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Safety Guide Lines

Introduction

In the laboratory individuals are exposed to hazards not found in a regular classroom. It is essential that students follow all rules established by the lab instructor, lab manager, or lab assistant to ensure the safety of all individuals in the class.

Failure to follow established rules may result in dismissal of the individual from the class. Laboratories have certain standard safety equipment. These typically include a general-purpose fire extinguisher, eyewash, safety shower and cut off switches for electrical and gas outlets. It is the responsibility of the student to locate and know how to use the general safety equipment in the laboratory. Additionally, students should be aware of exits from the room in case of emergency, the location of the nearest fire call box, how to summon Campus Security, and how to obtain emergency medical assistance.

The microbiology lab has some additional safety considerations. Since individuals work with potentially pathogenic organisms care must be taken to prevent possible infection or transmission of the organisms from the laboratory. Students must wear protective clothing (lab coats) while working the laboratory. Lab coats may not beworn outside the laboratory. Intact skin is an adequate barrier against microorganisms
so gloves are not necessary in lab. Gloves will be provided and students may wear gloves when handling cultures if they so desire. Tabletops must be disinfected before and after lab using the disinfectant provided. Instruction in aseptic technique will be provided. Aseptic technique must be followed while working with microorganisms.

Hand washing is a simple and effective way to prevent the transmission of disease. While antibacterial soap may provide some additional protection the major effect of hand washing is the mechanical removal of microbes from the skin. Friction when washing hands is important to mechanically remove organisms from the surface of the skin. Using a paper towel to turn off the water prevents recontamination of the hands with microorganisms. Hands must be washed whenever the student leaves the lab.

Two copies of the Laboratory Safety Rules are included. One must be signed and returned to the laboratory instructor at the end of class. The additional copy is for your reference.

Microbiology Laboratory Safety Rules

1. All materials and clothes other than those needed for the laboratory are to be kept away from thework area.

2. A lab coat or other protective clothing must be worn during lab. The lab clothing is not to be worn outside of the laboratory.

3. Clean the lab table before and after lab with the disinfectant solution provided

4. Wash hands before leaving lab.

5. Any item contaminated with bacteria or body fluids must be disposed of properly. Disposable items are to be placed in the BIOHAZARD container. Reusable items are to be placed in the designated area for autoclaving prior to cleaning. Sharps are to be disposed of in the appropriate container

6. Reusable items should have all tape and marks removed by the student before being autoclaved.

7. Because organisms used in this class are potentially pathogenic, aseptic technique must be observed at all times. NO eating, drinking, application of cosmetics or smoking is allowed. Mouth pipetting is not allowed.

8. Cuts and scratches must be covered with Band-Aids. Disposable gloves will be provided on request.

9. Long hair should be tied back while in lab.

10. All accidents, cuts, and any damaged glassware or equipment should be reported to the lab instructor immediately.

11. Sterilization techniques will involve the use of Bacticinerators that are fire and burn hazards. Bacticinerators reach an internal temperature of 850^{o} C or 1500^{o} F. Keep all combustibles away from the Bacticinerators. Do not leave innoculating loops or needles propped in the Bacticinerator.

12. Microscopes and other instruments are to be cared for as directed by the instructor.

13. It is the responsibility of the student to know the location and use of all safety equipment in the lab (eyewash, fire extinguisher, etc.)

14. Cultures may not be removed from the lab. Visitors are not allowed in the lab.

15. Doors and windows are to be kept closed at all times.

16. For the best lab experience, read labs before coming to class. Make notes as necessary. Wait for a laboratory introduction by the instructor before starting work.

1. Microbiological Media Preparation

Bacteria and fungi are grown on or in microbiological media of various types. The medium that is used to culture the microorganism depends on the microorganism that one is trying to isolate or identify. Different nutrients may be added to the medium, making it higher in protein or in sugar. Various pH indicators are often added for differentiation of microbes based on their biochemical reactions: the indicators may turn one color when slightly acidic, another color when slightly basic. Other added ingredients may be growth factors, NaCl, and pH buffers which keep the medium from straying too far from neutral as the microbes metabolize

Nutrient broth

Bacteria and fungi are grown on or in microbiological media of various types. The medium that is used to culture the microorganism depends on the microorganism that one is trying to isolate or identify. Different nutrients may be added to the medium, making it higher in protein or in sugar. Various pH indicators are often added for differentiation of microbes based on their biochemical reactions: the indicators may turn one color when slightly acidic, another color when slightly basic. Other added ingredients may be growth factors, NaCl, and pH buffers which keep the medium from straying too far from neutral as the microbes metabolize. In this exercise, your table will make all-purpose media called nutrient broth.

Composition of Medium

1. Beef Extract 5 grams
2. Peptone 3 grams
3. NaCl 5 grams
4. Distle Water 1000 ml

Requirements

1. Electronic or beam balances.
2. 10 ml nonsterile pipettes.
3. pH paper or pH meter with standard buffers.
4. 1000ml Erlenmeyer Flasks (conical flask)
5. Nonabsorbent cotton and gauze to make cotton stoppers.

Procedure

To 1000 ml of distilled water add the above composition of the medium and add 20 grams of agar before autoclaving it .

Sterilization

As a general rule, the dissolved medium should be heated for the shortest time possible. The vessels used for sterilization should therefore contain not more than 1 liter of the medium, and preferably less. If not otherwise indicated, the medium should be autoclaved at 121 °C for 15 minutes, excluding the time required for heating and cooling

After autoclavation, cool the media in a 55^{o} waterbath.

The prepared Nutrient Broth is used for the purpose

2. PREPARATION OF NUTRIENT AGAR

Principle:
Bacteriological Nutrient Agar is a **complex** medium because it contains ingradients such as Beef Extract (0.3%), Peptone (0.5%) and Agar (1.5%) in water. Beef extract is the commercially prepared in dehydrated form of autolysed beef and is supplied in the form of a paste. Peptone is casein (milk protein) that has been digested with the enzyme pepsin. Peptone is dehydrated and supplied as a powder. Peptone and Beef Extract contain a mixture of amino acids and peptides. Beef Extract also contains water soluble digest products of all other macromolecules (nucleic acids, fats, polysaccharides) as well as vitamins and trace minerals The advantage of complex media is that they support the growth of a wide range of microbes. Agar is purified from red algae in which it is an accessory polysaccharide (polygalacturonic acid) of their cell walls. Agar is added to microbiological media only as a **solidification agent**. Agar for most purposes has no nutrient value. Agar is an excellent solidification agent because it dissolves at near boiling but solidifies at 45^{o}C. Thus, one can prepare molten (liquid) agar at 45^{o}C, mix cells with it, then allow it to solidify thereby trapping living cells. Below 45^{o}C agar is a solid and remains so as the temperature is raised melting only when >95^{o}C is obtained.

Composition of Medium

1. Beef Extract	5 grams
2. Peptone	3 grams
3.NaCl	5 grams
4.Agar	20 grams
5. Distle Water	1000 ml

Requirements
1. Electronic or beam balances.
2. 10 ml nonsterile pipettes.
3. pH paper or pH meter with standard buffers.
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After autoclavation, cool the media in a 55 degree waterbath. Do not allow the solution to cool below this temperature as the agar gets solid at that temperature. At this step, one can leave the agar/media mixture for a long time

Checking the pH

Usually, the specified final pH of the medium is obtained automatically after autoclaving. However, it is advisable to check the pH, especially if the material has been stored for some time..

Casting plates

The culture medium should not be poured into Petri dishes until it has cooled down to about 45 °C. Otherwise condensation of water on the covers of the dishes makes the medium too concentrated.

The prepared plates are used for the cultivation of the bacteria

3. Staining Technique

Introduction

Bacteria have almost the same refractive index as water. This means when you try to view them using a microscope they appear as faint, gray shapes and are difficult to see. Staining cells makes them easier to see.

Simple stains use only one dye that stains the cell wall of bacteria much like dying eggs at Easter. Differential stains use two or more stains and categorise cells into groups. Both staining techniques allow the detection of cell morphology, or shape, but the differential stain provides additional information concerning the cell. The most common differential stain used in microbiology is the Gram stain.

3.1 Simple Staining

In direct staining the positively charged color portion of the basic dye combines with the negatively charged bacterium and the organism becomes directly stained.

Simple stain

Materials

Heat-fixed bacterial smears

Methylene blue, Crystal violet, or Safranin to act as simple stain

Bibulous paper or paper towels

Microscope

ORGANISMS

Your pure cultures of *Staphylococcus epidermidis* (coccus with staphylococcus arrangement) or *Micrococcus luteus* (coccus with a tetrad or a sarcina arrangement) and *Escherichia coli* (small bacillus) or *Enterobacter aerogenes* (small bacillus)

PROCEDURE

1. Heat-fix a smear of either *Escherichia coli* or *Enterobacter aerogenes* as follows:

a. Using the dropper bottle of distilled water found in your staining rack, place a small drop of water on a clean slide by touching the dropper to the slide.

b. Aseptically remove a small amount of the culture from the agar surface and touch it several times to the drop of water until it just turns cloudy

c. Burn the remaining bacteria off of the loop. (If too much culture is added to the water, you will not see stained individual bacteria.)

d. Using the loop, spread the suspension over the entire slide to form a thin film

e. Allow this thin suspension to completely air dry

f. Pass the slide (film-side up) through the flame of the bunsen burner 3 or 4 times to heat-fix

2. Place the slide on a staining tray and cover the entire film with safranin. Stain for one minute.

3. Pick up the slide by one end and hold it at an angle over the staining tray. Using the wash bottle on the bench top, gently wash off the excess safranin from the slide . Also wash off any stain that got on the bottom of the slide as well.

4. Use a book of blotting paper to blot the slide dry . Observe using oil immersion microscopy.

1. Cover the label on the slide with tape.
2. Place the slide on the staining rack and flood the slide with stain for 1 minute.
3. Rinse the slide with tap water, tilting the slide slightly to rinse all the stain from the slide. Tap the slide gently to remove excess water.
4. Place a piece of bibulous paper or paper towel on the lab table and put the slide on it. Fold the paper over the slide and gently blot the slide to remove the water.
5. Examine the stained smear with the microscope and record your results

3.2 Gram Staining

Principle

The Gram stain is a differential stain. Four different reagents are used and the results are based on differences in the cell wall of bacteria. Some bacteria have relatively thick cell walls composed primarily of a carbohydrate known as peptidoglycan. Other bacterial cells have thinner cell walls composed of peptidoglycan and lipopolysaccharides. Peptidoglycan is not soluble in organ solvents such as alcohol or acetone, but lipopolysaccharides are nonpolar and will dissolve in nonpolar organic solvents Crystal violet acts as the primary stain. This stain can also be used as a simple stain because it colors the cell wall of any bacteria. Gram's iodine acts as a mordant. This reagent reacts with the crystal violet to make a large crystal that is not easily washed out of the cell. At this point all cells will be the same color. The difference in the cell walls is displayed by the use of the decolorizer. A solution of acetone and alcohol is used on the cells. The decolorizer does not affect those cell walls composed primarily of peptidoglycan but those with the lipid component will have large holes develop in the cell wall where the lipid is dissolved away by the acetone and alcohol. These large holes will allow the crystal violet-iodine complex to be washed out of the cell leaving the cell colorless. A counter stain, safranin, is applied to the cells which will dye the colorless cells. The cells that retain the primary stain will appear blue or purple and are known as Gram positive. Cells that stain with the counter stain will appear pink or red and are known as Gram negative. The lipo polysaccharide of the Gram negative cell not only accounts for the staining reaction of the cell but also acts as an endotoxin. This endotoxin is released when the cell dies and is responsible for the fever and general feeling of malaise that accompanies a Gram negative infection. When reporting a Gram stain you must indicate the stain used, the reaction, and the morphology of the cell. Round, purple (blue) cells would be reported as Gram positive cocci and rod-shaped, purple (blue) cells would be reported as Gram positive bacilli. There are standard abbreviations that may be used for these reports.

GPC Gram positive cocci
GNC Gram negative cocci
GPB Gram positive bacilli
GNB Gram negative bacilli

The spiral-shaped bacteria of medical importance do not Gram stain well and are usually demonstrated using a dark-field microscope. There are no standard abbreviations for Gram stain reactions for the spirilla.

Materials required

Heat-fixed bacterial smears
Gram stain reagents

Crystal violet
Gram's iodine
Acetone-alcohol decolorizer
Safranin
Bibulous paper or paper towels
Microscope

Procedure

1. Cover the label on the slide with tape.
2. Place the slide on the staining rack and flood with crystal violet for 1 minute.
3. Rinse the slide with tap water, tilting the slide slightly to rinse all the stain from the slide.
4. With the slide slightly tilted, drop a few drops of Gram's iodine on the slide to rinse off the last of the rinse water. Place the slide flat and flood with Gram's iodine for 1 minute.
5. Rinse the slide with water as in step 3.
6. With the slide tilted slowly drop acetone-alcohol decolorizer on the slide. Blue color will run from the smear. Continue to apply decolorizer drop-by-drop until the blue stops running from the smear.
7. Immediately rinse with water.
8. With the slide slightly tilted add safranin to the slide to replace the rinse water then lay the slide flat and flood the slide with safranin for 30 seconds.
9. Rinse safranin from the slide with tap water. Gently tap the slide to remove excess water.
10. Place a piece of bibulous paper or paper towel on the lab table and put the slide on it. Fold the paper over the slide and gently blot the slide to remove the water.
11. Examine the stained smear with the microscope and record your results

DISCUSSION

The gram stain is the most widely used staining procedure in bacteriology. It is called a differential stain since it differentiates between gram-positive and gram-negative bacteria. Bacteria which stain purple with the gram staining procedure are termed gram-positive; those which stain pink are said to be gram-negative. The terms positive and negative have nothing to do with electrical charge, but simply designate two distinct morphological groups of bacteria.

Gram-positive and gram-negative bacteria stain differently because of fundamental differences in the structure of their cell walls. The bacterial cell wall serves to give the organism its size and shape as well as to prevent osmotic lysis. The material in the bacterial cell wall which confers rigidity is peptidoglycan.

In electron micrographs, the gram-positive cell wall appears as a broad, dense wall 20-80 nm thick and consisting of numerous interconnecting layers of peptidoglycan Chemically, 60 to 90% of the gram-positive cell wall is peptidoglycan. Interwoven in the cell wall of gram-positive are teichoic acids. Teichoic acids, which extend through and beyond the rest of the cell wall, are composed of polymers of glycerol, phosphates, and the sugar alcohol ribitol. Some have a lipid attached (lipoteichoic acid). The outer surface of the peptidoglycan is studded with proteins that differ with the strain and species of the bacterium.

The gram-negative cell wall, on the other hand, contains only 2-3 layers of peptidoglycan and is surrounded by an outer membrane composed of phospholipids, lipopolysaccharide, lipoprotein, and proteins. Only 10% - 20% of the gram-negative cell wall is peptidoglycan. The phospholipids are located mainly in the inner layer of the outer membrane, as are the lipoproteins that connect the outer membrane to the peptidoglycan. The lipopolysaccharides, located in the outer layer of the outer membrane, consist of a lipid portion called lipid A embedded in the membrane and a polysaccharide portion extending outward from the bacterial surface. The outer membrane also contains a number of proteins that differ with the strain and species of the bacterium.

3.3 Spore staining

Principle A spore is a dormant form of the bacterium that allows it to survive non-optimal environmental conditions. Spores have a touch outer covering made of the protein keratin and are resistant to heat and chemicals. The keratin also resists staining, so extreme measures must be taken to stain the spore. A primary stain of malachite green is forced into the spore by steaming the bacterial emulsion. Malachite green is water soluble and has a low affinity for cell material, so vegetative cells and spore mother cells may be decolorized with water and counterstained with safranin. Spores may be located in the middle of the cell, at the end, or between the end and the middle of the cell. Spore shapes may be round or oval.

ORGANISMS

Trypticase Soy agar plate cultures of *Bacillus / Clostridium*

PROCEDURE

1. Heat-fix a smear of *Bacillus megaterium* as follows:

a. Using the dropper bottle of distilled water found in your staining rack, place a small drop of water on a clean slide by touching the dropper to the slide

b. Aseptically remove a small amount of the culture from the edge of the growth (see Fig. 14) on the agar surface and generously mix it with the drop of water until the water turns cloudy

c. Burn the remaining bacteria off of the loop.

d. Using the loop, spread the suspension over the entire slide to form a thin film

e. Allow this thin suspension to completely air dry

f. Pass the slide (film-side up) through the flame of the bunsen burner 3 or 4 times to heat-fix

2. Place a piece of blotting paper over the smear and saturate with malachite green (see Fig. 19).

3. Let the malachite green sit on the slide for one minute and proceed to the next step.

4. Holding the slide with forceps, carefully heat the slide in the flame of a bunsen burner until the stain just begins to steam (see Fig. 20). Remove the slide from the heat until steaming stops; then gently reheat. Continue steaming the smear in this manner for five minutes. As the malachite green evaporates, continually add more. Do not let the paper dry out.

5. After five minutes of steaming, wash the excess stain and blotting paper off the slide with water . Don't forget to wash of any dye that got onto the bottom of the slide.
6. Blot the slide dry.
7. Now flood the smear with safranin and stain for one minute
8. Wash off the excess safranin with water, blot dry, and observe using oil immersion microscopy. With this endospore staining procedure, endospores will stain green while vegetative bacteria will stain red
9. Observe the demonstration slide of *Bacillus* . With this staining procedure, the vegetative bacteria stain blue and the endospores are colorless. Note the long chains of rod-shaped, endospore-containing bacteria.
10. Observe the demonstration slide of *Clostridium* . With this staining procedure, the vegetative bacteria stain blue and the endospores are colorless. Note the "tennis racquet" appearance of the endospore-containing *Clostridium*

DISCUSSION

A few genera of bacteria, such as *Bacillus* and *Clostridium* have the ability to produce resistant survival forms termed endospores. Unlike the reproductive spores of fungi and plants, these endospores are resistant to heat, drying, radiation, and various chemical disinfectants (see Labs 19 and 20)

Endospore formation (sporulation) occurs through a complex series of events. One is produced within each vegetative bacterium. Once the endospore is formed, the vegetative portion of the bacterium is degraded and the dormant endospore is released.

First the DNA replicates and a cytoplasmic membrane septum forms at one end of the cell. A second layer of cytoplasmic membrane then forms around one of the DNA molecules (the one that will become part of the endospore) to form a forespore. Both of these membrane layers then synthesize peptidoglycan in the space between them to form the first protective coat, the cortex. Calcium dipocolinate is also incorporated into the forming endospore. A spore coat composed of a keratin-like protein then forms around the cortex. Sometimes an outer membrane composed of lipid and protein and called an exosporium is also seen

Finally, the remainder of the bacterium is degraded and the endospore is released. Sporulation generally takes around 15 hours. The process is summarized in

The endospore is able to survive for long periods of time until environmental conditions again become favorable for growth. The endospore then germinates, producing a single vegetative bacterium.

Bacterial endospores are resistant to antibiotics, most disinfectants, and physical agents such as radiation, boiling, and drying. The impermeability of the spore coat is thought to be responsible for the endospore's resistance to chemicals. The heat resistance of endospores is due to a variety of factors:

- Calcium-dipicolinate, abundant within the endospore, may stabilize and protect the endospore's DNA.
- Specialized DNA-binding proteins saturate the endospore's DNA and protect it from heat, drying, chemicals, and radiation.

- The cortex may osmotically remove water from the interior of the endospore and the dehydration that results is thought to be very important in the endospore's resistance to heat and radiation.
- Finally, DNA repair enzymes contained within the endospore are able to repair damaged DNA during germination.

3.4 Capsule Staining

Principle

Very few dyes stain capsules, so they are usually viewed using a negative stain. However, a slightly different procedure will be used to visualize the cells as well as the surrounding medium. In this procedure crystal violet, which is positively charged, will stain the CELLS as well as the MILK in the medium. (It's important to have some substance in the medium that can bind the stain, such as milk, for this staining method to work.) We will wash with 20% $CuSO_4$ to decolorize the capsule and stain it faint blue on a purple background.

ORGANISM

Skim Milk broth culture of *Enterobacter aerogenes*. The skim milk supplies essential nutrients for capsule production and also provides a slightly stainable background.

PROCEDURE (to be done individually)

1. Stir up the Skim Milk broth culture with your loop and place 2-3 loops of *Enterobacter aerogenes* on a slide.
2. Using your loop, spread it out over the entire slide to form a thin film.
3. Let it completely air dry. Do not heat fix. Capsules stick well to glass, and heat may destroy the capsule.
4. Stain with crystal violet for one minute.
5. Wash off the excess stain with copper sulfate solution. Do not use water!
6. Blot dry and observe using oil immersion microscopy. The organism and the milk dried on the slide will pick up the purple dye while the capsule will remain colorless.

7. Observe the demonstration capsule stain of *Streptococcus pneumoniae* (the pneumococcus), an encapsulated bacterium often with a diplococcus arrangement.

RESULTS

1. Recognize capsules as the structures observed when microscopically viewing a capsule stain preparation.

DISCUSSION

Many bacteria secrete a slimy, viscous covering called a capsule or glycocalyx (see Fig. 3). This is usually composed of polysaccharide, polypeptide, or both. The ability to produce a capsule is an inherited property of the organism, but the capsule is not an absolutely essential cellular component. Capsules are often produced only under specific growth conditions.Even though not essential for life, capsules probably help bacteria to survive in nature. Capsules help many pathogenic and normal flora bacteria to initially resist phagocytosis by the host's phagocytic cells. In soil and water, capsules help prevent bacteria from being engulfed by protozoans. Capsules also help many bacteria adhere to surfaces and thus resist flushing

4. Agarose Gel Electrophoresis

Agarose Gel Electrophoresis is a simple and highly effective method for separating, identifying and purifying DNA fragments. The sepration is carried out undear an electric field applied to gel matrix. DNA molecules migrate towards anode due to negatively charged phosphates along the back bone. The rate of migration of aperticular linear DNA fragment is inversely proportional to its molecular weight . The larger molecules travel through the gel matrix at much lower speed compared to the smaller ones. Several parameters like agarose concentration, voltage applied , electrophoresis buffer and DNA concentration affect the migration of DNA through agarose and therefore should be critically selected. EtBr (Ethidium Bromide) intercalates between the bases and emits fluorescent radiation on trans-illuminator.

Requirements
TAE(Tris Aceate EDTA)buffer (50X), Agarose, EtBr (Ethidium Bromide), UV Trans-illuminator, Wather Bath , Electrophoretic unit, Gel casting tray/platform , Combs , Adhesive Tape , Micropipettes and Power pack.

Preparation of 50X TAE(Tris Acetic acid EDTA)buffer:
(For preparation of one liter buffer)

Tris base		121 gms
Glacial acetic acid		57.1ml
0.5M EDTA		100ml
Distle water	up to	1000ml

Preparation of EtBr solution
Weigh 100mg of EtBr and dissolve in 10 ml of distilled water

Gel loading buffer (6X)

(For preparation of 10 ml)

Sucrose	4gms
Bromophenol blue (1%)	2.5ml (25mg)
Dist water	10ml

Procedure:

1. Seal the ends of a suitable plastic gel casting platform with adhesive tape.
2. Place the appropriate comb (8/10/15 teeth) in the ridges on either side of the platform. Ensure that the comb does not touch the surface of the platform . the teeth of the comb from the wells for loading the DNA sample.
3. Take 50ml of 1X TAE buffer in to a conical flask and add 400mg of agarose (0.8%)
4. Heat in a boiling water bath/microwave oven and dissolve agarose by swirling the flask until it becomes transparent.

5. Cool the agarose solution to 50^{o} and add 5ml of EtBr (also see gel staining) . Pour the agarose solution into the gel casting tray without air bubbles.
6. Allow the gel to set for 30-40 min at room temperature
7. Carefully remove the comb, adhesive tape and place the gel in the electrophoresis tank containing 1X electrophoresis buffer. The gel should completely submerged in the buffer.
8. Load the DNA sample with the loading buffer (5ml of loading buffer + 10 μl of DNA sample)

9. Turn on the power and set the voltage to 50 . put off the power supply as the dye reaches the bottom of the gel.

10. View the gel under the Trans-illuminator and photograph the gel.

Gel staining:

1. Addition of 5ml of EtBr solution to agarose is sufficient for staining the gel

Or

2. Remove the gel from electophoresis tank and immerse it in water containing Etidium Bromide (0.5ug/ml) for 15 minutes

Note: Ethydium Bromide is a powerful mutagen and therefore handle the gel only after wearing the gloves. Use seprate trays and glassware for handling EtBr.

5. Estimation of DNA by diphenylamine reaction

Principle:

This is a general reaction given by deoxypentoses. The 2-deoxyribose of DNA, in the presence of acid, is converted to *W* – hydrxylevulinic aldehyde which reacts with diphenylamine to form a blue coloured complex with absorbance maxima at 600nm. Compounds such as furfural alcohol and arabinal, which can be converted in to *W*-hydroxylevulinic aldehyde will also give this reaction. In DNA since only deoxyribose of purine nucleotides is released, the value obtained represents half of the total deoxyribose in the sample. The reaction leading to the formation of the coloured complex is as follows:

2-deoxy-D-ribose ---HClO4---→ (w- Hydroxylevulinic aldehyde) ---Diphenylamine--- Blue coloured complex

Materials and Reagents:

1. Colorimeter or Spectrophotometer
2. Standard DNA solution: Dissolve DNA 100 (µg /ml) in 1N $HCLO_4$ by heating at 70^o for 15 min. Make different dilutions of this stock solution ranging from 20 – 100 µg DNA/ml using 0.5N $HCLO_4$.
3. 1.6% (*w/v)* acetaldehyde: Prepare by dissolving 1ml of ice cold acetaldehyde in 50 ml of distilled water.
4. Diphenylamine solution: Dissolve 1.5 g of diphenylamine in 100 ml of glacial acetic acid and 1.5 ml of conc. H_2SO_4
5. Diphenylamine Reagent: Prepare by mixing 0.5 ml of 1.6% of acetaldehyde and 00ml of diphenylamine solutions. This solution must be prepared fresh.

Procedure:

1. Take 2.0 ml of the sample in which DNA has to be estimated in a test tube.
2. In another set of test tubes, pipette 2.0 ml of standard DNA SOLUTION OF DIFFERENT DILUTIONS. In one of the test tube take 2.0 ml of 0.5 N HCLO4 as reagent blank.
3. Add 0.4 ml of diphenylamine reagent (Reagent no 5) to all the tubes, mix the contents properly and keep at room temperature in dark for 16 to 18 hrs or over night.
4. Record the absorbance at 600nm.
5. Draw a standard curve at Absorbance 600 *vs* DNA concentration. From absorbance of the sample, determine the amount of DNA in it. Express the results as mg of DNA /g.

6. Estimation of RNA by orcinol method

Principle:

This is a general method for estimation of Pentoses. Acid hydrolysis of RNA release ribose which in the presence of strong acid undergoes dehydration to yield furfural. Orcinal, in the presence of ferric chloride as a catalyst reacts with furfural producing green coloured compound with absorbance maxima at 665 nm. DNA gives limited positive reaction with orcinal test. The reactions leading to the formation of green coloured complex are as follows:

	Acid		Orcinol	
Ribose	----------	furfural	----------	green colored complex

Materials and Reagents:

1. Colorimeter or Spectrophotometer
2. Boiling water bath.
3. 5% HCLO4
4. Standard RNA solution: Dissolve yeast RNA (500µg/ml) in 5% HCLO4. Make different solutions containing 100µg to 500µg RNA /ml with 5% HCLO4
5. Orcinol reagent: Dissolve 100mg of ferric chloride (fecl3.H2O) in 100 ml of HCl and then add 3.5ml of 65 solution of Orcinol prepared in alcohol.

Procedure

1. Take 2.0 ml solution of each of the dilutions of RNA standard solutions, test sample and 2.0ml of 5% HCLO4 as blank, in different test tubes.
2. Add 3.0 ml of Orcinol reagent to all the tubes and mix properly.
3. Keep the test tubes in boiling water bath for 20 min.
4. After cooling them add 7.0 ml of *n*-butanol to each tube and measure at A600nm against blank.
5. Plot agar between Absorbanceal 600 vs. amount of RNA in the provided sample.

7. Restriction enzyme digestion

Principle:

Bacteria are under constant attack by bacteriophages. To protect themselves, many types of bacteria have developed defense mechanism in the form of enzymes called endonucleases chop up any foreign DNA. Since these enzymes restrict the infection of bacteriophages they are termed as "Restriction endonucleases". These molecular scissors found in bacteria l cytoplasm can prove dangerous to the cell, so bacteria protect their own DNA by methylating the adenine or cytosine bases. The methyl groups block the binding of restriction enzymes, but not the normal reading and replication of genetic information. DNA from an attacking bacteriophage will not have these protective methyl groups and will be destroyed.

Together restriction enzymes and its modification methyl transferase from a restriction modification (rm) system. Four kinds of RM systems are known. These are distinguished based on subunit composition, kinds of sequences recognized and cofactors needed for activity. Most characterized enzymes belong to type II class. They comprise the commercially available restriction enzymes used for DNA analysis and other manipulations.

Each restriction enzyme has a unique name, derived from genus, species and strains of bacteria that produce them, fallowed by a number that refers to discovery order. e.g EcoR1 and EcoR V are both from Escherichia coli, and strain R, 1 and V are the order in which they were discovered.

Restriction enzymes are powerful tools of molecular genetics used to:

- Map DNA molecules
- analyze population polymorphisms
- rearrange DNA molecules
- prepare molecular probes
- create mutants

The Type II restriction enzymes recognize specific DNA sequences and cleave the DNA at fixed locations at or near the recognizing sites. They act as dimers, each subunit recognizing the same 5'-3' nucleotide sequences in complementary DNA strands and hence are said to recognize palindromic sequences. Before the restriction enzymes cuts at its specific recognition site on a long DNA, it binds to a site amidst a very large number of non-cognate sites. This protein is then translocated from its initial site to specific, which occurs by "jumping or sliding". At this recognition site in presence of Mg^{2+}, enzyme under goes a conformational change, which kinks the helix and cleaves the DNA producing 'blunt' or 'sticky' ends.

For example:

Hae III produced by *Haemophilus aegypticus* cleaves straight across the double helix when it encounters the following sequence to p

5'- GGCC-3' ⟶ 5'-GG CC-3'

3'-CCGG-5' 3'-CC GG-5'

How ever, many restriction enzymes cut in an offset fashions to give sticky ends, which have protruding 5 or 3 ends with unpaired bases depending upon the restriction enzyme, for e.g. EcoR I recognizes the following sequence and cleaves each backbone between G and A base residue giving 5' protruding ends.

5'- GAATTC-3' ⟶ 5'-G AATTC-3'
3'-CTTAAG-5' 3'-CTTAA G-5'

Pst 1 recognizes the following sequence, cleaves between A and G residue to give 3' protruding ends.

5'- CTGCAG-3' ⟶ 5'-CTGCA G -3'
3'-GACGTC-5' 3'-G ACGTC-5'

The resulting sticky ends can base pair with any DNA molecule that has the complementary sticky ends to give a recombinant DNA molecule.

Any restriction endonuclease will cut only a specific base sequence, no matter what DNA molecule it is acting on. However, a given recognition sequence can be recognized by multiple enzymes called **Isoschizomers.**e.g.: Sma 1 and Xma 1. These can cleave the DNA at same or different position with in the recognition sequence. Frequency of cleavage depends on the probability of occurrence of recognition sequences. Thus enzyme with longer sequences cut less frequently and consequently produces large fragments than do enzymes with shorter recognition sequences.

Factors effecting Restriction Enzyme Activity:
Temperature:
Most digestions are carried out at 37^0 C. However, there are a few exceptions e.g.: digestion with Sma 1 is carried out at lower temperatures (25^oC), while with Taq 1 at higher temperaturei.e.65^0 C.

Buffer systems:

Tris-HCl is the most commonly used buffering agent in incubation mixtures which is temperature dependent. Most restriction enzymes are active in the pH range 7.0-8.0.

Ionic conditions:
Mg^{+2} is an absolute requirement for all restriction endonucleases,but the requirement of other ions(Na+/K+)varies with different enzymes.

Methylation of DNA: Methylation of specific adenine or cytidine residues within the recognition sequence of the restriction enzyme affects the digestion of DNA.

Materials required:
The list below provides information about the materials required.

- 2X assay buffer λ/Mlu 1 digest,2.5 X gel loading buffer, Lambda DNA (substrate), restriction enzymes: EcoRI, HindIII, Agarose, 6X staining dye, 50X TAE, 1.5ml vials.

- Equipment: Dry bath, Gel rocker (optional)
- Glass ware: Beaklers, conical flasks, measuring cylinders, staining tray
- Reagent: Distilled water
- Other requirements: Crushed ice/genei cooler, Tips, micro pipette.

Note:

- Enzymes are temperature sensitive; hence place the vials containing enzyme on ice.
- Ensure thorough mixing by gently tapping the vial, after the addition of buffer and substrate to the enzyme vial.
- Set the dry bath at 37 ^{0} C prior to starting the experiment.
- For preparation of gel, staining and destaining, refer agarose gel electrophoresis.

Procedure:

1. Place the vials containing restriction enzyme (EcoRI, HindIII) on ice.
2. Thaw the vials containing substrate(Lambda DNA) and assay buffer
3. Add 20 µl of λDNA (substrate) to each of the enzyme vials.
4. Add 25µl of 2X assay buffer to the enzyme and DNA mixture, mix by tapping the tube.
5. Incubate the vials at 37^{0}C for 1 hour.
6. Meanwhile, prepare a 1% agarose gel for electrophoresis.
7. After an hour, add 5µl of gel loading buffer to each of the enzyme vials.
8. Label a 1.5ml vial as S; add 10µl of substrate and 1µl of gel loading buffer to it.
9. Label a 1.5ml vial as M; add 10µl of marker and 1µl of gel loading buffer to it.
10. Load the digested samples, substrate and marker, note down the order of loading.
11. Electrophorese the samples at 50-100 V for1-2 hours.
12. Stain the agarose gel with 1X staining dye.
13. Destain to visualize the DNA bands.

8. The Folin-Lowry method of protein assay

Principle:
Protein reacts with the Folin coecalteau reagent to give a colour complex. The colour is so formed is due to the reaction of the alkaline copper with the protein as in the biuret test and the reduction of phosphor molybdate by tyrosine and tryptophan present in the protein. The intensity of the colour depends on the amount of these aromatic amino acids present and will thus vary for different proteins.

Materials:

1. Alkaline sodium carbonate solution (20g/lit Na_2CO_3 in 0.1 moles/lit NaOH).
2. Copper sulphate –sodium potassium tartarate solution (5g/lit $CuSO_4.5H_2O$in 10g/lit Na, K tartarate).Prepare fresh by mixing stock solutions.
3. 'Alkaline solution'. Prepare on day of use by mixing 50 ml of (1)and 1ml of(2).
4. Folin- coecalteau reagent (Dilute the commercial reagent with an equal volume of water on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids).
5. Standard protein (Albumin solution 0.2mg/ml).

Method:
Add 5ml of alkaline solution to 1ml of test solution. Mix thoroughly and allow to stand at room temperature for 10 min. or longer. Add 0.5ml of diluted Folin- coecalteau reagent rapidly with immediate mixing. After 30 min read the extinction against the appropriate blank at 750nm.

Estimate the protein concentration of an unknown solution after preparing a standard curve.

9. Estimation of carbohydrate by Anthrone method

Principle

The anthrone reaction is the basis of a rapid and convenient method for the determination of hexose, also pentoses and hexuronic acids, either free or present in poly-saccharides.The blue green solution shows an absorption maximum at 620 nm, although some carbohydrates may give other colours the reaction is not suitable when proteins containing a large amount of tryptophan are present, since red colour is obtained under these conditions.

The extinction depends on the compound investigated, but is constant for a particular molecule.

Materials:

1. Anthrone reagent(2g/lit in conc.H2SO4)
2. Glucose(0.1g/lit)
3. Glycogen(0.1g/lit)
4. Other carbohydrates of the same concentration if desired.

Method:

Add 4ml of anthrone reagent to 1ml protein free carbohydrate solution and rapidly mix. Place the tubes in a boiling water bath for 10 min with a marble on top to prevent loss of water by evaporation cool and read the extinction at 620nm against a reagent blank.

Prepare standard curves for the glucose and glycogen solutions and compare them. Remember that glucose exists as the glycoside form ($C_6H_{10}O_5$) in glycogen of molecular weight 162, *not* 180.Examine the purity of a number of samples of commercial glycogen.

10. Estimation of blood cholesterol

Principle

Acetic anhydride reacts with cholesterol in chloroform solution to produce a characteristic blue-green colour.The nature of the chromophore is not known but the reaction probably includes esterification of the hydroxyl grouping in the 3rd position as well as other rearrangements in the molecule.

Blood or serum is extracted with an alcohol-acetone mixture which removes cholesterol and other lipids and precipitates protein. The organic solvent is removed by evaporation on a boiling water bath and the dried residue is dissolved in chloroform. The cholesterol is then determined colorimetrically using the Liebermann-Burchard reaction. Free cholesterol is equally distributed between the cells and plasma while the esterified form occurs only in plasma.

It is essential to use absolutely dry glassware for this estimation.

Materials:

1. Serum or blood-25ml
2. Alcohol-acetone mixture-(1:1)-2lit
3. Chloroform-500ml
4. Acetic anhydride-Sulphuric acid mixture(30:1,mix just before use)-930ml
5. Stock cholesterol solution(2mg/ml in chloroform)-250ml
6. Working cholesterol solution(Dilute the above solution i1 in 5 with chloroform to give a solution of 0.4mg/ml)-1lit

Method:

Place 10ml of the alcohol-acetone solvent in a centrifuge tube and add 0.2 ml of serum or blood. Immerse the tube in a boiling water bath with shaking until the solvent begins to boil. Remove the tube and continue shaking the mixture for a further 5min.Cool to room temp. and centrifuge. Decant the supernatant fluid into a test tube and evaporate to dryness on a boiling waterbath. Cool the dissolve d residue in 2ml chloroform. At the same time, set up a series of standard test tubes containing cholesterol and a blank with 2ml of chloroform.

Add 2ml of acetic anhydride-sulphuric acid mixture to all test tubes and thoroughly mix. Leave the tubes in the dark at room temp. and read the extinction at 680nm.

11. Transformation

Principle:
The process of uptake of free or external DNA from the surrounding medium by bacterial cells is known as bacterial transformation. The process of uptake is limited in nature while in-vitro uptake can be created by competent conditions such as use of Calcium chloride.
The uptake of DNA by bacterial cells is known as competent and the phenomenon of competence is not understood well. The process involves, initial binding of DNA to the cell wall. (This complex is resistant to DNAse). The cells are then briefly exposed to a temperature of 42^0C(Heat-shock) where in pores are created and DNA is taken up. The cells are said to be transformed.

Screening of transformants:
The pUC plasmid used has ampicillin resistant factor and the transformed cells are able to grow on ampicillin containing medium.

Screening of recombinants:
The transformed cells contain few recombinants they are identified by insertional-inactivation a process by which inactivated gene formed is due to insertion and no longer displayed. The pUC-18 a high copy number plasmid has information for first 146 amino acids (NH_2-terminal) of β-galactosidase gene.

The E.coli strain used lacks this portion and it synthesizes an inactive C-terminal fragment. The plasmid encoded fragment and the C-terminal combine to form an enzymatically active protein. This type of complementation is known as α-complementation.

Lac+ bacteria that results from α-complementation can be recognized as blue coloured colonies in the presence of X-Gal. Insertion of fragment of foreign DNA into a poly cloning site of plasmid results in no α-complementation. The colonies are white in the presence of X-Gal (Blue-White screening).

Procedure:
- The host is revived using LB medium.
- Inoculate a single colony of host which is grown on LB plate into 5ml of LB medium and incubate at 37^0C on a shaker for overnight.
- Inoculate 1ml of overnight culture into 100ml LB medium and incubate at 37^0C on shaker. Grow, until the O.D reaches to 0.23-0.26 at 600nm.
- Chill the culture flask on ice for 10-20 min, transfer the culture aseptically into sterile centrifuge tubes and centrifuge at 6000rpm for 2 min.
- Discard the supernatant and to the pellet add approximately 15ml of cold 1M $CaCl_2$.
- Suspend the pellet gently into the solution.
- Place the tubes on ice for 30 min. and centrifuge these tubes at 3000rpm for 8 min at 4^0C.

- Discard the supernatant and suspend gently in 0.6ml of cold 0.1 M CaCl2 solution.
- Aseptically add aliquots 100µl of competent cells into pre-chilled vials.

Transformation procedure:

- Add 5µl of plasmid DNA to 5 aliquots of 100µl competent cells.
- Gently tap and incubate on ice for 10min.
- Heat shock the cells by placing the cells at 42^0C on water bath for 2min.
- Immediately return the vials to chill for 5min.
- Add 1ml of LB broth aseptically to the vials and incubate at 37^0C for an hour.
- Prepare LB plates and label 3 LB ampicillin plates with X-Gal and IPGT as A, B, C.
- Pipette 100µl of LB broth onto each plate and add 25, 50 and 100µl of transformed cells to the plates, A, B, C.
- Mix well and spread by using a spreader.
- Incubate the plates at 37^0C.

12. Isolation of bacteria from soil

Principle:
The main principle in the isolation of bacteria is the serial dilution technique. In this, the dilution of the sampling is done at various levels, of the order10^{-1},10^{-2},10^{-3}, 10^{-4} and so on. This increase in dilution of the sample will isolate individual colonies.

Procedure:

1. 1gm of the soil sample was weighed and it was diluted with 100ml of distilled water.

2. The requirements which are involved in the experiment such as test tubes, petri plates, and media are sterilized in an autoclave at 121^0C, 15lbs pressure for 15-20min.

3. Then in a sterilized room, under laminar flow hood then experiment was conducted.
4. The test tubes were serially diluted with soil sample. In this, to all the test tubes,9ml of the sterilized water was added, and to then first tube,1ml of the soil sample was added and fro this,1ml was taken and diluted to 9ml of water containing in another tube, that gives10^{-1} dilution. Similarly, the dilutions of 10^{-2},10^{-3},and so on were made.

5. Then from these dilutions, 0.1ml of the sample was taken and it was spreaded over the agar media in a Petri plate which has already been solidified.
6. Then finally, they were incubated in an incubator at 37^0C for 24-48 hours.

13. Isolation of Fungi from soil

Principle:
Soil, water, air are the richest sources of fungi. The technique mainly employed for isolation of fungi includes serial dilution technique, soil plate method, inversion tube method etc. of then dilution plate and soil plate methods are most widely used .Isolation of fungi from soil is important because of its biological importance.

Procedure:

1. Medium: Potato-150gm, Dextrose-40gm, agar-20gm, Distilled water 1000ml, pH-5.5.
2. 1gm of the soil sample was weighed and it was diluted with 100ml of distilled water.
3. The requirements which are involved in the experiment such as test tubes, Petri plates, and media are sterilized in an autoclave at 121^0C, 15lbs for 15-20min.
4. Then in a sterilized room, under laminar flow hood then experiment was conducted.
5. The test tubes were serially diluted with soil sample. in this, to all the test tubes,9ml of the sterilized water was added, and to then first tube,1ml of the soil sample was added and fro this,1ml was taken and diluted to 9ml of water containing in another tube, that gives10^{-1} dilution.Similarly,the dilutions of 10^{-2},10^{-3},and so on were made.
6. Then from these dilutions, 0.1ml of the sample was taken and it was spreaded over the agar media in a petri plate which has already been solidified.
7. Then finally, they were incubated in an incubator at 37^0C for 24-48 hours.

14. A, B, O Blood grouping

Principle:

Karl Land Steiner was the first person to report four different immunological human blood types that are, A, B, O and AB. The A,B,O blood groups are genetically controlled ,type-A blood has A antigen(glycoproteins)on the R.B.C's,Type-B has B-antigens, Type-AB has both A&B antigens and Type-O has neither antigens. These A, B, O antigens are inherited as to (one from each parent).Of the three alternative alleles A,B or O.The interaction between antibody and a particulate antigen results in visible clumping called agglutination. The agglutination reactions are useful for determining blood groups.

Procedure:

1. The ring finger was sterilized with cotton which is moistened with 70% alcohol and allowed to dry.
2. Then punctured the finger with the help of a sterile hypodermic needle and squeezed the finger in order to allow the drops of blood.
3. Two different individual drops were placed on a clean slide.
4. To these drops anti-A antibodies and anti-B antibodies were added.
5. With the help of separate glass rods, mixed the sera and the blood drops.
6. A visual clumping or agglutination was observed.

15. Rh factor determination

Principle:

Land Steiner and Wiener were the persons first reported the Rh factor (Rhesus monkey) and designated as D antigen. The Rh factor is a complex of many antigens. The persons processing the Rh agglutinogens were called as Rh positive.Generally, a person Rh type results from a combination of two possible alleles, a dominant one that produces the factor and a recessive one does not. A person inheriting at least one Rh gene will be Rh positive. Only those persons inheriting two recessive genes are Rh negative. The presence of Rh factor in human blood is determined by agglutination reaction between anti-B serum and RBC.

Procedure:

1. One drop of the anti-D serum was placed on a cleaned glass slide.
2. To this, four drops of blood was added.
3. With the help of a glass rod the serum and the blood were mixed thoroughly.
4. And allowed to stand for 2 min.
5. A visible clumping was observed.

16. Analysis of water quality

The three basic tests to detect the quality of water are-

- Presumptive test
- Confirmation test
- Completed test

Presumptive test:

Principle:

Presumptive test is specific for detection of coli form bacteria. Measured aliquots of the water are added to the Lactose fermentation broth containing an inverted glass vial. Because the bacteria are capable of using Lactose as carbon source their detection is specified by use the use of this medium. In addition, to lactose media also contains surface tension, depresent bile salts used to suppress the growth of organisms, other than coli forms. Of these lactose medium are inoculated with 10, 1, 0.1 ml aliquots of water sample. The greater the number of tubes, the greater the sensitivity. The presumptive test enables top obtain the number of coli forms by means of the most probable number. The most probable number is estimated by determining the number of tubes that shows gas following the incubation period.

Lactose fermentation broth:

Composition:	Single strength (g)	Double strength (g)
Beef extract	3	6
Peptone	5	10
Lactose	5	10
Distilled water	1000ml	1000ml

Procedure:

1. Arranged three test tubes series consisting of three groups to a total of nine tubes per series 0.1, 1,10ml.

2. Label the water sources and volume of the sample was inoculated

3. Durham tubes were dropped into the medium in inverted position.

4. Care should be taken to avoid the gas bubbles in the Durham tubes while dropping.

5. The medium was sterilized and innoculated the sample under sterilized conditions.
6. Then finally the tubes were incubated at 37^0C for 24-48 hours.

Confirmation test:

Principle:

In the confirmation test the samples from the positive presumptive lactose broth tubes are streaked on a selective differential medium for coli forms. The medium is eosin methylene blue that is selective in nature. Becuase of the presence of the dye methyline blue which inhibits the growth of gram +ve bavcteria allowing the growth of gram –ve bacteria. Lactose fermenting bacteria gives colour colonies due to the formation of complex in the EMB.

Procedure:

- Innoculated the EMB agar plates with the +ve 24hr.lactose broth culture with a sterile inoculated loop by streak plate technique.
- Then the plates were inncubated for 24-48hrs at 37^0c in an inverted position.

Completed test:

Principle:

Completed test is used as a confirmatory test for the presence of E.coli in water sample. In this test, lactose +ve colonies from EMB agar are isolated and innoculated into a lactose broth tube and streaked on a nutrient agar plate to perform gram staining. If there is production of acid and gas in the innoculated lactose and there are rod shaped bacteria showing gram-ve reaction, these confirm the presence of E.coli in the water.

Procedure:

- By using an innoculating loop the lactose fermentation broth was innoculated with the isolated coli forms.
- The nutrient agar slants were streaked with the colonies obtained from the EMB agar culture.
- Incubated both the inoculated broth and slants at 37^0C for 24hr.
- After incubation the organisms obtained from the agar slants were stained by gram staining.

17. Ouchterlony double diffusion

Principle:

Immunodiffusion in gels encompasses a variety of techniques which are useful for the analysis of antigen and antibodies. An antigen reacts with a specific antibody to form Ag-Ab complex. The composition of which depends on the nature, concentration and proportion of the initial reactants.
Immuno diffusion in gels are classified a s single and double diffusion. In ouchterlony double diffusion both antigen and antibody are allowed to diffuse into the gel.Thus, assay is frequently used for comparing different antigen preparation. In this test, different antigen preparation, each containing single antigenic species are allowed to diffuse from separate wells against the anti serum. Depending on the similarity between the antigens, different geometrical patterns are produced between antigen and antiserum wells. The patterns of lines that formed can be interpreted to determine whether the antigens are same or different as illustrated.

Pattern of identity:

The antibodies in the antiserum react with the both the antigens resulting in a smooth line of precipitate. The antibodies can not distinguish between the two antigens i.e. the two antigens are immunologically identical.

Pattern of partial identity:

In the pattern of identity the antibody in the anti serum react with the one of the antigens than the other. This spur is thought to result from the determinants present in one antigen but lacking in the other antigen.

Pattern of non identity:

In the pattern of non identity none of the antibodies in the antiserum react with the antigenic determinants that may be present in both the antigens i.e. the two antigens are immunologically un related as for as the antiserum is concerned.

Procedure:

1. Prepare 25ml of 1.2%agarose (0.3g/25ml) in 1X assay buffer by boiling to dissolve the agarose completely.
2. Cool the solution to 55-60^{0}C and pour 4ml per plate on to 5 grease free glass plates and place on a horizontal surface.
3. Allow the gel to set for 30min.
4. Punch the wells by keeping the glass plates on the template.
5. Fill the wells with 10µl each of the antiserum and corresponding antigens.
6. The glass plate was placed in a moist chamber for over night at 37^{0}C.
7. Plates were incubated for the precipitin lines between antigen and antisera wells.

18. Antiseptic test (phenol co-efficient method)

Principle:

The phenol co-efficient test compares the anti microbial activity of a chemical compound to that of phenol. Equal quantities of a series of dilutions of the chemical being tested and of the pure phenol are placed into sterile tubes. A standardized quantity of pure culture of the test microorganism was added to each of the test tubes. Sub- cultures of the test microorganisms are made from each dilution of the test chemicals into a sterile broth media at intervals of 5, 10, 15 min. after introduction of the organism. and the sub cultures are incubated .the phenol co-efficient is determined by dividing the highest dilution of the chemical being tested that destroyed the microorganisms in 10 min. but not in 5 min. by the highest dilution of phenol that destroyed the micro organisms in 10 min. but not in 5 min.
If the phenol co-efficient ratio is 1, it is more effective, and if it is less than 1, indicates the chemical agent is less effective.

Procedure:

1. Label the 30 nutrient broth tubes with the name and dilution of the disinfectant and the time interval of the sub culturing (ex: phenol 1:20 5 min.).
2. All the test tubes are placed in a test tube rack place one test tube of each of the different phenol and dettol dilutions.
3. One drop of the S.aureus culture was introduced in each of the test tubes with disinfectants and time of the introducing the microorganisms into the disinfectant was noted.
4. Agitate all the test tubes so as to ensure contact of the disinfectant and the microbes.
5. By using a sterile loop at intervals of 5,10,15 transfer 1 loopful from each of the test tubes containing thee disinfectant and the microorganisms into the approximately labeled sterile nutrient broth tube.
6. Incubate all the nutrient broth cultures at 37^0C for 48 hrs.

19. Chromatography

19.1 Column chromatography

Separation of compounds by column chromatography must be one of the most widely used techniques in biochemical work. It is there fore appropriate to consider some of the general precautions to be taken when preparing and running columns before dealing with the various types of chromatographic separations.

Columns:

Chromatography columns are usually glass and, generally, long columns give good resolution of components but wide columns are better for dealing with large quantities of material. The essential features of a chromatography column are shown in fig:

Preparation of the material:

Wide range of materials are used in chromatographic separations and all need to be equilibrated with the solvent before preparing the column. In addition, some form of pre treatment is often required; for example, some gel filtration materials need to be swollen, absorbents need to be obtained in the required ionized form by washing.

During the equilibration with solvent the material is allowed to settle and the fine particles remaining in suspension are removed by decantation. If this is not carried out, the flow rate of solvent down the column will be considerably reduced due to clogging by these fine particles.

The pouring of the column:

The chromatography column is packed with material by filling it about one third full with solvent and slowly adding a slurry of the material in the solvent. This is carefully poured down a glass rod, to stop air bubbles being trapped in the column. The suspension is allowed to settle and excess solvent run off. This process is repeated until the column is the required height. The column is then washed thoroughly with solvent and the level of the liquid kept just above the surface of the material.

Application of the sample:

The sample is first dissolved in the solvent or dialyzed against the eluting buffer before loading it on the column. In most class experiments the concentrated sample is carefully pipetted on to the surface and the tap opened until the top of the column is just bellow the level of the meniscus. The solvent reservoir is connected, and a constant head of liquid maintained at the top of the column from a pressure reservoir.

Elution:

The next stage is to remove the material from the column in order by eluting with an appropriate solvent .In displacement development; the solvent interacts more strongly with the chromatographic material than the compound on the column, thus displacing the bound molecules. Large quantities of the material can be separated in this way since about 50 percent of the total column capacity is used. The separation is adequate but for better resolution of peaks elution development is preferred .In this case, no more than 10 percent of the total capacity is loaded on to the column. The solvent interacts with the column more weakly than the solute molecules and overrides the bound

molecules, gradually eluting them from the column. This is probably the most commonly used means of eluting and different molecules are removed from the column by changing the strength or pH of the eluting solvent in a step wise fashion or by means of a gradient which can be linear, concave or convex.

The collection and analysis of fraction*:*
The effluent from the column is collected into a series of test tubes, either manually or with a fraction collector. Each fraction is then analyzed for the presence of the compounds being examined and an elution profile prepared of the amount eluted against the effluent volume.

19.2 Paper chromatography
Principle:
Cellulose in the form of paper sheets makes an ideal support medium where water is absorbed between the cellulose fibers and forms stationary hydrophilic phase. The mixture is spotted onto the paper, dried and the chromatogram developed by allowing the solvent to flow along the sheet. The solvent front is marked and, after drying the paper, the positions of the compounds present in the mixture are visualized by a suitable staining reaction. The ratio of the distance moved by a compound to that of moved by the solvent is known as the R*f* value and is more or less constant for particular compound, solvent system and paper under carefully controlled conditions of solute concentration, temperature and pH.
The Rf is related to the partition co-efficient α.

$$\alpha = \frac{A1}{As}(1/Rf - 1)$$

A1=area of cross section of liquid phase,
As=Area of cross section of solid stationary phase
Preparation of the sample:
Biological materials should be desalted before chromatography by electrolysis and electrodialysis. Excess salt results in a poor chromatogram with spreading of spots and changes in their Rf values. It can also affects the chemical reactions used to detect the compounds being separated. Macro molecules such as proteins are also removed prior to chromatography by ultra filtration or gel filtration. The sample (10-20µl) is then applied to the paper with a micro pipette.
Paper: Whatman no: 1 is the paper most frequently used for analytical purposes. Whatman no: 3 MM is a thick paper and is best employed for separating large quantities of material; the resolution is however inferior to Whatman no: 1.for a rapid separation, Whatman no: 4 and 5 are convenient although the spots are less well defined. In all cases, the flow rate is faster in the machine direction which is normally noted on the box containing the papers. The paper may be impregnated with a buffer solution before use are chemically modified by acetylation. Ion exchange papers are also available commercially.
For the separation of lipids and similar hydrophobic molecules, silica impregnated papers are available commercially.
Solvent:
This choice, like that of the paper is largely empirical and depends on the mixture investigated. If the compounds move close to the solvent front in solvent A, then they

are too soluble, while if they are crowded around the origin in solvent B, then they are not sufficiently soluble. A suitable solvent for separation would therefore be an appropriate mixture of A and B, so that the Rf values of the components of the mixture are spread across length of the paper.

Ascending chromatography:

The sheet of the paper is supported on a frame with in the bottom edge in contact with trough filled with solvent, alternatively the paper can be rolled into a cylinder fastened with a paper clip and stood in the solvent. The arrangement is contained in an air tight tank lined with paper saturated with the solvent to provide a costatnt atmosphere and separations are carried out in a constant room temperature.

Descending chromatography:

This method is convenient for compounds which have similar Rf values since the solvent drips off the bottom of the paper, thus giving a wider separation.

Two dimensional chromatography:

The mixture is separated in the first solvent which should be volatile then after drying, the paper is turned through 90^0 and separation is carried out in the second solvent .After location a map is obtained and compounds can be identified by comparing their position with a map of unknown compounds developed under the same conditions.

Detection of spots:

Most compounds are colourless and are visualized by specific reagents. The location reagent is applied by spraying the paper or rapidly dipping it in a solution of the reagent in a volatile solvent. Viewing under UV light is also useful since some compounds which absorb strongly show up as dark spots against the fluorescent background of the paper.

19.3 Thin layer chromatography

Principle:

Separation of compounds on a thin layer is similar in many ways to paper chromatography, but has added advantage that a variety of supporting media can be used so that separation can be by absorption, ion exchange, partition chromatography or gel filtration depending on the nature of the medium employed. The method is very rapid and many separations can be completed in an hour. Compounds can be detected at a lower concentration than on paper as the spots are very compact. Further more separated compounds can be detected by corrosive sprays and elevated temperatures with some thin layer materials, which of course is not possible with paper.

Production of thin layer:

The RF value is affected by the thickness of the layer below 200 μm and a depth of 250μm is suitable for most separations. Several good spreaders and commercially available plates are available in the market. Calcium sulphate is some times is incorporated into the absorbent to bind the layer to the plate.

Development: It is essential to make sure that the atmosphere of the separation chamber is fully saturated; otherwise RF values will vary wisely from tank to tank. This can be ensured by using as small a tank as possible and lining the walls with paper soaked in the solvent. Development of the plate is usually is by the ascending technique and is very rapid.

Location: The compounds are located as for paper chromatography by spraying with the appropriate reagent or by scanning, in the case of radioactive substances.

20. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

INTRODUCTION

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the nett charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique.

Principal

The landmark improvements to protein electrophoresis were the use of polyacrylamide for control of separation by molecular size, and the use of sodium dodecyl sulfate (SDS; or lauryl sulfate) to denature proteins in order to ensure reproducibility of the technique. SDS is an anionic detergent, meaning its molecules have a net negative charge. It binds to most soluble protein molecules in aqueous solutions over a wide pH range. Polypeptide chains bind amounts of SDS that are proportional to the size of the molecules. The negative charges on SDS destroy most of the complex (secondary and tertiary) structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field.

A polyacrylamide gel with acrylamide content above a critical density restrains larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in molecular weight (MW) of polypeptides. In a gel of uniform density the relative migration distance of a protein (Rf) is negatively proportional to the log of its MW. If proteins of known MW are run simultaneously with the unknowns, the relationship between Rf and MW can be plotted, and the MWs of unknown proteins determined. Protein separation by SDS-PAGE is used to determine the relative abundance of major proteins in a sample, their approximate molecular weights, and in what fractions they can be found. The purity of protein samples can be assessed.

You will need the following reagents:

1. 5x Sample Buffer

10% w/v	SDS
10 mM	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenolblue

Should add up to 8M urea for really hydrophobic proteins

2. 1x Running Buffer:

25 mM	Tris-HCl
200 mM	Glycine
0.1% (w/v)	SDS

1x Running Gel Solution

For different applications increase your desired percentage acrylamide, make up thirty ml of running gel by selecting one of the following percentages and mixing the ingredients shown below. After adding TEMED and APS your gel will polymerize fairly quickly, so do not add these until you are sure you are ready to pour.

	7%	10%	12%	15%
H_2O	15.3 ml	12.3 ml	10.2 ml	7.2 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	7.5 ml	7.5 ml
20% (w/v) SDS	0.15 ml	0.15 ml	0.15 ml	0.15 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	6.9 ml	9.9 ml	12.0 ml	15.0 ml
10% (w/v) ammonium persulfate (APS)	0.15 ml	0.15 ml	0.15 ml	0.15 ml
TEMED	0.02 ml	0.02 ml	0.02 ml	0.02 ml

Stacking Gel Solution (4% Acrylamide):

H_2O	3.075 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
20% (w/v) SDS	0.025 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (APS)	0.025 ml
TEMED	0.005 ml

Pouring the Gels:

Choose a percentage acrylamide based on the molecular weight range of proteins you wish to separate:

% Gel	M.W. Range
7	50 kDa - 500 kDa
10	20 kDa - 300 kDa
12	10 kDa - 200 kDa
15	3 kDa - 100 kDa

Now mix the ingredients needed for the chosen percentage and pour the solution quickly into your gel casting form - be sure to leave a some room for the stacking gel - I usually leave about 2 centimeters below the bottom of the comb for the stacking gel. You can do this by inserting the comb into the dry form, and marking a region below the comb for the height of the stacker you want. Look for bubbles and remove them, then layer the top of the gel with water or saturated butanol very carefully, with water. This will remove bubbles at the top of the gel and will ensure this part does not dry out. Wait for about 30 minutes for the gel to polymerize completely. (If you always use fresh ammonium persulfate, you're gel may polymerize more quickly and reliably.)

While waiting mix the reagents for the stacking gel, but LEAVE OUT the APS and TEMED until you are ready to pour this gel; stacking gels will polymerize more quickly than desired sometimes while one is trying to add combs to make wells.

When the running gel is polymerized wash out the butanol completely or your stacker may separate from the gel and you will get ugly running artifacts. Mix in the polymerizing reagents and pour the stacking gel on top of the running gel. Insert your combs trying not to get bubbles stuck underneath and allow another 30 min - 1 hour for complete polymerization. Your gels are ready!

Preparing your Sample:
Mix your protein 4:1 with the sample buffer. Heat your sample by either:
a) Boiling for 5-10 minutes (Works for most proteins)
b) 65^{O}C for 10 minutes (If you have smearing using the above procedure)
c) 37^{O} for 30 minutes (Membrane proteins or others that do not enter the gel otherwise may benefit from this type of sample preparation)

Running your gel:
Clamp in your gel and fill both buffer chambers with gel running buffer according to the instructions for your specific apparatus. Pippet your sample into the gel adjusting the volume according to the amount of protein in your sample. If you are going to stain using Coomassie, don't use much more than 5μg of your protein of interest to get a nicely defined band. Be sure to include a lane with molecular weight standards. Now attach your power leads and run the gel until the blue dye front reaches the bottom. I prefer to run at 250 V constant which in a four to twenty percent mini gel needs about 30 minutes total run time, but adjust to the thickness of your gel, the power supply used and the resolution desired. Remove the gel for the power supply and process further - Visualize your proteins using Coomassie Brilliant Blue, Silver stain, or any of the other protein stains. Use a carbohydrate stain for glycoproteins, or blot your gel for N-terminal sequencing or Western blotting.

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