert the plates and incubate at 37°C for 48 h.
6. At the end of incubation, count the number of plaques in each dilution and calculate the concentration of phage in the sewage sample. Record the size and shape of plaques.

Questions

1. What are the factors that determine the size of plaque?
2. Why did we use young growing culture?
3. Why did the plaques vary in size and shape?
4. Based on morphological characteristics of plaques can you speculate the kind of bacteriophages present in sewage sample?

Exercise 27: Determination of viable counts of bacteria

Bacterial viable counts in culture or bacteriological sample can be determined using different techniques. In contrast to direct microscopic count that gives the total number of bacteria (dead and live) present in sample, viable count determines only the number of bacteria, which can produce colonies. The accuracy of viable counts depends on several factors. Some bacteria that may be present in clumps, chains or pairs may not be separated and may produce single colony while others may not grow on the plating medium. In spite of this fallacy, the method is valuable in bacteriological examination of food, water and even clinical samples. Any of the following techniques can be used for determining viable counts of bacteria:

(a) Drop method or Miles and Misra method
(b) Pour plating
(c) Spread plate technique
(d) MPN method
(e) Roll tube technique

In first three methods the sample is serially diluted to obtain 10^{-1} to 10^{-10} dilutions using sterile blanks. One may use 0.9 ml, 9.9 ml or 99 ml blanks to skip in between dilutions (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}). Plating can be done using bacteriological pipettes (1.1 ml pipettes for transfer of 0.1 ml and 1 ml by pipetting once). Then 0.1ml of each dilution is spread on surface dried nutrient agar plates. In pour plating, 2.2 ml pipette may be used for plating in duplicates. 1.0 ml diluted sample is mixed with 20-25 ml melted and cooled (50^0C) nutrient agar in sterile petri plate. Miles and Misra (1938) method is economical as single drop from each dilution is dropped from fixed height from syringe or pipette on the same plate. Number of drops delivered /ml are calculated. MPN (most probable number) is an alternate method to standard plate count (SPC). The sample dilutions made in nutrient media until the volume transferred contain one viable cell are incubated overnight. Next day tubes are examined for turbidity. Highest dilution tube showing turbidity is the MPN. Instead of petri dishes roll tubes and shake tubes are used for the isolation and estimation of viable population of anaerobes.

Requirements

a. Dilution blanks,
b. Nutrient broth tubes (9 ml/tube),
c. Sterile bacteriological pipettes (1.1 ml and 2.2 ml),
d. Melted and 50°C cooled nutrient agar
e. Sterile petri plates and culture or bacteriological sample.

Procedure

Making dilutions

1. Arrange the 9.0 ml dilution blanks and label as 10^{-1}, 10^{-2}, 10^{-3},10^{-4}, 10^{-5} and 10^{-6}.
2. Transfer 1 ml sample to first dilution blank and mix by vortexing. From this tube, 1 ml is transferred to second tube and mix.
3. From second tube, 1ml is transferred to third and mixed. This sequential transfer and mixing is continued to the last dilution.
4. This sequential transfer gives10^{-1}, 10^{-2}, 10^{-3},10^{-4}, 10^{-5} and 10^{-6} dilutions of the original sample.

Pour plating technique

1. Transfer 1ml of each dilution (as prepared above) to separate petri dishes using separate sterile pipette for each dilution. Mix it with 15-20 ml melted nutrient agar by rotating.
2. Let the agar solidify.
3. Incubate the plates in inverted position at 37°C for overnight. Next day count the colonies on the plates showing colonial count 30-300 only.

Spread plate method (surface viable count)

1. Transfer 0.1ml diluted sample to surface dried nutrient agar plate, using separate pipette for each dilution.
2. Spread the sample on the agar surface with sterile spreader. Always flame and cool the spreader in between spreading next dilution.
3. Incubate the plates under inverted condition at 37°C. Select the plate with 30-300 colonial counts.
4. Calculate the viable count in sample by multiplying the colonial count at a dilution with the dilution factor.

Drop method (Miles and Misra method)

1. Prepare the dilutions as above. Drop 0.02 ml from each dilution from 2.5 cm height onto the medium so that it spreads over an area of 1.5-2.0cm.
2. Each drop is added in separate numbered sectors on the nutrient agar plate.
3. Incubate the plates in inverted position for 24-48 h.
4. Count the largest number of colonies without confluence (20 or more). Mean of triplicate gives the viable count per 0.02 ml of dilution.
5. Calculate the number per ml by multiplying count in 0.02 ml by 50 and respective dilution.

MPN method

1. Arrange nutrient broth tubes in sets (15 tubes/set i.e.5 tubes/ dilution) and label as set 1 (10^0, 10^{-1},10^{-2}), set 2 (10^{-3},10^{-4},10^{-5}) and set 3 (10^{-6},10^{-7},10^{-8}).
2. Dilute the given sample serially 10^{-1},10^{-2},10^{-3},10^{-4},10^{-5} and label them appropriately.
3. Take 1 ml aliquots from each dilution of samples separately and inoculate into the respective tubes containing growth medium.
4. Incubate the tubes at 37°C for 24 hrs.
5. Note the number of tubes showing growth in each set for each dilution.
6. Determine the number of positive tubes in three successive ten fold dilutions (set 1) and refer to the MPN Table.
7. If all the tubes in the range exhibit growth, determine the number of the positive tubes in the successive ten fold dilutions (10^{-3}, 10^{-4}, 10^{-5} set 2) and refer to the corresponding MPN numbers from the table considering 10^{-3}, 10^{-4}, 10^{-5} dilutions relating to 10^0,10^{-1}, 10^{-2} dilutions respectively. Multiply the number of viable cells obtained by 10^3 (dilution factor).
8. If all tubes still show growth, dilute the sample further to 10^{-6}, $10^{-7}$and 10^{-8} and repeat the experiment. Refer the MPN table considering these dilution relating to 10^0, 10^{-1}, 10^{-2} dilutions respectively. For calculation multiply the number of cells obtained by 10^6 as dilution factor.

Roll tube technique

1. Dispense the molten nutrient agar medium in anoxic tubes and autoclave. Allow it to cool to 45-50°C.
2. Transfer the specimen dilutions made in pre-reduced diluents or medium.
3. Gently mix the dilutions with molten medium carefully to avoid any frothing.
4. Roll the tubes horizontally under cold-water tap until the medium solidifies uniformly along the wall of the tube.
5. Incubate the tubes under anaerobic conditions and count the number of colonies appearing after incubation and calculate the viable count per ml as above by multiplying the count with effective dilution.

Questions

1. What do you understand by colony forming units?
2. How would you determine the number of enteric microorganisms in a food sample?
3. What is SPC? Why is a new pipette used for each procedure?
4. Which method did you find easy to perform and why?
5. Design an experiment to determine the number of spore formers in flour.
6. Why do you go from the highest dilution to the lowest dilution?

MPN Table

Dilutions (No. tubes positive)			MPN	Dilutions (No; tubes positive)			MPN
10^0	10^{-1}	10^{-2}		10^0	10^{-1}	10^{-2}	
0	1	0	0.18	5	0	0	2.3
1	0	0	0.20	5	0	1	3.1
1	1	0	0.40	5	1	0	3.3
2	0	0	0.45	5	1	1	4.6
2	0	1	0.68	5	2	0	4.9
2	1	0	0.68	5	2	1	7.0
2	2	0	0.93	5	2	2	9.5
3	0	0	0.78	5	3	0	7.9
3	0	1	1.10	5	3	1	11.0
3	1	0	1.10	5	3	2	14.0
3	2	0	1.40	5	4	0	13.0
4	0	0	1.30	5	4	1	17.0
4	0	1	1.70	5	4	2	22.0
4	1	0	1.70	5	4	3	28.0
4	1	1	2.10	5	5	0	24.0
4	2	0	2.20	5	5	1	35.0
4	2	1	2.60	5	5	2	54.0
4	3	0	2.70	5	5	3	92.0
4	3	0	2.70	5	5	4	160.0

MPN- most probable number per liter

Exercise 28: Study of bacterial growth cycle by determining viable counts

Growth in bacteria may be defined as an orderly increase in all the components of living cell resulting in multiplication of cells. Based on nutritional requirements microbes are classified in two categories: **autotrophs** and **heterotrophs**. Among autotrophs, we have photoautotrophs and chemoautotrophs. Among heterotrophs we have chemoorganotophs and chemolithotrophs depending on the energy sources used by these organisms for their metabolic activities. Organisms are highly versatile as far as energy utilization is concerned. Utilize the easily utilizable substrate first. Two log phases separated by lag phase can be seen with *E.coli* culture grown in medium containing glucose and lactose. Presence of glucose in the medium for which *E.coli* is constitutive represses the adaptive utilization of lactose (called **diauxic growth** or catabolic repression). Typical bacterial growth curve depicts four phases when grown in closed system. The duration of these phases may vary with respect to the availability of nutrients and other environmental factors.

Most organisms grow faster in complex medium that provides amino acids, nucleotide precursors, vitamins and other metabolites that the cell has to synthesize otherwise. Growth in microorganisms is dependent on several physicochemical factors. All these factors can influence the cell yield, metabolic pattern and chemical composition of bacteria. Growth in bacteria may be compared in terms of **generation time, growth rate** and **growth rate constant**. The generation time refers to the time taken by population to double in number. Growth rate refers to the number of generation per hour. It is usually more for organisms growing in enriched media. Growth rate constant refers to the rate of growth during the growth. **Growth factor** is the organic compound that the organisms require from exogenous source for growth and cannot synthesize of their own. Growth in microbes can be determined by determining cell numbers, cell activity and cell mass. Cell numbers: *Total cell counts*: DMC, Direct cell counts using counting chambers, coulter counter an electronic device, spectrophotometrically. *Viable counts:* pour plating (SPC), spread plate technique, roll tube technique and drop method. Cell activity: Utilization of sugars, substrate, enzyme production, respiration rate or any other metabolic activity. Cell biomass: nitrogen content, weight determination, gravimetric method.

Requirements

a. Sterile nutrient broth (50 ml/250 ml flask)
b. 24 hr old culture of *E.coli*
c. Sterile bacteriological pipettes (1.1 ml and 2.2 ml)
d. Dilution blanks (9 ml and 9.9 ml)
e. Nutrient agar: melted and cooled to 50°C
f. Sterile petri plates.

Procedure

1. Inoculate nutrient broth tubes in duplicate with 0.1 ml of the 24 h old culture.
2. Incubate the flask at 37C in the incubator.
3. Aseptically withdraw the samples immediately at 0, 2, 4, 8, 12, 16, 20 and 24 h. Each time transfer 1 ml sample to first dilution and vortex it and then make subsequent suitable dilutions.
4. Transfer 1ml of each dilution (as prepared above) to separate petri dishes using separate sterile pipette for each dilution. Mix it with 15-20 ml melted nutrient agar by rotating.

5. Let the agar solidify. Incubate the plates in inverted position at 37°C for overnight. Next day count the colonies on the plates showing colonial count 30-300 only.
6. Calculate the viable count in sample by multiplying the colonial count at a dilution with the dilution factor.
7. Plot a graph between the viable count and the time of incubation and calculate the generation time of *E.coli*.

Questions

1. Enlist the physicochemical factors that may affect the bacterial growth?
2. Can the same protocol be used for study of growth in anaerobes?
3. Why the colony count is restricted to plates showing colonial count 30-300 only?

Exercise 29: Study of bacterial growth cycle by measuring turbidity and biomass determination

Growth in bacteria may be defined as an orderly increase in all the components of living cell resulting in multiplication of cells. Bacterial growth curve requires the transfer of known inoculum to sterile nutrient media followed by incubation at specified temperature and gaseous condition. The growth is monitored by determining the increase in turbidity because of multiplication of cells during incubation, indicated by increase in absorbance spectrophotometrically versus the control (uninoculated medium) or monitoring the increase in cell number versus time of incubation.

Requirements

a. Sterile nutrient broth (5ml/tube)
b. 24 hr old culture of *E.coli*
c. Sterile 1 ml pipette
d. Colorimeter or spectrophotometer and cuvettes

Procedure

1. Inoculate nutrient broth tubes in duplicate with 0.1 ml of the 24 h old culture. The tubes used should have matched transmittance fitting to cuvette slot for reading transmittance directly. Or Inoculate tubes labeled as 0, 2, 4, 6, 8, 12, 16, 20, 24 h.
2. Retain the uninoculated as blank for adjusting the spectrophotometer or the colorimeter to 0 or 100% transmittance. Incubate all the tubes at 37°C.
3. Arrange to read the tubes at 0, 2, 4, 6, 8, 12, 16, 20, 24 hr intervals.
4. Switch on the spectrophotometer or colorimeter and allow it to warm up 3-5 min. Set the wavelength at 600 nm. Adjust to zero transmittance. Transfer the sterile blank to the cuvette and wipe off the cuvette from outside with tissue paper to remove the droplets and fingerprints.
5. Set the spectrophotometer at 100% transmittance. Thoroughly mix the contents of inoculated tube and transfer it to cuvette and read the absorbance or percent transmittance. Alternatively put the inoculated tube after mixing in cuvette slot and read the absorbance against control at set intervals.
6. Add 2 ml of culture at intervals in pre weighed watch glass or aluminum dish and dry in oven for 16 h and find out the differences in weight from the uninoculated broth dried similarly.
7. Plot the readings in terms of absorbance versus the incubation time. Correlate the transmission and dry weight growth curve

Questions

1. What are some limitations of determining bacterial density using the colorimeter?
2. Compare the direct and indirect methods for determining bacterial growth.

Exercise 30: Demonstration of catabolic repression in bacterial culture

Bacterial cells inoculated in complex medium utilize usable substances or substrates in a sequential manner. Therefore, the presence of specific substrate may lead to repression of the enzyme/s for metabolism of other substrates. The enzyme/s for utilization of other substrate are elaborated only when the concentration of repressing substrates has been reduced significantly. Specific regulation of bacterial physiology thus leads to an aberrant growth cycle that shows one or more intermediates but transient stationary phase. This response to a changing environment is termed as **"diauxic growth".** A classical example of diauxic growth is that of *E.coli* grown in presence of glucose and lactose. Subsequent to rapid growth until the point of glucose exhaustion, a deflection in the biomass curve occurs and there may even be a decline in biomass. After some lag, a new set of enzymes for metabolism of lactose is induced and the cells once again start growing luxuriently.

Requirements

a. *E.coli* culture.
b. Peptone water tube containing glucose and lactose
c. Fehling solution for estimation of glucose
d. Sterile pipettes nutrient agar plates and spreader dilution blanks

Procedure

1. Inoculate a flask of sterile peptone water (50 ml/250 ml flask) containing glucose and lactose with 0.1 ml 24 h old culture of *E.coli.* Make an arrangement to withdraw aliquots at 0, 2, 4, 6, 8, 12, 16 and 24 h.
2. Incubate the flask in the incubator and withdraw samples at the intervals aseptically. Make ten fold serial dilution and spread plate it on nutrient agar plate for determination of bacterial viable counts.
3. Plot the values on a graph between viable count at interval and time of incubation. Also determine the amount of glucose and lactose in the aliquots.
4. Correlate the viable count with the sugars present in the medium and comment on the kind of sugar in the medium and bacterial growth.

Questions

1. What is diauxic growth?
2. What is catabolic repression?
3. Why *E.coli* utilize glucose first?
4. What kind of difference in growth of organisms was there when it was grown in medium containing either of the sugar?

Exercise 31: Effect of oxygen on the growth of bacteria.

Based on the oxygen requirement bacteria can be grouped as **obligate aerobes** that require oxygen for growth and **obligate anaerobes** that fail to grow in presence of oxygen. The absence of catalase in anaerobes results in the accumulation of hydrogen peroxide to lethal levels. Facultative anaerobes or microaerophilic organisms can grow optimally under **microaerophilic** conditions, yet their growth is not deterred by the presence of oxygen. These organisms are devoid of cytochrome system; hence, there is no hydrogen peroxide generation. Unlike most nutrients oxygen is relatively insoluble in water (<10 mg/l) and quickly becomes limiting factor in liquid cultures unless special precautions are taken for its availability during growth.

Some microbes even grow better under 5-10% CO_2 atmosphere (in candle jar). Inoculated tubes and plates are kept in a large jar with a lighted candle. The burning candle consumes the available oxygen and thus raises the carbon dioxide concentration in closed jar. In the laboratory, oxygen concentration can be reduced by adding small amount of agar that reduces the diffusion of air into the medium or using reducing agents such as sodium thioglycolate that directly combines with oxygen. Reducing agents like phenosafranine (0.05%), sodium thioglycolate (0.05%), cysteine hydrochloride (0.025%), sodium sulfide (0.025%), FeS (4 ppm) and dithiothretol (0.02%) are added to most of anaerobic media to create low redox potential. Addition of indicator dye (methylene blue or resazurin) indicates the presence/absence of oxygen. Alternatively, non-reducing media may be incubated in Brewer anaerobic jar or Flides and McIntosh jar by adding a gas pack and palladium catalyst.

Requirements

a. Blood agar plates,
b. Thioglycolate broth tubes,
c. Anaerobic jar and hydrogen peroxide.
d. Bacterial cultures: *Alcaligenes fecalis, Clostridium perfringens, Streptococcus fecalis and Escherichia coli.*

Procedure

1. Label one tube each of thioglycolate broth for *Alcaligenes fecalis, Clostridium perfringens, Streptococcus fecalis and Escherichia coli* and transfer aseptically a loopful of respective culture to the tubes.
2. Place the tubes in 37°C incubator and examine the tubes for bacterial growth after 16-18 h of incubation.
3. With marker, divide each blood agar plate into four sectors on the bottom and label one quadrant each as *Alcaligenes fecalis, Clostridium perfringens, Streptococcus fecalis and Escherichia coli.*
4. Streak the labeled quadrant with respective culture. Place one plate in the anaerobic jar in inverted position. Heat the palladium catalyst, place the lid in position, and tighten it with clamps. Evacuate the air using vacuum pump. Replace the evacuated

air with hydrogen- nitrogen mixture and incubate the jar in 37°C incubator. Invert other plate and keep it at 37°C.

5. Observe both the plates after 16-18 h. of incubation for bacterial growth, catalase activity and hemolytic zone around the colonies. Note the characteristic double zone of hemolysis around *Clostridium perfringens* colonies on blood agar plates incubated anaerobically. Group the bacterial cultures based on oxygen requirement in different groups.

Questions

1. Sketch the bacterial growth of each culture in thioglycolate broth.
2. Which bacterial culture (s) did not show catalase activity? Did the culture lacking catalase activity grow aerobically?
3. Did you note the change in color of the indicator in thioglycolate broth?

Exercise 32: Effect of physical factors (pH and temperature) on growth

Purpose of this exercise is to reveal the diverse nature of microbial kingdom to grow in varied environments. Bacteria are capable to grow over a wide range of pH (highly acidic to highly alkaline), and temperature (-5°C to > 100°C) Specific group of organisms however grow within narrow range exhibiting optimal, maximal and minimal growth relative to the stability and activity of enzyme systems to temperature and pH. With increasing temperature enzyme activity increases until the structural changes denature the protein molecules. Proteins are amphoteric substances which can behave as acids or bases whose properties are affected by hydrogen ion in the environment. At optimum pH or temperature the rate of catalyzed reaction is greatest and growth rate most rapid. Organisms differ widely in their optimal growth temperature and pH. Optimal point is very near to maximal growth temperature and pH (highest temperature - pH at which the organism is able to grow). Organisms growing at these extremes are sometime called the extremophiles.

Based on the cardinal temperature of growth bacteria have been divided into three major groups: **Psychrophiles** able to grow at –5-20°C; **Mesophiles** 20-45°C and **hermophiles** 45-60°C. The purpose of this exercise is to demonstrate the influence of physical factors like the incubation temperature and hydrogen ion concentration (pH) of growth medium and the application of this phenomenon for the isolation of microbes. In some cases the ideal temperature of specific enzymatic activities may not coincide with the optimum growth temperature for given organism e.g. *Serratia marcescens* produces endogenously red or magenta pigment at 30°C while it grows optimally at 37°C but does not produce pigment, *S flexnerii* is hemolytic at 37°C but not at 30°C, *Y enterocolitica* is motile below 30°C but non motile if incubated at 37°C.

Requirements

a. Nutrient broth cultures: *Escherichia coli, Bacillus stearothermophilus, Pseudomonas aeruginosa* and *Serratia marcescens*
b. Sabouraud's broth culture of *Saccharomyces cerevisiae.*
c. Trypticase soy agar plates, glucose broth tubes
d. Incubator set at 30°C, 37°C and 60°C and refrigerator set at 4°C

Procedure

1. Mark the underside of four petriplates into four equal quadrants with marker and label one quarter each as *Escherichia coli, Bacillus stearothermophilus, Pseudomonas aeruginosa* and *Serratia marcescens.*
2. Aseptically streak each plate with the labeled culture and incubate the plates in inverted position at 4°C, 30°C, 37°C and 60°C in the incubator. Observe the plates after 24-48 h incubation for growth and pigment production.
3. Similarly label a set of four glucose broth tubes. Inoculate each of them with the test strains and incubate at 4°C, 30°C, 37°C and 60°C in the incubator as above. Observe the tubes after 24-48 h of incubation for acid and gas production.

4. Repeat the experiment with same set of organisms including *Saccharomyces cerevisiae* and inoculated on Sabouraud s agar plates having pH 3.5, 5.0, 7.0 and 9.0. Incubate the plates in inverted position and observe the plates visually at the end of incubation 24-48h for growth of the test strains.
5. Record the observations as +, ++, +++ depending on the amount of growth appearing on each plate after incubation at different temperature and pH against each strain in tabulated form and find out the optimal growth temperature and pH for each strain.

Questions

1. What are extremophiles?
2. What kind of medium is used for isolation of alkalophiles and acidophiles?
3. Compare the pH requirements for isolation of actinomycetes, bacteria, fungi, yeasts and molds.
4. Why is 37°C selected as the normal temperature of incubation of clinical samples and 28°C for environmental samples?
5. What are thermoduric and thermophilic organisms?

Exercise 33: Study of biochemical characteristics of bacteria

For identification of bacteria, certain general characteristics are of primary importance for determining the major group to which the new isolate is most likely to belong. Know about the nature of the organism: whether it is fastidious, non-fastidious, phototropic, heterotrophic, aerobic, anaerobic, microaerophilic or facultative, its morphological features like Gram reaction, endospores, capsule, rod, cocci, curved, spiral, acid fastness, motility, arrangement of cells occurring in pairs, chains, groups or in packets. Score the isolate for physiological traits like catalase, oxidase, glucose oxidation-fermentation reaction, and nitrate reduction. One should avoid shot-gun approach in which all sorts of tests are performed in desperate hope that some of them may be helpful. After preliminary analysis, fit the organism in a particular group and then apply the group specific tests to identify the genus and species. In some, cases such as clinical samples where the occurrence of suspected pathogen is more or less confined to a group of organisms one can make use of antisera for presumptive identification but the absolute characterization should be based on phenotypic characters. It requires culturing of organism on several media for correct evaluation of its physiological characteristics.

There are two reasons to study the biochemical characteristics of organisms. First: the property can be used to demonstrate the exceptional metabolic diversity amongst prokaryotes. The range of metabolic activity is very large, indicating their diverse nature. Some of the activities are unique to bacteria. The second reason is that biochemical characteristics of bacteria represent additional phenotypic traits that can be easily examined. The biochemical characteristics make it possible to identify isolates by matching the phenotype with that of a known reference organism. Reference organism is the type species of that genus.

The reactions of carbohydrates employed for identification are usually the catabolic reactions used by the bacteria as part of energy producing metabolism. The reactions fall in two groups: utilized and not utilized, if utilized how it is utilized, via oxidation or fermentation. Organisms are screened for their ability to utilize variety of sugars. The break down products of glucose in particular is examined for the nature of product, acid and gas production and its utilization through oxidation or fermentation. There are differences in breakdown products as well, which can be tested and nature ascertained, is helpful in diagnosis. In order to determine an organism's biochemical characteristics you must use a medium that induces or enhances that characteristics and you must use some chemical test to measure the activity. Carbohydrates present in medium form complexes if heated with phosphate, peptone or under alkaline conditions. *Caution*: Always sterilize the basal medium and sugars separately. The latter often involves the use of pH indicators in medium to detect the production of either acid or bases but may also depend upon added chemical that react with the products to give colored compounds.

Physiological characteristics

Catalase test: This test determines the ability of microorganisms to produce catalase that degrades hydrogen peroxide. During respiration many microorganisms produce hydrogen peroxide and other reactive oxygen intermediates like super oxide and halite ions. Accumulation of these is toxic unless inactivated enzymatically. The enzyme catalase inactivates highly toxic H_2O_2 to water and oxygen. Lack of catalase is a diagnostic characteristic of *Streptococci* and anaerobes.

Catalase is tested by adding a small growth of organisms to a drop of hydrogen peroxide (3% v/v). If the gas bubbles or the effervescence arise from the drop then the test is positive. This test can differentiate *Staphylococcus* from *Streptococcus* and *Bacillus* from *Clostridium*.

Oxidase: It is an enzyme involved in electron transport system of aerobic bacteria. Oxidase activity is present in aerobic, facultative anaerobes and microaerophilic microbes. It is an important test to identify *Pseudomonas*, *Alcaligenes*, *Neisseria*, *Vibrio*, *Pasteurella*, *Flavobacterium* and *Aeromonas* species, all of which are oxidase positive. The members of family *Enterobacteriaceae* are exclusively negative for this characteristic. The oxidase test is based on the oxidation of pinkish reagent tetra methyl-p-phenylenediamine dihydrochloride (an electron donor) to dark purple color. Change in color of the indicator dye confirms the presence of cytochrome oxidase that catalyses the oxidation of reduced cytochrome by molecular oxygen. High acidity is inhibitory for the activity of oxidase enzyme. Hence, never perform oxidase on crowded colonies growing on carbohydrate containing media such as *V.cholerae* grown on thiosulphate citrate bile salt sucrose (TCBS) medium.

1. Using straight inoculating needle aseptically take a part of colonial growth of test organism and macerate it on oxidase strip.
2. Examine the strip for any color change for 10-15 seconds. Alternatively, mix the bacterial growth in freshly prepared dye solution.
3. Oxidase positive strains turn the oxidase reagent (tetra methyl-p- phenylenediamine dihydrochloride) dark blue to purple color within 10 seconds. Color change is not observed with oxidase negative strains.

Coagulase: It is the most useful test for differentiation of *Staphylococcus aureus* from *S.epidermidis*, *S.saprophyticus* and *Micrococcus* species. The enzyme converts fibrinogen to fibrin. Fibrin coat surrounding the bacterial cell protects the organisms against host non-specific defenses. The test can be performed with rabbit, human or pig plasma. Sheep plasma is not suitable for the test as it lacks coagulase reacting factor 6 (CRF 6).

Two techniques are used for coagulase detection. Slide method is used for determining cell bound coagulase and the tube test for cell free coagulase.

Slide method

1. Place two drops of normal saline one on each end of slide.
2. Emulsify a loopful of growth from the colony in both the drops.
3. Transfer a loopful normal saline to control and loopful plasma to test drop.
4. Mix the contents with needle and rock the slide gently and observe the suspension for aggregation of cells or flakes of coagulated plasma. Clumps or flakes of coagulated plasma are observed if the strain is coagulase positive, otherwise the suspension will remain free flowing like the control. The strains found negative for cell bound coagulase must be examined for cell free coagulase by tube test.

Tube test

1. Take 0.5 ml diluted plasma in a sterile test tube (1 drop plasma + 9 drops normal saline).
2. Emulsify small amount of growth from the colony or a loopful culture from broth to the plasma tube. Incubate at 37°C for 4 h and examine it hourly.
3. Clot formation within 4 h is interpreted as positive test for cell free coagulase.

Phenyl pyruvic acid (PPA) test : This is a test to study the oxidative deamination of phenylalanine to phenylpyruvic acid. Phenylpyruvic acid carboxylic group on reaction with $FeCl_3$

forms a complex that is green in color. PPA test is exclusively positive for *Proteus, Morganella* and *Providencia* species amongst the members of family *Enterobacteriaceae*.

Medium contains: Yeast extract-3 g, DL phenylalanine-2 g, Disodium hydrogen phosphate-1 g, Sodium chloride-5 g, Agar-12 g, distilled water-1000 ml, dispense the medium and autoclave at 121°C for 15min and allow to solidify as long slants.

1. Inoculate heavily the slope of phenylalanine medium and incubate at 37°C for overnight.
2. Add 4-5 drops of ferric chloride solution (10%w/v) over the surface. Observe the reaction at the slant surface.
3. Results: development of green color in slant and free fluid indicates the test is positive (*Proteus, Morganella* and *Providencia*), negative: no change in color (*Salmonella, Yersinia, Shigella*).

Esculin hydrolysis : It is definitive test for the differentiation of hemolytic *Enterococcus*. All the strains of enterococci hydrolyze esculin. The breakdown of esculin is indicated when the medium which is brownish in color turns black.

Medium : Dissolve 10 g esculin, 0.5 g ferric citrate and 40 g nutrient agar base in 1000 ml distilled water. Autoclave the medium at 121°C for 15 min. Dispense the medium in tubes and cool in slanted position.

Inoculate the medium streaking on the slope and incubate at 37°C for 24 h and look for the blackening of medium an indication for positive test.

ONPG test: This test differentiates true non-lactose fermenters (NLF) from late lactose fermenters (LLF). LLF lack enzyme permease that transports lactose across the cell membrane but possess beta galactosidase and hence utilize slowly the lactose that diffuses across the cell membrane. ONPG is a rapid test for detection of beta galactosidase activity. This enzyme degrades orthonitrophenyl-β- D-galactopyranoside (ONPG) to orthonitrophenyl a yellow color product.

ONPG medium: Dissolve 0.6%w/v ONPG in 0.01M Na_2HPO_4 and filter sterilize. Mix 25 ml ONPG solution with 75 ml sterile peptone water and dispense 0.5 ml per tube in sterile tubes.

1. Inoculate heavily from TSI slants and incubate for 1h at 37°C.
2. Note the color of the broth. Test is positive and strain is LLF if yellow color develops in broth and if it remains colorless the test is negative and the strain is NLF.

Motility: Examine using motility agar or with hanging drop technique.

Fermentation of sugars: It contains a basal medium, which normally is peptone water, or any suitable medium that allows the growth of test organisms. It is supplemented with carbohydrates and indicator to detect the acid production. The carbohydrates added include:

Monosaccharides : arabinose, xylose, rhamnose (pentoses), glucose, fructose, mannose, sorbose, galactose (hexoses). Disaccharides: sucrose, maltose, lactose, trehalose, cellobiose,

Trisaccharides : raffinose, **Polysaccharides:** starch, inulin, dextrin and glycogen. **Sugar alcohols:** glycerol, erythritol, adonitol, mannitol, dulcitol, sorbitol and inositol. **Glycosides:** salicin and esculin. Glucose broth tubes in addition contain a small glass tube (Durham tube) for the entrapment of gas bubbles. If the bacteria do not utilize sugar, they will often use amino acids contained in the medium. When amino acids are used, ammonia is produced as a by-product, causing the pH of the medium to rise. Sugar fermentation results in TSI tube are reported as acid (A), acid and gas (AG), alkaline (K), no change (NC).

Sugar medium: peptone water (basal medium) - peptone 10g, sodium chloride 5g, water 1000ml, pH 7.2. Autoclaved medium is supplemented with sugar solution (0.5-1.0% level) sterilized separately. Andrade's indicator (0.5% acid fuchsin in 1N NaOH until the color is just yellow) is added at 1%v/v concentration to basal medium prior to autoclaving.

Indicator	pH range	color change	Concentration
Methyl red	4.4-6.0	red- yellow	0.005%
Phenol red	6.8-8.4	yellow to red	0.005%
Brom thymol blue	6.0-7.6	yellow to blue	0.005%
Bromocresol purple	5.2-6.8	yellow to violet purple	0.005%

Amino acid decarboxylation test: Amino acid decarboxylation test is widely used for differentiation of members of family *Enterobacteriaceae*. The breakdown of amino acid is demonstrated by the indicator color change of medium supplemented with amino acid occurring consequential to test strain growth under anaerobic condition. Bacteria growing under anaerobic condition ferment glucose and produce acid that lowers the pH and the indicator turns yellow thus making the environment (pH) optimal for the activity of decarboxylases. Decarboxylase in turn changes the indicator dark violet because of alkaline condition produced from the breakdown of amino acid.

Lysine + enzyme→ Cadaverine + CO_2→Bromothymol blue changes to blue as the pH rises.

Medium : Basal medium: peptone-5 g, lab lamco-5 g, glucose-0.5 g, distilled water 1000ml, pH –6.0, indicator bromocresol purple (1:500)- 5 ml and cresol red (1:500)- 2.5 ml.

Arginine (Arg) medium (red) : Add L-arginine monohydrochloride 1 g/100ml basal medium.

Lysine (Lys) medium (blue) : DL-lysine hydrochloride 2 g/100ml basal medium.

Ornithine (Orn) medium (violet) : L-ornithine 1 g or 2 g DL mixture/100ml basal medium.

Dispense 3 ml /sugar tube and sterilize by autoclaving.

1. Inoculate the tube and add about 5 mm thick layer of liquid paraffin and nurture.
2. Examine daily for 4 days. A positive test is indicated by dark violet or reddish violet color as a result of increase in pH. Amino acid decarboxylation reaction of major enteropathogens are shown in table below :

Organism	Arg	Lys	Orn	Organism	Arg	Lys	Orn
S.sonnei	-	-	+	*Citrobacter*	+	-	+
Shigella sp	-	-	-	*Klebsiella*	-	+	-
A-D group	-	+	-	*Hafnia*	-	+	+
E.coli	D	D	D	*E.cloacae*	+	-	+
S.typhi	-	+	-	*E.aerogenes*	-	+	+
S.paratyphi A	-	-	+	*E.tarda*	-	+	+
Salmonella sp	+	+	+	*Serratia*	-	+	+
Arizonae	+	+	+	*Morganella*	-	+	+
Proteus sp	-	-	-	*P.mirabilis*	-	+	+
Pseudomonas	+	-	-	*Providencia*	-	-	-
Y. enterocolitica	-	-	+	*Yersinia sp*	-	-	-

Citrate utilization : This test is useful for identification and differentiation of enterobacteriaceae. The test determines the strain's ability to use citrate as exclusive source of carbon and ammonia as its only source of nitrogen. Test strain is cultured in Koser's citrate medium or Simmon's citrate medium that is a modified form of Koser's citrate containing sodium citrate, an ammonium salt, bromothymol blue an indicator and agar.

Medium : sodium chloride-4.5 g, ammonium chloride-0.5 g, magnesium sulfate- 0.2 g, sodium dihydrogen phosphate 1g, dipotassium phosphate 1g, are added to boiled distilled water1000 ml, add sodium citrate and adjust pH 6.8. Add 20g agar and 40 ml bromothymol blue (1:500) solution and dispense the medium in tubes and autoclave at 10 lbs for 20 min. Avoid over heating. Solidify in slanting position to 3.5 cm slants and 2 cm butt.

1. Inoculate the tubes with straight wire and incubate at 37°C for 24 – 48 h.
2. Observe for color change. Light green to dark blue in Simmon's citrate medium. Turbidity and blue color development in Koser's citrate medium. The test is negative if there is no growth.

MRVP test: This is a set of two tests. Methyl red (MR) reaction determines the acid producing ability of an organism in a buffered glucose broth and Voges-Praskuer (VP) test identifies the production of acetyl-methyl carbinol from glucose.

Medium : Buffered glucose broth: peptone-5 g, KH_2PO_4 – 5.0 g, glucose-5 g / 1000 ml distilled water. Dispense 2.5 ml per tube and autoclave at 121°C for 15 min.

Methyl red (MR) reagent : Methyl red -0.1g/100 ml absolute alcohol, made to 250 ml with distilled water.

VP reagents : (O'Meara)—0.3 g creatine in 40% potassium hydroxide (KOH). Barritt's modification: 5% alpha-naphthol in absolute alcohol. Keep in dark bottle.

1. Inoculate the tubes with test organism at 30°C for *Hafnia* and for other at 37°C.
2. Check for MR test by adding 5-6 drops of MR, appearance of red color indicates MR positive and yellow color MR negative.

3. VP test: Make the culture highly alkaline by adding 2 drops of 40% KOH. Shake the tubes well and then add 0.5 ml O'Meara reagent or 6 drops of Barritt's reagent (alpha-naphthol 5% in absolute alcohol). Shake and keep tubes at 37°C for 10 min. A positive reaction is indicated by eosin-pink color developing from top, which later on darkens to crimson red.

Nitrate reduction test : It is the property of several organisms to utilize NO_3 as the final electron acceptor in anaerobic respiration and reducing it to NO_2.

Medium : Potassium nitrate 0.2 g, peptone 5 g, water 1000 ml and dispense 5 ml per tube and autoclave.

Test reagent : Solution A - 8 g sulphanilic acid in 1000 ml 5N acetic acid.
Solution B- 5g alpha naphthylamine in 1000 ml 5N acetic acid.

A drop each of solution A and B is added to nitrate utilization medium after incubation. The medium turns red if the nitrate has been reduced to nitrite otherwise the test is negative and the medium color remains straw color. If the test is negative for nitrite it may convey three things: organism does not reduce nitrate, nitrate has been converted to other nitrogenous product via nitrite reductase to ammonia or gaseous nitrogen or denitrification of nitrate has occurred in the reduction of nitrite to nitrogen gas. If nitrite test is negative add small amount of zinc dust and shake the tube well. If the medium turns red on adding zinc the test is negative as zinc reduces the nitrate to nitrite. If no change in color is noticed it indicates that nitrate has been reduced beyond nitrite to gaseous nitrogen.

Triple sugar iron (TSI) test: TSI is a modification of Kligler's iron agar (KIA). This test is particularly useful in differentiation of genera of *Enterobacteriaceae* that ferment glucose with acid and gas production. Differentiation is based on carbohydrate fermentation and hydrogen sulfide production by intestinal flora particularly those associated with diarrhoeal diseases. It is more useful than Kligler iron agar because of the added screening value of the third sugar, sucrose. TSI contains 1% sucrose and lactose and 0.1% glucose with phenol red as indicator. Some organisms may be differentiated from others based on hydrogen sulfide production. H_2S is a by product of cysteine breakdown by bacteria, which produce cysteine desulfurase. It reacts with silver, iron or lead present in medium and form black precipitates. Sugar fermentation results in TSI tube are reported as A (acid production indicated by yellow color), acid and gas (AG), alkaline (K red color) and no change (NC). Different types of TSI reactions observed with different strains are as under:

1. Alkaline slant and acid butt with and without gas (K/A or AG) reactions indicate that only glucose fermentation has occurred. Since glucose is present in minimal concentration hence a limited amount of acid is produced throughout the medium initially. But later with the exhaustion of glucose and rapid oxidation acid at surface of slant leads to neutralization of acid resulting in acid butt and an alkaline slant (K/A or AG) after 24 h of incubation.
2. Acid slant and acid butt (A/A or AG) reaction indicates the fermentation of lactose and/or sucrose which are present in high concentration and hence enough acid is produced that is sufficient for maintaining acidity throughout the medium.
3. Some times slow fermentation of sucrose may result in little acid production that is neutralized at the surface so that the slant is alkaline (red) and butt acidic (yellow).

4. Alkali slant and no change in butt (K/NC or K/K) reaction occurs if the organism failed to ferment any of the sugar rather drop in pH may occur due to breakdown of peptones and amino acids. Both the butt and slant turn alkaline.

Medium : peptone 20 g, beef extract 3 g, yeast extract 3 g, sodium chloride 5 g, lactose 10 g, glucose1g, sucrose10 g, ferrous ammonium sulphate 0.2g, sodium thiosulphate 0.2g, agar 20 g, phenol red 0.025 g and distilled water1000 ml pH 7.3. Dispense and autoclave at 15 lbs for 15 min. Medium is slanted to give a slant of 2.5 cm and a butt of 4-5 cm deep.

1. Following aseptic condition inoculate TSI tubes by stabbing the butt and streaking the slant of medium with straight wire.
2. Incubate at 37°C for 24 h and note the color of butt and slant.
3. Blackening of butt as a result of H_2S reaction with ferrous ammonium sulfate forming ferrous sulfide indicates H_2S production.

Hydrogen sulfide production : With the help of a cotton plug hang a sterile filter paper strip soaked in saturated solution of lead acetate over the indole production medium. H_2S production from amino acids is indicated by the blackening of strip due to the formation of lead sulfide.

Urease test : This test is very useful in identification of *Proteus* species and *Helicobacter* species. Other strains also decompose urea but the rate of hydrolysis is slower as compared to *Proteus* sp. Urease is a hydrolytic enzyme that targets carbon and nitrogen bonds in amide compounds such as urea and liberates alkaline end product ammonia and carbon dioxide.

$$NH_2CONH_2 + H_2O \text{ ----------} \rightarrow 2NH_3 + CO_2$$

The alkaline product of this reaction is detected by color change of phenol red indicator. For urease test organism is grown in urea broth or slants.

Medium : Basal medium: peptone1g, sodium chloride 5g, KH_2PO_4 2 g, agar 20 g, phenol red 0.2% solution 6 ml, glucose 1g and urea 20 g, pH 6.8. Urea solution is filter sterilized and added to sterilized basal medium prior to pouring in tubes for making slants. Make slants.

1. Inoculate aseptically the urease medium slant with test organism.
2. Incubate the tubes at 37°C for 24 h.
3. Next day note the change in color of the medium. Appearance of red color in the inoculated medium indicates the urease production and the strain is said to be urease positive.

Indole test: Some organisms possess tryptophanase enzyme that degrades tryptophan to pyruvate and indole. Indole gives a colored product when treated with para-dimethyl-amine benzaldehyde solution (Kovac's reagent). The reagent is immiscible with water, hence forms a ring or layer on the medium surface.

Medium : Tryptone 10 g or peptone 20 g, sodium chloride 5 g, distilled water 1000 ml, pH 7.4. Dispense and autoclave at 15 lbs for 20 min.

1. Inoculate the peptone water tube and incubate.
2. At the end of incubation test the culture medium for indole production by adding 2-3 drops of Kovac's reagent.
3. Red color appearing in reagent ring indicates the test is positive otherwise negative. Alternatively extract indole by mixing it with 1-2 drops of xylene and then add 2-3 drops of reagent (Ehrlich reagent or Kovac's reagent). A red color ring will form over the culture surface, if the test is positive.

Alternate test : Hang a strip of filter paper soaked with saturated oxalic acid solution over the culture and held in place by the cotton plug. Development of pink color on the strip indicates indole production. Important indole producer genera are *E.coli* and *Proteus* and exclusively negative are *Salmonellae*.

Gelatin liquefaction test : Gelatin is a protein colloid. Hydrolysis of peptides by gelatinase producing bacteria causes the destruction of colloid. Liquefaction of gelatin is a routine test used as index of proteolytic activity. Inoculated tubes of nutrient gelatin medium after 24 h of incubation are examined for gelatin solidification when the incubated tubes are cooled to <20°C. Failure to solidify is a positive test for gelatin hydrolysis.

Medium : beef extract 3 g, peptone 5 g, gelatin 120 g, distilled water 1000ml. Inoculate the medium by stabbing with straight needle soon after it is removed from refrigerator in summer. In winter it may be equilibrated to room temperature. Incubate at 20°C for 30 days. Liquefaction is observed at intervals.

Organic acid fermentation medium : It is used in identification and biotyping of *Salmonella*.

Base medium contains peptone 1g, Bromothymol blue 0.2% solution 1.2 ml, 0.1 N NaOH 8.5 ml and distilled water to make 100 ml. Autoclave it. Add 1g mucic acid to hot autoclaved medium and adjust the pH 7.4 with 5N NaOH that also dissolves the mucic acid. Dispense 3-4 ml per tube and autoclave at121°C for 10 min.

1. Inoculate with a loopful of overnight broth culture.
2. Incubate the tubes for 14 days at 37°C and examine the tubes daily.
3. Development of an acid reaction indicates utilization of mucate. Positive reaction is shown by shift of color from blue to green or yellow, followed by a reversion to blue. Positive: *E.coli*. Negative: *Shigella sonnei*.

Hugh Leifson medium : The medium distinguishes between aerobic and anaerobic breakdown of sugars. Two fundamental processes can accomplish bacterial metabolism of carbohydrates: oxidation and fermentation. The degree of acidity produced by oxidation is generally less than that produced during fermentation. If acid is produced only at surface of medium, the attack on sugar is oxidative as the conditions at surface are clearly aerobic and if throughout, it is fermentation.

Medium : Peptone 2g, NaCl 5g, K_2HPO_4 0.3g, agar 3 g, distilled water 1000ml, dissolve and adjust pH to 7.1 and add 4ml indicator (Bromo thymol blue 1%) and 10g glucose sterilized separately. Dispense in tubes and autoclave.

1. Inoculate in duplicate by stabbing with straight needle.
2. Cover the broth surface in one tube immediately with sterile mineral oil and incubate the tubes at 37°C for 24 h.
3. Next day observe the tubes for color change at the surface and in the butt and for the type of bacterial growth. *Pseudomonas* sp, *Bacterium anitratum*, *Flavobacterium* +/-, *Alcaligenes* -/-. Medium can also be used for recording motility.

Sugar fermentation medium for fastidious bacteria

Hiss's serum water- pathogens like *Streptococcus pyogenes, S.pneumoniae* and *Neisseria* do not grow well on ordinary medium unless it is enriched. One part of ox or sheep serum is mixed with 3 parts of peptone water and 0.005% phenol red, pH 7.6. Filter sterilize, and use. For

carbohydrate metabolism, add glucose, mannose and arabinose at 0.5% concentration with Bromo thymol blue as indicator.

Hiss's Serum Sugars : 0.1% peptone 1part, sheep serum 1 part, add Andrade's indicator and sugar one percent each. Sugars required for *C.diphtheriae* are glucose, maltose and sucrose. Mix peptone water and serum and adjust pH to 7.6. Add Andrade's indicator and mix well; distribute 1.8ml to sterile Pyrex 12x100mm tubes. Sterilize by steaming for 30 min. Cool and add 0.2ml 10% sterile sugar solution to each tube. Steam for 30 min at 100°C for two successive days.

Questions

1. Name the pathogen that does not ferment glucose?
2. Enlist three salient characteristics of family *Enterobacteriaceae*?
3. Amongst the enteropathogens which is anaerogenic?
4. What are the visible manifestations of TSI fermentation tube?

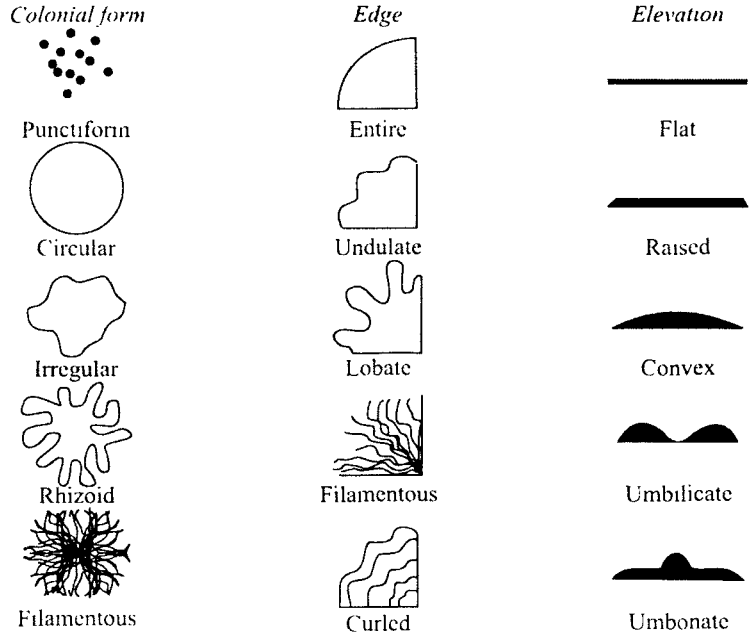
Exercise 34: Identification of unknown bacteria

Once an organism has been isolated in pure culture, it can be definitively identified. This is done by ascertaining a number of characteristics of an organism, and then trying to fit these characteristics to the members of a known species and genus. For identification of bacteria, certain general characteristics are of primary importance for determining the major group to which the new isolate is most likely to belong. Isolate is systematically examined for physiological traits like catalase, oxidase, glucose oxidation-fermentation reaction, and nitrate reduction. After preliminary analysis, fit the organism in a particular group and then apply the group specific tests to identify the genus and species. In some cases such as clinical samples where the occurrence of suspected pathogen is more or less confined to a group of organisms one can make use of antisera for presumptive identification but the absolute characterization should be based on phenotypic characters. It requires culturing of organism on several media for correct evaluation of its physiological characteristics.

Staining :

Growth on culture media : Colonial characteristics on

(i) Enrichment media
(ii) Differential or selective media and
(iii) Special media used



Colonial characteristics

Gram reaction : Gram positive or negative, cocci/rods, cell arrangement-present in singles, pairs, tetrads, chains, packets, bunches regular or irregular.

Other characteristics : Motility, capsule, spores, and volutin granules

Cultural characteristics: Growth- aerobic, anaerobic or microaerophilic, fastidious non fastidious

Biochemical characteristics:

Ability to utilize sugars:

Glucose : by oxidation or fermentation (Hugh-Leifson Test)

Mannitol : aerobic or anaerobic (cocci in bunches), lactose, sucrose, maltose, mellibiose, arabinose, xylose, raffinose, adonitol, sorbitol and dulcitol.

Ability to utilize carbon source other than sugars

Tartarate, mucate and citrate

Ability to utilize nitrogen

Nitrate reduction

Deamination of amino acids e.g. Phenyl Pyruvic Acid test

Decarboxylation of amino acids: arginine, lysine and ornithine

Gelatin liquefaction

Hydrolysis of urea (urease test)

Other tests

Indole production

MRVP test

TSI reaction

Growth in KCN broth

Serological characterization

It is very important in diagnosis and control of microbial infection especially when:

a. Pathogen is not found in routine specimen examinations (rheumatic fever, acute glomerulonephritis).

b. Pathogen present in samples but not easily isolated and identified with other laboratory techniques e.g. persons suffering from syphilis, rickettsial, leptospiral infections, infectious mononucleosis, brucellosis, hepatitis and rotavirus infections.

c. Procedure provides early diagnosis or presumptive diagnosis of diseases e.g. meningitis, cholera, AIDS etc.

d. For identification of serotype and characterization of isolates.

e. Study the prevalence, spread and control of infections.

Common serological tests used for diagnosis of infections include: precipitation, agglutination, complement fixation test, haemagglutination, haemagglutination inhibition, radio immuno assay, fluorescent antibody technique and ELISA.

An experienced bacteriologist may use only one or two tests of many available. But for the novice conduct systematic series of tests that aid in the identification of bacteria. Initially use a few basic primary tests of the listed above and on ascertaining these decide about the additional confirmatory tests to positively identify the unknown organism. The primary tests selected can identify roughly to a generic level and the secondary tests further to characterize species. In this exercise primary characteristic of *E.coli* and *S.aureus* as unknown cultures will be discussed

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Unit three
Bacterial genetics and molecular biology

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Exercise 35: Isolation of bacterial mutants

The set of genetic determinants carried by a cell is called its genotype and its observable properties the phenotype. Bacteria undergo genetic changes due to mutation or recombination. Microorganisms grow rapidly producing millions of progeny in hours of incubation. During rapid multiplication spontaneous mutation may occur. Such random mutations can only be recognized if it brings about an observable phenotypic change. Metabolic mutants can be easily identified and isolated. The frequency of mutation can be enhanced using deliberately induced mutations with mutagenic agents. Wild type bacteria are called **prototrophs** and the mutants lacking particular characteristic **auxotrophs**. An **auxotorph** is a bacterial mutant that requires one or more growth factors that the wild type or the prototroph can synthesize. Auxotrophs fail to catabolise certain organic-substrates or require growth factor for growth. In this exercise UV rays will be used for inducing mutations in bacterial population. Auxotrophs that fail to grow on minimal medium will be identified.

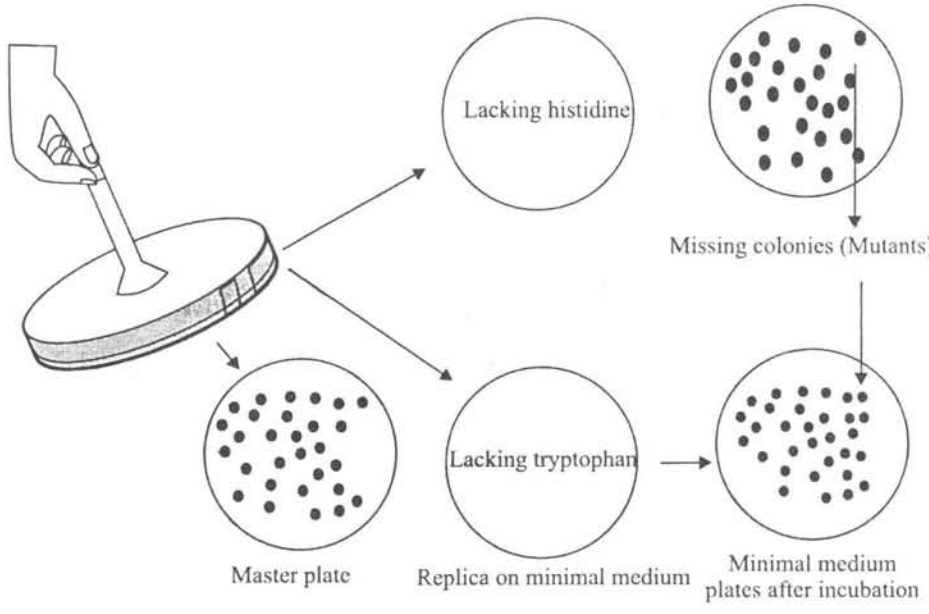
Requirements

a. Nutrient agar petri plates
b. Dilution blanks (9.0 ml)
c. Sterile 1 ml pipettes
d. UV lamp, Spreader, Alcohol
e. M9 glucose minimal medium agar plates
f. Replica-plating block
g. Bacterial cultures: *Serratia marcescens* and *Escherichia coli*

Procedure

1. Label three nutrient agar plates A, B and C and dilution blanks 1,2 and 3.
2. Aseptically transfer 1ml broth culture to dilution blank 1 and mix well. With another pipette, transfer 1ml from dilution blank 1 to dilution blank 2 and mix well. In the same way, transfer 1ml from blank 2 to dilution blank 3 and 1ml to the surface of plate A.
3. Mix blank 3 and transfer 1ml to the surface of plate B with a sterile pipette and 0.1ml to the surface of plate C.
4. Disinfect the spreader by dipping in alcohol and igniting the alcohol in a Bunsen burner flame. Let it cool.
5. Spread the liquid on the entire surface of plates. Disinfect the spreader before and after spreading the culture on each plate.
6. Expose the plates with lid off to ultraviolet light rays positioning the plate at 30 cm distance directly under the lamp placed in the dark hood.. Turn on the U.V. source for 30 to 60 seconds. Place the lid back on the plates and cover them with aluminum foil. This is done so as to avoid reversion of mutants (i.e. photo reactivation).
7. Incubate the plates in dark until the next day: *Escherichia coli* at 35°C; *Serratia marcescens* at 30°C.
8. Select the plate with 25 - 50 isolated colonies. Mark the bottom of the plate with a reference mark. This is the master plate. Mark the uninoculated complete and minimal media with a reference mark on each plate.

9. Assemble the replica-plating block by placing the replicator block on the center of sterile velveteen. Pick up the four corners of the cloth and secure it tightly on the handle with a rubber band.
10. Hold the replica-plating block firmly with velveteen surface up. Invert the master plate selected in step 1 on the block, and allow the master plate agar to lightly touch the block. Remove the cover from the minimal medium, align the reference marks, and touch the uninoculated minimal agar with the inoculated replica-plating block. Replace the cover. Remove the cover from the uninoculated complete medium and inoculate with the replica-plating block, keeping the reference marks the same. Replace the covers and incubate at respective temperature.
11. At the end of incubation period, compare the number and location of colonies appearing on each plate. Note the position of the colonies present on master plate but missing on the minimal medium. These are the colonies of auxotrophs.



Replica plate technique

Questions

1. Compare the results of *E.coli* and *S.marcescens*.
2. Encircle the auxotrophs on the diagram of complete medium.
3. Why the cultures were exposed for shorter duration to UV light?
4. What is the effect of longer exposure on culture?
5. How can you use this technique to find growth factor for auxotrophs?

Exercise 36: Study of mutagens by Ames test

An increased understanding of the mechanisms of mutation and cancer induction has stimulated efforts to identify environmental carcinogens so that these can be avoided. With the information that most carcinogenic agents are also mutagenic is the basis for detecting potential carcinogens. Ames test developed by Bruce Ames in 1970s is a test that uses a special *Salmonella* strains to test chemicals for mutagenicity and potential carcinogenicity. It is a mutational reversion assay carried out with special strains of *Salmonella typhimurium*. Each strain has a different mutation in the amino acid histidine biosynthesis operon. *S.typhimurium* the indicator organism is *his*$^-$ i.e. histidine auxotroph which has leaky cell walls that permit the rapid entry of chemicals. The test strain (*his*$^-$ mutant strain of *Salmonella typhimurium*) and the test compound are mixed in dilute molten mix, which is then poured on top of minimal agar plates and incubated. The test strain (*his*$^-$ auxotrophs) cannot grow on minimal medium, as it cannot synthesize this amino acid. Only the revertants (*his*$^+$) that have regained the ability to synthesize histidine will grow. Number of visible colonies appearing on minimal medium are counted and compared to control gives an estimate about the relative mutagenicity of the compound.

Requirements

a. *S.typhimurium* Ames strain TA98 or TA 1538
b. Three minimal agar plates
c. Three tubes with molten 2 ml top agar and sterile biotin-histidine solution.
d. Sodium phosphate buffer (0.2M, pH 7.4)
e. 2- nitrofluorene dissolved in ethanol
f. Commercial hair dye
g. Sterile Pasteur pipettes
h. Serological pipette 1 ml.
i. Water bath
j. Mechanical pipetting devices

Procedure

1. Culture *Salmonella typhimurium his*$^-$ strains in nutrient broth for overnight. Centrifuge the cells and suspend in a buffer.
2. Melt the three tubes of top agar and cool to 45-50°C.
3. To each molten agar tube add 0.01 ml overnight culture of *S typhimurium his*$^-$ auxotroph and 0.2 ml sterile biotin-histidine solution.
4. Mix gently and thoroughly by rotating the tube between the palms. Add 0.5 ml of test compound, solvent and buffer to tubes marked 1,2 and 3 respectively. Solvent is chosen depending upon the solubility of the test compound. Mix the contents immediately by rotating the tube between palms of your hands and pour the contents over the surface of a minimal glucose agar plate (unknown). *Caution*: Mixing bacterial suspension with mutagen and pouring over the plate should be completed as quickly as possible taking not than 20 seconds.
5. Rotate the plate gently to distribute the top agar evenly on the surface of plate.
6. Similarly plate the other two sets of top agar tubes, one with bacteria alone (negative control) and other with bacteria plus mutagen (Aflatoxin B_1 dissolved in methanol 10ug/ml positive control).

7. Allow the agar to harden in the dark for a min. incubates all the plates in an inverted position in the dark at 37°C for 24 –48 h Examine the negative control plate for the appearance of the colonies of histidine revertants (prototrophs) and the test plate for the his$^+$ colonies induced by chemical. Count the number of colonies in all the three plates and record the data in a tabular form. *Caution*: Ignore the presence of few scattered revertants in the negative control indicate spontaneous back mutation. A chemical that induces back mutation at higher frequency are considered carcinogens.
8. Count the revertant colonies on the test plates and on the control plates. Plot the number of *his*$^+$ revertants *per* plate *versus* the dose of the compound. Determine the mutagenic potency of the compound.

Ames test by spot method

Spot method is slightly different from the pour plate method. In this method sterile filter paper discs saturated with mutagen solution are placed once the top agar containing the test bacterial inoculum poured onto minimal E medium has solidified. The mutagen can be added directly as a few crystals. Solvent solution is placed in the center of the test plate and plates are incubated. Diffusion of the test compound from the disc or crystal creates a concentration gradient of chemical thus inducing the reversions Carcinogenic potential of test chemical can be determined by noting the number of colonies present on the plate. Spot test is widely used for screening of chemical compounds for mutagenicity.

Requirements

a. 100 ul of aflatoxin B_1 (0.001mg/ml).
b. Test compound.
c. Dimethyl sulfoxide (DMSO).
d. VB medium (minimal glucose plates)
e. Sterile filter paper disc
f. Forceps

Procedure

1. Melt the three tubes of top agar, cool and place in water bath at 50°C.
2. Label the bottom of the three minimal agar plates as negative control, positive control and hair dye.
3. To each molten agar tube aseptically transfer 0.01 ml overnight culture of *S typhimurium* his auxotroph and 0.2 ml sterile biotin-histidine solution and thoroughly mix the contents by vortexing.
4. Immediately pour culture mixed top agar onto the minimal agar plate uniformly and allow it to solidify.
5. With a sterile forceps dip sterile filter paper disc into chemical solution and drain the excess fluid by touching the side of the container. Place the chemical impregnated disc in the center of the respectively labeled E minimal glucose agar plates. Gently press down on the disc. Insert a sterile disc dipped in sterile water on the negative control plates. Incubate all the plates at 37°C for 24-48h.
6. Observations examine all the plates for the appearance of colonies around the disc count the number of colonies in each plate and record the results in tabular form.

7. Results: the positive test plates having (2 nitrofluroene) a strong mutagen will have high density of revertants around the disc. If the hair dye used is mutagenic only than revertant colonies will be seen around the disc and the negative control plate will have either no or a few scattered revertants produced as a result of spontaneous back mutation.

Questions

1. What is the function of biotin-histidine solution in the Ames test?
2. What is the relationship between chemical carcinogenicity and mutagenicity?
3. What is Ames test and how is it carried out?
4. Why is mutant selection technique preferred to direct isolation and detection of mutants?

Reversion rate of test strains.

Test strain	Mutagenic agents									
	Control		4-NQO	B(a)P	AFB1	MMS	2-AF	ICR	MMC	EMS
			Concentration plate							
			0.5	1	0.1	1	10	1	0.5	10
S. typhimurium TA 1535	20	20								6000
S. typhimurium TA 1537	4							380		
S. typhimurium TA1538	27	27	283				2250			
S. typhimurium TA 97	148	157	550			297		4000		
S. typhimurium TA 98	30	40	390	180	1820		2200			
S. typhimurium TA 100	130	188	1522		1350	2010	1000			
S. typhimurium TA 102	270			420		3600			1860	

The Number of His revertants per plate are indicated.

4-NQO:	4 nitroquinoline-1-oxyde	(50µl sol. 0.01mg/ml)
B(a)P:	Benzo-(a)-pyrene	(100µl sol. 0.01mg/ml)
AFB 1:	Aflatoxin B1	(100µl sol 0.001mg/ml)

MMS:	Methyl-methane-sulfonate	(1µl pure compound)
2-AF:	2-Aminofluorene	(100µl sol. 0.1mg/ml)
ICR:	ICR191 (acridine)	(100µl sol. 0.01mg/ml)
MMC:	Mitomycine C	(50µl sol. 0.01mg/ml)
EMS:	Ethyl-methane- sulfonate	(10µl pure compound)

Exercise 37: Genomic DNA extraction from bacteria

Deoxyribonucleic acid (DNA) is the genetic material of most cells. In order to study structure, functions, sequencing and cloning of genomes, it is imperative to isolate DNA from test organisms. Genomic DNA comprises of chromosomes and extracellular DNA present in plasmids, mitochondria and chloroplasts. The procedure requires the disruption of cell walls and cell membranes either with sodium dodecyl sulphate (SDS) or cetyl trimethyl ammonium bromide (CTAB) for releasing DNA into the extraction buffer. Unwanted substances like proteins, carbohydrates etc. are precipitated out from aqueous solutions using organic solvents like chloroform, isoamyl alcohol and phenol. Alcohal and Isopropanol help in precipitating DNA out of aqueous solutions. By enlarge prokaryotes contain single copy of chromosome. Prokaryotes and lower order eukaryotes like *Giardia* lack introns.

Requirements

a. TE buffer: Tris-HCl buffer (10 mM, pH 8.0), EDTA (1mM, pH 8.0). Used to suspend cell pellet as well as to dissolve precipitates of DNA.
b. 10% (w/v) sodium dodecyl sulfate (SDS): Used for cell lysis.
c. Proteinase K (20 mg/ml): Helps in degradation of proteins. Store it at –20°C.
d. 5 M NaCl
e. CTAB/NaCl solution: Dissolve 4.1g NaCl in 80 ml H_2O and slowly add 10g CTAB (hexadecyltrimethyl ammonium bromide) while warming and stirring. If necessary, heat up to 65° C till it dissolves. Adjust the final volume to 100 ml. This solution aids in lysis of cells.
f. Chloroform: iso amyl alcohol (24:1): Helps in precipitating proteins and carbohydrates present in aqueous solutions.
g. Phenol. Chloroform: iso amyl alcohol (25:24:1): Phenol denatures proteins.
h. Isopropyl alcohol: Helps in precipitating DNA out of aqueous solutions.
i. 70% ethanol: Used to wash out extra salts from precipitates of DNA.

Procedure:

1. Grow 5 ml of *E.coli* culture (overnight, 37°C, shaking). Take out 1.0ml culture in 1.5ml capacity Eppendorf tubes. Centrifuge the culture (10,000 g for 2 min.) in a microcentrifuge. Discard the supernatant.
2. Resuspend the cell pellet in 567 µl TE buffer by repeated pipetting. Add 30 µl SDS (10%w/v) and 3µl proteinase K (20 mg/ml) mix and incubate for1 hr at 37°C.
3. Add 100 µl of 5 M NaCl and mix properly. Add 80 µl of CTAB/NaCl solution mix and incubate for10 min at 65°C.
4. Add equal volume of chloroform/isoamyl alcohol and mix gently. Centrifuge the sample (10,000g for 5 min.). White precipitate will appear at the interface of two aqueous layers.
5. Transfer the upper aqueous layer (it contains DNA) to a fresh tube.
6. Add equal volume of phenol/chloroform/isoamyl alcohol. Mix it well and centrifuge (10,000 g for 5 mins.). Again transfer upper aqueous layer to a fresh tube.
7. Add 0.6-volume isopropanol and mix gently until white thread like material appears (it is genomic DNA). Centrifuge at 10,000 g, for few min. and discard the supernatant.
8. Wash the precipitate with 1 ml ethanol (70 %v/v) for a few seconds. Centrifuge the tube for a minute and discard the supernatant.

9. Air-dry the DNA pellet to evaporate ethanol (it takes 5-10 minutes).
10. Dissolve the DNA pellet in 100 µl of TE buffer for further use.

Questions

1. Why do we extract genomic DNA?
2. Differentiate between chromosomal DNA and genomic DNA.
3. Name some organisms that have RNA as genotype.

Exercise 38: Agarose gel electrophoresis for DNA

In order to visualize DNA on a solid matrix and also to determine its size, liquid DNA samples are run on agarose gel. Agarose is a complex carbohydrate and the movement of DNA in it depends on its molecular size. Small size DNA moves faster in gel matrix than large size DNA. The dye helps in detecting how far the DNA samples have moved on the agarose gel. Migrated DNA can be visualized after staining with ethidium bromide under UV transilluminator and the molecular weight markers are used to determine the size of test DNA samples.

Requirements

a. Electrophoresis buffer: TAE : pH 8.0, 0.04M Tris-acetate, 0.001M EDTA; or TBE: pH 8.0, 0.045 M Tris borate, 0.001M EDTA.
b. Ethidium bromide solution (0.5 ug/ml).
c. Agarose.
d. 6x-loading dye Bromophenol blue, 0.25%; Xylene cyanol FF, 0.25%, Sucrose (40%, w/v) or Glycerol (30%, w/v). Store at 4°C.
e. DNA molecular weight markers: Lambda Hind III digest or 1.0 Kb ladder.
f. Gel casting platform: to cast the agarose gel.
g. Gel comb (slot formers): to form wells in agarose gel.
h. DC power supply: to run DNA samples in the agarose.
i. UV transilluminator: for detection of DNA and RNA in agarose gel.

Procedure

1. Add enough agarose (0.7-1.0%, w/v) in the electrophoresis buffer and boil it in a microwave oven to dissolve the agarose. Note: The volume of agarose added should be enough that provides a thickness of around 1 cm. thick gel.
2. Cool it to about 55°C.
3. Pour the agarose solution in a sealed (using tape) gel-casting platform. Insert the gel comb close to one end of the gel-casting platform. After gel has hardened, remove the seal from gel casting platform and withdraw the gel comb.
4. Place the gel in electrophoresis tank containing sufficient electrophoresis buffer to cover the gel up to 1mm.
5. Prepare DNA samples with an appropriate amount of 10x loading dye. Total volume of the sample should be around 10-20ul. Load the samples into wells with auto pipette. Be sure to include appropriate DNA molecular weight markers in a separate well.
6. Connect the gel with power supply so that DNA migrates from cathode to anode. Usually DNA gels are run between 70-90 V and a current of around 50mA.
7. When dye reaches the end of the gel turn off the power supply.
8. Place the gel in ethidium bromide solution for about 10 min. **Caution:** Use gloves, as ethidium bromide is carcinogenic.
9. Place the gel on UV transilluminator. Wear UV-protecting goggles. Turn on the transilluminator.
10. DNA and RNA will appear as orangish-red colored bands. Note their size by comparing their movement as compared to the movement of DNA bands present in the DNA marker.

Precautions

1. Always wear gloves while handling ethedium bromide, as it is a carcinogen.
2. Always wear UV protecting glasses, as UV rays are mutagenic.

Questions

1. Give the merits/demerits of using TAE or TBE buffers in agarose gel electrophoresis.
2. What is the principle behind separation of DNA bands using agarose gel electrophoresis?
3. Why Ethidium bromide is mutagenic in nature?

Exercise 39: Plasmid DNA isolation

Plasmids are extra chromosomal DNA elements. These are non-essential as far as cell viability is concerned but may carry genes that may enhance their survival in the environment e.g. antibiotic resistance genes, toxin-producing genes etc. In addition, plasmids have an important place in molecular biology research. They are used for cloning of genes or DNA fragments.

Requirements

Solution 1

50 mM Glucose
25 mM Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)

Solution 1 should be autoclaved for 15 min at 10 lb/sq on liquid cycle and stored at 4^0C. Lysozyme powder is added to this solution before the start of the experiment at a concentration of 2.0 mg/ml. Lysozyme is heat labile and should be kept in refrigerator (4^0C). Lysozyme breaks the cell wall of bacteria. Glucose maintains the osmolarity of the solution. EDTA prevents the digestion of DNA by nucleases.

Solution 2

0.2 N NaOH (Freshly diluted from 10 N stock)
1% SDS

It has to be prepared fresh. Solution 2 is to lyse the cells.

Solution 3

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

The resulting solution is 3M with respect to acetate. Solution 3 precipitates unwanted organic material from the sample.

Procedure

1. Grow the culture in 20 ml of liquid media overnight. Next day pellet one ml of culture in 1.5 ml tube.
2. Discard the supernatant and add 150µl solution 1 in each tube.
3. Add 300µl of solution 2 mix gently and keep it at 37°C for 5 min.
4. Add 225µl of solution 3 in each tube and leave it on ice for 15-20 min.
5. Centrifuge it at 10000g for 10 min.
6. Separate the supernatant into fresh tubes, avoid any junk material collected at the interface.
7. Add equal volume of Phenol: Chloroform: Iso amyl alcohol::25:24:1.
8. Shake it gently and centrifuge it at 10000g for 5 min.
9. Separate the aqueous phase in fresh tubes. Add equal amount of chloroform into it.
10. Centrifuge as in step 8. Separate the aqueous phase.
11. Add 0.7 volume of iso-propanol and leave it for 5 min.
12. Centrifuge it at 10000g for 20-25 min.
13. Remove the supernatant and add 250 µl of chilled 70% ethanol. This is called alcohol wash. Centrifuge it at 5000 g for 2-3 min at room temperature.

14. Drain out 70% ethanol and leave it for air-drying till the alcohol evaporates completely from the tubes.
15. Dissolve the pellet in 20-25 µl of TE buffer and leave it at 37°C for 10 min.
16. Run the samples on 0.7% agarose gel and check the results.

Questions

1. Name some naturally occurring and synthetic plasmids.
2. Differentiate between plasmid and a vector.
3. Differentiate in the mobility of linear, open circular and circular forms of any given plasmid.

Exercise 40: Bacterial transformation

Transfer of genetic material, via homologous recombination, in bacteria occurs through three processes namely transformation, transduction and conjugation. Transformation is a process whereby naked DNA from the medium enters the competent cells and alters the inheritable genotype. Griffith discovered transformation in 1928 in *Diplococcus pneumoniae* that causes pneumonia in children. Prerequisite for introduction of foreign DNA into the recipient bacterial cells include the preparation of competent cells, transformation of competent cells (binding, uptake and integration of DNA) and selection of transformants. Transformation efficiency is 10^5 10^7 for most strains of *E.coli*. Transformation efficiency depends on many factors DNA size and its configuration: closed, linear or circular, and antibiotic selection marker etc. *E. coli* soaked in ice cold salt solution or treated with $CaCl_2$ helps in DNA binding and not actual uptake. Cells can be stimulated for DNA uptake by raising the temperature to 42°C. Heat destabilizes the lipids and increases the transfer rate.

Requirements

a. *E.coli* DH5α overnight old culture
b. Insert DNA (pBR322)
c. LB medium
d $CaCl_2$ solution (50 mM- 100 mM)
e Tris-EDTA buffer (10 mM Tris and 1 mM EDTA)
f. Antibiotics –ampicillin and tetracycline

Procedure

1. Transfer 0.1 ml *E.coli* culture to 25 ml fresh LB medium.
2. Place the flask on rotary shaker and allow the culture to grow to 0.5 O.D at 600nm.
3. Chill the culture by keeping in ice-cold water for 30 min.
4. Spin the culture at 5000g for 15 min. discard the supernatant and suspend the cell pellet in 5 ml $CaCl_2$ solution (50-100 mM).
5. Place the tubes in crushed ice bath for 30 min. Centrifuge the culture and discard the supernatant. Suspend the pellet in 100 mM $CaCl_2$ solution. Take 0.2 ml aliquot and mix it with 50-500 ng of DNA in Tris-EDTA buffer (10 mM Tris and 1 mM EDTA) and keep in ice for 30 min. Run the control similarly but add buffer without DNA.
6. Give a heat shock at 42°C for 2 min and transfer it to ice and keep again for 5 min.
7. Add 0.8 ml sterile LB broth and incubate at 37°C for 45 min for cells to resume growth and express marker.
8. Take an aliquot of 0.1 ml each of these cells and plate on LB agar containing ampicillin (50 ug/ml) or tetracycline (15 ug/ml) or both and incubate the plates at 37°C for overnight. Also plate 0.1ml of the control culture on the selective plate and incubate at 37°C along with the test culture plates.
9. Count the number of colonies appear on selective plate from test and control tubes. Calculate the number of transformants obtained per ug of DNA.

Exercise 41: Bacterial transduction

Transduction is a process of homologous genetic recombination in which genetic material is transferred via bacteriophages from donor to recipient bacteria. Generalized transduction: any gene of donor bacteria can be transferred with equal frequency to recipient strain by a phage (e.g. P1 or P22 phage of *Salmonella*) Specialized transduction: a particular gene adjacent to temperate bacteriophage is transferred to a recipient strain (e.g. Lambda phage of *E.coli*).

In brief, a phage lysate of donor bacterial strain is prepared. Lysate is mixed with recipient *E coli* strain and transductants are selected on a selective medium.

Requirements

1. Donor strain: *E.coli* (rifamycin resistant, streptomycin sensitive)
2. Recipient strain: *E coli* (rifamycin sensitive, streptomycin resistant).
3. P1 phage lysate

Procedure:

1. Inoculate 5 ml LB with donor *E.coli*. Incubate at 37°C, overnight.
2. Transfer 0.1ml of culture into 5 ml LB broth and incubate (37°C shaking, till mid log phase). Add sterile $CaCl_2$ solution to give a final concentration of 1.5 mM.
3. Infect the culture with 0.1 ml of high titer P1 phage lysate and incubate (37°C, shaking) till the culture lyses completely (indicated by increase in the viscosity of the medium).
4. Add a few drops of chloroform (to kill the cells; no harm to phages). Shake the lysate for about 5-10 min. Centrifuge the broth. Discard the debris and save the supernatant (containing phages) in a fresh tube. Add a few drops of chloroform, mix and store it at 4°C.
5. Inoculate 5 ml of LB broth with recipient *E.coli* strain. Incubate at 37°C, overnight. Centrifuge the culture and discard the supernatant. Resuspend the cells in 5 ml normal saline containing 3-5 mM concentration of $CaCl_2$. Transfer 1ml of the cells into each of 4 tubes.
6. Add 0.1ml of phage lysate (original, 1:10 and 1:100 dilution) into three tubes. Add 0.1ml saline in the fourth tube (control).
7. Mix well and keep at 37°C for 15min. for phage to adsorb to the cells.
8. Add 0.1ml of 1M sodium citrate to each tube. Centrifuge the cells, wash once with normal saline, centrifuge and resuspend the cell pellet in 0.2 ml saline and spread the entire amount on nutrient medium containing rifamycin and streptomycin. Incubate the plates at 37°C for 48 h Count the number of transductants on control and test plates.

Questions:

1. Differentiate between conjugation and transduction
2. Explain the function of $CaCl_2$ and sodium citrate in the process of transduction.
3. What do you infer if you get transductants on a control plate?

Exercise 42: Bacterial conjugation

In nature, homologous recombination in the genome of bacteria can occur by the process of conjugation, transduction and transformation. In conjugation, there is direct contact between donor and recipient cells via a thin proteinaceous tube called "sex pillus". The genes responsible for synthesis of sex pilus reside on a plasmid called "F" factor. F factor is a large (about 100 kb in size) double stranded, covalently closed circular DNA plasmid. Cells harboring F factor are termed F^+ cells. F factor can integrate at any position in the chromosome. Such a cell is called Hfr (high frequency of recombination). F' is a F factor carrying a part of chromosomal fragment. Conjugation is quite common in family *Enterobacteriaceae*.

Requirements

1. Donor strain: Hfr strain of *E coli*, streptomycin sensitive
2. Recipient strain. Lysine auxotroph of *E coli*, streptomycin resistant
3. M9 medium
4. Streptomycin

Procedure

1. Inoculate donor and recipient strains of *E.coli* in 5.0 ml Luria broth (LB). Incubate overnight at 37°C.
2. Transfer 0.1 ml of cultures in 4.9 ml LB. Incubate the tubes at 37°C with mild shaking (vigorous shaking will break the sex pili of donor strain). Grow the cultures till mid log phase (i.e O.D 0.6-8).
3. Transfer 4.5 ml of recipient strain and 0.5 ml of donor strain to 100ml conical flask. Mix gently. Keep the flask in incubator (37°C) for two h without shaking.
4. Transfer the culture to a sterile glass tube and vortex (to break the union of donor and recipient strains).
5. Transfer 0.1 ml of original and diluted (1:10, 1:100, 1:1000 and 1:10,000) cultures to M9 medium + Streptomycin (25 ug/ml) plates. Incubate at 37°C for 24-48 h.
6. Count the number of *lys*$^+$ transconjugants.

Questions:

1. Explain homologous and heterologus genetic recombinations.
2. What is the fate of recipient *E coli* strain in terms of F^+ or F^- if genetic recombination occurs using F factor or Hfr strain of *E.coli*?
3. Name a few bacteria that show conjugation phenomenon.

Exercise 43: Estimation of protein by biuret method

The biuret reaction (Robinson and Hogdon, 1940) owes its name to the intense reddish violet chelated compound formed by cupric ions with biuret (H_2N-CO- NH-CO- NH_2). The intensity of this colored complex is directly proportional to the protein concentration in solution. Protein copper sulphate linkages form similar copper chelates in alkaline solution. It is interesting that the capsular polypeptide of *Bacillus anthracis* does not give a biuret reaction presumably the peptide linkages are γ and not α. This method is readily adopted in the determination of total protein in whole microbial cell. Hot sodium hydroxide treatment helps in complete extraction of protein from bacteria, yeasts and molds. Biuret gives extremely reproducible results and the protein values agree with those obtained by acid hydrolysis of protein followed by amino nitrogen determination. DNA, RNA, polysaccharides, glucans and mannan do not interfere. However, interference due to glucose and reducing sugars cannot be ruled out owing to caramalization and cuprous oxide formation on heating with alkali. The only serious draw back is its sensitivity.

Reagents

a. Biuret reagent: Dissolve 0.75 g $CuSO_4.5H_2O$ and 2.25 g Sodium potassium tartarate in 125 ml of 0.25 N NaOH. Then add 1.25 g of potassium iodide, dissolve and make the volume to 250 ml by adding 125 ml 0.2 N NaOH.
b. Protein solution (bovine serum albumin 2.5 mg/ml)

Procedure

1. Arrange six clean tubes in a row in test tube stand. Add 0 ml, 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.2 ml BSA standard solution.
2. Make the volume to 2 ml in each tube by adding distilled water.
3. Add 5 ml biuret reagent to each tube and mix properly.
4. Place the tubes in boiling water bath for ten min.
5. Switch on the spectrophotometer and set the wavelength at 530nm. Using blank set its absorbance zero record the absorbance of each standard. Plot a graph between the concentration and absorbance.

Questions

1. What is the sensitivity range for protein estimation by biuret method?
2. What is biuret reaction?

Exercise 44: Estimation of protein by Lowry's method

Protein estimation by Lowry's method is based on the detection of aromatic amino acids. Final color development is due to biuret reaction of proteins with copper ion in alkali and reduction of phosphomolybdic phosphotungstic reagent by aromatic amino acids (tyrosine, phenylalanine and tryptophan) present in protein. Most proteins have aromatic amino acids that have absorption maximum at 280 nm. Hence the results correlate well with protein estimation spectrophotometrically at 280 nm. The method is more sensitive than biuret method.

Requirements

a. 4% Na_2CO_3
b. 0.5% $CuSO_4.5H_2O$ in 1% potassium sodium tatrate
c. Alkaline copper solution: Mix 50 ml of reagent A with 2 ml of reagent B. Prepare always fresh before use.
d. 0.2 N NaOH
e. Dilute Folin's reagent: Mix 1:1 ratio 0.2 N NaOH and Folin's reagent.
f. Standard protein solution: Bovine serum albumin aqueous 0.1 mg/ml.

Procedure

1. Arrange six tubes in row in test tube stand. Add 0.3 ml, 0.5 ml, 0.7 ml. 1.0 ml, and 1.5 ml of BSA stock solution and in tube six add test protein sample containing protein concentration 50-100 ug/ ml.
2. Add 0.1 N NaOH to all the tubes to make the volume 1.5 ml. Add 1.5 ml reagent C to all the tubes. Shake the tubes well to mix the contents and keep the tubes for 10 min at room temperature.
3. Add 0.2 ml Folin's reagent to each tube. Immediately mix by vigorous shaking. Leave the tubes undisturbed for 30 min at the bench.
4. Measure the absorbance of standards and test sample at 500 nm or at 750 nm in the spectrophotometer set to zero with blank.
5. Plot a standard curve and find out the concentration of the unknown sample from the standard curve.

Questions

1. Why is it necessary to mix the sample vigorously after adding Folin's reagent?
2. Some proteins do not give color reaction with Folin's reagent. Why?
3. Why is the protein solution need to be alkaline and pure for estimating protein by Lowry's method?

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Unit four
Environment microbiology

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Exercise 45: Study of micro flora of soil

Soil is the richest source of different kinds of microorganisms. Predominance of a particular microflora is related to the prevailing soil conditions i.e. organic and inorganic content, soil pH, moisture content, and other environmental conditions. Number of organisms in garden soil per gram has been reported to contain millions of actinomycetes, fungi, bacteria, molds, algae, protozoa, yeasts and viruses. It is difficult to culture *in vitro* all the microorganisms present in soil using any one culture medium. Therefore, it is obligatory to use a specific medium keeping in view the growth requirement of organism of choice. Microbial content in soil can be determined using serial dilution and plating on nutrient media.

Requirements

Sterile petri plates, pipettes, sample bottles, spatula, Nutrient agar, potato dextrose agar, soil samples and dilution blanks

Procedure

1. Sampling: Remove all the vegetation from the surface of soil. Dig the soil 2-3 inches deep with sterile spatula. Collect about 20-50 g soil in sterile bottle. Grind a small portion of it in pestle and mortar.
2. Weigh 11 g soil and transfer it to 99 ml dilution blank. Mix it thoroughly and make serial dilution 10^{-2} onward to 10^{-6}.
3. Transfer 1ml of each dilution in duplicate to separate sterile petri plates bearing corresponding label. Now, add melted and cooled nutrient agar to one set for bacterial viable count and to another set pour melted potato dextrose agar (yeast and mould count) and mix the contents immediately in each plate.
4. Incubate the plates at 30°C for 3-5 days. At the end of incubation (3 day bacterial and 5 days for yeast and moulds), observe the plates for viable counts. Include the plates for viable counts where the colonial count is 30-300.
5. Note the morphology and staining characteristics of typical colonies.

Questions

1. Why were the plates incubated at 30°C and not at 37°C?
2. Is the soil an excellent media for vast majority of microbes? If yes, why?

Exercise 46: Isolation of nitrogen fixing organisms

(a) *Azotobacter* from soil and
(b) *Rhizobium* from root nodules of legumes

Nitrogen fixing organisms convert atmospheric gaseous nitrogen into nitrogenous compounds (fixed nitrogen) that can be assimilated by other microbes and plants. Nitrogen fixation is mediated by two microbial systems classified into two families: *Azotobacteriaceae* and *Rhizobiaceae*. Some other groups of bacteria that can fix nitrogen are *Klebsiella* sp, *Clostridium* sp, *Cyanobacteria* and photosynthetic bacteria. Non-symbiotic free-living microorganisms such as *Azotobacter* sp, *Beijerinkia* sp, *Clostridium* sp, *Klebsiella* sp and *Cyanobacteria* sp which can use atmospheric gaseous nitrogen as their nitrogen source. These organisms form thick cell walls and hence can resist drying and UV radiation but fail to withstand much heat. The symbiotic nitrogen fixers, *Rhizobium* species grow in tumor like nodules in the roots of leguminous plants.

Ability of these organisms to fix atmospheric nitrogen can selectively be used as enrichment procedure for isolation of these organisms from soil. Soil sample is added to selective medium devoid of nitrogenous compounds, so that only the microbes that can utilize atmospheric nitrogen as nitrogen source and glucose as carbon source are able to grow. These are free-living nitrogen fixers such as *Azotobacter*, *Azomonas* and others. *Rhizobium* species are symbiotic nitrogen fixers and they fix atmospheric nitrogen only under symbiotic conditions. Such organisms usually form nodules in leguminous plants. In nodules these bacteria exist as pleomorphic rods (bacteroids) and other shapes.

Requirements

a. Garden soil samples,
b. Nitrogen free medium: broth and nitrogen free mannitol agar plates (asparagine mannitol agar).
c. Freshly picked leguminous plants with root nodules
d. Gram stain and methylene blue,

Procedure

Azotobacter

1. Transfer one g garden soil to 100 ml sterile nitrogen free medium in a Erlenmeyer 250 ml flask. Shake the flask vigorously.
2. Incubate the flask at room temperature 25-30°C for 4-7 days.
3. At the end of incubation period examine the flask for the presence of a thin film.
4. Aseptically withdraw a loopful of inoculum from the surface film and streak the inoculum on nitrogen free agar medium. Alternatively serially dilute the culture and plate the diluted sample on nitrogen free agar medium.
5. Incubate the plate at 25-30°C for 4-5 days and note the colony morphology and color of the pigment produced.
6. Select the colonies differing in appearance for pigmentation and place a loopful of each colony on separate glass slides. Observe each slide under UV source for the presence and absence of green fluorescence. Caution: use safety glasses while looking under UV light.
7. Gram stain each isolate and make a record of size, shape and arrangement of the cells.

Rhizobium

1. Select out the young pink colored nodules from the leguminous plant. Initially wash with water to remove dirt and soil.
2. Then give 2-3 washing with 70% alcohol and then wash it with sterile distilled water. Place the nodules in sterile petriplate.
3. Either crush the nodules by placing a nodule between two sterile slides or crush the nodules with the help of teasing needles.
4. Make a smear from the teased material on the clean glass slide. Let the smear air dry and Gram stain the slide.
5. Examine the slide under oil immersion objective and note down and sketch the different shapes of organisms seen.
6. Take a loopful of the crushed nodule and streak it on asparagine mannitol agar plate. Incubate the plate for 24-48h at 37°C.
7. Observe the type of growth appearing on the plate. Make a smear from the colony and stain it with methylene blue. Record the observations with respect to size, shape and morphology of the organisms.

Questions

1. How do rhizobia form root nodules?
2. Why are young nodules pink in color and old nodules brownish?
3. Does the *Rhizobium* sp exhibit any specificity or any specie can cause nodulation in different kinds of cereals?
4. Distinguish between nitrogen fixation and denitrification.
5. Distinguish between symbiotic and non-symbiotic nitrogen fixation.

Exercise 47: Preparation of *Rhizobium* inoculants and inoculation of seeds

Leguminous plants such as soybeans, grams, beans, alfalfa form root nodules that contain bacterial population called *Rhizobium* sp. Root nodulation is a symbiotic process whereby *Rhizobium* sp bind to lectins on to the surface of the root hair which is then penetrated by microbes to infect the root cells. The infected root cells divide and form a nitrogen-fixing nodule. Nodules provide the anaerobic environment necessary for nitrogen fixation. *Rhizobium* sp vary in their selectivity about the nodulation in leguminous plants. *Rhizobium* inoculants used as biofertilizers include large population or combinations of several strains of *Rhizobium* for a group of leguminous plants that produce efficiently root nodules on relevant plants and thereby enhance nitrogen fixation, crop growth and yield. Root inoculants usually contain 10^7 viable rhizobia/g of carrier on dry mass basis. Cultures are screened for purity on yeast extract mannitol agar. The carrier material such as peat, lignite, peat soil, humus or similar material favoring growth is neutralized with calcium carbonate and sterilized and powdered to 150-212 microns in size. Seeds are mixed with *Rhizobium* culture sugar-gum arabic slurry for seed inoculation. This gives a uniform coating of *Rhizobium* culture around the seeds.

Requirements

a. *Rhizobium* sp culture or *R. phaseoli* Bean Rhizobia
b. *Cicer arietinum* (Chick pea or Bengal gram)
c. *Phaseolus vulgaris* (Kidney beans)
d. Yeast extract mannitol agar
e. 10% sugar solution boiled for 15 min
f. 40% gum Arabic solution

Procedure

1. Inoculate selected *Rhizobium* sp on yeast extract mannitol agar plates and incubate the plates at 28°C for 5 days.
2. Scrap the growth of bacterium with scalpel and suspend in water to 10^6 to 10^8 per ml. For viable counts spread plate 0.2 ml aliquot from 10^6 to 10^8 dilutions on plating medium Incubate plates in inverted condition at 28°C for 3-5 days for fast growing strains and 7-10 days for slow growing strains.
3. Take 500 ml sugar solution and add 200 ml gum arabic solution and allow it to cool to room temperature.
4. Mix the *Rhizobium* suspension and add kidney beans or grams and mix in slurry with hands so as to give uniform coating to seeds with inoculant.
5. Transfer the seeds to filter paper and dry in shade and sow the seeds in fields.
6. Observe the extent of nodulation on inoculated and non-inoculated plants after 10-20 days of sowing. Compare the yields of rhizobia and record the yields.

Questions

1. What is the concentration of *Rhizobium* in RI?
2. Describe the colony characteristics of *Rhizobium* growing on asparagine mannitol agar.

Exercise 48: Isolation of phosphate solubilizing microbes (PSMs) from soil

Phosphorus is one of the essential inorganic nutrients needed for variety of plant metabolic activities. By and large, Indian soils are poor in phosphorus. Even most of the phosphorus present in the chemical fertilizers gets immobilized in the agricultural soil i.e. becomes insoluble, hence roots cannot absorb it. There are microbes (bacteria and fungi), which have the ability to convert insoluble phosphorus into soluble form, which is easily taken up by the plants. Such microbes are being used as Biofertilizers e.g. *Acinetobacter, Rhizobium, Bacillus* etc.

Requirements

a. Agricultural soil
b. Pikovskaya's medium.

Procedure

1. Aseptically collect 5-10 g of rhizospheric soil from the agricultural land.
2. Transfer aseptically 1-2 g of soil in a tube containing 10 ml of sterilized water.
3. Shake the soil vigorously for 2-3 min. Let it stand for 5-10 min. for the soil particles to settle down.
4. Take 1.0 ml of the liquid suspension and centrifuge at 2000-3000 rpm at room temperature. Discard the debris and transfer the supernatant in a fresh sterile tube.
5. Place 0.1 ml of original and 1:10 and 1:100 dilution of the supernatant on Pikovskaya's medium contained in petri plates.
6. Spread out the samples using sterile spreader.
7. Incubate the plates at 25-30°C for 24-72 h Colonies showing zone of clearance are of phosphorous solubilizers.

Questions:

1. What is the morphology and Gram character of phosphorous solubilizers?
2. Describe the mechanism of phosphorous solubility by microbes when tricalcium phosphate is added to the nutrient medium.
3. What are the advantages of PSMs over chemical phosphorous fertilizers?

Exercise 49: Isolation of bacteria from soil:

(a) Saccharolytic microorganisms
(b) Proteolytic microorganisms and
(c) Lipolytic microorganisms

Microorganisms are metabolically highly versatile and can utilize variety of complex organic compounds. Some of these compounds are quite large in size and thus can't be transported inside the cells in native state. Such compound necessitates the synthesis and secretion of extra cellular enzyme(s) for the breakdown of complex substrate into simpler one, which can be easily transported and assimilated inside the cell. Most of these enzymes are inducible enzymes and their producers are usually Gram-positive organisms. Gram-negatives are highly conservative in this regard. The complex compound(s) is often supplemented to the medium and the medium is inoculated with the sample to be screened for microbes capable of utilizing the added substrate. The expression of enzyme or substrate utilization can be quantified with the conversion of substrate.

Requirements

c. Starch agar
d. Milk agar
e. Tributyrin agar
f. Sample from garden soil, sterile petri plates, pipettes.

Procedure

1. Weigh 10g garden soil. Suspend it in 100 ml sterile distilled water and mix thoroughly.
2. Now, transfer 0.1ml of this suspension to starch agar, milk agar and tributyrin agar.
3. Spread the soil sample with spreader on the surface of each plate.
4. Invert the plates and incubate at 37°C for 24 h.
5. **Saccharolytic or amylase producer:** At the end of incubation period flood the starch agar plate with Gram's iodine for 30 sec. Decant off iodine and observe the plate for colorless zones around the colonies in blue background. These are **amylase** enzyme producer's colonies.
6. **Proteolytic organisms**: Observe the milk agar plates for clear transparent zone appearing around the proteolytic organisms
7. **Lipolytic organisms**: Observe a clearing zone around the colonies of each lipolytic organism.

Questions

1. Design an experiment to isolate pectin-degrading organism from forest soil.
2. What are enrichment media? Why are these so important for isolation of organism of interest from samples containing large number of native flora as well?
3. How would you isolate bacteria that produce phospholipase?
4. What is the role of microbial enzymes in soil fertility?
5 What is rancidity? What kinds of organisms are responsible for it?

Exercise 50. Determination of dissolved oxygen of water

The dissolved oxygen can be determined using either the titrimetric method or the electrometric method. In titrimetric method, divalent manganese salt in solution is precipitated by strong alkali to divalent manganese hydroxide. It is rapidly oxidized by dissolved oxygen present in the sample to form trivalent or higher valence hydroxide. Iodide ions are added and acidified which reduce tetravalent hydroxides back to their stable divalent state thereby liberating equivalent amount of iodine. This iodine is equivalent to dissolved oxygen present in the sample.

Requirements

a. Manganese sulphate solution: Dissolve 480 g of $MnSO_4$ in freshly boiled and cooled water, filter and make up to 1000 ml. The solution should not give blue color by addition of acidified potassium iodide solution.
b. Alkaline iodide solution: Dissolve 500 g NaOH and 135 g of sodium iodide in freshly boiled and cooled water, filter and make up to 1000 ml.
c. Concentrated sulphuric acid
d. Starch indicator
e. Standard sodium thiosulphate solution: Make stock solution by dissolving 25 g sodium thiosulphate in freshly boiled and cooled water and make volume to 1000ml and add 1 g sodium hydroxide to preserve it. Dilute it 1:3 with distilled water and standardize against known standard before use.

Procedure

1. Collect the sample in 300 ml stoppered bottle. With 2 ml separate pipettes add 2 ml manganese sulphate solution and then 2 ml alkaline iodide solution taking care that the tip of pipette is dipped well below the liquid surface.
2. Replace the stopper without the inclusion of any air bubble and mix the content thoroughly by shaking the bottle several times. Allow the precipitate formed to settle.
3. After 5 min of settling of precipitate, carefully remove the stopper and quickly add 2 ml conc. H_2SO_4 by running the acid down the neck of the bottle Stopper the bottle and mix thoroughly to dissolve the liberated iodine.
4. Take 200 ml solution and titrate it immediately against standard sodium thiosulphate solution, adding 3-5 drops of indicator starch solution. The end point is pale blue to colorless.
5. Calculate the dissolved oxygen in mg per litre. Dissolved oxygen in mg per litre is equivalent to the volume in ml of 0.025 N thiosulphate solution used for titration

Questions

1. Is the procedure given above suitable for all kinds of samples?
2. Which is the method most suitable for effluent containing suspended solids and samples containing ferric ions?

Exercise 51.Biochemical oxygen demand (BOD) of water

Biochemical oxygen demand of sewage or sewage effluent represents the oxygen expressed in parts per million (mg/litre) required to accomplish stabilization of organic matter by aerobic bacteria. BOD test is based on mainly bioassay procedure that measures the dissolve oxygen consumed by microorganisms while assimilating and oxidizing the organic matter under aerobic conditions. The standard test conditions include incubating the sample in an airtight bottle in dark at a specified temperature for specific time. When incubated at 20°C the demand is usually satisfied at the rate of 33% in two days, 68% in five days, 90% in ten days and 100% in twenty days.

Requirements

a. Incubation bottles: 300 ml capacity narrow neck special .BOD bottle with planed mouth with ground glass stoppers.
b Thermostatically controlled incubator 27± 1°C.
c. Phosphate buffer solution: dissolve 8.5 g potassium dihydrogen phosphate, 21.75 g potassium hydrogen phosphate, 33.4 g disodium hydrogen phosphate and 1.7 g ammonium chloride in about 500 ml distilled water and dilute to 1000 ml. Solution pH should be around 7.2 without any further adjustment.
d. Magnesium sulphate solution: dissolve 22.5 g $MgSO_4.7\ H_2O$ in distilled water and dilute to one litre.
e. Calcium chloride solution: dissolve 27.5 g $CaCl_2$ in distilled water and dilute to one litre.
f. Ferric chloride solution: 0.25 g $FeCl_3.6H_2O$ in distilled water made to one litre.
g. 1N NaOH and 1N H_2SO_4

Reagents for dissolved oxygen measurement:

a. Manganese sulphate solution: Dissolve 480 g of $MnSO_4$ in freshly boiled and cooled water, filter and make up to 1000 ml. The solution should not give blue color by addition of acidified potassium iodide solution.
b. Alkaline iodide solution: Dissolve 500 g NaOH and 135 g of sodium iodide in freshly boiled and cooled water, filter and make up to 1000 ml.
c. Concentrated sulphuric acid.
d. Starch indicator.
e. Standard sodium thiosulphate solution: Make stock solution by dissolving 25 g sodium thiosulphate in freshly boiled and cooled water and make volume to 1000 ml and add 1 g sodium hydroxide to preserve it. Dilute it 1:3 with distilled water and standardize against known standard before use.

Procedure

Determination of dissolved oxygen of distilled water

1. Siphon two narrow necked, glass-stoppered bottles of 250-300 ml capacity full of distilled water. Remove the stopper and add 0.7 ml concentrated sulphuric acid and 1ml potassium permanganate solution (6.32 g $KMnO_4$ diluted to 1000 ml). Introduce all reagents well below the surface. Mix by inverting several times. Permanganate should be slightly in excess if not add another 1 ml permanganate solution after 20 min After 20

min destroy excess $KMnO_4$ by adding 1 ml potassium oxalate solution. Restopper the bottle and mix.

2. Add 2 ml manganese sulphate solution and then 2 ml alkaline iodide solution.
3. Replace the stopper without the inclusion of any air bubble and mix the content thoroughly by shaking the bottle several times. Allow the precipitate formed to settle.
4. After 5 min of settling of precipitate, carefully remove the stopper and quickly add 2 ml conc. H_2SO_4 by running the acid down the neck of the bottle Stopper the bottle and mix thoroughly to dissolve the liberated iodine.
5. Take 200 ml solution and titrate it immediately against standard sodium thiosulphate solution, adding 3-5 drops of indicator starch solution. The end point is pale blue to colorless.
6. Calculate the dissolved oxygen in mg per litre. Dissolved oxygen in mg per litre is equivalent to the volume in ml of 0.025 N thiosulphate solution used for titration of 200 ml water. Oxygen content of distilled water is 6-9 ppm at room temperature.

Preparation of dilution water

1. Aerate the required volume of distilled water in a container by bubbling compressed air for 8-12 hour to attain dissolved oxygen saturation. Let it stabilize for 4hr at room temperature around 27°C.
2. At the time of use add 1 ml each of phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride for each litre of dilution water. Add 2-5 ml treated sewage per litre of dilution water or use commercially available microbial seed mixture as per manufacturer's direction for seeding purposes.

Dilution of sample and incubation

3. Adjust the pH to 7.0 with strong alkali or acid solution so that it may not affect dilution more than 0.5%.

Sample volume and dilution technique

4. Based on COD determine the expected BOD using the following formula for calculating sample volume: Sample volume (ml/ litre dilution) = 1000 X/ expected BOD
   For keeping two dilutions take X =2.5 and 4.0
   For single dilution take X= 3.0 or 3.5.Round off to nearest convenient volume fraction. In case of high BOD samples prepare primary dilutions with distilled water and then make the final dilution.
5. Take requisite quantity of sample in one litre volumetric flask. Dilute to the mark with the dilution water by siphoning from the container. Mix well. Rinse three BOD bottles with the diluted sample. Stopper the bottles immediately after removing the air bubbles. *Caution*: Samples from river lakes and marine have native microbial population. BOD most likely is less than 5 mg/litre hence do not require dilution and BOD determination may be carried out directly without any further dilution of sample.
6. Determine initial dissolved oxygen for one bottle and keep two bottles for incubation at 27± 1°C for three days. At the end of incubation period determine the final dissolved oxygen in the incubated bottles.
7. Calculate the BOD as below:

Undiluted sample

BOD mg/litre=DO before incubation-DO after incubation

Unseeded dilution water

BOD mg/litre $D_1 - D_2 \times 1000/ P$

Seeded dilution water

BOD mg/litre $= D_1 - D_2 - (B_1 - B_2) \times f \times 1000/ P$

D_1= Initial dissolved oxygen of sample in mg/ litre

D_2 = DO after incubation in mg /litre

B_1 = DO of seed control before incubation mg/l

B_2 = DO of seed control after incubation mg/l

f = ratio of seed in diluted sample to seed in control i.e. ml seed in diluted sample/ volume of seed in seed control

P = sample volume (in ml) diluted to 1000 ml with dilution water.

BOD is expressed as mg/L, 3 days at 27± 1°C, round off the values above 10 to whole number and to first decimal value between 0-10.

Questions

1. Why are the samples from surface bodies not seeded and diluted?
2. What types of organisms are frequently present in surface water bodies?
3. Enlist some water borne diseases?

Exercise 52: Chemical oxygen demand (COD) of water

In recent years the boom in industrialization has revolutionized the countries but has also added much to the sufferings. There is tremendous increase in the water and air pollution. Large amounts of effluents from industries containing organic substances of different nature are discharged or enter the aquatic systems resulting in water pollution. Determination of biochemical oxygen demand only is insufficient to give a clear picture of organic content of water bodies and the presence of toxic chemicals in the sample are also an interference to BOD. Alternate to this chemical oxygen demand that does not differentiate between biologically oxidisable and nonoxidisable organic matter gives satisfactory estimate of organic matter present. The method is simple and less time consuming as compared to BOD. The method uses known amount of chemical oxidant like potassium permanganate or potassium dichromate for oxidation of organic matter present in water. Then excess of oxygen (left after oxidation of organic matter) present is allowed to react with potassium iodide to liberate iodine in amounts equal to the excess oxygen that is estimated by titration with sodium thiosulfate solution using soluble starch as indicator.

Requirements

a. Water sample
b. Potassium dichromate solution: 0.1N in distilled water
c. Sodium thiosulfate solution: 0.1 M in distilled water
d. Sulphuric acid 2M
e. Potassium iodide solution 10% , starch solution 1%

Procedure

1. Add 50 ml water sample and distilled water (control) to 100 ml flasks in triplicates.
2. Simultaneously run distilled water blank standards.
3. Add 5 ml potassium dichromate solution to each flask.
4. Keep the flasks in water bath at 100°C for 1 h.
5. Allow the sample to cool for 10 min.
6. Add 5 ml potassium iodide and 10 ml sulphuric acid to each flask.
7. Titrate the contents of each flask with 0.1M sodium thiosulfate solution until the appearance of pale yellow color.
8. Add 1 ml starch solution to each flask and titrate it again with 0.1M sodium thiosulfate solution until the blue color disappears completely.
9. Find out the volume of sodium thiosulfate used for each titration. Taking the mean of the three readings find the volume of sodium thiosulfate used for control and test.
10. Results : COD mg/l of water sample = COD+ 8xCx(B_A)/S
    Where S = volume of water sample taken (ml)
    C = concentration of titrant (mol/L)
    A = volume of titrant used for control blank
    B = volume of titrant used for test sample

Questions

1. How do BOD and COD differ?
2. Explain the functions of KI, $K_2Cr_2O_7$ and $Na_2S_2O_3$ in COD?

Exercise 53: Microbiological testing of water for its potability

Water is well known vehicle for transmission of water borne diseases. Water polluted with fecal matter or sewage is likely to contain many intestinal pathogens and may transmit diseases like giardiasis, amoebiasis, hepatitis, typhoid, cholera, dysentery etc. to susceptible consumers. Natural waters may also be contaminated with microbes from soil, vegetation and other sources. Bacteriological quality of water evaluation is based on total bacterial content (membrane filter technique), coliform count (MPN) and the presence of fecal streptococci. Coliforms are aerobic or facultative anaerobic Gram negative, non-spore forming, rods that ferment lactose with acid and gas production within 48 h at 35-37^{0}C. Coliform group includes *Enterobacter, Klebsiella* and *Citrobacter* in addition to *E.coli*. Detection of fecal *E.coli* an indicator of water fecal contamination is considered to be the best method to judge the potability of drinking water. The presence of typical intestinal organisms in water serves as an index of fecal contamination. Most probable number (MPN) test is conducted in three stages: presumptive test, confirmatory test and completed test. Membrane filter technique relies on passing a known amount of water through sterile membrane filter and the filter transferred to selective culture medium is incubated.

Requirements

a. Lactose bile broth double strength (LBDS) and single strength (LBSS)
b. Eosin Methylene Blue agar (EMB),
c. Peptone water tubes,
d. Methyl red Voges Prausker test (MRVP) tubes
e. Brilliant green, phenol red,
f. Sterile glass stoppered bottle
g. Sterile 10ml and 1ml pipettes

Procedure

Sample collection

1. Water must be collected in sterile container, preferably glass stoppered bottle, of 200 ml capacity.
2. For sampling chlorinated water, add about 0.1-0.2 ml of 3% w/v sodium thiosulphate per bottle to neutralize chlorine or chloramines.
3. **Sample from tap**: Remove the splash nozzle. Clean it carefully from outside. Turn the tap full and let it run for 1min Sterilize the tap with spirit lamp flame. Cool the tap by running water for few sec. Fill the sample bottle from gentle flow of water and replace the stopper.
4. **From tube well**: Operate the pump for 3-5 min. Flame the mouth of pump and let it run for another min Aseptically collect water sample by allowing water to flow directly into the bottle. While collecting sample from river, aseptically remove the cap and face the mouth of bottle upstream.

MPN method

Presumptive test

1. Arrange 5 double strength lactose bile broth (LBDS) tubes and 10 single strength lactose bile broth (LBSS) tubes in a test tube stand.
2. Label 10 ml on 5 LBDS tubes and 1 ml and 0.1 ml on each set of 5 LBSS tubes.

3. Aseptically, transfer 10 ml water sample to LBDS tubes, 1 ml, and 0.1 ml to LBSS tubes having corresponding label.
4. Mix the tube contents gently and keep at 37°C for 24-48 h.
5. Examine the tubes for gas collection in Durham tube and lactose fermentation indicated by color change of broth. Note the number of tubes in each set showing these changes. No gas production in any of the tubes indicates that the coliform test is negative and no further testing is required.
6. Determine the MPN from the MPN tables under respective tube settings. A sample showing more than two coliforms per 100 ml of water is considered unsatisfactory. As the coliform could be fecal or non-fecal origin, test is extended to confirmatory test.

Confirmed test

1. Select the positive acid and gas tube from presumptive test preferably the highest dilution.
2. Take a loopful from this tube and transfer it to peptone water tube. Incubate this at 44.5^0C for 24 h. At the end of incubation period, check the contents for Indole production. Indole production confirms the presence of fecal *E.coli* in the water sample. A loopful from indole positive tube streak on EMB agar and incubate the plate at 37°C. Check the plates for greenish luster or metallic sheen after incubation.
3. Streak a loopful from LBSS tube onto EMB agar or Endo's agar medium. Invert and incubate the streaked plates at 37°C for 24h. Note colonial characteristics. Green metallic sheen in reflected light confirms the presence of *E.coli.*

Completed test

1. Indole and methyl red test is positive for *E.coli* and VP and citrate negative. Select the colony showing greenish metallic luster. Put up IMViC (indole, methyl red, Voges Prauskuer, citrate) tests taking inoculum from the colony showing metallic sheen or luster.
2. Incubate the inoculated biochemical media at 37°C except indole test, which is incubated at 44.5°C.
3. Next day, check the tubes for respective metabolites. Add a drop of Kovac's reagent to indole tube: development of red ring indicates indole production. Development of red color in MR tube on adding one drop of methyl red indicator indicates mixed acid production.
4. For VP test add 2 drops of 40% KOH and shake the tube contents and then add 6 drops of alpha naphthol, red to crimson red color developing indicates test is positive otherwise negative.
5. No change in color of Simmon's citrate medium indicates citrate has not been utilized. Indole production.
6. Similar procedure is followed for three tube test except that water sample is added to a set of 3 tubes in each sets instead of five. 10 ml water sample is transferred to LBDS, 1 ml and 0.1 ml to LBSS.

Questions

1. Why is *E.coli* used as an indicator of fecal contamination in water?
2. Why is lactose used in preference to glucose in presumptive test?
3. How do you confirm the *E.coli* presence in water?

4. How would you verify a positive presumptive test for feces contamination?
5. Why the number of fecal type is more important than the other types present in water?
6. What other indicator organisms can be used as index of fecal contamination?

MPN index table : three tubes inoculated with 10ml, 1ml and 0.1ml water sample

No; of inoculated tubes positive			MPN Index	No; of inoculated tubes positive			MPN Index
10 ml	1 ml	0.1ml	/100ml	10	1	0.1	/100ml
0	0	0	<3	3	0	0	23
0	0	1	3	3	0	1	39
0	1	0	3	3	0	2	64
1	0	0	4	3	1	0	43
1	0	1	7	3	1	1	75
1	1	0	7	3	1	2	120
1	1	1	11	3	2	0	93
1	2	0	11	3	2	1	150
2	0	0	9	3	2	2	210
2	0	1	14	3	3	0	240
2	1	0	15	3	3	1	460
2	1	1	20	3	3	2	1100
2	2	0	21	3	3	3	>2400
2	2	1	28				

MPN index table : five tubes inoculated with 10ml, 1ml and 0.1ml water sample

No; positive tubes			MPN/ 100ml	No; positive tubes			MPN/ 100ml
10ml	1 ml	0.1ml		10 ml	1 ml	0.1ml	
0	0	0	<2	4	3	0	27
0	0	1	3	4	3	1	33
0	1	0	3	4	4	0	34
0	2	0	4	5	0	0	23
1	0	0	2	5	0	1	30
1	0	1	4	5	0	2	40
1	1	0	4	5	1	0	30
1	1	1	6	5	1	1	50
1	2	0	6	5	1	2	60
2	0	0	4	5	2	0	50
2	0	1	7	5	2	1	70
2	1	0	7	5	2	2	90
2	1	1	9	5	3	0	80
2	2	0	9	5	3	1	110
2	3	0	12	5	3	2	140
3	0	0	8	5	3	3	170
3	0	1	11	5	4	0	130
3	1	0	11	5	4	1	170
3	1	1	14	5	4	2	220
3	2	0	14	5	4	3	280
3	2	3	17	5	4	4	350
4	0	0	13	5	5	0	240
4	0	1	17	5	5	1	300
4	1	0	17	5	5	2	500
4	1	1	21	5	5	3	900
4	1	2	26	5	5	4	1600
4	2	0	22	5	5	5	>1600
4	2	1	26				

Exercise 54: Coliform count using membrane filter

Membrane filtration method can be used to enumerate coliforms and *E.coli* in beverages, waters and some rinses. In this method a fixed amount of water is passed through a sterile membrane filter, which is kept in a special filter apparatus contained in a suction flask. The disc is aseptically transferred to a sterile petridish containing absorbent pad saturated with selective differential liquid medium and the colonies that develop following incubation are counted. This method is valuable in many respects (i) the method enables the examination of large volume of water to be tested (ii) it is more economical (iii) results obtained are more accurate (iv) less time consuming than the multiple tube technique.

Requirements

a. Membrane filters of 0.45 um porosity
b. Sterile millipore membrane apparatus assembly
c. Water sample, peptone water and lactose bile broth
d. MacConkey's broth or Endo broth and sterile absorbent pad
e. EMB agar plates
f. Suction flask, Forceps sterile water

Procedure

1. Set up membrane filter assembly with sterile membrane filter (0.45um).
2. Shake the water sample and pour 100 ml water sample into the funnel housing the membrane filter and fitted on to suction flask. Filter under vacuum. After filtering sample water rinse the funnel inner part with 100 ml sterile water. *Caution*: depending upon the coliform count it may be necessary to dilute some waters or rinses with 0.1% peptone water. In each case 100 ml of dilution shall be examined.
3. Remove the membrane from the filter assembly using sterile forceps and carefully place it on the surface of EMB or endo agar plate or transfer it to a pad of sterile MacConkey's membrane broth.
4. Invert the plates and incubate at 37°C for 24 h.
5. Count the number of typical colonies formed using magnifying glass (5-10x) and record as presumptive *E coli*. Typical *E coli* colonies show a greenish metallic sheen. Check the selected colonies for acid and gas production in lactose bile broth and indole production at 44.5°C.

Questions

1. What is the genesis behind selecting *E coli* as indicator organism of water pollution?
2. What are the disadvantages and advantages of membrane filter technique?
3. Can this technique be used elsewhere?

Exercise 55: Demonstration of associative activities of bacteria

Microorganisms in nature live in close associations as symbionts (parteners). Symbiotic relationship may be beneficial, harmful or neutral. These associations have been named as mutualistic both being benefited, commensals one is benefited but other is not harmed and parasitic where one thrives at cost of other. Because of shared activities some organisms growing together succeed in utilization of a substrate, which none can utilize if grown individually. Hence, the coexistence is obligatory for such symbionts. Present exercise shows it with sucrose utilsation by *Staphylococcus aureus* and *Proteus vulgaris*.

Requirements

a Bacterial culture: *Staphylococcus aureus* and *Proteus vulgaris*
b. Sucrose fermentation medium

Procedure

1. Take three sucrose fermentation tubes and label *S.aureus* on tube one, *P.vulgaris* on tube two and third tube as *S.aureus* and P.*vulgaris*.
2. Flame the inoculating needle red-hot and cool it. Aseptically transfer a loopful culture to respective tubes as per label.
3. Incubate the tubes at 37°C for overnight. Examine the tubes for sugar utilization indicated by change in indicator color. Record the observation. Color change is observed in tube three only inoculated with both the cultures. No color change in tube inoculated with either of the culture alone.

Questions

1. What is infection?
2. Define: commensals, parasite, normal flora and host.
3. What is microbial ecology?

Exercise 56: Study of micro flora of air

Air is not a natural environment for the growth and reproduction of microorganisms however, some organisms may be found associated with dust particles and in aerosols suspended in air. The density of microbes in air is high near cattle sheds, dairies and fermentation industries and the atmosphere may carry several types of bacteria, yeasts, molds spores and bacteriophages. Air is also an important medium for transmission of number of respiratory tract infections. The air inside bacteriological laboratory and operation theatres requires to be sterilized frequently by irradiation or fumigation by bactericidal chemicals.

Requirements

Sterile nutrient agar plates and Sabouraud's agar plates

Procedure

1. Take 3 plates each of nutrient agar and Sabouraud's agar plates and label one each as laboratory, rooftop and tuck shop.
2. Remove the tops from plates and place the plates as per label to respective places for 10 min. immediately replace the tops.
3. Incubate nutrient agar plates at 37^0C for 24-48 h and Sabouraud's agar plates at 22^0C for 3-4 days
4. At the end of incubation period, count the number of colonies on each plate.
5. Note the different types of colonies appearing on each plate. Prepare the smears from representative colonies and Gram stain. Examine molds colonies under dissecting microscope and record the observations.

Questions

1. Is air a good medium for the growth of *Bacillus* sp?
2. What are the major groups of organism present in air?
3. What are aerosols, fomites and droplets?
4. What is biosphere? What climatic conditions may increase the bacterial load in air?
5. Enlist any three air borne infections.

Exercise 57: Direct microscopic count (Breed count of milk)

Several kinds of tests are recommended for detection of contaminants, degree and type of bacterial contaminants and physical and chemical changes in milk. These are broadly categorized as routine tests for grading milk on hygienic conditions using standard plate count (SPC), direct microscopic count (DMC), dye reduction tests and presumptive coliforms tests. Rapid tests or platform tests for detection of unsatisfactory supply of milk comprise of organoleptic test, 10 min resazurin reduction test (RRT), clot on boiling (COB) test, acidity determination and alizarin alcohol test. There are special tests for detection of miscellaneous pathogens and keeping quality tests for judging the suitability of milk for processing and manufacture of milk products.

SPC though gives direct assessment of microbes present but the method is costly, time consuming (24-72 h), laborious and prone to error due to sampling and bacteria present in clumps and chains. Coliform test is part of standard plate count mainly indicates the degree of contamination by coliforms.

The method is simple, quick and gives rapid estimation of total bacterial count in milk sample. It is also useful in tracing the source of contamination. It enables the worker to analyze large number of samples in short time unlike the tedious pour plate method where the results are available after 24 h-48 h. This method is not suitable for pasteurized milk samples. The ratios between plate counts and direct microscopic count is 1:4 approximately. The other rapid tests used for testing milk include: titration of developed acidity and dye reduction methods and quantitative estimation of coliforms and determination of specific pathogens. In DMC, a known volume of test milk sample is spread over 1 sq.cm marked area on clean glass slide. The smear is stained and examined under microscope. The number of cells or clumps of cells in specific number of microscopic fields are counted depending on the bacterial load and the average number per field calculated. The microscopic fields (MF) are calculated by dividing the milk spread area by the area of one microscopic field. From these values, the number of bacterial cell or clumps of cells per ml of culture suspension are calculated.

Requirement

a. Microscopic slides with marked area 1sq.cm
b. Breed's pipette (0.01 ml division),
c. Inoculating wire bent at 90° at tip for spreading milk,
d. Compound microscope
e. Newman's stain.

Procedure

1. *Area of microscopic field*: Measure the diameter of microscopic field with a stage micrometer using 1.8 mm objective and 10x eyepiece. Calculate the area of the field as πr^2 where r is the radius of the microscopic field. Microscopic factor is the number of microscopic fields per square centimeter i.e. $1/\pi r^2$.
2. *Preparation of milk film or smear*: Mix the milk sample vigorously and draw milk into the breed pipette just above the graduation mark. Wipe off milk adhering to the tip of the pipette with a blotting paper and adjust the volume to exactly 0.01 ml mark. Touch the tip in the center of marked area on slide and expel the entire volume of milk. Spread the milk uniformly over the entire area with sterile needle. Dry the film by placing the slide on a level surface under table lamp or warm place (40-45°C) protected from dust.

3. *Fixing and staining*: Stain the smear with Newman's stain for 30-60 sec. Wash off the excess strain. Dry in air or in an incubator and examine the slide under oil immersion objective.
4. *Counting the organisms in microscopic fields*: Count the number of organisms in several fields covering the entire area of the film. The total number of fields counted is as per the following observations:

No. of clumps/ field	No. of fields to be counted
> 0.5	50
0.5-1	25
1-10	10
10-30	5

5. *Calculate the average number* of clumps per field and multiply this by microscopic factor to get direct microscopic count per ml milk. Alternatively, count individual cells in each field for total count per ml of milk sample. Normally the clump count agrees more closely with plate count.
6. *Quality of milk* : The following standards are tentatively suggested for the bacteriological quality of milk supplies based on DMC.

DMC clump count/ ml	Bacteriological quality
Less than 500,000	Good
500,000- 4,000,000	Fair
4,000,000- 20,000,000	Poor
> 20,000,000	Very poor

Questions

1. What are the advantages and disadvantages of direct microscopic count?
2. What is the relation between direct microscopic count direct microscopic count and standard plate count?
3. Why Newman's stain is preferred for staining in direct microscopic count?
4. Is this method suitable for platform test?

Exercise 58: Methylene blue reduction test

The fresh milk oxidation-reduction potential is about +300 mv, which decreases and becomes negative with the growth of contaminating bacteria. The change in oxido-redox potential is directly proportional to the bacterial population. This change can be measured by adding oxidation-reduction dye like methylene blue or resazurin. Methylene blue (Eh 11mV) almost colorless (Eh of –49mV) in reduced state or under anaerobic conditions and it is blue in color in completely oxidized state (at Eh 71mV). The milk sample that is heavily contaminated with metabolizing microorganisms contains markedly low concentration of dissolved oxygen i.e. oxidation redox potential of sample is lowered. The speed at which the methylene blue is reduced indicates the quality of milk and is proportional to the bacterial load in milk sample.

Requirements

a. Sterile test tubes (150x16mm) and boiled water sterilized rubber stopper to fit in the tubes or Screw cap test tubes.
b. Water bath with rack for holding tubes set at 37°C.
c. Raw milk sample and pasteurized milk sample stored at room temperature for 48h.
d. Methylene blue aqueous solution (1:300,000 dilution)
e. Sterile 10 ml and 1ml pipette

Procedure

1. Mix the milk sample thoroughly. Take two tubes and mark one as test and the other as control.
2. Transfer 9 ml milk sample to both the tubes.
3. Add 1 ml methylene blue solution to each tube and stopper the tubes either with lock stopper or rubber stopper. Mix the tube contents thoroughly inverting slowly twice or thrice.
4. Keep the control tube for 3 min in boiling water to destroy the natural reducing system of milk. Allow 5 min time to tubes for equilibration, invert gently and replace both the control and test tubes in water bath set at 37°C. Note the decolorisation of milk at 30 min interval for 6hr and time for complete decolorisation of milk.
5. Based on your results find the quality of milk: good, fair, poor or very poor.

Questions

1. Explain the principle of MBR and resazurin reduction test.
2. Why the milk sours faster in summer at room temperature and slower when kept in refrigerator?
3. How the milk becomes contaminated while it is said to be a sterile body fluid?
4. Can the good quality milk be a source of infection to humans?

Exercise 59: Resazurin reduction test

Dye reduction tests are simple and do not require any special instrument. MBR requires the minimum equipment. Dye reduction time is proportional to the number of bacteria present in milk. Occasional heavy contaminated milk with inert bacteria may give false results. Short reduction time for milk containing leukocytes (mastitis milk and late lactation period) does not give true picture of bacterial contamination. However, the dye reduction tests are best-relied tests for detection of poor hygienic quality milks. Unlike methylene blue that is reduced to colorless leuco dye, resazurin dye is reduced through series of color changes: blue, lilac, mauve, pink mauve, mauve pink, pink and finally colorless state. Resazurin is reduced to colored formazan in two stages: blue colored resazurin changes---irreversibly rapidly through series of shades to→ pink colored resorufin (Eh –51mV) and finally→ to colorless dihydroresorufin (Eh -110 mV) - a reversible reaction. Resazurin is widely used redox dye, being nontoxic to bacteria and is effective at 1-2ug/ml concentration. The latter reaction requires additional reducing agent (biological or chemicals) in the medium. Thus making it possible to record the reduction at any stage or specified time after the test has been put. Series of color changes are compared with comparator disk. Milk is graded according to the amount of reduction that has occurred. Alternatively the result may be expressed as the time required for complete reduction of dye. Ten min. resazurin reduction test is the best among the platform test for segregation of poor quality milk. One-hour test can be used to determine the bacteriological quality of milk. The test is read using resazurin comparator that measures the stages of reduction of milk with the help of standard color disk. Rapid reduction is relative to heavy microbial contamination and abnormal collection. Protocol set up is same for both the tests.

Requirements

a. Sterile test tubes (150x16mm) and boiled water sterilized rubber stopper to fit in the tubes or screw cap test tubes.
b. Water bath with rack for holding tubes set at 37°C.
c. Raw milk sample and pasteurized milk sample stored at room temperature for 48h.
d. Resazurin dye (1:180,000 dilution)
e. Sterile 10 ml and 1ml pipette

Procedure

1. Take two tubes and label one as test and other as control.
2. Transfer 9 ml milk sample to both the tubes.
3. Add 1 ml resazurin dye solution to each tube and stopper the tubes either with lock stopper or rubber stopper. Mix the tube contents thoroughly inverting the tubes slowly twice or thrice.
4. Keep the control tube for 3 min in boiling water to destroy the natural reducing system of milk. Allow 5 min time to tubes for equilibration, invert gently and replace both the control and test tubes in water bath set at 37°C.
5. Note the color changes at 10 min interval for 60 min.
6. Based on your results find the quality of milk as good, fair, poor or very poor.

Disk color changes	Milk quality	Remarks
4 or more	Satisfactory	Accept
3.5-1	Doubtful	Check further
0.5-0	Unsatisfactory	Reject

Questions

1. Does dye reduction test always give true picture of bacterial contamination?
2. When does dye reduction test give false results and why?
3. Explain the utility of resazurin reduction test as platform test.
4. Explain the importance of 10 min and 60 min resazurin reduction test (RRT).

Exercise 60: Phosphatase test for milk

This test is based on the detection of phosphatase enzyme that is inactivated at pasteurization temperature. Commonly used test is the Aschaffenburg and Mullen phosphatase test. This test determines the presence of phosphatase enzyme normally present in milk but rendered inactive in pasteurized milk. The enzyme present in milk releases p-nitrophenol from disodium-p-nitrophenyl phosphate. Release of p-nitrophenol imparts yellow color to milk that can be measured qualitatively by comparing color visually with p-nitrophenol standards.

Requirements

a. Comparator
b. Sterile test tubes
c. Pipettes
d. Substrate solution
e. Milk sample
f. Carbonate bicarbonate buffer: Dissolve 3.5 g Sodium carbonate and 1.5 g Sodium bicarbonate in distilled water and make volume to1000 ml.
g. Substrate solution: Dissolve 150 mg Disodium-p-nitrophenyl phosphate in carbonate bicarbonate buffer and make the volume to 100 ml.

Procedure

1. Take two tubes. Label one as control and other test. Transfer 5 ml substrate solution to each tube with sterile pipette. Place the tubes in water bath set at 37°C for equilibration.
2. Add 1 ml milk sample untreated to test and boiled milk sample to control.
3. Incubate the tubes in water bath for 30 min.
4. Observe the tubes for p-nitrophenol release from the substrate indicated by the development of yellow color in the tube containing unpasteurised milk sample.
5. No yellow color in the test and the yellow color is less than or equal to 10 ug/ ml p-nitrophenol released in milk after 30 min incubation then the milk is considered to be pasteurized. Yellow color matching with standard p-nitrophenol (p-nitrophenol standard 10, 20, 30, 50 ug/ ml milk prepared by mixing suitably diluted stock solution of p-nitrophenol 4 ml and 1 ml milk) exceeding 10 ug per ml the milk is an indication that the milk is not pasteurized. Record the observations and grade the milk.

Questions

1. What are HTST and LTHT?
2. What is the source of phosphatase in milk?
3. Name the organism usually present in raw milk that is phosphatase positive.
4. Which is the index organism for pasteurization temperature?

Exercise 61: Sterility test for milk

This test is based on the principle that boiling milk for 5 min. coagulates the albumins and the milk is said to be free from albumin. The milk boiled at least for 5 min passes the test. Fractional precipitation of proteins other than albumin is achieved by adding ammonium sulfate just sufficient to precipitate other proteins in milk leaving behind albumin. Subsequently when the filtrate is boiled turbidity is seen in filtrate containing albumin and absent in albumin free boiled milk. The test detects the contamination of raw milk. It gives no indication of the keeping quality of "sterilized" milk and sterility of milk from microbes.

Requirements

a. Ammonium sulfate
b. Weighing balance
c. 50 ml conical flask
d. Whatman No. 12 filter paper
e. Funnel
f Test tubes, burner and milk sample

Procedure

1. Take 20 ml of well-mixed milk to 50 ml conical flask.
2. Weigh 4 g ammonium sulfate and add this slowly while shaking the flask in rotary motion. Dissolve all the ammonium sulfate and let it to stand for 5 min at room temperature.
3. Filter milk through Whatman No 12 folded filter paper. Collect at least 5 ml filtrate in a tube and boil the filtrate for 5 min.
4 Cool in cold water and examine the tube for turbidity, holding it against light source. Compare the turbidity with a tube of milk boiled for 10 min and then treated with ammonium sulfate in the same way.

Questions

1. What do you understand by sterility of milk? Is it free from all kinds of pathogens?
2. What is the basis of sterility of milk?

Exercise 62: Stormy clot fermentation test

Contamination of milk may occur from various agencies. Microorganisms may come from diseased animal udder, from environment, milking under unhygienic conditions, dirty utensils, handlers and soil borne organisms from animal excreta. Milk contaminated with animal fecal matter may contain *Clostridium welchii*, which is regularly present in the intestine of animals and man. *Clostridium* group of organisms has been known to cause many serious diseases. *Clostridium welchii* though present as resident flora of man and animals is associated with most cases of gas gangrene and also causes the food poisoning. Stormy clot fermentation test has been designed to indicate the presence of clostridial group of organisms in milk. This group includes spore forming, anaerobic, metabolically highly active organisms that can utilize proteins and sugars with equal efficiency and generate large amount of gas. Production of acid and gas in milk on incubation breaks the casein into pieces and gas produced in abundance blows off the wax plug. Heat treatment of milk at 80°C for 10 min destroys selectively the vegetative bacteria sparing the heat resistant spore bearing microbes.

Requirements

a. Milk sample
b. Test tubes
c. Water bath set at 80°C
d. Melted wax
e. Incubator

Procedure

1. Transfer 10 ml milk sample into 25 ml sterile test tube. Place the tube for 10 min in water bath at 80°C.
2. Cool the tube in cold water and add melted wax about 2 cm thickness and replace the cotton plug. Let the wax solidify. Incubate the tube at 37°C in incubator for 48-72 h.
3. Examine the tube daily for acid and gas production.
4. Record your observations. Anaerobic conditions created by wax plug favor the growth of anaerobes.
5. If anaerobes are present these will grow in large numbers producing enormous amount of gas, which will blow off the wax plug and the cotton plug. Gas bubbles can be seen arising from microbes trapped in acid coagulated protein particles.

Questions

1. A positive stormy clot fermentation test indicates what?
2. Why is the milk heated at 80°C?
3. Name the diseases caused by *Clostridia*?
4. What is the source of anaerobes in milk?

Exercise 63: Microbes in foods

Raw foods are likely to be contaminated with a variety of microbes. Number and types of contaminants depend on the sanitary conditions, processing, handling and storage temperature. Many of these organisms grow and bring about the spoilage of foods (e.g. souring putrefactive changes and gas production). The methods used for preserving foods from microbial spoilage include: heating, canning, addition of chemical preservatives and fermentation. Even processed and canned foods are liable to spoilage by microbial activities due to inefficient processing or subsequent contamination from the containers and other resources. Foods and food materials are also potential vehicles for transmission of infection. The numbers and types of microorganisms present in the foods are useful parameter in determining the hygienic quality and causes of spoilage of food. Large numbers of organisms are undesirable in most foods and food involved is likely to be spoiled. Hence, standard plate count is considered an important test in routine to judge the quality of most foods besides other specific tests.

Requirements

a. Food sample,
b. Molten nutrient agar,
c. Sterile 1ml pipette and 99 ml blanks

Procedure

1. Label four sterile petri dishes at the bottom with the dilutions $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$.
2. Aseptically, transfer 1ml milk sample to 99 ml dilution blank using sterile 1ml pipette, and label the bottle $1:10^2$ and discard the pipette. Shake the bottle at least 20 times upside down and up vigorously.
3. Now, transfer 1ml and 0.1 ml diluted sample to petri plates labeled 1: 10^2 and 10^3 respectively and 1 ml to 99 ml dilution blank to prepare next dilution using the same pipette.
4. Again, mix the blank contents thoroughly and discard the pipette. From this dilution transfer with fresh sterile pipette 1ml and 0.1ml diluted sample to petri plate labeled $1:10^4$ and $1:10^5$ respectively.
5. Pour about 20 ml melted and cooled nutrient agar (50^0C) to each plate in sequence and mix the dilutions by rotating the plates on the working bench to distribute the milk sample evenly in the agar.
6. Let the agar solidify in each plate than invert the plate and incubate at 37°C for 24-48 h.
7. Next day arrange the plates from lowest to highest dilution and note the plates where the colony count is between 30-300. Count the number of colonies on the selected plate. Find out the count per ml milk sample as below:
   Count/ ml milk sample = colony count x sample dilution

Questions

1. Why do we select plate count ranging between 30-300?
2. Why does freezing and thawing alters the bacterial count in meat?
3. How can you ensure that organisms present in food do not pose health problem?

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Unit five
Medical microbiology & immunology

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Exercise 64: Koch postulates

Absolute proof that a microorganism is the cause of disease rests upon the fulfillment of certain criteria indicated by Henle and enunciated by Robert Koch. This criteria is known as Koch's postulates. As per postulates laid down, an organism can be accepted as causative agent of an infectious disease if it satisfies the following conditions:

1. The organisms must be associated in every case of disease but absent from healthy individuals.
2. It must be isolated and grown in pure culture.
3. The organisms must, when introduced or inoculated into susceptible animal, cause the same disease.
4. The organisms must be recovered from the experimental animal. The isolate must resemble in all respects with the previous isolate.

Although it was not possible to satisfy all the postulates by each infectious agent but these were extremely useful in resolving the distrustful claims.

Requirements

a. Pus from a case of abscess or purulent lesion.
b. Plating media for isolation, differentiation and identification.
c Gram staining set.
d. Blood agar plate and MacConkey agar plate.
e. Biochemical media.
f. Sterile normal saline, centrifuge tubes, syringe and centrifuge.
g. Laboratory animal- mice

Procedure

1. Make a smear of the sample taken from diseased animal and Gram stain it. Study the morphology of organisms.
2. Culture it on suitable laboratory media. Study its biochemical characteristics.
3. Check the pathogenicity by injecting bacterial suspension in normal saline to mice subcutaneously or intraperitoneally.
4. Reisolate from mouse lesions and compare its morphological and biochemical feature with the previous isolate.

Questions

1. State Koch's postulates.
2. What are Koch's modified postulates?
3. Name some of the diseases, which do not fulfill the Koch's postulates.
4. Define infectious agent, carrier, disease and virulence.
5. How do microbes produce disease?

Exercise 65: Study of micro flora of skin

Skin in general is most inhospitable and largest organ for most organisms. Continual shedding, skin pH, oxygen concentration, skin secretions, moisture present and perspiration are important factors that determine the type and concentration of native flora of skin. Some organisms utilize these secretions and produce propionic acid thus reducing skin pH and making it highly acidic (pH 3-5). Acidic pH also suppresses the growth of other bacteria. Most are concentrated in moist area of skin e.g. axilla, armpit, and sides of nose. *Propionobacterium* live in hair follicles on sebum and produce propionic acid. Yet the common microorganisms that naturally reside as normal flora of skin include mainly the Gram-positive organisms: *Staphylococcus* predominantly *S.epidermidis* and *Streptococci:* enterococci, alpha hemolytic, non-hemolytic streptococci , *Diphtheria* sp and some *Bacillus* sp, yeasts and fungi.

Requirements

Blood agar, salt mannitol agar, sterile cotton swab, sterile saline, hydrogen peroxide, Gram staining set, rabbit plasma, Hugh Leifson medium

Procedure

1. Moisten the swab in normal saline. Swab any area of skin preferably the moist areas such as behind pinna, skin folding, armpit where the organisms are found in large numbers.
2. Swab about one fourth of plate with swab. Than using inoculating needle, distribute these to the whole plate, moving away from the inoculum.
3. Invert the plate and incubate at 37^0C.
4. Examine the type of colonies appearing on the plate. Select a representative colony of each type and score it for Gram staining, catalase activity, and opaque colonies for coagulase and mannitol fermentation.
5. For coagulase test, make heavy suspension of bacteria to be tested in two drops of normal saline, one drop on each end of slide. Add a drop of rabbit plasma to test and a drop of normal saline to control and mix the suspension in both the drops with inoculating wire. Observe for clumping of bacterial cells in test drop. If clumping occurs, test bacteria is coagulase positive and no clumping or the cell suspension appears similar to control, it is negative for coagulase.

Questions

1. What is the use of salt mannitol agar for finding normal skin flora?
2. What is coagulase and how is it detected in the laboratory?
3. Why do large number of organism fail to colonize skin?
4. Which antimicrobial factors of skin protect it from infection?
5. How can you differentiate *Micrococcus* and *Staphylococcus*?

Exercise 66: Laboratory diagnosis of urinary tract infection (UTI)

UTI is the active infection of any site of the urinary tract beyond the distal urethra, which normally is bacteriologically sterile. Causal agents include a number of agents that gain access to urinary tract. These may reach the UT through ascending route or by haemotegenous route. Mechanical factors that disrupt the urine flow or complete emptying of the bladder, capsular antigens, hemolysins, urease, adhesion to uroepithelium, introital colonization, renal calculi, ureteric reflux, tumors, pregnancy, urinary bladder stones, loss of sphincter control, prostatic hyper trophy and short urethra in females are some predisposing factors to UTI. The diagnosis of urinary tract infection can be made on the basis of clinical findings and microscopic examination of urinary sediment and quantitative results from culture of urine. Mid stream urine (MSU) is ideal specimen for diagnosis of UTI. First part of urine washes away the surface commensals from the distal urethra and hence MSU indicates the actual bacteriological picture of urinary tract.

Urine may be obtained by the clean catch voided mid stream procedure or suprapubic aspiration directly from the bladder. The transport of sample to the laboratory must be immediate or store at 4°C, since the urine is a very good culture medium. Keeping urine at room temperature for several hours may increase the bacteria many folds and may result in erroneous results, count being reported as significant (bacterial count greater than 10^5/ ml urine). Three consecutive early morning specimens or 24-hour urine samples are to be collected and delivered to laboratory for the diagnosis of urinary tract tuberculosis. Urine is centrifuged and deposit is examined for the presence of pus cells, erythrocytes and bacteria. Presence of bacteria and pus cells indicate UTI while bacteria in absence of pus cells may be due to contamination. Detection of erythrocytes is suggestive of damage or trauma to the urinary tract making it more prone to bacterial infection. Conventionally urine sample is streaked on plating media with special calibrated loop. Following incubation an estimate is made about the number of organisms per ml. On determining bacteriuria, identification of infectious organism is accomplished.

Requirements

a. Mid stream urine sample
b. Blood agar plates
c. MacConkey's agar plates
d. Urine dip slides
e. Calibrated inoculating loop
f. Centrifuge, microscope and microscopic glass slides
g. Dilution blanks and sterile pipettes

Procedure

Calibrated loop streak method

1. Collect the MSU sample in sterile container after the periurethral area has been cleaned with mild detergent. Macroscopic examination: soon after the specimen is received in the laboratory, note down its color, deposit or sediment, pH and turbidity if any prior to centrifugation. The development of microbes such as *Proteus* may make the pH alkaline that is injurious to white blood cells.
2. Inoculate and streak the blood agar plate and MacConkey's agar plate with 0.01 ml well mixed urine sample using calibrated loop.
3. Invert the plates and incubate at 37°C for 24 h.

4. Examine the plates for the kind of bacterial growth and count the number of colonies on each plate. If the count is significant proceed for identification and antibiogram determination of isolate.

Pour plate method

1. Macroscopic examination: soon after the specimen is received in the laboratory, note down its color, deposit or sediment, pH and turbidity if any prior to centrifugation. The development of microbes such as *Proteus* may make the pH alkaline that is injurious to white blood cells. In case delay is unavoidable, refrigerate the sample.
2. Mix the urine sample by swirling the liquid and then pipette 1ml into 9ml sterile saline solution, mix it and transfer 1ml to another 9ml blank and mix. Dilute the urine once more this way; it would give serial dilutions of 1:10, 1:100 and 1: 1000.
3. Using Eppendorf set transfer 100 µl onto preincubated or surface dried MacConkey and trypticase soy agar of 1:10, 1:100 and 1: 1000 dilutions. Spread it onto the surface of agar with sterile glass spreader.
4. Incubate the plates at 37°C for 24h. Count the colonies on each plate where the count is between 30-300 colonies.

Using rapid screening methods (UTI)

Microstix^{3R} is a reagent strip manufactured by Ames Company. The strip tests nitrate and total bacterial load and gram-negative bacterial counts in urine. Bactercult^{R} manufactured by Wampole labs, Bactercult is a sterile disposable plastic tube that is coated on inner surface with a special nutrient indicator culture medium suitable for detection of bacteriuria and presumptive identification of urinary enteric bacteria. Urine dip slides contain differential medium on one side and CLED (cystine lactose electrolyte deficient) a non-inhibitory on other side

Procedure

1. Mix the urine sample well, remove the cap from Bactercult^{R} tube and place it on a clean surface upside down.
2. Pour approximately 10ml urine into tube care not to touch the inside of the cap or tube, pour out the urine into the discard jar kept on the bench, allowing a few seconds to drain the tube completely.
3. Rotate the tube to spread the sample to the entire surface. After drainage of urine from the tube, discard the residual urine with slight jerk of twist while holding the tube in inverted position. Replace the cap and incubate in 37°C incubator.
4. Count the colonies on each side. Find out the average count. Count 25 or less the urine is normal, between 25-50 it is of doubtful significance and exceeding 50 indicates positive bacteriuria.

Questions

1. What is significant bacteriuria?
2. Differentiate amongst vaginitis, cystitis, glomerulonephritis, pyelitis and pyelonephritis.
3. What is haematuria?
4. How can the urine sample be preserved in case sample is delayed for more than one hour?
5. Which are the organisms usually encountered as causal agent of UTI?

Exercise 67: Animal bleeding

The following procedures can be used for bleeding the animals depending upon the type of animal and the blood requirement: retro orbital plexus puncture, venous puncture and cardiac puncture.

(i) Retro orbital plexus puncture : This method is particularly used for obtaining blood from small rodents like rats, mice, hamsters and guinea pigs etc. The procedure yields about 0.5-5 ml blood repeatedly at 1-2 weeks interval from the immunized animal.

Requirements

a. Experimental animal: mice or rat
b. Ether for anaesthizing rat
c Heparin
d. Capillary tubing or Pasteur pipette with long capillary and test tubes
e. Jar with lid and platform for anaesthesia.

Procedure

1 Hold the mice against the wire grid under the left hand and restrain by holding tail with right hand. With the help of fore finger and thumb of left-hand hold the scalp of neck.
2. Place the animal in the jar containing a small swab of cotton soaked with ether and cover the jar with lid immediately. Wait until the animal is anaesthetized and remove it immediately.
3 Hold the animal in left hand by the skin on the neck, using the left index finger and thumb pull the loose skin on the head and adjacent to the right eye tightly so that the eye protrudes slightly from the socket.
4. Now insert the end of the capillary tubing into the orbital cavity at the lower inside of the corner of the right eye.
5. Slide it along the eyeball at 45° angle gently in the inner angle of the eye with a little rotation to the ophthalmic venous plexus that lines the back of the orbit.
6 Withdraw the capillary tubing slightly freeing the end. The blood will rush in the tubing by the capillary action. Transfer the blood in the test tube.

(ii) Venous puncture: This method is used to draw blood from rabbit (ear vein), rats and mice (tail vein), sheep (jugular vein) and chickens (wing vein).

Requirements

a. Rabbit
b. Xylene
c Tubes for collection of blood
d. Razor blade
e. Box for holding rabbit syringes and needles

Procedure

1. Place the rabbit in the box so that its head is held outside the collar hole of the box.
2. Hold the ear with left hand and shave the skin over the marginal vein of the right ear. For successful bleeding, dilate the vein by rubbing the ear with cotton swab soaked in xylene or by warming the ear.

3. With a quick stroke of the razor cut the marginal vein transversely. Allow the blood to drip in the collecting tube. If the blood stops flowing, a flick of finger against the vein may start its flow again.
4. The more relaxed the rabbit the more rapid will be the blood flow. Approximately 3-4 ml blood can be collected by this procedure.
5. Stop the flow by placing a small piece of rolled cotton over the cut and firmly pressing the area of the cut between thumb and index finger. Bleed the chickens by puncturing the wing vein or the jugular vein.

(iii) Cardiac puncture: This procedure is used for collection of the blood from rabbit, chickens and guinea pigs for withdrawing large amount of blood.

Requirements

a. Rabbit, chicken or guinea pig
b. Ethyl alcohol 70%
c. Sterile centrifuge tube
d. Sterile syringe and needle 18G
e. Razor for shaving, rabbit board, cotton or square gauze

Procedure

1. Secure the rabbit in supine position on special board by fastening the hind legs by a slip knot placed above the anklebone or take the help of another person so that rabbit is held in an immobile position. This is because slight mobility of rabbit may rupture the heart and result in death of the animal.
2. Now shave the rabbit over the thorax about 5 cm below the sternum. Wipe the area with 70% ethyl alcohol.
3. Prepare the syringe for bleeding by fixing 21G needle on it and ensure that the needle is not blocked and working properly. Holding the syringe parallel to the mid line insert the tip of the needle through the gap between the last sternal rib on left side of mid line and xiphoid process.
4. Press the needle into the heart with a quick thrust as soon as pulse of heart is felt. Withdraw or advance the needle till the blood starts flowing into the syringe. Maintain the needle in the same position and with draw the syringe plunger to collect the desired amount of blood.
5. Now remove the syringe and transfer the blood into the test tube.

Questions

1. Why do you rub the rabbit ear prior to taking blood from marginal vein?
2. Name the sites of venous puncture in chickens, sheep, human and rat?
3. What precautions are necessary for cardiac puncture?
4. What is the maximum amount of blood that can be taken from orbital plexus?

Exercise 68: Preparation and preservation of plasma and serum

Bleeding of animal should be done prior to feeding for preparation of clear plasma or serum that has low lipid content. Blood is collected with or without anticoagulant. Anticoagulants like sodium citrate (3.8%), EDTA (2%), ammonium oxalate (1.2%) or potassium oxalate (0.8%) dissolved in physiological saline are added at one-tenth volume of blood. Heparin is added at 10-15 µl / ml blood. Serum is the fluid part of the blood and is collected after the blood has been allowed to clot. It differs from plasma that is prepared from unclotted blood containing anticoagulant. Serum is devoid of all blood clotting factors. Prior to storing plasma or serum it should be decomplemented by incubating it at 56°C for 30 min. Prepare serum or plasma from sterile blood under aseptic conditions or preserve it by adding preservatives like sodium azide-0.1%, merthiolate-0.01% or hydroxyquinoline sulfate-0.00001%. It may be stored after filter sterilization, by freezing or lyophilization. Serum can be prepared from plasma by clotting fibrin present in plasma by adding calcium chloride and warming it to 37°C. Two ml of 10% anhydrous $CaCl_2$ is added to 10 ml, 12.5 and 16.5 ml plasma containing sodium citrate, EDTA and oxalate as anticoagulant respectively. Break the clot after 2-4h and centrifuge at 3,000g for 30 min at 4°C.

Requirements

a. Blood sample with and without anticoagulant
b. Sodium azide or merthiolate
c. Centrifuge
d. Pasteur pipette

Procedure

Serum

1. Draw the blood and allow it to clot at room temperature for 1-2 h by keeping the tubes in slanting position.
2. Carefully separate the clot from the wall of the test tube by using either an applicator stick or Pasteur pipette or thin metal spatula. Avoid hemolysis as it leads to degradation of immunoglobulins by enzymes
3. Aspirate the fluid collected above the clot. Centrifuge it at 2000-3000 g for 10 min. Aspirate the supernatant and collect it in another tube.
4. Serum can be stored in frozen condition for months. It is advisable to add preservative in serum to avoid any microbial or fungal growth. Repeated freezing and thawing destroys many components of serum. Hence the serum must always be stored in small lots in deep freeze (-80°C). It can be stored without any preservative in deep freeze. Serum can also be lyophilized and stored as powder.

Plasma

Similar procedure is employed for preparation of plasma.

1. Blood-containing anticoagulant is centrifuged directly at 2000-3000 g for 10 min.
2. Aspirate the supernatant and collect it in sterile test tube. The deposit may be discarded or may be used for preparing red blood cell suspension.
3. Store and preserve plasma using the preservative and conditions applicable for serum.

Questions

1. How does the blood clot?
2. What is the difference between serum and plasma?
3. Can the serum be prepared from plasma? If yes, how?
4. Name the blood-clotting factor that initiates the clotting process.
5. Why do we store the serum in small lots?

Exercise 69: Separation of immunoglobulins

The immune system has two major tasks: location and elimination of the infectious agents or altered cells of the body and prevent it from subsequent attacks. It is called immunity and the molecules that induce an immune response are called antigens. When a person or animal becomes immune to a disease, the immunity is largely due to the development within the body of substances capable of destroying or inactivating the causative agent of the disease.

There are two types of immune responses; humoral and cell mediated. Humoral is mediated through antibodies (**immunoglobulins**) and the cellular mediated by immune cells, the T lymphocytes that locate and destroy the abnormal cells i.e. cell harboring pathogen or expressing altered cell surface antigens. Cells of immune system comprising B-lymphocytes and T lymphocytes respond specifically against organisms that bypass the host defenses by producing **humoral** and **cellular immune** responses. Antibodies are the proteins that appear in serum following exposure of animal to foreign substances such as infectious agents. Immunoglobulins can be separated from serum using fractional precipitation using ammonium sulphate or sodium sulphate.

Requirements

a. Saturated ammonium sulphate
b. Serum

Procedure

1. Take 5 ml serum in a test tube. Add 5 ml saturated ammonium sulphate to it and mix thoroughly.
2. Let it stand at room temperature 5 min and then centrifuge at 3000 g for 10 min. Discard the supernatant containing albumin and suspend deposit in 5 ml distilled water and again add 5 ml ammonium sulphate and mix again.
3. Now, centrifuge again. Discard the supernatant, dissolve the deposit in distilled water, and dialyze it against sufficient distilled water overnight.
4. Take out the contents in centrifuge and centrifuge it. The deposits represent the pure immunoglobulins.

Questions

1. What are immunoglobulins?
2. Which is the immunoglobulin present at mucosal surfaces?
3. What are reagenic antibodies and what is their significance?
4. What do you understand by hypersensitivity reactions?
5. What are autoimmune diseases?
6. What percentage of ammonium sulfate is used for separation of immunoglobulins?
7. How can you ascertain the purity of immunoglobulins?
8. From these immunoglobulins how can IgG and IgM be separated?

Exercise 70: Agglutination reaction

The phenomenon of agglutination (clumping) is a visible proof of interaction that occurs between particulate antigen and homologous antibody. It involves a system whereby antibody reacts with particulate antigen held in suspension e.g. erythrocytes, antigen coated on latex particles and bacteria. The test either be performed as slide agglutination or in tubes referred as tube agglutination. The test is called **direct agglutination**, if it involves the interaction of antibody with particulate, insoluble or antigens present on cell surface. **Indirect agglutination** (passive agglutination): the soluble antigen is coated on bentonite or latex particles or any other particulate matter. **Reverse passive agglutination** reactions: In this case antibodies are coated on particulate matter e.g. bacteria, red blood cells or latex particles. **Hemagglutination** represents the reaction of antibodies with antigens present on erythrocytes cell surface. Direct agglutination test can be demonstrated by reacting bacterial suspension with homologous antiserum. The phenomenon is the basis of Widal test for typhoid fever caused by *S typhi*, Brucellergen test for brucellosis caused by *Brucella* and characterization of strains using antiserum.

Requirements

a. Polyvalent "O" *Salmonella* antiserum
b. Overnight grown *Salmonella typhi* culture on slant or in nutrient broth
c. Phosphate buffered saline

Procedure

1. Place one drop of PBS on each side of microscopic slide.
2. Take a loopful of culture from the slant and mix it uniformly.
3. Place a drop of antiserum to one drop and a drop of PBS to other.
4. Mix gently and swirl the slide or rock the slide for 1-2 min.
5. Observe the agglutination in the test drop.
6. In case of broth culture, the test can be performed directly by mixing loopful of culture with a drop of antiserum.

Questions

1. What is the primary limitation for the agglutination reaction?
2. Where is this phenomenon used for diagnosis of infection?
3. Why do the clumps settle to the bottom?

Exercise 71: Haemagglutination and blood grouping

Agglutination reactions occur when particulate antigens react with specific antibodies. Surface antigens present on cells or antigens coated on particulate matter such as latex particles are particulate antigens. Agglutination of these antigens results in clumping of cells. When the cells involved are red blood cells, the reaction is called haemagglutination. Haemagglutination reactions are used in blood group typing.

Presence or absence of blood group (carbohydrate) antigen A, B or both antigens located on red blood cells is determined with specific antiserum. Haemagglutination occurs only when homologous antigens and antibodies react. Individuals with blood group O do not show any haemagglutination when reacted with either anti-A or anti-B serum. Individuals possess antibodies to alternate antigen.

Another important blood antigens present on human red blood cells is Rh antigen (a complex of many antigens) also called Rh factor. Individual is Rh positive if this antigen is present and Rh negative if it is absent. This antigen is detected by haemagglutination reaction between red blood cells and anti-D antiserum. Antibodies against Rh factor are not present in Rh-negative individuals.

Requirements

a. Cotton moistened with 70% alcohol
b. Anti-A, Anti-B and Anti-D antisera
c. Glass slides
d. Toothpicks or sterile lancets

Procedure

1. With a glass marking pencil mark circles at both the end and in the center of a clean glass slide and label as A, B and D.
2. Disinfect your middle finger with cotton saturated with alcohol. Pierce the disinfected finger with sterile lancet.
3. Put a drop of blood into each circles marked with pencil on the slide (at both ends and in center). Stop the bleeding with sterile cotton and apply adhesive bandage.
4. In sequence add one drop each of Anti A, B and Anti D antiserum respectively to circle A, B and D.
5. Mix each suspension with separate toothpick, look for agglutination, and find out the blood group.

Questions

1. Compare haemagglutination and agglutination?
2. What is the hemolytic disease of newborn and how does Rhogam prevent it?
3. Why the blood group O persons are considered universal donor?
4. What is the origin of antibodies to ABO blood antigens other than present on individual's red blood cells?

Exercise 72: Ouchterlony's immunodiffusion technique

A precipitation reaction involves the reaction between antibody and soluble antigen. Amongst antigen antibody reactions, it is the least sensitive technique for the detection of antigens. The precipitation reaction may be demonstrated by various techniques: ring test in which one reagent is layered over the other in a test tube and precipitate in the form of ring occurs at the interface. Precipitation or flocculation produces a flocculent precipitate in tube or on glass slide. Precipitation reaction can be seen in gels and the technique is called immunodiffusion. Immunodiffusion carried out using electric current is called immnunoelectrophoresis. Counter current immunoelectrophoresis (CIEP) involves the electrophoretic diffusion of antigen and antibodies placed in opposite direction.

Precipitation reaction most commonly used in laboratory is the double diffusion Ouchterlony's technique. Antigens and antibodies diffusing from opposite direction form precipitin lines at points where antigen and antibodies accumulate at optimum concentration. The technique is useful in finding identity, partial identity and non-identity among antigens when immunodiffused against antiserum. The reaction shows precipitin lines in gels, which can be preserved and stained for future reference and better visibility Both the agglutination and precipitin reactions are affected by pro zone and post zone phenomenon.

Requirements

a. Molten 1% agarose in phosphate buffered saline (0.05M, pH 7.2).
b. Gel punch with template, humid chamber
c. Gel stain: coomassie blue or amidoblack
d. Test samples: Antigen and antibody

Procedure

1. Clean the glass slides with alcohol and place them on horizontal surface.
2. Pour 3 ml molten Agarose solution on each slide avoiding any bubble formation. Let the agarose (5-10 min) set.
3. Punch out the wells 3 mm diameter from the agarose layer by placing the slide on the pattern of the wells.
4. Load the wells with antiserum in the central well and antigen in the side wells.
5. Keep the slide in humidified diffusion chamber for 48-72 h. Note the development of immunoprecipitate bands daily. Stain them with 0.1% amidoblack or coomassie brilliant blue for improved sensitivity.
6. When the precipitates become optimally visible, wash the slides in normal saline with several changes to remove unprecipitated proteins. Dry the gel at room temperature by keeping Whatman paper over it without trapping the air in-between or dry the strip at 120-130°C for 15 min.
7. After drying remove the filter paper by slightly wetting and immediately immerse in staining solution for 10 min. transfer the slides to destaining solution (5% acetic acid) and change the destain 2-3 times till dark sharp bands are clear.

Questions

1. What are precipitins?

2. What kind of serological reaction will occur if homologous antibodies are mixed with bacterial cell suspension, flagella, leucocytes, capsular polysaccharides and bovine serum albumin as antigens?
3. What kind of antibody you think will be produced if a horse is injected tetanus toxoid?
4. What will happen if antigen antibody concentration is very high?
5. Why is humid chamber used?

Exercise 73: Electrophoretic separation of serum proteins

Immuno-electrophoresis combines electrophoretic separation, diffusion and immuno-precipitation of proteins. Both identification and fixation or approximate quantification can be accomplished for individual proteins in serum, urine and other biological fluids. Proteins carry charge at pH other than their isoelectric point. Above the isoelectric point they are negatively charged and below that they are positively charged. Hence in an electric field they move differently depending upon the net charge they carry in a given buffer system. The technique introduced by Graber and Williams (1955) is often used in the analysis of serum for detection of abnormal serum components.

Requirements

a. Tris-HCl buffer, pH 8.6 (Tris 15 g, glycine 25 g, adjust pH with HCl and make volume to 3 L).
b. Molten 1% agarose prepare in the same buffer
c. Human serum and rabbit anti human serum samples (50-60 µl)
d. 1% amido black prepared in 1% acetic acid
e. 10% glacial acetic acid
f. Wicks of filter paper and gel puncher with template
g. Electrophoresis apparatus and DC power supply

Procedure

1. Carefully layer 3 ml molten agarose on clean glass slide keeping pipette vertically in the center of slide. Allow agarose to solidify. Punch two wells 1 cm apart towards cathodic side with gel punch.
2. Fill the tank with Tris-HCl buffer (pH 8.6) keeping the level same in both the compartments. Arrange the slides in the electrophoretic chamber and connect the ends of the slide with the buffer tank with the help of filter paper wicks (2x5 cm each).
3. Start the pre run for 15 min by applying constant current (7.5 mA per slide). Stop the current and apply serum sample to cathodic end. Connect the electrodes to power supply and run electrophoresis for 45-90 min at constant current of 7 5 mA per slide
4. Disconnect the power supply. Cut a trough 1mm width along the center of the slide leaving the two wells on either side of the trough. Fill the trough with antiserum and leave the slide in humid chamber for 24-48 h for precipitin lines to develop.
5. *Staining*: Pre rinse the slide with normal saline and then with distilled water. Cover the slide with moistened filter paper and dry in oven at 40°C. Peal off the paper carefully and stain with amidoblack for 30 min without agitation.
6. *Destain* by giving washings of six min each with 10% glacial acetic acid till background is clear but dark sharp bands are clear. Dry the slide and store.

Questions

1. Why is high pH 8.6 buffer used during electrophoresis?
2. Why are holes punched towards cathode side?
3. How does IEP of normal serum, serum from agammaglobulinemia and myeloma patients differ?

Exercise 74: Counter current immnoelectrophoresis (CIEP)

In Counter current immnoelectrophoresis (CIEP) two parallel wells on either end of agarose gel are cut and filled one with antigen and other with antibody solution. Then voltage is applied across the gel so that the antigen and antibody move towards each other at a faster rate. The antigen migrating towards anode and antibody moving in opposite direction interact with each other and form precipitin lines.

Requirements

a. 1% agarose in Tris-HCl buffer pH 8.6 (Tris 1.21g, 12.2 ml HCl 0.2M, NaCl 4.0 g dissolve in 50 ml distilled water and made to 200 ml).
b. Normal saline.
c. 1% amidoblack or 0.1% coomassie blue.
d. 2% acetic acid
e. Humid chamber
f. Electrophoretic apparatus with power supply
g. Whatman filter paper No 3
h. Template and gel punch

Procedure

1. Pour 3 ml of molten agarose solution on each clean microscopic slide and allow it to solidify.
2. Fill the tank with Tris HCl buffer pH 8.6 keeping the level same in both the compartments. Arrange the slides in the electrophoretic chamber and connect the ends of the strip with the buffer tank with the help of filter paper wicks.
3. Start the pre run for 15 min by applying constant current (7.5 mA per slide). Stop the current. Apply 5-10µl each of antiserum to anodic well and antigen in cathodic well respectively. Connect the electrodes to power supply and run electrophoresis for 45-60 min at constant current of 7.5 mA per slide.
4. Disconnect the power supply and place the slides for 12 h in humid chamber.
5. Examine the slides for precipitin band. For better results the slides may be stained as explained earlier.

Questions

1. Is this technique suitable for all the detection of all kinds of antigens?
2. What are the advantages of using CIEP over IEP?
3. What will happen if antigen and antibodies are misplaced in holes?

Exercise 75: Enzyme linked immunosorbant assay (ELISA)

Enzyme linked immunoassays are versatile and sensitive and provide an important analytical procedure that can be used either for identification and titration of antigen or antibodies. Assay can be performed on tissues, plates; strips and nitrocellulose strips (dot ELISA). In these assays an enzyme is attached to an antibody molecule and the presence of bound antibody is detected by adding the chromogenic substrate for the enzyme. The antibody linked enzyme in immune complex releases colored product from chromogenic substrate that can be detected using colorimeter. The test is performed as a double antibody technique for the detection of test antigens or as an indirect immunosorbent assay for the detection of the test antibodies. In double antibody system the unlabeled antibody is adsorbed onto the inner surface of the plastic well in the microtiter plate. Unbound antibody is washed and then specific test antigen is added to the well. If the antigen is homologous it will bind and form immune complex with antibody adhering to the wall of the well and this complex will not be removed in subsequent washings for removal of any unbound antigen. Then enzyme-linked antibody specific for the antigen is added. This labeled antibody will bind to the test antigen in immune complex (antibody-antigen) forming an antibody-antigen-antibody-enzyme complex. Unbound enzyme labeled antibody is again removed by washing. This is followed by addition of a substrate that is capable of producing a colored end product upon its reaction with the enzyme. That may be compared visually or measured spectrophotmetrically.

The indirect immunosorbant assay is similar to double antibody technique in that it requires the use of an enzyme-linked antibody. Antigen is adsorbed onto the inner surface of the well. ELISA is used for the detection of AIDS, influenza, respiratory syncytial viral infections, rubella, syphilis, brucella , cholera, salmonellosis and detection of drugs in tissues.

Requirements

a. Antigen
b. Microtiter plate
c. Antisera
d. Carbonate bicarbonate buffer, 0.05M, pH 8.6
e. PBS, pH 7.4 containing 1% BSA and PBS, pH 7.4 containing 0.05% Tween 20 (PBST)
f. Enzyme conjugate peroxidase conjugated to anti rabbit immunoglobulins diluted in PBST
g. Substrate O-phenylenediamine (OPD) solution: Substrate O-phenylenediamine 2mg/ ml plus hydrogen peroxide (0.01% in citrate phosphate buffer pH 5.0 - 28 ml 0.1M citric acid + 22 ml 0.2M $Na_2 HPO_4$ + 50 ml H_2O_2)
h. 2 N H_2SO_4 to stop reaction.

Procedure

1. Adjust the antigen concentration 0.5-5 µg/ ml in carbonate-bicarbonate buffer.
2. Add 100µl of diluted antigen in each well and incubate it for 2 h at 4°C or at room temperature in a humid chamber.
3. Remove the unbound antigen and wash the wells thrice thoroughly with wash buffer (PBST) and invert the plate.
4. Add 100 µl PBS containing 1% BSA (casein. or skimmed milk) solution to each well to block non-specific protein binding sites. Incubate plate for 2 h at room temperature.
5. Wash the plates thrice with PBST.

6. Add 100-µl antibodies to each well and incubate the plates for 2 h at room temperature in a humid chamber. Wash plate thrice with PBST.
7. Add 100-µl enzyme (peroxidase) conjugate. Incubate plate at room temperature for 2 h. Wash plate thrice with PBST.
8. Add 100 µl of diluted freshly prepared substrate to each well and incubate at room temperature for 30 min. in dark.
9. Stop the reaction by adding 50 ul of 2N sulphuric acid.
10. Measure the absorbance in a micro ELISA reader at 492 nm.

Questions

1. Name some of the enzymes, which are suitable for enzyme immunoassays.
2. List the merits and demerits of ELISA over other immunodiagnostic techniques.
3. What reaction is catalyzed by peroxidase enzyme used in this exercise?

Exercise 76: Routes of immunization

An antigen can be administered into experimental animal through various routes considering the nature of antigen and the kind of immune response expected. Antigen may be introduced via various routes e.g. intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous. Experimentally the rabbits give excellent IgG response to variety of antigens with long term Freund's adjuvant immunization. It is advisable to always screen the animals prior to immunization for the immune response and inject animal in-groups to avoid any variance in animals. Animals must always be maintained in proper conditions without stress. Polyclonal antisera are the conventional serum product of an immunized laboratory animal usually the rabbit, mice, rat, sheep and horses. Larger animals are preferred for bulk production of antisera.

Intradermal route: This route is used for injecting viscous and slowly dispersing antigens e.g. antigens emulsified with Freund's adjuvant. It provides rapid access to the lymphatic. Antigen is injected into flank or back of rodents, side of neck in sheep/goat, into ears in pigs and forearm of higher primates.

Procedure

1. Prior to injection shave the site of injection with fresh blade avoiding any abrasions.
2. Disinfect the area with 70% alcohol. Hold the fold of skin between the forefinger and thumb. Insert needle into the dermis parallel to the skin surface about 5-7 mm deep taking care that the tip of the needle is just visible below the surface.
3. Release the skin fold holding the needle firmly; inoculate a maximum volume of 0.05 ml antigen. Withdraw needle compressing simultaneously along the track of the needle with fore finger and thumb.
4. *Caution*: While withdrawing the needle after inoculation do not relax the plunger of the syringe as some of the inoculated antigen may be withdrawn in the syringe. A hard pea like swelling at injection site indicates true intradermal injection.

Subcutaneous: This route is suitable for emulsions, precipitates and viscous material and the antigens are spread a little more as compared to intradermal mode of injections. Antigens are absorbed slowly as compared to intraperitoneal or intravenous route. Free movement of needle in an arc below skin ensures subcutaneous injection. After injection pinch the area to avoid antigen leakage.

Procedure

1. Remove the hairs from the thorax area by clipping shaving or plucking.
2. Scrub the area with 70% alcohol. Raise a tent with thumb and index finger of one hand.
3. With other hand insert the needle deep underneath the skin just parallel to the underlying muscles.
4. Inject the material and withdraw the needle.

Intramuscular : This route is commonly used for injecting alum-precipitated antigen and absorbed antigen. Antigen is deposited in muscular region of thigh. Needle is inserted from the rear to midway along the femur. After the injection gently massage the injection site.

Intraperitoneal : Injection into intraperitoneal cavity immediately attracts the attraction of macrophages and monocyte. It is most suitable site for injecting particulate antigens and Ag that need processing. Hold the mice as in case giving subcutaneous injection and make injection into abdomen about 6 mm. deeper injections may cause damage to organs. Pinch the injection site after withdrawl of needle.

Intravenous : In this case antigen is injected directly into vein. This route is preferred for particulate antigens but not suitable for viscous and non-miscible antigens. In rodents tail vein is used for intravenous injections.

Procedure

Rabbit

1. Marginal ear vein is used. The hair is removed from the region by clipping, shaving or plucking.
2. Scrub the area with 70% alcohol. Flicking the ear a few times to produce hyperemia.
3. Compress the vein at the base of the ear and hold the ear horizontal in one hand with other hand insert the needle as parallel as possible to the vein toward the head of the animal.
4. Inject slowly, the material will pass freely into the vein and will cause blanching in the vein. If the needle is not in the vein a blanched area will result in the neighboring tissue. For initial inoculation, start as near to the tip of the ear as possible. After inoculation withdraw needle compress puncture with cotton pledget for some time.
5. If abnormal breathing or collapse should occur during inoculation or within 5 min thereafter notify the instructor immediately.

Footpad

This method is also suitable for injecting particulate and cellular antigen in mouse, rat, and guinea pig but less preferred than other routes, because some antigens given by this route may cause granuloma formation that renders the animal immobile. Inoculations are made in hind foot as the animal uses the forepaws for holding food. Clean the foot with 70% alcohol prior to injection and inject the material (25-50µl) either from the distal or proximal end to about 5 mm deep into footpad.

Questions

1. Explain the factors that may affect the immune response?
2. What criteria will you consider prior to selecting animal for immunization?
3. Why do antigens administered by different routes?
4. Why is the immune response different to same antigen given by different route?

Exercise 77 : Differential leukocyte counts of blood

The differentiation of leukocytes is based on the size and shape of nucleus and cytoplasmic contents staining. Out of several diagnostic tests differential leukocyte counts of blood is the most common. The test provides valuable information about the differential diagnosis of bacterial and viral infections. Neutrophil count is usually higher with some exception during bacterial infection and monocyte counts in viral infections. The normal leukocyte blood profile contains 50-70% neutrophils, 20-30% lymphocytes, 2-6% monocyte, 1-3% eosinophils and about 1% basophils. A blood smear is stained either with Wright's stain or Leishman stain. These stains differentiate the granuulocytes (polymorphonuclear leukocytes, basophils and eosinophils), agranulocytes (mononuclear leukocytes) and thrombocytes (platelets).

Requirements

a. 70% ethyl alcohol
b. Sterile lancet for pricking
c. Container with disinfectant
d. Wright's stain or Leishman stain

Procedure

Wright's staining

1. Disinfect the tip of middle finger with cotton moistened with 70% ethyl alcohol.
2. Prick the end with sterile lancet and press it gently while keeping the finger in downward position. Touch the blood drop oozing out from the prick to one end of clean glass slide.
3. Place the slide on the table with blood drop side up. Wipe the punctured area with cotton dipped in alcohol and hold it gently until the bleeding stops.
4. Take another slide and place it along the drop of blood and allow the blood to spread to edges of the spreading slide. Hold the spreading slide slightly slanting position ($45–50^0$) and push the spreading slide other end of blood drop slide rapidly to form a thin smear with no ridges.
5. Allow the smear to dry. Cover the smear with Wright's stain and let it stain for 4 min.
6. Dilute the stain 1:1 with distilled water and continue staining for another 10 min. with occasional shaking in-between.
7. Wash the slide gently under running tap water. Blot dry.
8. Examine under 40x or using oil immersion objective.
9. Count at least 100 white blood cells and make a record of each type of leukocyte seen. Calculate the percentage of each cell types recorded by you.

Leishman staining

1. Place the slide on the staining rack.
2. Cover the smear with Leishman stain and allow it to act for 2 min.
3. Dilute the stain on the smear with distilled water and allow the diluted stain spread upon the edges and leave the stain for 10 min.
4. Wash the slide with distilled water. Dry and examine under microscope at 40x and 100x using immersion oil.

Questions

1. What is the importance of differential leukocyte count of blood?
2. How does the DLC count differ in viral and bacterial infections?
3. What are granulocytes? How do they differ from macrophages?

Exercise78: Use of hemocytometer for counting blood cells

Hemocytometer (counting chamber) developed for counting blood cells is also used for counting different kinds of cells including bacterial and yeast cell suspension. Neubauer or Petroff Hausser hemocytometer is 0.1mm deep and can hold 0.1 cubic mm fluid spread over an area of 1 sq cm. The counting chamber has 9 squares, each having an area of $1mm^2$. One square per microscopic field is visible under 100x Objective. Each corner squares has sixteen subdivisions. Each central square ($1mm^2$) has 25 subdivisions (0.2x0.2mm) wherein each subdivision is further subdivided into 16 small squares (0.05x.0.05mm). Each subdivision square in central area is further subdivided into 16 small squares. Each corner i.e. 16 big squares consist of $1mm^2$ area and similarly the central 25 squares in total occupy $1mm^2$. The depth of field in the space between the counting grid and the cover is 0.1mm. Each $1mm^2$ is divided into 25 medium sized squares.

Charging of hemocytometer: Prepare the hemocytometer by placing the cover slip on the counting grid so that sides of the cover slip touch on both sides of the lateral support of the chamber. From one side put the suspension and count the number of the cells in the squares. For large cells e.g. WBC counting is done in corner big squares. For small cells e.g. RBC counting is done in small squares.

Questions

1. Can this technique be used for determination of bacterial or yeast counts?

Exercise 79: Total leukocyte count in peripheral blood

Blood cell count determination provides valuable information about the ongoing disease processes in the body. In some infections the cell count dwindles indicating alteration of physiological conditions. The test is very simple but provides significant information about disease states like leukemia and leukocytosis even in persons apparently looking very healthy.

Requirements

a. Thoma pipette for counting WBC
b. Hemocytometer
c. Turk's fluid (2% acetic acid containing methylene blue)
d. Microscope

Procedure

1. Draw the blood in a thoma pipette containing white bead up to the mark I and dilute it with Turks fluid up to mark II. This effects a dilution of 10 times to the blood.
2. Shake the pipette and fill the hemocytometer with a cover slip on it with the above-diluted fluid. Count the number of the cells in the corner chambers (in all the16 big squares).
3. Calculate the cell concentration per ml by the following formula:

$$\frac{\text{Total number of cells counted} \times \text{Dilution factor} \times 10^{4}}{4}$$

Questions

1. What is leukopenia and leukocytosis?

Exercise 80: Red blood cells count in blood

Blood contains three main components the fluid, the clotting agents and the cells. The cells present broadly are grouped as WBC and RBC. Red color of the blood is due to the presence of red blood cells. These cells originate in body from two different pathways and perform important functions. During certain ailments in body the number of these cells dwindles. Hence counting the number of these cells constitutes an important diagnostic test.

Requirements

a. Thoma pipette with red bead for counting RBC
b. Hemocytometer
c. Microscopic slides
d. Gover's fluid
e. Microscope

Procedure

1. Fill the thoma type blood-diluting pipette for red cells to 0.5 mark. Wipe off the excess blood and dilute the blood in pipette with Gover's fluid to 101 mark above the bulb.
2. Remove the rubber tubing from the pipette and shake the pipette to form a uniform suspension. This dilutes the blood 1:200. Charge the hemocytometer as explained above with a drop of RBC suspension.
3. Set the hemocytometer under the microscope and focus the central square. Count the cells in large squares of central area (squares a, b, c, d and e shown in fig).
4. Calculate the cell suspension concentration per cubic centimeter as below:
   Total number of RBC counted (in 5 squares) x 5 x 200x 10^4 = RBC / ml blood
   RBC in central square consisting of 25 squares x dilution x10^4 = RBC / ml blood

Questions

1. What is anemia?
2. What is leukopenia and leukocytosis?
3. How do RBC generate in body?
4. What will happen to RBC if instead of PBS distilled water is added as diluent?

Exercise 81: Separation of lymphocytes from peripheral blood

Lymphocytes are the immune cells that comprise the basic unit of immune system. These are present in billions in the body in blood and lymphoid organs. The number, distribution, the lymphocyte products and the type of lymphocyte present in body reflect the immune status of the individual. Immune surveillance is the function of circulating lymphocytes. Different kinds of lymphocytes small and large granular lymphocytes perform different functions. Morphologically lymphocytes are distinct from other cells. Lymphocytes possess a distinct large dark stainable nucleus with just a rim of cytoplasm. Lymphocyte separation constitutes an important exercise useful in many diagnostic procedures and research. Most common technique used for separation of lymphocytes from blood is the density gradient method beside cell electrophoresis using flow cytometry.

Requirements

a. Minimum essential medium Eagle (MEM) pH 7.4, 10ml
b. Human blood 10 ml and Heparin 250 IU.
c. Gradient containing 1 ml 33% sodium metrizoate and 2.4 ml 8% Ficoll (density 1.08) 3.4 ml or Ficoll hypaque density gradient 1.077 g/m or Histopaque from Sigma)
d. Trypan blue 0.1% in NS
e. Centrifuge
f. Laminar flow
g. Microscope,
h. Hemocytometer
i. Pasteur pipette with long capillary

Procedure

1. Collect the blood in a tube containing heparin as an anticoagulant. Dilute it 1:2 with MEM.
2. Put Ficoll hypaque in centrifuge tube. Keep the centrifuge tube slightly in slanting position.
3. Add three volume of diluted blood along the wall of centrifuge tube gently so that it overlays on the Ficoll hypaque layer.
4. Centrifuge at 400g for 30 min at room temperature. The tube contents are separated into four distinct layers: i. plasma layer, ii. White interface lymphocyte layer, iii. Ficoll hypaque and iv. Pelleted erythrocytes and platelets.
5. Carefully separate the interface white layer with Pasteur pipette and collect white ring of lymphocytes in separate test tube. Suspend the lymphocytes in 2 ml MEM.
6. Wash the lymphocytes thrice with MEM medium before use.
7. Count the lymphocytes in a hemocytometer. Adjust the concentration to 10^6- 10^7 per ml.
8. Check the viability of cells with trypan blue.

Questions

1. How can live and dead lymphocyte be distinguished?
2. Suggest an alternate method to gradient centrifugation or cell electrophoresis for separating lymphocytes?
3. Why are lymphocytes suspended in MEM and not in PBS?

Exercise 82: Determination of viability of lymphocyte preparation

Live and dead cells can be differentiated by dye exclusion method. The dead cells are stained blue with trypan blue where as live cells resist the entry of trypan blue and remain unstained.

Requirements

a Lymphocyte preparation
b. 1% trypan blue

Procedure

1. Mix one drop of cell suspension with one drop of trypan blue.
2. Incubate for 5 min. at room temperature.
3. Prepare a slide out of this suspension and check it under microscope.
4. Count the cell, which are stained blue, and also those cells that remained unstained or colorless.
5. Calculate the percent viability of cells by dividing the number of unstained cell with total number of stained and unstained cells and multiplying it by 100.

Questions

1. Why were only dead cells stained and not the live ones?
2. Can the live and dead cells be differentiated using any other test?
3. What is practical importance of this test?
4. What do you mean by morphological index?

Exercise 83: Production of antibody in experimental animals

Immune response to any foreign element involves three phases: the cognitive phase, reactive phase and the effecter phase. It is expressed either in terms of activated cells and or as antibodies The information for anamnestic response is stored in the lymphoid cells. Choice of animal, form and dose of antigen, adjuvant use, route of immunization and the immunization schedule are some factors that affect the magnitude of immune response. For raising antibodies usually inbred laboratory animals (rats, mice, chickens, rabbit and Guinea pigs) are preferred to avoid variations in immune response because of strain differences. Antibodies against toxoids and viruses for therapy are raised in larger animal like horses and sheep. Phylogenetically unrelated animals recognize and respond only major antigenic differences but for recognition of minor differences like those present in isoantigens (Rh factor human volunteer (phylogenetically related) are used who supplied the antigen.

Immune response is usually better against particulate antigens injected intravenously without adjuvant as compared the soluble antigen administered with adjuvant subcutaneously, intramuscularly or intradermally. Administered dose may vary from1ug-1mg/animal (proteins) first dose is given with adjuvant and booster dose after 1-3 months later. Frequent repeated injection of 1-100 million cell/animal of particulate antigen give good immune response. Bleed the animal 5-7 days after booster dose. Short immunization schedule generally produces highly specific antibodies and lacks immune response to antigen present in traces. Long immunization produces less specific antibodies to potent cross-reacting antigens including the antibodies against minor components of antigen mixture.

Requirements

a. Antigen: *E.coli* inoculate 50 ml broth heavily with test strain and incubate for 24 h. Add 50 ml of 0.6% formalin saline solution and store in refrigerator.
b. Horse serum
c. Freund's complete adjuvant
d. Rabbit, sheep.
e. Syringe and needles, alcohol, cotton, grease, markers, rabbit box, scalpel with fine surgical blade

Procedure

Particulate antigen

1. Immunize the rabbit as per the following schedule using inactivated *E.coli* cell suspension as antigen as an example of particulate antigen. Prior to beginning of immunization schedule bleed the animal for pre immune serum from the marginal vein.

Day	Antigen	Route
1	0.2 ml	Intravenous
4	0.4 ml	Intravenous
8	1.0	Intravenous
12	1.5	Intravenous
16	Rest	Rest
20	2.0	Intravenous
27	Bleed	Marginal vein

2. Rabbits are injected using syringe fitted with 26-gauge needle in the marginal vein slowly. Injection into vein can be ascertained by blanching in the vein.

Soluble antigen

1. Inject soluble antigen (tetanus toxoids/ BSA/ kidney antigen) using 26-gauge needle in rabbit as per schedule given below:

Kidney antigen

Time (week)	**Volume Ag**	**Route**
First	0.05 ml Ag +0.5 ml FCA	Intra peritoneally
Second	1.0 ml Ag	Subcutaneously
Third - fourth	1.5 ml Ag	Subcutaneously
Sixth	2.0 ml Ag	Subcutaneously
Seventh	-	Bleed

2. **Bovine serum albumin**: Inject rabbit with 1% BSA in saline, 0.2 ml intravenously using 26 gauge needle from day 1-9. Rest for one week and bleed on day 15th.

Questions

1. For raising antibodies why the antigens are given by different routes?
2. Why the first soluble antigen injection is given along with adjuvant and it is eliminated from the subsequent injections?
3. Why do you bleed the animal prior to using it for raising antibodies?
4. How will you immunize the animal for cellular immune response?

Exercise 84 : Isolation of pathogens from sore throat

Respiratory tract comprises two main compartments upper respiratory tract that houses a rich population of indigenous flora and the lower respiratory tract, which has its own ciliary escalator for keeping the internal parts larynx, bronchial tubes and alveoli sterile. Despite the presence of pathogens in upper respiratory tract (URT) and inhalation of large number of organisms in breath daily, very few organisms can ensue the disease process because of microbial antagonism and highly efficient respiratory efflux process of upper part, until bronchus is covered with mucous layer. The upper respiratory tract flora is never static; it is liable to change as result of environmental conditions. Throat swab microscopy is of no value unless Ludwig angina (a mixed infection caused by spirochetes and a Gram negative anaerobic fusiform is suspected.

Likely pathogens include β-Hemolytic Streptococci Lancefield group A, Staphylococci and yeasts are the major cause of sore throat (acute pharyngitis). *Hemophillus, Corynebacterium, Bordetella, Yersinia* and *Francisella* are also encountered occasionally in throat samples from children below 5 years of age and aged persons (over 50 years). Alpha and gamma hemolytic streptococci constitute the predominant normal flora of throat. *S.pneumoniae* causal agent of pneumonia in children can be differentiated from other α hemolytic streptococci based on its colonial characteristics, optochin and ethyl hydrocuprein sensitivity and bile solubility tests.

Requirements

a. Blood agar plate.
b. Blood potassium tellurite agar (BPTA).
c. Sterile swabs, spatula and candle jar.

Procedure

1. *Sample collection*: Throat cultures are taken from the rear part of the mouth, behind uvula by rotating sterile swab in the tonsil inflamed area over the mucosal surface. Avoid touching uvula or tongue. The tongue may be depressed with sterile spatula while sampling throat swab.
2. Transfer the charged swab in the same sterile test tube and send it immediately to laboratory for processing.
3. Charge the blood agar plate by firmly rolling the swab over an area of 8-10 sq cm and then swab equivalent area on Mac Conkey agar plate. Inoculate BPTA plate as well for suspected case of *Diphtheria* and prepare smear and look for volutin granule examination.
4. Using sterile inoculating loop spread the primary inoculum to entire plate to get isolated colonies. Streak heavily *S.aureus* culture in the center of blood agar across the primary and subsequent streaks.
5. Incubate blood agar plate in candle jar and MacConkey's agar in the incubator at 37°C.
6. Examine the blood agar plates for pinpoint and transparent colonies surrounded by complete hemolytic zone (β-hemolysis), slightly bigger opaque colonies with β-hemolysis and smooth shining α hemolytic colonies. Carefully look for translucent, smooth colonies around the *S.aureus* streak exhibiting satellitism. Examine MacConkey agar plate for enteric pathogens.

7. Check α hemolytic colonies for catalase, optochin sensitivity and bile solubility and β-hemolytic for catalase and bacitracin sensitivity. Opaque colonies for coagulase and mannitol fermentation.
8. *S.pyogenes* is β-hemolytic, catalase negative and bacitracin sensitive. Pneumococcus is optochin sensitive and bile soluble α hemolytic streptococcus. *S.aureus* is coagulase positive and catalase positive cocci forming irregular bunches morphologically.

Questions

1. Is blood agar a differential medium?
2. What is satellitism? Which pathogen grow as satellites around *S.aureus* streak?
3. Identify the organism isolated from 6-year-old child, which is catalase negative, α hemolytic, optochin sensitive cocci?

Exercise 85: Study of biochemical properties of *Staphylococcus*

Organisms comprise the normal micro flora of human skin, mouth, nose and throat. It causes extensive disease in patients of all groups when they penetrate the skin barrier or mucous membranes and cause abscess, boils, carbuncles, scalded skin syndrome, Reiter's disease seen in infants and impetigo contagiosum is wide spread skin disease in school going children. Toxic shock syndrome (TSS) is characterized as blood disorder leading to fever and circulatory collapse. It is caused by toxin producing strains.

Requirement

a. Bacterial culture: *Staphylococcus aureus, S.epidermidis* and *Micrococcus luteus* on nutrient agar slants.
b. Gram staining set, rabbit plasma, blood agar plate, salt mannitol agar plates. MacConkey agar plates, candle jar, hydrogen peroxide, microscopic slide, biochemical media (glucose broth, MRVP broth, mannitol, Hugh Leifson's medium, nitrate broth).

Procedure

1. Make a smear on clean glass slide from all the three cultures and Gram stain. Examine each smear for Gram reaction and morphological arrangement of cocci. In which culture did you notice tetrads and regular bunches?
2. Culture each on the plating medium provided. Incubate sheep blood agar in candle jar and other two plating media in the incubator at 37°C for 18-24 h. Transfer the inoculum from the slant to peptone water and let it incubate at 37C for 2-4 h. Use this peptone water for inoculating the various biochemicals and incubate these at 37°C.
3. Make hanging drop slide from the peptone water and examine it for motility.
4. Next day examine the colonial morphological characteristics of each on plating media, especially the β-hemolysis around the colonies on blood agar and mannitol fermentation on mannitol salt agar. Did all the three strains grow on MacConkey agar? Score each biochemical test for metabolite as described earlier under biochemical tests.
5. From the colonies, perform catalase, oxidase and coagulase test. Which culture did you find coagulase positive? Did you find the strain coagulase positive for both, slide as well as tube coagulase? Did you find any difference in their oxidase and catalase activity as well?
6. Record results of all the tests and identify the cultures based on their biophysical and physiological characteristics.

Questions

1. How would you differentiate *S.epidermidis* from *S.saprophyticus* and *Micrococcus* sp?
2. Which is positive for Voges-Prausker test: *S.aureus, S.albus or Micrococcus* sp?
3. Why all the cultures did not grow either on MacConkey agar or on mannitol salt agar?
4. Which component in mannitol salt agar makes it selective medium?

Exercise 86: Isolation of pathogens from stool samples

In gastrointestinal infections number of pathogens is much less as compared to the resident flora. Hence it is often difficult to fish out a pathogen amongst the large resident flora. Therefore, to facilitate the pathogen isolation number of selective and differential media have been devised along with enrichment media, which contains the chemicals that inhibit the growth of non-pathogens with no effect on the growth of suspected pathogens. Differential media, inoculated lightly, provide primary presumptive information regarding the pathogen. Media are also inhibitory to Gram positives and some coliforms as well. Pathogens likely to be present in fecal specimen are *Salmonella, Shigella, E.coli* (enterotoxigenic, enteroinvassive, enteropathogenic, Vero toxin producer), *Yersinia, Campylobacter, Vibrio* and *Clostridium welchii etc* On differential/selective medium like MacConkey agar, *Salmonella* and *Shigella* produce white, grey or black colonies and *E.coli* pink colonies. *Proteus* colonies are black to brownish without any swarming. *S.choleraesuis* do not grow in Selenite F broth. Tetrathionate formed by oxidation of thiosulphate with iodine in TTB is bacteriostatic to *E.coli*.

Requirements

a. Fecal sample
b. Tetrathionate broth (TTB) and Selenite F broth, GN broth, phosphate buffer saline, alkaline peptone water
c. MacConkey agar plate (differential medium), SS agar, deoxycholate agar (DCA) or Wilson and Blair medium plate (selective medium) and biochemical media.
d. Special media for specific pathogen: Columbia agar (*Campylobactor*), sorbitol MacConkey agar (Vero toxin producing *E.coli*), CIN agar (*Yersinia*), TCBS medium (*Vibrio*) and Neomycin blood agar (*C.welchii*)

Procedure

1. Streak one plate each of differential medium (MacConkey agar) and selective medium heavily (DCA, Wilson and Blair medium, XLD or BSA) with fecal sample and transfer about 1g feces in each enrichment media.
2. Incubate the inoculated plates and enrichment medium at 37°C and examine the plates for non-lactose fermenting colonies resembling *Salmonella, Shigella* or *Yersinia*. Subculture from enrichment media onto differential and selective medium if no NLF colony is found on primary culture medium. Some time you may find pure growth of *Staphylococcus* on differential medium. Under these conditions, it may be reported as causal agent.
3. Inoculate these on TSI, PPA and Urease biochemical medium and incubate at 37°C.
4. Discard the isolate, which is urease positive and PPA positive and also those, which give A/AG or A/A reaction on TSI. Select the isolates with K/A, K/A H_2S^+, K/AG $H_2S\pm$, urease ± and PPA negative. Identify these based on further biochemical tests and preferably serologically for *Shigella, Salmonella or Yersinia.*
5. Find out the antibiotic sensitivity of the isolate using Stoke's method or Kirby Bauer method and accordingly send the report.

Questions:

1. At what stage in life is the intestinal micro flora established?
2. Is this micro flora static? Is it relatively easy to alter this flora?
3. What is differential enrichment medium? How does it differ from selective medium?
4. What is the pigment produced by *Serratia marcescens*?
5. Why do *Proteus* species swarm all over the plate?

6. What measures might be taken to inhibit swarming?
7. Name three species each of *Shigella* and *Salmonella* that cause disease in human.
8. How do *Proteus*, *Morganella* and *Providencia* differ from one another?

Unit six
Control of microbial activities

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Exercise 87: Phenol coefficient determination

Terms as antiseptics and disinfectants are generally applied to different chemical substances used for disinfecting inanimate and animate objects respectively. Sometime the same chemical is grouped under both the categories. The antiseptics are mild in their activity that prevent the multiplication of bacteria but may not kill them, with no injurious effect at the site of application. On the contrary, disinfectants are harsh to act and are used in much higher concentrations to destroy microorganisms associated with inanimate objects. These are marketed as germicides, bactericides, fungicides, insecticides etc. Another term sanitizer is used for chemicals that reduce the bacterial load to a safe level as judged by public health requirements.

The selection of disinfectants depends upon the purpose and the conditions under which it is to be used. The disinfectants available in market vary in their effectiveness, potency or ability to kill all microorganisms. Ideally, the disinfectant should: kill all types of microbes, rapid in action, not inactivated by organic matter, penetrate the material to be disinfected, be miscible with water, no unpleasant odor, no decomposition under unfavorable conditions etc. The most common test used designed for comparing effectiveness of disinfectants is the phenol coefficient method or the Rideal-Walker test.

Requirements

a. Nutrient broth tubes
b. Stock solution phenol (4%)
c. Lysol or any other disinfectant (5 %)
d. Bacterial culture *Salmonella typhi* NCTC 786

Procedure

1. Arrange six sterile test tubes in a test tube rack and label them as 1:75, 1:100, 1:125, 1:150 and 1:175 for phenol.
2. Arrange another set of ten tubes in another rack for test disinfectant and label as 1:80,1:100, 1:120, 1:140, 1:160,1:180, 1:200, 1:220 and 1: 240.
3. Make phenol and test disinfectant dilutions as shown in table.
4. Arrange nutrient broth in a test tube stand taking two tubes for each dilution of phenol and test disinfectant. Label one 5 min and another 10 min. for each dilution.
5. Transfer 0.5ml 16-18 h old *S.aureus* culture aseptically using sterile pipette to each dilution of phenol and test disinfectant taking care that pipette does not touch the tube wall. Mix gently. Keep the tubes in water bath kept at 25^0C.
6. At 5 and 10 min interval withdraw a loopful sample from each tube and transfer it to respective nutrient broth.
7. Incubate the inoculated nutrient broth tube at 37^0C for overnight.
8. Next day observe the tubes for turbidity and record the results as growth and no growth at 5 and 10 min against each dilution.

Phenol				Test disinfectant			
Dilution	**4% phenol (ml)**	**Sterile water (ml)**	**Discard Volume (ml)**	**Dilution**	**5% Test (ml)**	**Sterile water (ml)**	**Discard Volume (ml)**
1:75	2	4	1	1:80	2	6	2
1:100	2	6	3	1:100	1	4	-
1:125	1	4	-	1:120	1	5	1
1:150	1	5	1	1:140	1	6	2
1:175	1	6	2	1:160	1	7	3
1:200	1	7	3	1:180	1	8	4
1:225	1	8	4	1:200	1	9	5
1:250	1	9	5	1:220	1	10	6
1:275	1	10	6	1:240	1	11	7

9. Select the highest dilution of phenol and test disinfectant killing the test organism in 10 min but not in 5 min. Calculate the phenol coefficient as below:

$$\text{Phenol coefficient} = \frac{\text{Selected highest dilution of test disinfectant}}{\text{Selected highest dilution of phenol}}$$

Questions

1. Name the factors influencing the action of disinfectants.
2. How do chemical disinfectant act?
3. Define disinfectant, germicide, sanitizer and bacteriostatic.
4. List any other tests you know for comparing the efficiency of disinfectants.
5. What is "in use test"?

Exercise 88: Sterilization and disinfection

Sterilization means freeing an object from all kinds of living organisms including spores. An object from which all microbes have been killed or removed is said to be sterile. Sterility is of paramount importance to a microbiologist for varied reasons like isolation of pure cultures, fermentations with pure cultures and prevention of infection during surgical procedures.

Methods of sterilization

The sterilization procedures in use can broadly be grouped in two groups:

(1). **Physical methods**:(a) **Heat**, dry heat and wet heat, (b) **Infra red rays**, (c) Radiations, ionizing and non ionizing radiations (d) **Filtration**

and

(2).**Chemical methods.**

Sterilization procedure is chosen from any of the following taking into consideration the nature and type of nutrient medium or the article to be sterilized.

Dry heat: It is applied mainly in two ways. Direct incineration/ red heat/ flaming and heating at high temperature in a closed chamber called oven. Direct incineration or burning in naked flame is a technique used in routine for sterilizing inoculating needles before and after use. All contaminated objects that can't be reused are preferably incinerated. All the glassware used in the laboratory excluding those with rubber linings are sterilized in oven at 180°C for 20 min or160°C for 90 min The articles for sterilization are placed in clean and dry state in oven. Temperature of 160°C is generally used for sterilizing small quantities of powders, oils, petroleum jelly and petrolatum gauze etc. that resist wet heat penetration. Dry heat is not so effective as sterilizing agent as wet heat of same temperature.

(b) **Wet heat** : It is more effective than dry heat. It kills the organisms by denaturing enzymes and structural proteins. Factors affecting heat sterilization include temperature, time, container size, pH, bacterial load and nature of medium etc. Moist heat is applied in following ways:

(i) **Heating below 100°C**

(ii) **Boiling or heating at 100°C**:

(iii) **Autoclaving i.e. temperature above 100°C**

Heating below 100°C: (**Pasteurization** and **Inspissation**). **Pasteurization** technique employed for killing disease causing and food spoilage vegetative forms of organisms. It is applied in two forms. In batch process, the milk is heated at 63°C for 30min (**LTHT**) and at 71.2°C for 15-20 sec in flash process (**HTST**). This process ensures the killing of all kind of vegetative pathogens but not the spores. **Inspissation:** Technique is used for coagulation of egg component or serum proteins by heating medium containing these at 80°C in an inspirator. Serum is heated at 56°C for inactivating complement and one hour daily on successive eight days to ensure sterilization. Temperature above 59°C inspissation may occur.

Steaming, boiling, or heating at 100°C: Boiling at 100°C does not ensure sterility. Intermittent heating for one hour daily for three successive days is called "**Tyndallization**". The heating stimulates the spores germinating into vegetative form that are destroyed in successive heating the next day. The method is used for sterilizing media containing high sugar content (5% or more).

Heating under pressure or autoclaving: It is the most reliable method widely used for sterilization of culture media and surgical: Gloves, drapes, towels, gauze pads, instruments and

metal ware and glassware. Autoclaving is done at various temperature and pressure depending on the nature of the medium. The exact temperature attained depends on complete air discharged. Most common time temperature combinations used for heating are as below:

Pressure (lb/sq. in)	Temperature (°C)	Holding time (min.)
5	110	45
10	115	30
15	121	15
20	126	5

Two methods are used for testing efficiency of autoclaving: using chemical indicators and spore indicators. Chemical indicators: Browne's sterilizer tube contains a red solution which turns green when heated at 115° for 25 min (type1), 15 min (type2) or 160°C for 60 min (type3) and Bowie's-Dick tape. Alternatively, use *Bacillus stereothermophillus* spore suspension and test for viability, cultivating at 55-60°C.

(iii) **Infra red rays**: These are used for sterilizing syringes. Process is carried under vacuum followed by instant cooling to avoid oxidation. As the sterilization using infrared rays generates much heat.

Radiations: ionizing and non ionizing radiations: radiations differ in wavelength and energy. The shorter wavelengths have more energy. Ionizing radiation: X-rays and gamma rays ionize water into highly reactive free radicals that cause break in DNA strands. Non ionizing radiations between 15-390 nm are called UV rays. UV rays below 200nm are absorbed by air hence; fail to reach to living organisms. UV radiation is most lethal from 200-330 nm. Because of low penetrating ability, usage is limited to heat labile solutions. UV rays induce pyrimidine (thymidine) dimer (T=T) formation in nucleic acids. Dimers in critical gene result in death of cell unless it is repaired by pyrimidine dimerase (active in visible light-photo reactivation). In dark dimers are removed by endonucleases and DNA polymerase replacing the bases with the help of DNA ligase. Gamma rays generated from an isotope such as Cobalt 60 are very expensive, used commercially for sterilization of bulk pre-packed disposables like plastic syringes, petri plates, catheters which do not withstand heat.

Filtration : Method is used to separate soluble products that are damaged by heat e.g. serum, toxins, antibiotics, vitamins etc. Filtration efficiency depends on the porosity of filter. The efficient filter retains *S.marcescens*. The filtrate is bacteria free but may contain viruses hence not safe for clinical use. Various type of bacteriological filters in use are: (i) Berkefeld, Chamberland filters made from fossil diatomaceous earth, (ii) Seitz filters consists of an asbestos disc, (iii) Sintered glass filters made from finely ground glass fused particles (iv) Cellulose membrane filters made from nitrocellulose and cellulose acetate.

Chemical methods: Terms as disinfectants and antiseptics are generally applied to different chemical substances used for disinfecting inanimate and animate objects. Only few of them have effect on spores. Merthiolate is used for preservation of sera. Formaldehyde is a cheap, non-injurious, efficient in killing all kinds of spores and vegetative forms. It can be used for disinfecting rooms, stores, furniture, clothing etc. Ethylene oxide a gaseous disinfectant is of

particular value in sterilizing heat sensitive materials such as plastics, rubber articles, blankets, pharmaceuticals etc.

Disinfection and decontamination of laboratory waste:

Laboratory material must be made non-infectious prior to its disposal. Ideally, the material should be sterilized but it is not always possible. The reusable articles may be disinfected by physical means or using chemicals. The chemical disinfectants used in routine for this purpose include: 2-5 % phenolics [laboratory bench jars, hypochlorites (2500ppm) for disinfecting spills and surfaces, aldehydes for decontaminating safety cabinets [formaldehyde- (HCHO) generated by heating formalin or paraformadehyde] and alcohol [70-80%] for swabbing skin. Decontamination of all possible autoclavables by autoclaving, for efficient autoclaving put the things in shallow containers. Incineration and bone fire disposal is practiced in urban and district level laboratories.

Questions

1. How would you sterilize liquid paraffin, oils and talcum powder?
2. Why wet heat is more efficient than dry heat as sterilizing agent?
3. Why do we classify chemicals as disinfectant or antiseptics and not as chemical sterilent?
4. What are positive pressure and negative pressure filters?
5. Which time and temperature combination of autoclaving will you use to sterilize: peptone water, 5% dextrose solution, alcohol production medium and nutrient broth?

Exercise 89: Antibiotic sensitivity test

Some microbes produce substances that inhibit the growth of microbes belonging to other genera. This process is called antibiosis and the substance involved is called antibiotic. In 1928, Alexander Fleming, observed antibiosis around mold (*Penicillium*) growth on a culture of *Staphylococci*. He found that culture filtrates of *Penicillium* inhibited the growth of many Gram-positive cocci and *Neisseria* sp. The antibiotic substance produced may be specific or may have broad-spectrum activity inhibiting many organisms. Era of chemotherapy i.e. treating diseases with chemical substances began in 1930 with the discovery of sulfanilamide. Some of these chemicals are produced by microorganisms and are called antibiotics while other are synthesized in laboratory. Today we have large list of chemotherapeutic agents to choose from, differing in their mode of action and other properties. Some of these are broad spectrum, effective against wide range of organisms and the narrow spectrum effective against selected group of organisms. Selective toxicity of the agent is most important while recommending it for treatment. The agent should be toxic to parasite and not to the host. It is based on the physiological differences between parasite and host. Mere isolation and characterization of pathogen does not solve the problem unless the physician is provided the antibiotic sensitivity pattern of the isolate. The physician selects a correct combination of chemotherapeutics to which the isolated pathogen has been found to be susceptible in the clinical laboratory.

In most laboratories, disk diffusion method and tube dilution techniques are used for determining antibiotic sensitivity of the isolate. In disk diffusion method paper disk impregnated with antimicrobial agent are placed on the surface of agar. During incubation, the agent diffuses from the disk from an area of high concentration to an area of lower concentration creating a zone of inhibition around the paper disc. The concentration of agent at the edge of zone of inhibition represents its minimum inhibitory concentration (MIC). The zone size is affected by medium thickness, inoculum, the diffusion rate of agent and the growth rate of the organism. To minimize the variance between laboratories the standard Kirby-Bauer test for agar diffusion method is used in many laboratories.

Requirements

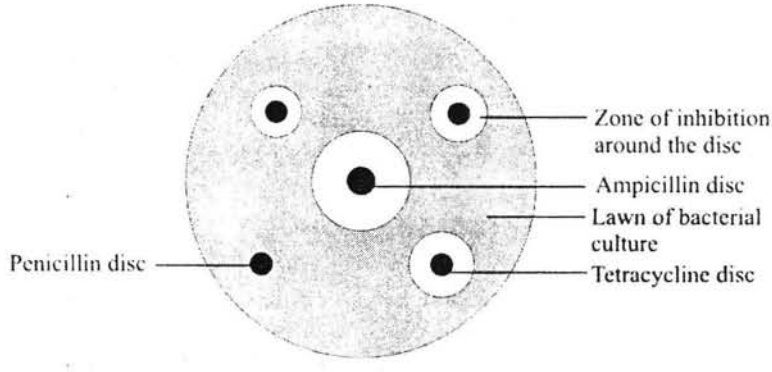
a. Mueller Hinton agar plate
b. Antibiotic dispenser and disks
c. Bacterial cultures: *S.aureus, E.coli, P.aeruginosa.*

Procedure

1. Aseptically swab the assigned culture onto the appropriate plate. Swab in three directions to ensure complete plate coverage. Let it stand at least 5 minutes.
2. Place the disc impregnated with antibiotic using automatic disc dispenser (A) or individual disc manually (B).
   A. Place the chemotherapeutic impregnated disks by pushing the dispenser over the agar. Press slightly each disk with sterile forceps to ensure better contact with the agar. Record the agents and disk code.
   B. Sterilize forceps by dipping in alcohol and burning off the alcohol. Obtain a disk impregnated with a chemotherapeutic agent and place it on the surface of the agar. Gently tap the disk to ensure better contact with the agar. Repeat, placing 5 to 6

different disks the same distance apart on the Petri plate (see the location of the disks). Record the agents and the disk code in your notebook.

3. Incubate the plate inverted at 35°C until the next period. Measure the zones of inhibition in millimeters, using a ruler on the underside of the plate. Record the zone size and compare with standard values to indicate whether the organism is sensitive, moderately sensitive and resistant. Observe the results of students using other bacteria.



Questions

1. What is MIC and MBC?
2. What is drug resistance?
3. How do the organisms acquire drug resistance?
4. What is the mode of action of penicillin, tetracycline, gentamicin, bacitracin and vancomycin?
5. What do the terms MRSA and PPNG mean?
6. What factors other than the agent being tested can affect the zone of inhibition?
7. How do you differentiate between sensitive and resistant organisms in antibiotic sensitivity test?
8. Can this technique be used for the detection of antibiotic producing strains?
9. What do the colonies present within zone of inhibition indicate?

Exercise 90: Lethal effects of ultraviolet (UV) radiations on microbes

This exercise is to explain the use of UV as an antimicrobial agent. Electromagnetic radiations differ in wavelength and energy. Radiations below or above visible range are lethal for microbes. These radiations are used for control of microbes. Electromagnetic radiations with shortest wave length below 300 nm have the greatest energy and therefore are highly lethal to microbes. X rays and gamma rays that represent the ionizing radiations bring about the effect though ionization of water into highly reactive free radicals the hydroxal ions (OH^- and H^+) hydrogen ions that can break strands of DNA and react quickly with SH containing compounds like proteins and inactivate them. High-energy ionizing radiations are highly effective in sterilization of plastic wares that don't withstand heat or any other material, which are heat sensitive. UV rays are non-ionizing radiations and are also used to control microbial growth.UV radiations have low penetration power and lower energy content than ionizing radiations. These radiations are capable of producing lethal effects in cells exposed to 210-310 nm. Most lethal wavelength is in the range of 265 that corresponds to the optimal absorption wavelength of DNA. UV light induces aberrant chemical bonds between adjacent thymine nucleotide bases in the nucleic acid that results in deletion mutation. Mutations in genes for essential function are lethal. In the presence of visible light, an enzyme, thymine dimerase gets activated and splits the thymine dimers. This process is called photoreactivation. Because of their low penetration power, UV rays are used for air and surface sterlization in hospital rooms, operation theatres, and pharmaceutical industries, in food packaging industries.

Requirements

a. Bacterial culture: *Serratia marcescens*
b. Nutrient agar plates
c. Glass spreader
d. Ultra violet lamp or UV source
e. Marker

Procedure

1. Add 5 ml culture in sterile petriplate. Place the petriplate containing culture under the UV source at a distance of 11-12 inches.
2. Remove the petridish cover and immediately withdraw an aliquot aseptically from the plate. Dilute the aliquot serially in sterile blanks and spread plate onto surface dried fresh nutrient agar plates as explained in earlier exercise.
3. Switch on the ultra violet light. Withdraw samples from the plate at regular interval after 5 min, 15min, 30 min, 60 min and 90 min of exposure to UV light. *Caution*: Be careful not to expose skin or eye to UV rays, as it is injurious. Use gloves and UV protective glasses or switch off the UV light prior to withdrawing the sample.
4. Make suitable serial dilutions and spread plate. Invert and cover the plates with used carbon paper and then incubate the plates at 37°C for 24-48 h.
5. At the end of incubation period count the number of colonies in control and UV exposed culture and plot the data between log viable count versus exposure time to develop death time curve for UV.

Questions

1. Why are X- rays and gamma rays are considered better germicidal agent than UV rays ?
2. What is the difference in the mode of action of UV rays and gamma rays?

Appendix-I

(Reagents/Stains)

Acetone/ ethanol

Mix ethanol (95%) and acetone in 1: 1 ratio.

Acid alcohol

Mix 3 ml HCl with 97 ml 95% ethyl alcohol.

Acid decolorizer

Add 0.5 ml HCl to 10 ml 70% ethanol.

Acridine orange

Dissolve 1g acridine orange in100 ml distilled water. Dilute it ten times with 0.2 M acetate buffer, pH 4.0.

Albert's stain

Dissolve 0.2 g malachite green and 0.15 g toluidine blue in 2.0 ml 95% ethyl alcohol. Add 100 ml distilled water and 1.0 ml glacial acetic acid.

Albert's iodine

Dissolve 6.0 g iodine and 9.0 potassium iodide in 900 ml distilled water.

Alsever's solution

Dissolve 20.5 g dextrose, 8.0 g sodium citrate, 0.55 g citric acid, 4.2 g sodium chloride and distilled water to make 1000 ml. Sterilize at 15 lbs for 20 min.

Amido black stain

Dissolve 1.0 g amido black in 500 ml acetic acid (1M) solution. Add it to 500 ml sodium acetate solution (0.5M) and mix.

Ammonical silver nitrate stain

Add 10% ammonia to 0.5% silver nitrate solution, prepared in distilled water, until precipitates form and redissolve. Now add more of silver nitrate solution drop wise until precipitates returns and do not dissolve.

Ammonium molybdate

100 g molybdic acid, 144 ml ammonium hydroxide, 271 ml distilled water. Dissolve molybdic acid in ammonium hydroxide and distilled water. With constant stirring add slowly 489 ml nitric acid and 1148 ml distilled water.

Barritt's reagent

Solution A-5 % alpha-naphthol in absolute ethanol.

Solution B- 40% KOH (aqueous)).

Biuret reagent

Dissolve 1.5 g copper sulfate and 5 g sodium potassium tatrate in 250 ml of 0.2N NaOH. Add 2.5 g potassium iodide and dissolve. Make the volume to 500 ml by adding 0.2N NaOH. Prepare fresh.

Bouin's fixative

Mix saturated picric acid solution 75 parts, formalin 25 parts and glacial acetic acid 5 parts

Bromophenol blue

0.25% xylene cyanol FF, 0.25%, sucrose (40%, w/v) or 30%, w/v, glycerol. Store at 4°C.

Cetyl pyridinium chloride

0.34% etylpyridinium chloride dissolved in distilled water.

Coomassie blue (gel staining)

0.5 g coomassie blue, 45 ml ethanol, 10 ml acetone and make the volume to 100 ml with distilled water.

Coomassie blue (IEP)

1.25 g coomassie blue, 50 ml acetic acid and 185 ml distilled water.

Congo red (aqueous)

Congo red 0.5 g dissolved in 10 ml ethanol and volume made to 100 ml with distilled water.

Copper sulphate

Dissolve 20 g copper sulphate in 100 ml distilled water.

Crystal violet

Gram's crystal violet: Dissolve 2 g crystal violet in 20 ml 95% ethanol and 0.8 g ammonium oxalate in 80 ml distilled water. Mix both and filter before use.

Crystal violet 0.2% aqueous: Dissolve 0.2 g crystal violet in 100 ml distilled water.

CTAB/NaCl solution

Dissolve 4.1 g NaCl in 80 ml H_2O and slowly add 10 g CTAB (hexadecyl trimethyl ammonium bromide) while warming and stirring. If necessary, heat up to 65° C till it dissolves. Adjust the final volume to 100 ml with distilled water.

Dilute carbol fuchsin

Dilute ZNCF stain 10-15 times with water.

Dorner's Nigrosine

Add 10 g nigrosine to 100 ml distilled water and boil for 20-30 min. Add 0.5 ml formalin as preservative. Filter twice through double layer of filter paper.

Eosin stain

Mix 10% aqueous eosin solution - 4 parts, inactivated serum - 1 part and a crystal of thymol. Keep at room temperature for a day, centrifuge and use.

Ferric chloride

10% Ferric chloride (aqueous).

Gelatin solution

3% Gelatin (aqueous).

Gover's fluid

12.2 g sodium sulfate, 33.3 ml glacial acetic acid and distilled water to make 200 ml.

Gram's iodine

Dissolve 1.0 g iodine and 2.0 g potassium iodide in 300 ml distilled water.

Gram's safranine

Dissolve 0.25 g safranine O in 10 ml 95% ethanol and then add 100 ml distilled water.

Giemsa's stain

Solution A: 0.5 g Giemsa stain powder, 33 ml glycerin and 33 ml methanol.

Solution B: Phosphate buffer 0.15 M, pH 7.0.

Working solution: Add 1.0 ml of solution A and 2.0 ml and solution B to 47 ml of distilled water.

Iodine solution

Dilute Gram's iodine 1:1 ratio with distilled water.

Intra-vital stain

Crystal violet 1: 120000 (aqueous).

Kinyoun's carbol fuchsin

Dissolve 4 g basic fuchsin in 20 ml ethanol (95%). Then slowly add 100 ml distilled water and 8 ml phenol while stirring.

Kovac's reagent

5.0 g paradimethylaminobenzadehyde, 75 ml amyl alcohol and 25 ml HCl. Dissolve paradimethylaminobenzadehyde in amyl alcohol heated to 50°C in water bath. Cool and add acid slowly and store in dark colored bottle.

Lactophenol cotton blue

20.0 g phenol, 20.0 g lactic acid, 40.0 ml glycerol and 20 ml distilled water. Dissolve phenol in warm distilled water and then add other ingredients. Add 0.05 g cotton blue dye.

Leifson's flagella stain

20 ml $NH_4Al\ (SO_4)_2$ (saturated solution), 2.0 g tannic acid, 3.0 ml basic fuchsin (saturated solution), 15 ml ethanol and 10 ml distilled water. Dissolve in sequence. Prepare fresh for use.

Leishman stain

0.2 g Leishman stain, 100 ml methanol. Warm methanol to 50°C to dissolve Leishman stain.

Loeffler's methylene blue

Make a saturated solution of methylene blue in 95% ethanol. Add 300 ml of it to 700 ml KOH (0.1%).

Malachite green

Dissolve 5 g malachite green (oxalate) in 100 ml distilled water.

Maneval's stain

5% phenol-30ml, 20% glacial acetic acid-10ml, 30% ferric chloride-40 ml, 1% acid fuchsin, 18 ml and 2 ml acid alcohol.

Methylene blue

Dissolve 0.3 g methylene blue in 100 ml distilled water.

Methyl red reagent

Dissolve 1.0 g methyl red in 300 ml ethanol. Make the volume to 500 ml with distilled water.

Mordant (for spirochetes)

Dissolve 1.0 g phenol and 5.0 g tannic acid in 100 ml distilled water.

Neisser methylene blue

1.0 g methylene blue, 90 ml ethyl alcohol, 50 ml glacial acetic acid and 1000 ml distilled water.

Neutral red solution

For modified Neisser method - Mix 1.0 g neutral red and 2.0 ml glacial acetic acid (1.0%) in 1000 ml distilled water.

Newman stain

Mix 1.0 g methylene blue chloride, 6 ml glacial acetic acid, 40 ml trichloroethane and 54 ml ethanol.

Nigrosine dye

10% nigrosine in distilled water containing 0.5% formalin.

Nitrate test reagent

Solution A: 8 g sulfanilic acid in 1000 ml of 5 N acetic acid.

Solution B: 5 g dimethyl alpha naphthylamine dissolved in 1000 ml of 5N acetic acid.

Polychrome methylene blue

300 ml of saturated solution methylene blue prepared in 95% ethanol. Bring the volume to 1000 ml with 0.1% KOH. Allow the stain to ripen slowly for 12 months at room temperature with occasional shaking for proper aeration

Rhodamine –auramine stain

Dissolve 1.5 g auramine, 0.75 g rhodamine, 75 ml glycerol, and 10 ml phenol in 50 ml distilled water. Filter through glass wool and store at room temperature. *Counter stain*: 0.5% $KMnO_4$ (aqueous).

Saline solution

0.15 M NaCl in distilled water 0r 0.85% NaCl (aqueous)

Schiff's fuchsin sulphate

(Schiff's base): Dissolve 20.0 g basic fuchsin in 400 ml boiling distilled water. Cool to 50°C and filter. Add 10 ml of 2 N HCl and then add 4.0 g potassium meta-bisulphite. Mix and leave it in stopper bottle for overnight in dark. Add charcoal, mix and filter at once. Add 20 ml of 2 N HCl and mix.

Shunk's mordant

Mix spirit 18 ml and aniline oil 4 ml and make volume to 100 ml with distilled water.

Sodium hydroxide (40%)

Dissolve 40 g NaOH in distilled water and make volume to 100 ml.

Sudan black stain

Dissolve 0.3 g Sudan black B powder in 70% ethyl alcohol.

Toluidine blue

Dissolve 0.1 g toluidine blue in 10 ml ethanol and make volume to 100 ml with distilled water.

Trypan blue

1%, w/v, (aqueous).

WBC diluting fluid

1.5 ml acetic acid, 1.0 g gentian violet and distilled water to make 1000 ml. Dilute 1:20 with distilled water before use.

Wright's stain

Grind 0.3 g Wright stain powder in mortar and add 3.0 ml glycerol and grind together. Add 97 ml methyl alcohol and store in stoppered flask and keep it for 2 weeks with occasional shaking daily.

Ziehl-Neelsen's carbol fuchsin

Solution A: Dissolve 3 gm basic fuchsin in 10 ml 95% ethanol.

Solution B: Dissolve 5 gm phenol in 95 ml distilled water.

Primary stain: Mix solution A and B and use.

Appendix-II

(Media)

Medium preparation

Nutrient media may be prepared by mixing individual component or by dissolving known amount of dehydrated medium being marketed by number of firms. Ingredients concentration has been given grams per liter medium unless specifically stated.

***Azotobacter* agar**

Dibasic potassium phosphate	1.0
Magnesium sulfate	0.2
Sodium chloride	0.2
Ferrous sulfate	0.2
Agar	15.0
Soil extract	100.0 ml
Tap water	900.0 ml

Dissolve and adjust contents to pH to 7.6 and autoclave. Add 1 ml sterile 10% glucose solution to each tube.

Blood agar

Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
Distilled water to make	1000 ml.

Dissolve the ingredients and autoclave. Cool to 50°C and aseptically add 50 ml sterile blood and mix the blood gently avoiding any bubble formation.

Buffered glucose broth (pH 7.2)

Dextrose	5.0
NaCl	5.0
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Distilled water up to	1000 ml

Chocolate agar

Peptone	20. 0
Dextrose	0.5
Sodium chloride	5.0
Disodium phosphate	5.0
Agar	15.0
Distilled water up to	1000 ml

Aseptically add 5% defibrinated blood to sterile and molten agar. Heat to 75-80°C until chocolate color develops.

Corn meal agar

Corn meal (ground yellow maize)	40.0
Agar	20.0
Distilled water up to	1000 ml

Czapek Dox Agar

Sucrose	30.0
Sodium nitrate	3.0
$K_2 HPO_4$	1.0
$MgSO_4$	0.5
KCl	0.5
$FeSO_4$	trace
Agar	15.0
Distilled water up to	1000 ml

Endo medium

Lactose	20.0
Peptone	20.0
Dibasic potassium phosphate	7.0
Sodium sulfite	5.0
Basic fuchsin	0.8

Dissolved and volume made to 1000 ml with distilled water.

Eosin methylene blue agar

Peptone	3.0
Lactose	5.0
K_2HPO_4	2.0
Agar	15.0
Eosin yellow	0.4
Methylene blue	0.065
Distilled water	1000 ml

Glycerol yeast extract agar

Glycerol	5.0 ml
Yeast extract	2.0
Dibasic potassium phosphate	1.0
Agar	15.0
Distilled water up to	1000 ml

Histidine/Biotin solution (0.5 M):

D- biotin	105 mg
L - histidine HCl-	122 mg
Distilled water	1000 ml

Dissolve biotin by heating. Autoclave for 20 minute at 121°C. Store at 4°C.

Hay infusion

Add 5.0 g wheat or rice husk in 100 ml water Boil it for 10 min. and let it stand for 24 h

Hugh–Leifson medium, pH 7.2 (0/F medium)

Glucose	10.0
Peptone	3.0
Sodium chloride	5.0
K_2HPO_4	0.3
Bromothymol blue	0.03
Agar	3.0
Distilled water	1000 ml

LB medium, pH 7.2

Bacto peptone	10.0
Bacto yeast extract	5.0
Sodium chloride	10.0
Distilled water	1000 ml

Add 1.5% agar for solid medium i.e. L agar.

Milk agar

Skim milk powder	5.0
Nutrient agar	100 ml

Mineral salt agar

Ammonium sulfate	0.5
Potassium chloride	0.5
Magnesium sulfate	2.0
Sodium nitrate	2.0
Agar	15.0
Distilled water up to	1000 ml

M 9 medium

KH_2PO_4	3.0
Na_2HPO_4	6.0
NH_4Cl	1.0
NaCl	1.0

Dissolve in 975 ml distilled water. Sterilize and mix aseptically with 25 ml sterile 20% glucose solution. 0.1ml calcium chloride (1M) and 1.0 ml magnesium sulfate (1M)

Minimal essential medium (Eagle MEM)

L - Arginine	105 mg
L Cystine	24 mg
L - Histidine	31 mg
L - Isoleucine	52 mg
L Leucine	52 mg
L - Lysine	58 mg
L – Methionine	15 mg
L – Phenyl alanine	32 mg
L – Threonine	48 mg
L – Tryptophan	10 mg
L – Tyrosine	36 mg
L – Valine	46 mg
Choline	1 mg
Folic acid	1 mg
Inositol	2 mg
Nicotinamide	1 mg
Pantothenate	1 mg
Pyridoxal	1 mg
Riboflavin	0.1 mg
Thiamine	1 mg
Dextrose	1 g
NaCl	6.8 g
KCl	0.4 g
$CaCl_2$	0.2 g
$MgCl_2$	0.2 g
Mono sodium phosphate	15 g
Sodium bi-carbonate	2 g
Triple distilled water	1000 ml

Nutrient agar, pH 7.2

Beef extract	3.0
Peptone	5.0
NaCl	5.0
Agar	15.0
Distilled water up to	1000 ml

Nutrient broth, pH 7.2

Beef extract	3.0
Peptone	5.0
NaCl	5.0
Distilled water up to	1000ml.

Autoclave at 15 lbs for 20 min.. Store at ambient temperature.

Nitrogen free mannitol agar (pH 7.3)

Mannitol	15.0
Dipotassium hydrogen phosphate	0.5
Magnesium sulfate	0.2
Calcium sulfate	0.1
Sodium chloride	0.2
Calcium carbonate	5.0
Agar	15.0
Distilled water to make	1000 ml

Peptone water, pH 7.2

Peptone	10.0
Sodium chloride	5.0
Distilled water	1000 ml

Pikovskaya's medium, pH 7.0

Glucose	10.0
$Ca_3 (PO4)_2$	5.0
$(NH_4) SO_4$	0.5
NaCl	0.2
$MgSO_4.7H_2O$	0.1
KCl	0.2
Yeast extract	0.5
$MnSO_4$	trace
$FeSO_4$	trace

Agar	15.0
Distilled water up to	1000 ml.

Sugar fermentation medium:

a. Peptone water basal medium

Peptone	10.0
Sodium chloride	5.0
Distilled water	1000 ml

b. Broth base

Meat extract	5.0
Peptone	10.0
Sodium chloride	3.0
Na_2HPO_4	2.0
Bromothymol blue	0.024
Distilled water	1000 ml

c. Hiss serum water, pH 7.6

Mix one part serum with three parts distilled water and 5 ml of 0.2% phenol red per 100 ml medium.

d. Buffered glucose peptone water

(For MR and VP test)

Peptone	7.0
Dextrose	5.0
K_2HPO_4	5.0
Distilled water	1000 ml

Sabouraud's agar, pH 5.6

Peptone	10.0
Glucose	40.0
Agar	15.0
Distilled water	1000 ml

Soil extract

Mix 500 g dry garden soil in 1300 ml water containing 1% sodium carbonate. Steam for one hour. Filter and make volume to 1000 ml.

Starch agar

Starch soluble	1.0
Peptone	5.0
Beef extract	3.0
Agar	15.0
Distilled water	1000 ml

Top agar

Agar	6.0
Sodium chloride	5.0
Distilled water	1000 ml

Autoclave for 20 minutes at 121°C. Let down the temperature at 50-60°C and add 0.5 M histidine/biotin solution. Mix and distribute 2.5ml aliquot in sterile glass tubes. Store at 4°C no longer than one month.

Tributyrin agar, pH 7.5

Peptone	5.0
Yeast extract	3.0
Tributyrin	10.0
Agar	15.0
Distilled water	1000 ml

Triple Sugar Iron (TSI), pH 7.3

Peptone	20.0
Beef extract	3.0
Yeast extract	3.0
Sodium chloride	5.0
Lactose	10.0
Glucose	1.0
Sucrose	10.0
$FeSO_4$	0.20
$Na_2S_2O_3$	0.30
Agar	20.0
Phenol red	0.025
Distilled water	1000 ml

Dispense in tubes and autoclave at 15 lbs for 15min. Medium is slanted to give a slant of 2.5 cm and a butt of 4-5 cm deep.

Trypticase soy agar, pH 7.3

Trypticase	20.0
Potassium nitrate	1.0
Na_2HPO_4	2.0
Agar	15.0
Distilled water up to	1000 ml

Vogel-Bonner medium E (x50)

Distilled water	670 ml
Magnesium sulfate	10.0
Citric acid monohydrate	100.0
K_2HPO_4	500.0
$Na_2(NH_4)PO_4$	175.0
Distilled water up to	1000 ml.

Allow each salt to dissolve before adding the next. Autoclave at 15 lbs for min at 121°C. Store at ambient temperature.

VB medium (minimal glucose plates)

Distilled water	930 ml
Agar	15.0

Dissolve the contents, dispense and autoclave at 121^0C for 30 minutes. Let the temperature fall to 50-60^0C and then add 50 ml glucose (40% solution) and 20 ml Vogel-Bonner medium E (x50).

Yeast extract mannitol agar, pH 7.0

Yeast extract	1.0
Mannitol	10.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Agar	20.0
Distilled water	1000 ml

Sterilize the medium by autoclaving at 121°C for 20 min.

Appendix-III

(Buffers and Solutions)

Carbonate bicarbonate buffer, pH 8.6
Dissolve 3.5 g sodium carbonate and 1.5 g sodium bicarbonate in distilled water and make volume to1000 ml.

CTAB/NaCl solution
Dissolve 4.1g NaCl in 80 ml H_2O and slowly add 10 g CTAB (hexadecyltrimethyl ammonium bromide) while warming and stirring. If necessary, heat up to 65° C till it dissolves. Adjust the final volume to 100 ml.

Glucose-6-phosphate (1M)
Glucose-6-phosphate 2.82g
Sterile distilled water 10ml
Store at –20°C.

Nicotinamide adenine dinucleotide phosphate solution (NADP) (0.1M)
NADP 76.6mg
Sterile distilled water 10 ml
Store at –20°C.

Phosphate buffer saline
Solution A: KH_2PO_4 2.78 g / 100 ml normal saline
Solution B: Na_2HPO_4 3.56 g/ 100 ml normal saline

pH 7.2 : 28 ml Solution A + 72.8 ml solution B.

pH 6.4: 73.5 ml solution A + 26.5 ml solution B.

Salt solution
Potassium chloride 61.5 (1.65M)
Magnesium chloride 40.7 (0.4M)
Distilled water 500 ml
Autoclave for 20 min. at 121°C. Store at 40°C.

Sodium phosphate buffer (0.2M, pH 7.4)
Sodium dihydrogen phosphate 0.2 M (13.8g/500ml) 60ml
Disodium hydrogen phosphate 0.2 M (14.2g/500ml) 440ml
Autoclave for 20 min. at 121°C.

TAE buffer
Tris-acetate buffer (0.04M, pH 8.0) containing 0.001M EDTA;

TBE buffer
Tris-borate buffer (0.045 M, pH 8.0) containing 0.001M EDTA.

TE buffer
Tris-HCl buffer 10 mM, pH 8.0
EDTA 1 mM, pH 8.0

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