

# **SAFI INSTITUTE OF ADVANCED STUDY (SIAS)**

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## **Laboratory Manual**

**Compiled by**



**Department of Biotechnology**

# Preface

*This laboratory manual is prepared to introduce both undergraduate and postgraduate students to techniques that are required to master various fields of Biotechnology. In this manual, we present most of the experiments prescribed by the University of Calicut for General Biotechnology course. The manual lists out protocols for Environmental Biotechnology, Cell Biology, Molecular Biology, Bioprocess Technology, Plant Biotechnology, and Genetic Engineering.*

*The manual categorizes the experiments under various fields of biotechnology. It provides structured guidance to both the lab instructor and the student. Each of the experiments provided in the manual has an in-depth analysis of the principle on whose basis the experiment is formulated. The materials required for carrying out the experiments, the detailed procedure for it as well as the expected observations and results are listed out.*

*These protocols have been compiled from various books and e-resources and we would also like to acknowledge the contributions of faculties of other departments of our college. We sincerely hope that the protocol manual will help both instructors as well as students in performing experiments without much difficulty.*

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## 1. ASEPTIC TECHNIQUES - STERILIZATION AND DISINFECTION

**Aim:** To learn different methods of sterilization and disinfection.

### **Introduction**

The need for techniques to control the growth of microorganisms has been shaped by both cultural and scientific advances. From the days of early food preservation using fermentation of milk products and smoking of meats to extend the shelf life of foods, practical needs have contributed to the development of these techniques. Formal development of clinical medical settings led to an awareness of the cause and effects of the disease. These observations contributed to the techniques developed by Joseph Lister, Oliver Wendell Holmes, and Ignaz Semmelweis that prevented disease transmission between patients. Our requirements for sterilization, antisepsis, and sanitizing thus go beyond the historical needs of the research and clinical laboratories and commercial production requirements. This chapter reviews commonly used laboratory techniques.

Sterilization is the removal of all microbes, including endospores, and can be achieved by mechanical means, heat, chemicals, or radiation. When using heat, it may be either dry heat or moist heat. Traditionally, moist heat under pressure is provided by autoclaving and dry heat by ovens. Disinfection is the process that eliminates most or all microorganisms, with the exception of endospores. Disinfectants can be further subcategorized as high-level disinfectants, which kill all microorganisms with the exception of large numbers of endospores with an exposure time of less than 45 min; intermediate-level disinfectants, which kill most microorganisms and viruses but not endospores; and low-level disinfectants, which kill most vegetative bacteria, some fungi, and some viruses with exposure times of less than 10 min. Antiseptics destroy or inhibit the growth of microorganisms in or on living tissues and can also be referred to as biocides. Disinfectants are used on inanimate objects and can be sporostatic but are not usually sporocidal. Steam sterilization or dry heat can be monitored by the use of biological indicators or by chemical test strips that turn color upon having met satisfactory conditions. These indicators are widely available. Usually the spores of species of *Geobacillus* or *Bacillus spp.* are used in either a test strip or suspension, as these organisms are more difficult to kill than most organisms of clinical interest. The growth of the spores in liquid media after the cycle of sterilization is complete indicates the load was not successfully sterilized. Only autoclave cycles of 120 min at 31.5 psig/275°F and 75 min at 45 psig/292°F effectively decontaminated the building decontamination residue (BDR) contaminated with 106 spores of *Geobacillus stearothermophilus*.

Other common methods of sterilization include gases such as ozone, radiation, or less commonly electronic accelerators (see Table 1.1). Solutions containing heat-labile components require a different approach. Filtration is generally the most accepted and easiest method. Solutions containing heat-labile components require a different approach.

Filtration is generally the most accepted and easiest method. The FDA and industry consider 0.22- $\mu$  filters sterilization grade based on logarithmic reductions of one of the smaller bacteria *Brevundimonas diminuta*.

### **Sterilization by heat**

The use of dry heat is based on the removal of the water content of microbes and subsequent oxidation. An open flame can be used for sterilization if the object is not directly exposed to flame damage. Different laboratory devices (e.g. scalpel, knife, inoculating loop or needle) can be sterilized quickly and safely by crossing over open flame or by ignition.

Dry heat sterilization is performed in a hot air sterilizer. It is an electric box with adjustable temperature like an incubator. In order to achieve uniform chamber temperature, hot air is circulated. Sterilization with dry heat is limited to devices made of metal, glass or porcelain, and other thermo-stable materials, like glycerol, soft paraffin, oils, and fats. In the dry heat sterilization system they have to withstand the temperature needed to kill the spore-forming bacteria (at 160°C for 45 minutes; at 180°C for 25 minutes; at 200°C for 10 minutes).

The heat conductivity of water is several times higher than that of the air, therefore heat sterilizes more quickly and effectively in the presence of hot water or steam than dry heat. Boiling is the simplest and oldest way of using moist heat. The temperature of boiling water does not exceed 100°C at normal atmospheric pressure. Heat resistant, endospore-forming bacteria can survive the 10-30-minute heat treatment of boiling, so no sterilizing effect can be expected from boiling.

Pasteurization is a widespread method – named after Louis Pasteur – to reduce the number of microorganisms found in different heat-sensitive liquids. Milk can be pasteurized by heating to 65°C for 30 minutes or to 85°C for 5 minutes. During ultra-pasteurisation milk is heat-treated at 135-150°C for 2 minutes in a heat exchanger. The temperature and time used for pasteurization are suitable to control the presence of some pathogenic bacteria, however endospores and cells of heat resistant bacteria e.g. *Mycobacterium* species can survive.

### **Autoclave**

The use of saturated steam under high pressure is the most effective method to kill microorganisms. In the laboratories, a sealed heating device called autoclave is used for this purpose. From the inside of the carefully temperature-controlled autoclave, the air is expelled by the less dense steam and sterilization takes place in a closed chamber at 121°C and overpressure. The household pressure cooker works on a similar principle but with lower temperature. Autoclaves are widely used in microbiological practice mainly for sterilization of culture media, glassware, and heat-resistant plastic products before their use, and also for contaminated materials prior to disposal as municipal solid waste. To achieve sterilization, generally 15 minutes of heat treatment at 121°C under 1.1 kg/cm<sup>2</sup> pressure has to be applied. Most microbes are unable to tolerate this environment for more than 10 minutes. However, the time used for sterilization depends on the size and content of the

load.

**Materials and equipment:**

- distilled water
- heat-proof gloves
- autoclave

**Procedure:**

1. Open the lid of the autoclave and check that there is a sufficient amount of distilled or deionisedwater in it. If necessary, refill.
2. Place the correctly packaged materials (e.g. laboratory equipment, culture medium in a flask) intothe chamber of the autoclave. Stick a piece of autoclave indicator tape onto the surface of materials.
3. Close the lid of the autoclave.
4. Make sure that the bleeder valve is open.
5. Turn on the heating of the autoclave (the indicator lamp is lit).
6. If an intense (a thick, milky white) steam outflow can be detected through the outlet tube of thebleeder valve (100°C on the built-in thermometer), wait for 4-5 minutes and close the bleeder valve(venting).
7. With the help of a built-in thermometer and manometer, check the temperature and pressure increaseinside the chamber of the autoclave.The sterilization time (15 minutes or more) begins only when the temperature equalization (to121°C) in the chamber has occurred. It is important that the operator stays with the device andcontrols the process of sterilization from the time it is turned on until the end of the sterilizationperiod.
9. Turn off the power switch of the autoclave when the sterilization cycle/period has ended.
10. Allow the device to cool down to at least 60-70°C.
11. For decompression, slowly open the bleeder valve. Thereafter, carefully open the lid of the autoclaveand remove the sterilized materials, using heat-proof gloves. Check the colour of sterilization indicator controls.

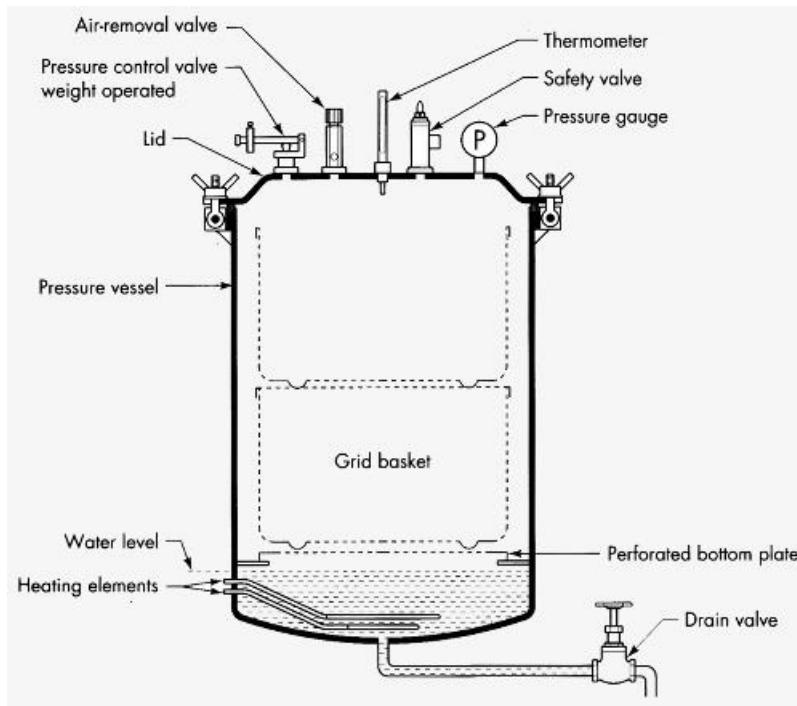


Figure: Autoclave.

### **Sterilization by radiation**

Other forms of energy [e.g. ultraviolet (UV) and ionizing radiation] are also used for sterilization especially for heat-sensitive materials. The full spectrum of UV radiation can damage microbes but only a small part is responsible for the so-called germicidal effect. A very strong "germicidal" effect can be achieved around 265 nm because maximum UV absorption of DNA occurs at this wavelength. The main cause of cell death is the formation of pyrimidine dimers in nucleic acids. Bacteria are able to repair their nucleic acid after damage using different mechanisms; however, beyond a certain level of damage, the capacity of the enzyme system is not enough and the accumulation of mutations causes death. UV (germicidal) lamps are widely used in hospitals and laboratories (e.g. in biological safety cabinets) for decontamination of air and any exposed surfaces. The disadvantage of the use of UV radiation is that it does not penetrate through glass, dirt films, water, and other substances.

Among the high-energy ionizing radiation,  $\gamma$ -rays from radioactive nuclides  $^{60}\text{Co}$  are generally used for sterilization of disposable needles, syringes, bandages, medicines and certain food (e.g. spices). The advantage of gamma radiation is its deep penetration through the packaging. Its disadvantage is the scattering in all directions, which requires special circumstances for application.

### **Filter sterilization**

The most commonly used mechanical method of sterilization is filtration. During

filtration, liquids or gases are pressed through a filter, which (depending on its pore size) retains or adsorbs (e.g. asbestos filter pads) microbes, thereby the filtrate becomes sterile. The pore diameter of filters should be chosen carefully so that bacteria and other cellular components cannot penetrate.

Earlier Seitz-type asbestos or different glass filters were commonly used for the filtration of microorganisms. The modern membrane filters are usually composed of high tensile-strength polymers (cellulose acetate, cellulose nitrate or polysulfone, etc.). Their operation is based partly on the adsorption of microbes, partly on a mechanical sieve effect. The pure sieve-based filters can be beneficial because they do not change the composition of the filtered solution. To remove bacteria, membrane filters with a poresize of  $0.22 \mu\text{m}$  are the best choice.

Membrane filters are biologically neutral; do not hamper life activities of microorganisms remaining on the filter and do not inhibit their enzyme functions. Furthermore, nutrients can diffuse through the membranes, so bacteria can be cultured in a variety of media also by placing the filters onto their surface.

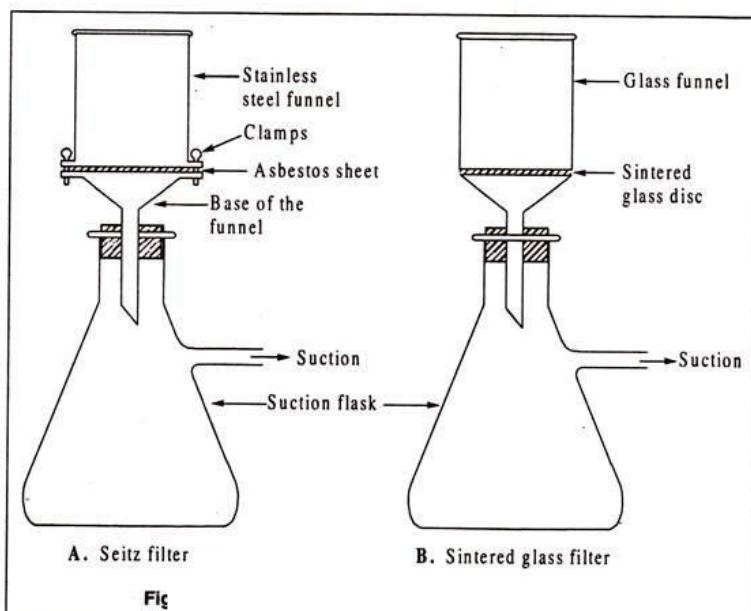


Figure: Filtration unit for sterilization of culture media.

### Laminar Air Flow cabinet

A laminar flow cabinet or tissue culture hood is a carefully enclosed bench designed to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive materials. Air is drawn through a High-efficiency particulate air (HEPA) filter and blown in a very smooth, laminar flow towards the user. Filters meeting the HEPA standard must satisfy certain levels of efficiency. Common standards require that a HEPA air filter must remove - from the air that passes through- at least 99.95% (European Standard) or 99.97% (ASME, U.S. DOE) of particles whose diameter is greater than or equal to  $0.3 \mu\text{m}$ .

μm. Laminar flow cabinets may have a UV-C germicidal lamp to sterilize the interior and contents before usage to prevent contamination of the experiment. Germicidal lamps are usually kept on for 15 minutes to sterilize the interior and no contact is to be made with a laminar flow hood during this time.

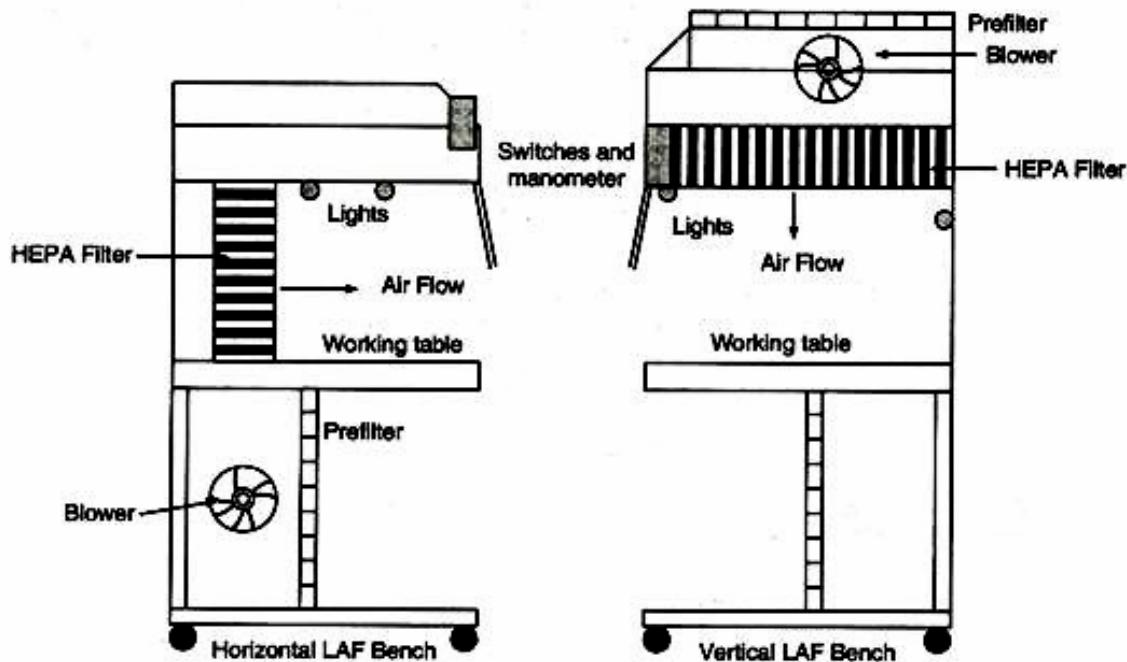


Figure: Horizontal and Vertical Laminar Airflow Bench.

### Sterilization by chemicals

A wide range of chemicals is suitable to inhibit or kill microbes. Some of the antimicrobial agents only inhibit the growth of microorganisms (e.g. bacteriostatic, fungistatic, and virostatic compounds) while others kill them (e.g. bacteriocidal, fungicidal, and virocidal agents). The -static or -cidal effect of a substance depends on the applied concentration and exposure time in addition to its quality. Only “-cidal” effect substances are used for chemical sterilization. These substances have the following requirements: they should have a broad-spectrum effect, they should not be toxic to higher organisms, they should not enter detrimental reactions to the materials being treated with, they should not be biodegradable, they should be environmentally friendly, easy to apply and economical. The materials used in chemical sterilization are liquids or gases. Liquid agents are used especially for surface sterilization. Among sterilizing gases, those working at low-temperature function by exposing the materials to be sterilized to high concentrations of very reactive gases (e.g. ethylene oxide, betapropiolactone or formaldehyde). Due to their alkylating effect, these compounds cause the death of microbes by damaging their proteins and nucleic acids. The chemical agents used for sterilization must be chemically compatible with the substances to be sterilized, therefore they have great importance in sterilisation of pharmaceutical and

thermoplastic materials. The chemicals used by the gas sterilizers are harmful to humans as well. Therefore, the application of gas sterilizers requires compliance with the precautions by the users.

## **Disinfection**

Any process aimed at destroying or removing the infectious capability of pathogenic microbes that generally occur on inanimate objects is called disinfection. The chemicals used for disinfection can be classified according to their chemical structure and their mode of action.

Among the alcohols, ethanol and isopropanol are widely used as disinfectants. 50-70% aqueous solution has excellent antiseptic properties. The action mechanism of alcohol depends on the applied concentration. Due to the solubility of lipids in 50-95% ethanol solutions, biological membranes are disintegrated. Alcohols pass through the cell membrane with altered permeability, denature the proteins inside the cell and have a dehydration effect as well. Absolute alcohol (100% ethanol) provides the best dehydration effect but does not coagulate the intracellular proteins. 70% dilution of alcohols is the most effective way to kill the vegetative forms of bacteria and fungi, but less effective against spores and lipid-enveloped viruses.

Phenol called carbolic acid was first used as a disinfectant by Lister. Phenol denatures proteins, and irreversibly inactivates the membrane-bound oxidases and dehydrogenases. Due to the unfavorable physical, chemical and toxicological properties, phenol is no longer used. However, substituted (alkylated, halogenated) derivatives are often used in combination with surfactants or alcohols (e.g. cresol, hexachlorophene, chlorhexidine).

The halogens (F, Cl, I, Br) and their derivatives are very effective disinfectants and antiseptic agents; mainly their non-ionic forms have antimicrobial activity. Chlorine gas is used almost exclusively for the disinfection of drinking water or other waters. In addition, different compounds (e.g. chloride of lime, chloramine-B, sodium dichloroisocyanurate) are among the most widely used disinfectant agents. Sodium hypochlorite ("household bleach") is a mixture of 8% NaClO and 1% NaOH) is one of the oldest high-bleaching and deodorizing disinfectant. The basis of the effect of chlorine and its derivatives is that during decomposition in aqueous solution, a strong oxidant, nascent (atomic state) oxygen ('O'), is released. Nascent oxygen is very reactive and suitable to destroy bacteria, fungi, and their spores as well as viruses.

Iodine is also a widely used disinfectant and antiseptic agent. There are two known preparations: tincture of iodine (alcoholic potassium iodide solution containing 5% iodine) and iodophors (aqueous solutions of iodine complexes with different natural detergents). It is applied in an alcoholic solution to disinfect skin or in aquatic solution for washing prior surgery.

Aldehydes, such as formaldehyde and glutaraldehyde, are broad-spectrum disinfectants. They are used for the decontamination of equipment and devices. Formalin is the 34-38%

aqueous solution of formaldehyde gas. Its effect is based on the alkylation of proteins. Heavy metals such as mercury, arsenic, silver, gold, copper, zinc and lead, and a variety of their compounds are highly efficient disinfectants but they are too damaging to living tissues to apply. They can be used as disinfectants at very low concentrations. Inside the cell, they bind to the sulphhydryl groups of proteins. Primarily, organic and inorganic salts of silver and mercury-containing products are commercially available, which have bactericidal, fungicidal and virocidal effects.

Detergents or surfactants are amphiphilic organic molecules that have a hydrophilic "head" and a long hydrophobic "tail". Detergents can be non-ionic, anionic or cationic according to the charge of the carbon chain. Nonionic surfactants have no significant biocidal effect and anionic detergents are only of limited use because of their poor efficiency. The latter group includes soaps, which are long-chain carboxylic acids (fatty acids) of sodium or potassium salts. They are not disinfectants on their own but are efficient cleaning agents due to their lipid-solubilizing effect. Cationic detergents, such as quaternary ammonium salts, are the best disinfectants.

X-----X-----X-----X

## 2. PREPARATION AND STERILIZATION OF MICROBIOLOGICAL CULTURE MEDIA

**Aim:** To prepare sterile microbiological culture media.

### **Introduction**

The survival and growth of microorganisms depend on availability of favorable growth environment. Culture media are nutrient solutions used in laboratories to grow microorganisms. For the successful cultivation of a given microorganism, it is necessary to understand its nutritional requirements and then supply the essential nutrients in the proper form and proportion in a culture medium.

The general composition of a medium is as follows:

- i. H-donors and acceptors (approximately 1-15 g/L)
- ii. C-source (approximately 1-20 g/L)
- iii. N-source (approximately 0.2-2 g/L)
- iv. Other inorganic nutrients e.g. S, P (50 mg/L)
- v. Trace elements (0.1-1 µg/L)
- vi. Growth factors (amino acids, purines, pyrimidines, occasionally 50 mg/L, vitamins occasionally 0.1-1 mg/L)
- vii. Solidifying agent (e.g. agar 10-20 g/L)
- viii. Solvent (usually distilled water)
- ix. Buffer chemicals

Microbiological culture media could be classified according to:

1. **Consistency**, which could be adjusted by changing the concentration of solidifying or gelling agents,  
e.g. agar, gelatine (liquid media do not contain such materials)
  - Cultures in liquid media (or broth) are usually handled in tubes or flasks and incubated under static or shaken conditions. This way, homogenous conditions are generated for growth and metabolism studies, (e.g. with the control of optical density and allowing sampling for the analysis of metabolic products).
  - Semisolid media are usually used in fermentation and cell mobility studies and are also suitable for promoting anaerobic growth.
  - Solid media are prepared in test tubes or in Petri dishes, in the latter case, the solid medium is called agar plate. In the case of tubes, the medium is solidified in a slanted position, which is called agar slant, or in an upright position, which is called agar deep tube. Solid media are used to determine colony morphology, isolate cultures, enumerate and isolate bacteria (e.g. using dilutions from a mixed bacterial population in combination with spreading), and for the detection of specific

biochemical reactions (e.g. metabolic activities connected with diffusing extracellular enzymes that act with insoluble substrates of the agar medium).

## **2. Composition**

- Chemically-defined (or synthetic) media are composed only of pure chemicals with defined quantity and quality.
- Complex (or non-synthetic) media are composed of complex materials, e.g. yeast extract, beef extract and peptone (partially digested protein), therefore their chemical composition is poorly defined. On the other hand, these materials are rich in nutrients and vitamins.

## **3. Function**

- All-purpose media do not contain any special additives and they aim to support the growth of most bacteria.
- Selective media enhance the growth of certain organisms while inhibiting others due to the inclusion of a particular substrate(s).
- Differential media allow identification of microorganisms usually through their unique (and visible) physiological reactions. In the detection of common pathogens, most practical media are both selective and differential.
- Enrichment media contain specific growth factors that allow the growth of metabolically fastidious microorganisms. An enrichment culture is obtained with selected media and incubation conditions to isolate the microorganisms of interest.

X-----X-----X-----X

## **PREPARATION OF AGAR SLANTS**

**Aim:** To prepare agar slants.

**Materials Required:**

1. distilled water
2. measuring cylinder
3. flask
4. bacteriological chemicals
5. laboratory scales
6. chemical spoons
7. 1N NaOH solution
8. 1N HCl solution
9. pH indicator paper or pH meter
10. cotton gloves
11. dispenser
12. test tubes
13. test tube caps
14. test tube basket
15. slanting stage
16. autoclave
17. incubator

**Procedure:**

1. Measure the components of the medium (e.g. TSA or nutrient, see Appendix) into a flask containing 9/10 volume of the solvent. Use a clean chemical spoon for every measurement. Dissolve the solid components and fill with the remaining solvent up to the final volume. If the medium contains heat-sensitive components (like sugars), they must be separately sterilized in solution (e.g. by filter sterilization), and then mixed with the already sterilized and cooled agar medium.
2. Close the flask with cotton plug and cover with aluminumfoil, put into the autoclave and start a sterilization cycle. This cycle could be intermittent when the internal temperature has reached 121°C, at that temperature every component (e.g. agar-agar) will be dissolved correctly.
3. Check the pH of the medium with an indicator paper or with a pH meter and adjust to the proper value with NaOH or HCl solution.
4. Pour the 60-70°C medium into the dispenser. Add 5-6 mL medium to each test tube, close themwith caps and place them into a test tube basket.
5. Place the tubes into the autoclave and complete a whole sterilization cycle for 20 min at 121°C.
6. Put the test tubes onto a slanting stage to let the medium solidify in the test tubes.
7. Label the slants according to the type of the medium and perform a sterility test: incubate the testtubes at 28°C for 24 hours, and check for sterility.
8. The prepared media can be stored for 1-2 weeks at 12-15°C, or longer in a refrigerator. (Do not store medium containing agar-agar under 4-5°C as it destroys

its structure!)

**Result:** The agar slants were prepared for inoculating isolated microorganisms.



Figure: Agar slant

X-----X-----X-----X

## **PREPARATION OF AGAR PLATES**

**Aim:**To prepare agar plates

**Materials Required:**

1. distilled water
2. measuring cylinder
3. flask
4. bacteriological chemicals
5. laboratory scales
6. chemical spoons
7. 1N NaOH solution
8. 1N HCl solution
9. pH indicator paper or pH meter
10. cotton gloves
11. sterile, empty Petri dishes
12. Bunsen burner
13. autoclave

**Procedure:**

1. Prepare a medium as in the previous experiment.
2. Cool the sterilized medium to 55°C.
3. Take out the cotton plug and flame the mouth of the flask over a Bunsen burner, and then pour the medium into sterile, empty Petri dishes (15-20 mL into each Petri dish).
4. Keep the Petri dishes horizontally until the medium completely solidifies. Turn dishes upside-down and stack them up for storage.
5. Label the plates according to the type of the medium.
6. In case of longer storage, Petri plates must be placed into plastic bags or boxes to avoid drying out.

**Result:** The agar plates were prepared for inoculating isolated microorganisms.



Figure: Agar plate

### 3.ISOLATION OF MICROORGANISMS FROM AIR, WATER, SOIL

#### QUANTIFYING HETEROOTROPHIC MICROBES USING THE SPREAD-PLATE TECHNIQUE

**Aim:** To isolate microorganisms from air, water, and soil samples.

#### **Introduction**

The diluted sample is pipetted onto the surface of a solidified agar medium and spread with a sterilized, bent glass rod (glass spreader) for the determination of heterotrophic plate count using the spread-plate method.

#### **Materials Required:**

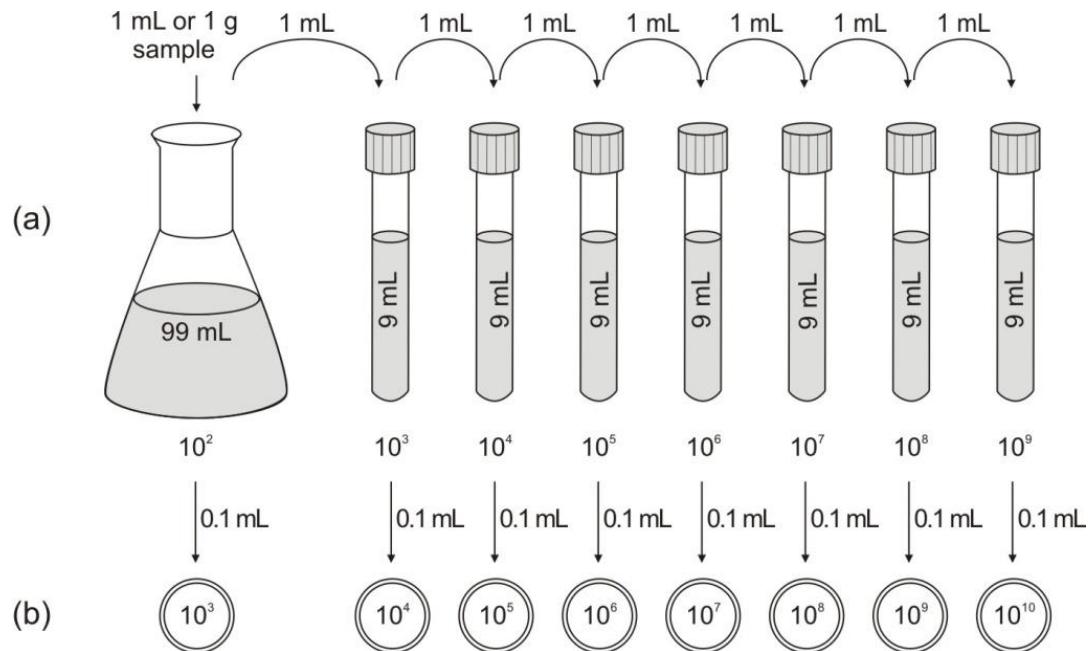
1. agar plates
2. glass spreader (alcohol for sterilization)
3. pipettes, sterile pipette tips
4. 99 mL sterile distilled water in a flask
5. 9 mL sterile distilled water in test tubes
6. vortex mixer
7. Bunsen burner
8. Incubator

#### **Procedure:**

1. Make a 10-fold dilution series from an environmental sample: measure 1 g soil sample into a flask containing 99 mL sterile water (or 1 mL water sample to 99 mL sterile water) mix thoroughly with vortex mixer, pipette 1 mL from this suspension into a test tube containing 9 mL sterile water, mix thoroughly with vortex, pipette 1 mL from this latter suspension into another test tube containing 9 mL sterile water, mix thoroughly, etc. (until the desired degree of dilution is reached) (Figure). Use unambiguous labeling throughout the practice (indicate sample name, degree of dilution, etc.).
2. Spread 0.1 mL from the given dilution onto the surface of agar plates: pipette 0.1 mL from the appropriate member of the dilution series onto the centre of the agar surface; rinse the glass spreader with alcohol (remove any excess alcohol) and sterilise the rod by flaming (take the rod away from the flame while the alcohol burns); cool down the glass spreader by touching the medium surface (without touching the liquid containing bacteria); spread the liquid evenly over the surface (while spreading dish should be opened only slit-like) (Figure).
3. Incubate Petri dishes at 28°C for one week.
4. Count the number of discrete colonies, in case of parallel plates, average the numbers and calculate the CFU value of the sample from the given formula. The results of different dilutions should also be averaged. Give the CFU values of the original sample in CFU/mL or CFU/g units.

**Result:** The CFU units from each dilution were calculated and are shown in the

observation table.



**Figure:** Germ count estimation using the spread plate technique. (a) The sample is diluted in sterilized distilled water, and a 10-fold dilution series is prepared. (b) Appropriate amounts of these dilutions are plated onto suitable growth medium in the Petri plate.

X-----X-----X-----X

## **4.STANDARD PLATE COUNT OF MICROORGANISM IN SEWAGE WATER SAMPLE**

### **QUANTIFYING HETEROTROPHIC BACTERIA USING THE POUR- PLATE TECHNIQUE**

**Aim:** To perform standard plate count of microorganisms from sewage water samples.

#### **Introduction:**

In the pourplate method, a sample from an accurate dilution of microbes/sample is pipetted into aPetri-dish, and then agar medium is poured over the liquid and mixed or the adequate volume of sampleis mixed with the melted agar medium and poured into Petri dishes.

#### **Materials and equipment:**

1. sterile, empty Petri dishes
2. pipette, sterile pipette tips
3. 99 mL sterile distilled water in a flask
4. 9 mL sterile distilled water in test tubes
5. melted growth medium in test tubes
6. Bunsen burner
7. Incubator

#### **Procedure:**

1. Make a dilution series from an environmental sample (10-fold, see spread plate technique).
2. Pipette 0.1-0.1 mL sample into the labeled Petri dishes from each dilution.
3. Pour 20-25 mL sterile, melted (ca. 50°C) medium into the inoculated Petri dishes and mix themsoftly. Let it solidify.
4. Incubate Petri dishes at 28°C for one week.
5. Count the number of discrete colonies, in case of parallel plates, average the numbers and calculatethe CFU value of the sample. The results of different dilutions should also be averaged. Give the CFUvalues of the original sample in CFU/mL or CFU/g units.

**Observation:** The number of colonies was found to decrease with increasing dilution.

**Result:** The CFU units from each dilution were calculated and are shown in the observation table.

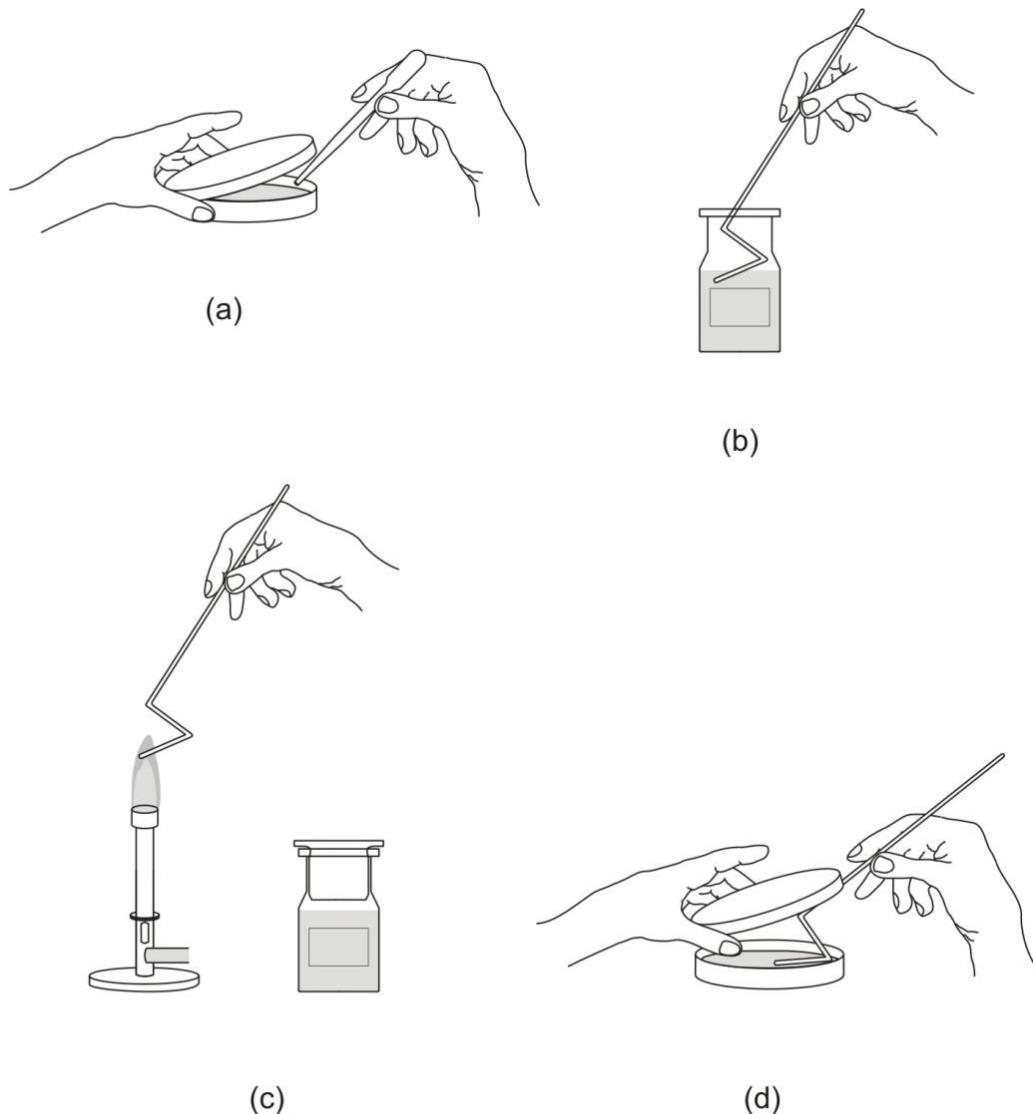
#### **Calculation:**

Initially 1mL in 9mL (test tube number 2) = Final Volume / Sample volume = 10/1 =

10.

$$\text{Dilution factor of Test tube 3} = 10 * 100 = 10^3$$

$\text{CFU/mL} = \text{cfu/ml} = (\text{Average no. of colonies} \times \text{dilution factor}) / \text{volume of culture plate}$



**Figure.** Spreading on the surface of an agar plate. (a) Pipette 0.1 mL from the appropriate member of the dilution series onto the centre of the surface of an agar plate. (b) Dip the L-shaped glass spreader into alcohol. (c) Flame the alcohol on the surface of the glass spreader over a Bunsen burner. (d) Spread the sample evenly over the surface of agar using the sterile glass spreader, carefully rotating the Petridish underneath at the same time.

**Observation table:**

Dilution	Plate 1	Plate 2 (duplicate)	Average count
1			
10			
100			
1000			
10000			
100000			
.....			

X-----X-----X-----X

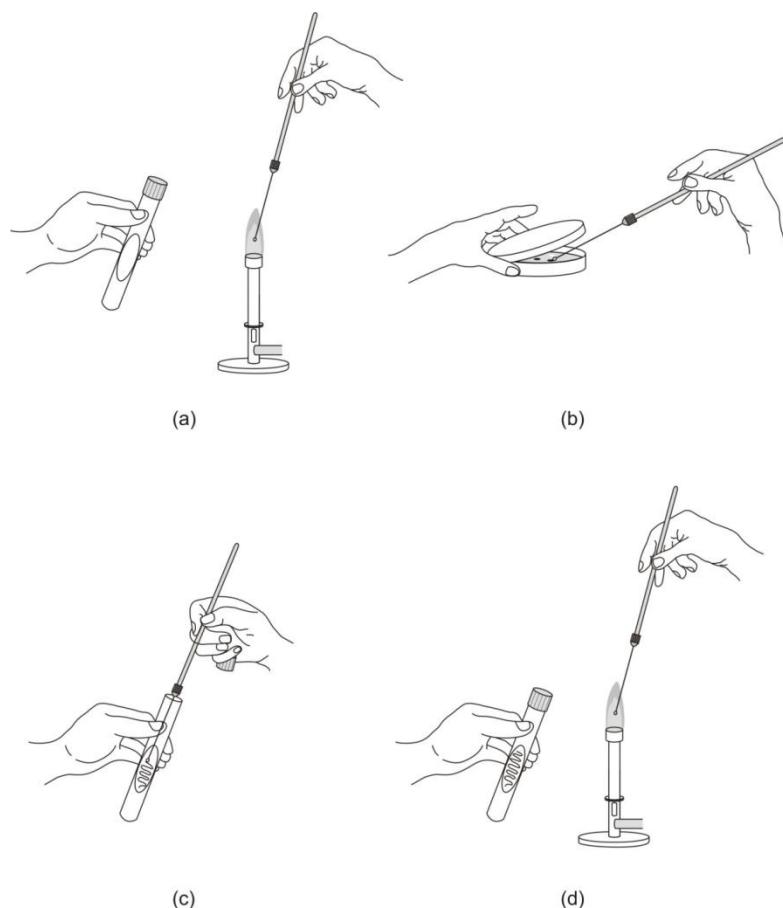
## 5. ISOLATION AND GROWTH OF MICROORGANISMS BY STREAK PLATE TECHNIQUE

**Aim:** Isolation and growth of microorganisms by streak plate method.

### **Introduction:**

During isolation, bacterial cells from a discrete colony that developed on the surface of an agar plate are transferred to an agar slant having the same composition. The culture developing on the surface of the agar slant after the isolation is called an isolate.

To aseptically transfer microorganisms from the broth, slant or agar cultures to another medium, inoculating needles or loops are used. They are made up of a handle, a shaft, a turret and a straight or a loop-ended nickel-chromium needle (Figure).



**Figure:** (a) Take the inoculating loop in one hand and hold it like a pencil. Flame the inoculating loop over a Bunsen burner until the wire becomes red-hot. (b) Make a gap on the Petri dish and choose a discrete colony to pick up a loopful of inoculum with the inoculating loop, then close the lid of the Petri dish. (c) After opening and flaming the neck of the test tube, inoculate the surface of the agar slant in zigzag streaks using the infected

inoculating loop. (d) Reflame the neck of the tube, closeit and sterilize the loop with reflaming as well.

### **Materials Required:**

1. Nutrient agar plates (see Appendix)
2. Inoculating loop
3. Bunsen burner
4. Incubator

### **Procedure:**

1. Label a Petri plate to be inoculated with the date, your name, and the mark of the isolate to be purified.
2. Take the inoculating loop and hold it like a pencil. Flame the inoculating loop over a Bunsenburner until the wire becomes red-hot.
3. Holding the inoculating loop in one hand, take the test tube containing the suspension of mixed bacterial cultures on the other hand.
4. Using the same hand that is holding the inoculating loop, remove the cap from the test tube, hold it between your fingers, and briefly flame the neck of the tube over a Bunsen burner by passing through the flame.
5. Take a loopful of inoculum from the suspension.
6. Flame the neck of the tube again and close it with the cap. Place the tube on the rack.
7. Inoculate approximately one-third of the agar surface (at the edge) using the infected inoculating loop (without scratching the agar).
8. Sterilize the loop again by flaming until the wire becomes red-hot.
9. Cool the loop by thrusting it into the sterile agar.
10. Cross over the streaks of the first inoculation when streaking the second part of the agar surface.
11. Flame and cool the loop again before repeating the streaking process on the third part of the agar surface.
12. Sterilize the loop again by flaming and place it on the rack.
13. Incubate the culture at 28°C for one week.
14. Check the growth of discrete colonies with different morphology after the incubation period. Perform re-isolation.

### **Observation:**

The bacterial colonies were observed on the agar plates following 24 hours and 48-hour incubation.

### **Result:**

The pure colonies were isolated from the last final streak of the agar plates and e-isolated for further study.

## 6. ISOLATION OF RHIZOBIUM FROM SOIL/ROOT NODULE

**Aim:** To isolate *Rhizobium* from soil/root nodule.

### **Introduction:**

*Rhizobia* are the Gram-negative and aerobic bacteria that symbiotically form nodules with roots or leguminous plants. They fix atmospheric nitrogen and render it into combined forms resulting in a high amount of proteins in roots. The proteins are transported along the plants and also secreted in rhizosphere region. In recent years, rhizobia are used as biofertilizers for selected crops.

### **Materials Required:**

1. Root nodules
2. YEM agar medium
3. Test tube with nylon mesh
4. Petri dishes
5. 0.1% acidified Mercuric chloride
6. Sterile tap water
7. Nichrome blade
8. Plates containing YEM agar medium
9. YEM agar (yeast extract mannitol agar) medium:
10. Distilled water

### **Procedure:**

1. Procure healthy root nodules of a young leguminous plant by cutting with a blade.
2. Wash the nodules thoroughly first with tap water and then with sterile distilled water keeping over the nylon mesh under aseptic conditions so as to remove contaminants and adhering soil particles.
3. Thereafter, immerse them in 0.19% acidified HgCl<sub>2</sub> for 5 minutes.
4. Transfer nodules in a sterile beaker containing 10 ml of 95% ethanol and wait for 2-3 minutes
5. Wash the nodules thoroughly for 5 times with sterile tap water, and blot dry by using sterile blotting paper.
6. Aseptically crush the nodules with glass rod or dissect the nodules by using nichrome blade and prepare dilutions.
7. Pour 1 ml suspension on YEM agar plates.
8. Incubate the inoculated plates at 28°C for 48 hours. Thereafter, observe the bacterial colonies which are gummy, translucent or white opaque.
9. Pick up a discrete colony and streak on a second YEM agar plates for better separation
10. However, if there is contamination, transferred the colony into a test tube containing 9 ml
11. sterile distilled water. Serially dilute it and then pour 1 ml bacterial suspension onto YEM agar plates.

12. Incubate the inoculated plates as above. Colonies of above features ensure the presence of Rhizobium.
13. Pick up a single colony and transfer on YEM agar slants for preservation.
14. Identify by Gram's staining and other molecular biological techniques.

**Observation:**

Large gummy colony were observed after 2-4 days of incubation.

**Result:**

The presence of *Rhizobium* bacteria in root nodules confirmed.

**Yem Agar**

	<u>g/L</u>
Yeast extract	1.00
Mannitol	10.000
Dipotassium phosphate	0.500
Magnesium sulphate	0.200
Sodium chloride	0.100
Agar	15.000
Final pH ( at 25°C)	7.0±0.2

X-----X-----X-----X

## 7.THE END POINT DILUTION TECHNIQUE (MPN METHOD)

**Aim:** To detect the number of cells in a given sample using the most probable number (MPN) technique.

### **Introduction**

The endpoint dilution method (MPN=Most Probable Number) is based on a series of dilutions prepared from a sample, where selected liquid media are inoculated with each dilution using 3-5 parallels . After the incubation period, estimation of microbial germ count is possible using statistical tables based on the number of positive tubes/wells (showing characteristic microbial growth: change in optical density, shifting of pH, changing of redox values and subsequently colour, etc. This method presumes that in the liquid medium the distribution of cells is uniform, cells do not cluster together and that there is detectable growth even if only one viable microbial cell is present in the tube/well.

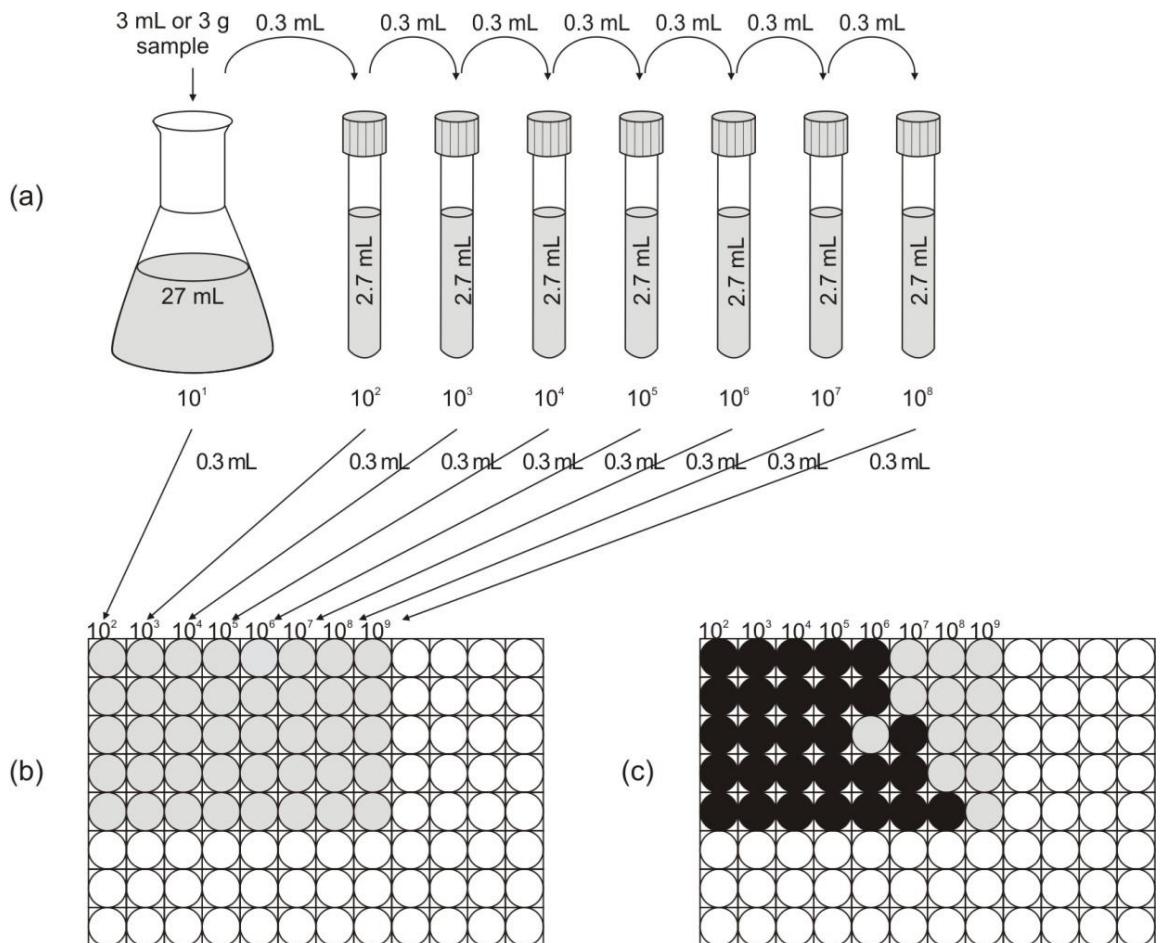
### **Materials Required:**

1. DRCM differential medium
2. pipette, sterile pipette tips
3. water bath
4. test tubes with sterile 9 mL distilled water
5. paraffin oil
6. incubator

### **Procedure:**

1. Prepare a soil suspension from 1 g soil in 9 mL sterile distilled water in duplicate and also from the Clostridium cultures.
2. Put one bacterial suspension and one soil suspension into an 80°C water bath for 5 min to inactivate the vegetative forms of bacteria.
- Make a 10-fold dilution series both from the heat-treated and from the untreated samples (see spread plate method).
4. Make 3-3 parallel subcultivations from all dilutions in DRCM-containing test tubes.
5. Cover the inoculated test tubes with 2-3 mm paraffin oil to assure anaerobic conditions.
6. Incubate the prepared tubes at 30°C for 44-48 hours.
7. Evaluate the germ counts of the original sample from the number of positive test tubes using the McCarty statistical table. Compare the different samples and treatments.

**Result:** From the McCarty statistical table the MPN of the given sample was found to be \_\_\_\_\_ MPN/ml.



**Germ count estimation with MPN method.** (a) The sample is diluted in the selected liquidmedium, and a 10-fold dilution series is prepared. (b) Appropriate amounts of these dilutions aremeasured into the wells of a microplate using 5 parallels. (c) Results can be read after incubationperiod. Black colour change indicates a positive test result. Germ counts can be determined by usingthe adequate McCardy statistical table.

**Table 1:**McCardy statistical table for determining the most probable number of bacteria from the number of positive test/wells using the MPN method.

Positive Tubes			MPN/mL	Positive tubes			MPN/mL	Positive tubes			MPN/mL
0.1	0.01	0.001		0.1	0.01	0.001		0.1	0.01	0.001	
0	0	0	<1.8	3	0	2	13	4	5	1	48
0	0	1	1.8	3	1	0	11	5	0	0	23
0	1	0	1.8	3	1	1	14	5	0	1	31
0	1	1	3.6	3	1	2	17	5	0	2	43
0	2	0	3.7	3	2	0	14	5	0	3	58

0	2	1	5.5	3	2	1	17	5	1	0	33
0	3	0	5.6	3	2	2	20	5	1	1	46
1	0	0	2.0	3	3	0	17	5	1	2	63
1	0	1	4.0	3	3	1	21	5	1	3	84
1	0	2	6.0	3	3	2	24	5	2	0	49
1	1	0	4.0	3	4	0	21	5	2	1	70
1	1	1	6.1	3	4	1	24	5	2	2	94
Positive Tubes			MPN/mL	Positive tubes			MPN/mL	Positive tubes			MPN/mL
0.1	0.01	0.001		0.1	0.01	0.001		0.1	0.01	0.001	
1	1	2	8.1	3	5	0	25	5	2	3	120
1	2	0	6.1	4	0	0	13	5	2	4	150
1	2	1	8.2	4	0	1	17	5	3	0	79
1	3	0	8.3	4	0	2	21	5	3	1	110
1	3	1	10	4	0	3	25	5	3	2	140
1	4	0	11	4	1	0	17	5	3	3	180
2	0	0	4.5	4	1	1	21	5	3	4	210
2	0	1	6.8	4	1	2	26	5	4	0	130
2	0	2	9.1	4	1	3	31	5	4	1	170
2	1	0	6.8	4	2	0	22	5	4	2	220
2	1	1	9.2	4	2	1	26	5	4	3	280
2	1	2	12	4	2	2	32	5	4	4	350
2	2	0	9.3	4	2	3	38	5	4	5	430
2	2	1	12	4	3	0	27	5	5	0	240
2	2	2	14	4	3	1	33	5	5	1	350
2	3	0	12	4	3	2	39	5	5	2	540
2	3	1	14	4	4	0	34	5	5	3	920
2	4	0	15	4	4	1	40	5	5	4	1600
3	0	0	7.8	4	4	2	47	5	5	5	>1600
3	0	1	11	4	5	0	41				

X-----X-----X-----X

## 8. SIMPLE STAINING

**Aim:** To identify shape of bacteria using simple staining method.

### **Introduction:**

Characteristics of cell morphology have great importance in the classification of bacteria using traditional taxonomical methods. Microorganisms cannot be identified solely by morphological characteristics since bacterial cells can only be assigned to a limited number of categories. Bacteria are  $\mu\text{m}$ -sized organisms, where cell size is an important aspect of a thorough morphological characterization. The size and shape of the cells are usually determined following staining. The circumstances of culturing, the age of the culture and the physiological condition of bacterial cells can alter cell size and shape. According to their shape, bacteria can usually be identified as rods, cocci or spirals. An average rod-shaped bacterium is 2-5  $\mu\text{m}$  long and 0.5-0.8  $\mu\text{m}$  wide in diameter. The average diameter of a sphere-shaped bacterium is 0.8  $\mu\text{m}$ . The size of some bacterial groups deviates from average values: spirochetes include some extremely thin (0.2  $\mu\text{m}$ ) bacteria, while there are some giants: *Thiomargarita namibiensis* (100-300 x 750  $\mu\text{m}$ ) and *Epulopiscium fishelsoni* (50 x 600  $\mu\text{m}$ ).

Most commonly, fixed and stained smears are used for the study of cell morphology, intracellular constituents, and structures. The chemistry of simple staining is based on the principle that different charges attract, while similar charges repel each other. In an aqueous environment, at pH 7, the net electrical charge produced by most bacteria is negative. Dyes applied for staining could be acidic, basic, and neutral dyes according to their chemical characteristics. Each dye contains a cation (positive charge) and an anion (negative charge) and either one could be the chromophore (the part of the molecule that is coloured). Since acidic dyes carry a negative charge on their chromophore, the bacterial cells (also negatively charged) reject these dyes. Negative staining could also be conducted with dyes having a colloidal particle size that therefore cannot enter the cell (e.g. the black coloured India Ink and Nigrosine). The chromophores of basic dyes have a positive charge and result in the staining of bacterial cells (positive dyes) since they bind to proteins and nucleic acids (around neutral pH carrying a negative charge). Basic dyes include safranin (red), methylene blue (blue), crystal violet (violet), malachite green (green).

**Table: Morphology of bacterial cells.**

Micrococcus	Following cell division, cells separate (singles)	<i>Micrococcus luteus</i>
Diplococcus	Following cell division, cells remain in pairs	<i>Neisseria gonorrhoeae</i>
Streptococcus	Chain of cocci	<i>Streptococcus lactis</i>
Staphylococcus	Grape-like cluster of cocci	<i>Staphylococcus aureus</i>
Tetragenus	Cell division on 2 planes, cocci in tetrads	<i>Planococcus</i>
Sarcina	Cell division on 3 planes, cocci in aggregates (packets) of eight	<i>Micrococcus luteus</i> (earlier "Sarcinalutea")
<b>Rod (bacillus)</b>		
	Shape and size very variable: long-short, wide-thin, coccoid, irregular	<i>Bacillus megaterium</i> <i>Pseudomonas</i> spp. <i>Haemophilus influenzae</i> <i>Corynebacterium</i> spp.
<b>Curved rod (spiral shape)</b>		
Vibrio	Cell with quarter or half a turn	<i>Vibrio cholerae</i>
Spirillum	Rigid cell wall, motility with flagella, cell with one or more returns	<i>Spirillum volutans</i>
Spirochaeta	Flexible cell wall, endoflagella, cell with one or more turns	<i>Treponema pallidum</i>
<b>Filamentous</b>		
	Actinomyces have branching cells, forming bacterial hyphae and their network (mycelium)	<i>Streptomyces</i> sp., <i>Nocardia</i> sp.
Variable		
	Intermediate forms (e.g. rod-coccus lifecycle)	<i>Rhodococcus</i> spp.

**Materials Required:**

1. Glass slide
2. Glass dropper dispenser
3. Inoculating loop
4. Bunsen burner
5. Wooden test tube clamps
6. Methylene blue dye solution
7. Light microscope
8. Immersion oil
9. Bacterial culture

**Procedure:**

1. Grip a glass slide with wooden test tube clamps, degrease the surface of a glass slide with alcohol over a Bunsen burner, put it down on a metal rack/staining stand

- with the degreased surface upwards, let it cool down.
2. Label the degreased slide adequately.
  3. Put a small drop of water onto the slide (a well-degreased slide will be wetted) and then mix a small loopful of bacterial culture in it. A thin suspension will be formed this way. Make a film layer(smear) with the needle of the inoculating loop and let it dry.
  4. Fix your preparation with heat over the Bunsen burner.
  5. Drop basic dye onto the fixed smear until it is fully covered and let it get stained for 1-2 minutes.
  6. Wash the smear with tap water to remove excess dye solution.
  7. Dry the slide.
  8. During microscopy, first, use 40x, then 100x objective lenses. In the latter case, use immersion oil. Make a drawing of the observed microscopic field.
  9. After finishing microscopic observation, clean all used objective lenses with benzene (do not use alcohol for this purpose as it can dissolve the lens' adhesives).

### **Observation and Result**

The microbial cells were observed as blue rods. The size, shape and arrangement of the bacteria were observed and drawn for further study.



Figure: Bacillus stained with methylene blue

X-----X-----X-----X

## 9.GRAM STAINING

**Aim:** To differentiate the given bacterial samples by Gram's staining.

### **Introduction:**

This important bacteriological staining procedure was discovered in 1884 by a Danish scientist, Christian Gram. The staining is based on the cell wall structure of bacteria. When bacteria are stained with crystal violet, the cells of most Gram-negative bacteria can be easily decolourised with organic solvents such as ethanol or acetone, while cells of most Gram-positive bacteria restrict decolourisation. The ability of bacteria to either retain or lose the stain generally reflects fundamental differences in the cell wall and is an important taxonomic feature. Gram staining is therefore used as an initial step in the identification of bacteria. The cells of some bacteria are strongly Gram-positive when young, but tend to become Gram-negative in aging cultures (e.g. *Bacillus cereus*, *Clostridium spp.*), which may reflect degenerative changes in the cell wall. Some bacteria give a Gram-variable reaction: they are sometimes Gram-positive, sometimes Gram-negative; this could reflect minor variation in the staining technique or changes in cell wall thickness, etc.

### **Materials Required:**

1. Glass slide
2. Glass dropper dispenser
3. Pipette
4. Inoculating loop
5. Bunsen burner
6. Wooden test tube clamps
7. Crystal violet dye solution
8. Iodine solution
9. 96 % ethanol
10. Safranin dye solution
11. Light microscope
12. Immersion oil
13. Wad of paper

### **Procedure:**

1. Prepare a fixed smear from the bacterial strains.
2. Stain with crystal violet solution (1 min).
3. Rinse with tap water.
4. Treat with iodine solution (1 min).
5. Rinse with tap water.
6. Decolourise with 96 % ethanol (drip with ethanol until the solvent runs down colorless).
7. Rinse with tap water.

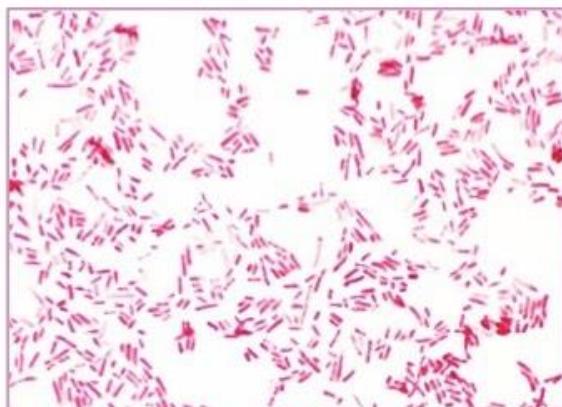
8. Counterstain with safranin solution (1 min).
9. Rinse with tap water.
10. Dry the slide.
11. Examine with microscope. Make a drawing of the observed microscopic field.

**Observation:**

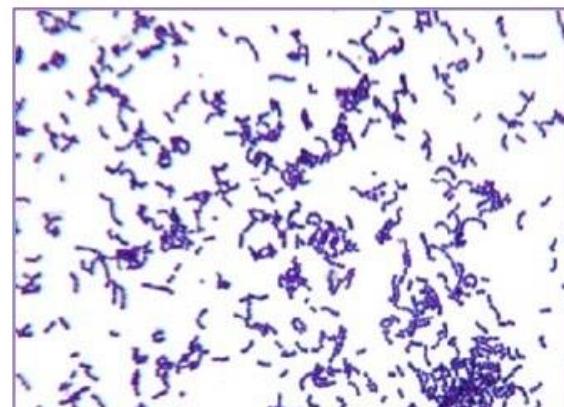
Gram-positive cells are purple, while Gram-negative ones are pinkishred.

**Result:**

The \_\_\_\_\_ spp. is Gram-positive, whereas \_\_\_\_\_ spp. is Gram-negative.



**Gram-Negative Bacteria**



**Gram-Positive Bacteria**

X-----X-----X

## 10.DETERMINATION OF DISSOLVED OXYGEN IN WATER

**Aim:** To estimate the amount of dissolved oxygen in the given water sample.

**Introduction:** Some amount of oxygen is dissolved in water which is used by aquatic plants and animals. The sources of dissolved oxygen in water are autotrophic aquatic plants which as a result of photosynthesis evolve oxygen, and air where oxygen is dissolved in water depending on salinity, temperature and water movement. Moreover, in an oligotrophic lake the amount of dissolved nutrient salts remains low, therefore, it supports sparse plant and animal lives. This results in high dissolved oxygen gradually increasing with depth. In addition, in eutrophic water reservoirs e. g. lakes, ponds, pools, etc. the organic nutrients accumulate abundantly which in turn are subjected to microbial decomposition. More growth of microorganisms, plants, and animals depletes oxygen. This depletion increases with increase in water depth. Dissolved oxygen is measured by titrimetric method. The theory behind this method is that the dissolved oxygen combines with manganese hydroxide which in turn liberates iodine (equivalent to that of oxygen fixed) after acidification with  $H_2SO_4$ . The iodine can be titrated with sodium thiosulfate solution by using starch indicator.

**Materials Required:**

1. BOD bottles (250 ml capacity)
2. Water sample from a water body, sewage or treated sewage water, etc.
3. Alkaline iodide-azide solution
4. Sodium thiosulfate (0.025 N)
5. Manganese sulfate solution
6.  $H_2SO_4$  (cone.)
7. Pipette (2 ml)
8. Titration set
9. Starch Indicator

**Preparations:**

- Alkaline iodide azide solution: Dissolve 500 g of NaOH and 150 g of NaI in 1000 ml of distilled water. To this solution add 10 g of sodium azide ( $NaN_3$ ) dissolved in 40 mL of distilled water.
- Manganese sulfate ( $MnSO_4 \cdot 4H_2O$ ) solution: Dissolve 400 g of manganese sulfate in 1000 ml of distilled water, filter and keep in a stoppered bottle.
- Starch indicator: Weigh approximately 2 gm of starch and dissolve in 100 ml of distilled water by boiling. For the preservation of starch indicator add 0.2 gm of salicylic acid as preservative.

**Procedure:**

1. Collect water samples in a BOD glass bottle (250 ml) in such a way that water bubble should not come out.
2. Pipette separately 2 ml of manganese sulfate and 2 ml of alkaline iodine-azide solutions

3. Add these solutions in succession at the bottom of bottle and place the stopper of bottle.
4. Shake the bottle upside down for about 6-8 times. There develops brown precipitate.
5. Leave the bottle for a few minutes, the precipitate settles down.
6. Add 2 ml of concentrated H<sub>2</sub>SO<sub>4</sub> in the bottle. Shake properly so that brown precipitate may dissolve.
7. Take a clean flask and pour 50 ml of this water sample. Titrate it against 0.025 N sodium thiosulfate solution taking in a burette until pale straw colour develops.
8. Add 2 drops of starch solution to the flask. Colour of contents changes from pale to blue.
9. Again, titrate against the thiosulfate solution until the blue colour disappears. Note the volume of sodium thiosulfate solution used in titration.

### **Results:**

The amount of dissolved oxygen in control (distilled water)-----mg/ml

The amount of dissolved oxygen in test (water sample)-----mg/ml

---

Calculate the amount of dissolved oxygen (DO) (mg/litre) by using the following formula:

$$DO \left( \frac{mg}{ml} \right) = \frac{8 \times 1000 \times N}{V} \times v$$

V= Volume of water sample used for titration

v = Volume of sodium thiosulfate (titrant)

N = Normality of titrant

8 = It is a constant since 1 ml of 0.025N sodium thiosulfate solution is equivalent to

0.2 mg oxygen.

X-----X-----X-----X

## 11.DETERMINATION OF DISSOLVED OXYGEN IN WATER BY BOD METHOD

**Aim:** To estimate the amount of dissolved oxygen in the given water sample by the Biological oxygen demand (BOD) method.

**Introduction:** BOD is the index of water pollution. In the presence of organic and inorganic waste products numerous microorganisms grow. These act as a food base for the microorganisms hence they decompose and utilize the substrates. However, the amount of oxygen required by bacteria during aerobic decomposition of organic compounds in sewage is called BOD. It is also represented by the amount of organic matter present in water or effluents. For more organic matter, more oxygen is required by bacteria for its decomposition. This results in release of organic nutrients in water (rivers, ponds, etc. resulting in death of fish (asphyxiation).

For measuring BOD, water samples are incubated at 20°C for 5 days in the dark under aerobic condition metabolic, whereas the same can be incubated at 27°C for 3 days during in tropical and subtropical regions where metabolic activities are higher. Oxygen is also consumed during nitrification, therefore, 1ml of 0.05% allylthiourea should be added to check over estimation of BOD.

### **Materials Required:**

1. BOD free water (Double distilled water passed through activated carbon or MilliQ water)
2. BOD bottles
3. Erlenmeyer flak
4. Pipette
5. BOD incubator
6. pH meter
7. Phosphate buffer solution (pH 7.4)
8. H<sub>2</sub>SO<sub>4</sub> 1 N (2.8 ml in 100 ml BOD-free distilled water)
9. Allylthiourea solution (0.5%)
10. All reagents required for estimation of dissolved oxygen as given in previous experiment.

### **Procedure:**

1. Add 1N acid/1N alkali in the water sample to adjust the pH to 7.0.
2. Gently transfer this water into BOD bottles so as bubbles should not come out.
3. Add 1 ml of allylthiourea to each bottle to avoid nitrification
4. Measure dissolved oxygen following the steps as described for dissolved oxygen
5. Incubate the other BOD bottle at 27°C for 3 days in a BOD incubator.
6. Collect water sample in a BOD glass bottle (250 ml) in such a way that water bubble should not come out.

7. Pipette separately 2 ml of manganese sulfate and 2 ml of alkaline iodine-azide solutions
8. Add these solutions in succession at the bottom of bottle and place the stopper of bottle,
9. Shake the bottle upside down for about 6-8 times. There develops brown precipitate.
10. Leave the bottle for a few minutes, the precipitate settles down.
11. Add 2 ml of concentrated H<sub>2</sub>SO<sub>4</sub> in the bottle. Shake properly so that brown precipitate may dissolve.
12. Take a clean flask and pour 50 ml of this water sample. Titrate it against 0.025 N sodium thiosulfate solution taking in a burette until pale straw colour develops.
13. Add 2 drops of starch solution to the flask. Colour of contents changes from pale to blue.
14. Again, titrate against thiosulfate solution until the blue colour disappears. Note the volume of sodium thiosulfate solution used in titration.

**Results:**

The BOD of the Tap water = \_\_ mg/ml.

The BOD of the Water sample = \_\_ mg/ml.

---

Calculate the BOD of water by using the following formula:

$$BOD \left( \frac{mg}{ml} \right) = DO_1 - DO_2$$

where, DO<sub>1</sub>= Initial dissolved oxygen (mg/l) in the first sample (mg/l)

DO<sub>2</sub>= Dissolved oxygen (mg/l) in the second sample after 3 days of incubation.

X-----X-----X-----X

## 12. DETERMINATION OF CHEMICAL OXYGEN DEMAND (COD) OF WATER (RAW/TREATED SEWAGE)

**Aim:** To determine the chemical oxygen demand of the given water sample.

**Introduction:** Due to a gradually increasing population the number of industries is also increasing. This results in increased pollution. The chemically oxidizable organic substances discharged in water depletes the amount of oxygen. The estimation of BOD alone could not give the exact idea of pollutants present in water. COD refers to the oxygen consumed by the oxidizable organic substances. The values of COD cannot be compared directly with that of BOD.

The chemical oxidants such as potassium dichromate ( $K_2Cr_2O_7$ ) or potassium permanganate ( $KMnO_4$ ) are used to measure the oxidizability of the organic matter of water where the oxidants oxidize the constituents (or the hydrogen but not the nitrogen). amount of oxygen reacts with KI and liberates iodine. Then potassium iodide (KI) is added. The excess amount of oxygen liberates equal amount of iodine. By using starch indicator, iodine is titrated with sodium thiosulphate and the amount is estimated.

**Materials required:**

1. Water sample
2. Conical flasks (100 ml capacity)
3.  $K_2Cr_2O_7$  solution (0.1 N, 3.67 g/l )
4.  $H_2SO_4$  solution 2M (10.8 ml conc.  $H_2SO_4$  /1000 ml distilled water)
5. Sodium thiosulfate 0.1M (15.811 g/1 litre distilled water)
6. Starch solution (1%)
7. 10% Potassium iodide
8. Water bath
9. Distilled water control blank (3)
10. Titration assembly

**Procedure:**

1. Pour 50 ml of water sample in a conical flask (100 ml capacity).
2. Similarly, take 50 ml distilled water in a flask.
3. Pour 5 ml  $K_2Cr_2O_7$  solution separately in both the flasks.
4. Mix 5 ml KI solution, and 10 ml of  $H_2SO_4$  solution in each flask.
5. Incubate the flasks at 100°C for one hour keeping in a water bath.
6. Thereafter, remove the flasks to cool for 10 minutes.
7. Transfer 0.1 M sodium thiosulfate solution in burette fitted in titration assembly, and titrate with both the samples in flasks till pale yellow colour disappears. In each case note the amount of sodium thiosulfate solution used.
8. Add 1 ml of starch solution to both the flasks. Colour turns blue.
9. Again, titrate with sodium thiosulfate as above till complete disappearance of blue colour.

Note the volume of sodium thiosulfate used for both the water samples.

**Results:**

The COD of the water sample = \_\_\_ mg/l.

---

Calculate the COD (mg/l) of water by using the following formula:

$$\text{COD (mg/l)} = \frac{8 \times C \times V_R - V_A}{V_S}$$

Where, C = Concentration of titrant (mM/l)

V<sub>A</sub>= Volume (ml) of titrant used for control

V<sub>H</sub> = Volume (ml) of titrant used for water samples

V<sub>S</sub> = Volume (ml) of water sample taken

X-----X-----X-----X

## 13. BACTERIOLOGICAL EXAMINATION OF WATER

- AIM:** i) Carry out a presumptive test for the presence of coliform bacteria in a water sample.  
ii) Determine the most probable number (MPN) of bacteria in a positive presumptive sample.  
iii) Carry out a confirmed test to begin isolation of bacterial colonies.  
iv) Carry out a completed test using a Gram stain and morphology

### **INTRODUCTION:**

We consider fresh-water streams, lakes, ground water or coastal ocean water to be polluted when some condition makes the water unsafe for human recreation or consumption. We usually think of two forms of pollution: toxic chemicals or pathogenic microorganisms. Probably the largest single source of potentially pathogenic microbes is animal faeces (including human), which contains billions of bacteria per gram. Although most intestinal microbes are non-pathogenic, some cause enteric disease. The organisms which cause typhoid fever (*Salmonella typhi*), cholera (*Vibrio cholera*), and bacterial dysentery (*Shigella flexneri*) are examples of enteric diseases caused by bacteria. In addition, some viral and protozoan pathogens are spread through water contaminated by faeces. Testing for each organism separately would be extremely costly and time-consuming. Therefore, a simple rule is followed: if a water sample contains any microorganism common to animal intestines, it should not be consumed, because it may contain enteric pathogens. Water testing for microbiological safety rests on the ability of microbiologists to detect coliform bacteria. The word “coliform” refers to any bacterium that is like *Escherichia coli* in the following characteristics:

- 1) it is a small, gram-negative rod;
- 2) it does not contain spores;
- 3) it ferments lactose with the production of acid and gas;
- 4) it produces a green metallic sheen on EMB agar.

*E. coli*, which is found in large numbers in the feces of all animals, lives longer in water than most intestinal pathogens do. Therefore, if no *E. coli* are present, there should be no intestinal pathogens present in the water sample. For this reason, testing for coliform organisms is performed daily by municipal water departments and waste-water (sewage) treatment plants. It is regularly tested for in coastal sea water samples, as well as runoff water. The bacterial examination of water has been standardized into three tests. The first, or presumptive test, is a screening test to sample water for the presence of coliform organisms. A series of lactose fermentation tubes are inoculated with the water sample. If the presumptive test is negative, no further testing is performed, and the water source is considered microbiologically safe. If, however, any tube in the series shows acid and gas, the water is considered unsafe and the confirmed test is performed on the tube displaying a positive reaction. The presumptive test is also designed to estimate the concentration of coliform organisms, called the most probably number (MPN) in the water sample. The confirmed test is a second screening procedure in which a gram-negative selective medium

is used (like EMB). This also allows for the differentiation of coliform (producing a green metallic sheen) from non-coliform colonies. The completed test is performed on a typical, well-isolated colony to reaffirm gas production in lactose, and to determine the morphology and gram reaction of the isolate from a nutrient agar slant.

### **Presumptive Test and MPN**

#### **PROCEDURE:**

1. Three double strength lactose broth and six single strength lactose broth with an indicator, bromothymol blue was prepared.
2. An inverted Durhams tube was also added into each tubes to check the gas evolution.
3. Collect approximately 50 ml of water sample to be tested in sterile bottle. Record the source and date of samples to be tested.
4. Always use aseptic technique in the water inoculations and label the tubes with the amount of water sample.
5. Transfer 10 ml of the sample into each of the three, sterilized double strength lactose broth in tubes. Transfer 1 ml of the sample into each of three single strength lactose broth in tubes and transfer 0.1 ml of water sample to each of the three remaining single strength lactose broth.
6. Incubate all tubes at 37° C for 24 hours.

### **Confirmed Test**

1. From any tube showing 10% of gas production or more, streak one loopful of the broth onto an EMB plate using the quadrant streaking technique. Incubate the plate at 37° C for 24 hrs until the next laboratory session.

### **Completed Test**

1. Carefully examine the plate, looking for well-isolated coliform colonies. Typically, *E. coli* colonies appear with a metallic green sheen on EMB. From one of these colonies, set up your completed test by inoculating a lactose fermentation tube and a NA slant. Incubate them at 37° C. until the next laboratory session.
2. After this incubation, check the lactose tube for acid and gas production. If no gas is present, this is a negative completed test.
3. Prepare a Gram stain from the NA slant.

#### **Observation**

Examine the tubes from the presumptive test and determine if any tube has produced an acid/gas reaction. If so, this is a positive presumptive test. If no gas is present in any of

the Durham tubes, this is a negative presumptive test.

Determine the number of tubes positive for acid/gas in each of the three volume categories. Determine the MPN of the water sample by comparing these numbers to the MPN Determination chart.

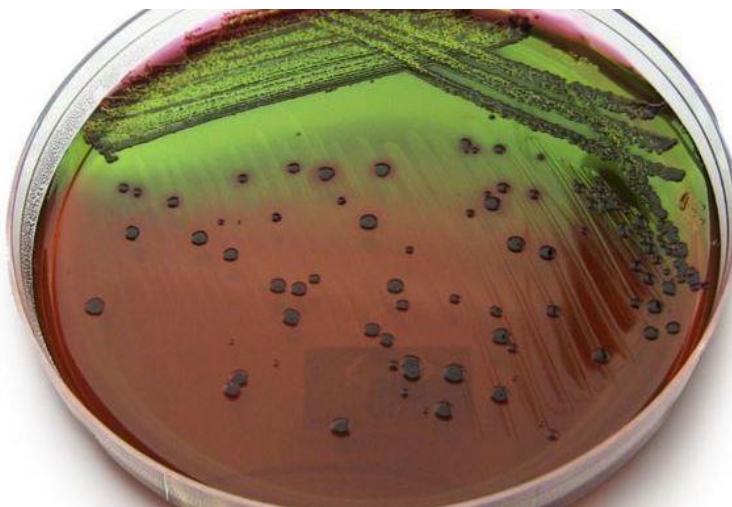
If from the positive presumptive test, the organism is identified as a non-spore producing Gram negative rod and the lactose broth shows an acid/gas reaction, this is a positive completed test.

### **RESULTS:**

The results of the Presumptive Test for the water are given as below and the MPN was calculated to be -----MPN/ml.

Sample Source	No of 10ml Tube	No of 1 ml Tube	No of 0.1ml Tube	MPN

---



**E coli on EMB agar with green metallic sheen**

**Table 1:**McCardy statistical table for determining the most probable number of bacteria from the number of positive test tubes/wells using the MPN method.

Positive Tubes			MPN/mL	Positive tubes			MPN/mL	Positive tubes			MPN/mL
0.1	0.01	0.001		0.1	0.01	0.001		0.1	0.01	0.001	
0	0	0	<1.8	3	0	2	13	4	5	1	48
0	0	1	1.8	3	1	0	11	5	0	0	23

0	1	0	1.8	3	1	1	14	5	0	1	31
0	1	1	3.6	3	1	2	17	5	0	2	43
0	2	0	3.7	3	2	0	14	5	0	3	58
0	2	1	5.5	3	2	1	17	5	1	0	33
0	3	0	5.6	3	2	2	20	5	1	1	46
1	0	0	2.0	3	3	0	17	5	1	2	63
1	0	1	4.0	3	3	1	21	5	1	3	84
1	0	2	6.0	3	3	2	24	5	2	0	49
1	1	0	4.0	3	4	0	21	5	2	1	70
1	1	1	6.1	3	4	1	24	5	2	2	94
Positive Tubes			MPN/mL	Positive tubes			MPN/mL	Positive tubes			MPN/mL
0.1	0.01	0.001		0.1	0.01	0.001		0.1	0.01	0.001	
1	1	2	8.1	3	5	0	25	5	2	3	120
1	2	0	6.1	4	0	0	13	5	2	4	150
1	2	1	8.2	4	0	1	17	5	3	0	79
1	3	0	8.3	4	0	2	21	5	3	1	110
1	3	1	10	4	0	3	25	5	3	2	140
1	4	0	11	4	1	0	17	5	3	3	180
2	0	0	4.5	4	1	1	21	5	3	4	210
2	0	1	6.8	4	1	2	26	5	4	0	130
2	0	2	9.1	4	1	3	31	5	4	1	170
2	1	0	6.8	4	2	0	22	5	4	2	220
2	1	1	9.2	4	2	1	26	5	4	3	280
2	1	2	12	4	2	2	32	5	4	4	350
2	2	0	9.3	4	2	3	38	5	4	5	430
2	2	1	12	4	3	0	27	5	5	0	240
2	2	2	14	4	3	1	33	5	5	1	350
2	3	0	12	4	3	2	39	5	5	2	540
2	3	1	14	4	4	0	34	5	5	3	920
2	4	0	15	4	4	1	40	5	5	4	1600
3	0	0	7.8	4	4	2	47	5	5	5	>1600
3	0	1	11	4	5	0	41				

X-----X-----X-----X

## 14. IMViC TESTS

### **INTRODUCTION:**

The IMViC series is a group of four individual tests that are commonly used to identify bacterial species, especially coliforms. The capital letters in 'IMViC' each stand for one of the four tests: I for Indole test, M for Methyl Red test, V for Voges-Proskauer test, and C for Citrate test.

#### **1. INDOLE TEST**

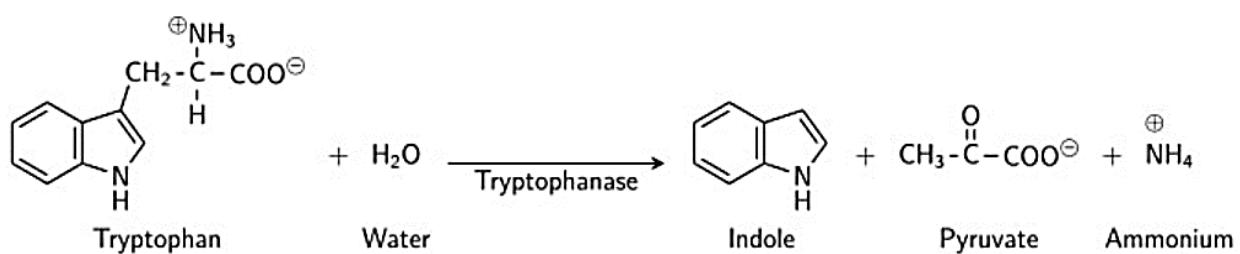
This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophane to indole, which accumulates in the medium. Indole production test is important in the identification of Enterobacteria. Most strains of *E. coli*, *P. vulgaris*, *P. rettgeri*, *M. morgani* and *Providencia* species break down the amino acid tryptophan with the release of indole. This is performed by a chain of a number of different intracellular enzymes, a system generally referred to as "tryptophanase." It is used as part of the IMViC procedures, a tests designed to distinguish among members of the family Enterobacteriaceae.

A variation on this test using Ehrlich's reagent (using ethyl alcohol in place of isoamyl alcohol, developed by Paul Ehrlich) is used when performing the test on non-fermenters and anaerobes.

### **PRINCIPLE:**

Tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria that express tryptophanase enzyme. Indole is generated by reductive deamination from tryptophan via the intermediate molecule indolepyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine (-NH<sub>2</sub>) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonium (NH<sub>4</sub><sup>+</sup>) and energy. Pyridoxal phosphate is required as a coenzyme.

When indole is combined with Kovac's Reagent (which contains hydrochloric acid and p-dimethylaminobenzaldehyde in amyl alcohol) the solution turns from yellow to cherry red. Because amyl alcohol is not water soluble, the red coloration will form in an oily layer at the top of the broth.



In the spot test, indole combines, in the filter paper matrix, at an acid pH with p-

Dimethylaminocinnamaldehyde (DMACA) to produce a blue to blue-green compound. Indole Spot Reagent has been reported to be useful in detecting indole production by members of the family Enterobacteriaceae and certain anaerobic species.

### **MATERIALS REQUIRED:**

1. Tryptone broth ( rich in tryptophan).
2. Sterile test tubes.
3. Kovac reagent

### **PROCEDURE:**

1. Take a sterilized test tubes containing 4 ml of tryptone broth.
2. Inoculate the tube aseptically by taking the growth from 18 to 24 hrs culture.
3. Incubate the tube at 37°C for 24-28 hours.
4. Add 0.5 ml of Kovac's reagent to the broth culture.
5. Observe for the presence or absence of ring.

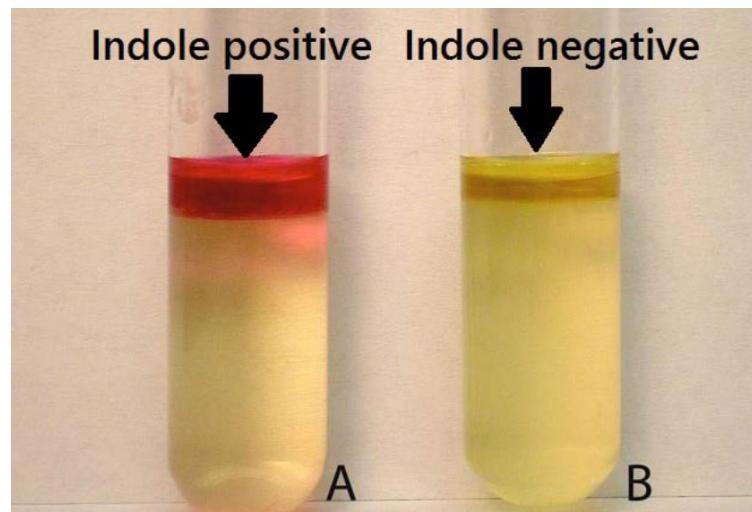
### **OBSERVATION AND RESULT:**

**Positive:** Formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent.

Examples: *Aeromonashydrophila*, *Aeromonaspunctata*, *Bacillus alvei*, *Edwardsiella sp.*, *Escherichia coli*, *Flavobacterium sp.*, *Haemophilus influenzae*, *Klebsiella oxytoca*, *Proteus sp.* (not *P. mirabilis* and *P. penneri*), *Plesiomonasshigelloides*, *Pasteurellamultocida*, *Pasteurellapneumotropica*, *Enterococcus faecalis*, and *Vibrio sp.*

**Negative:** No color change even after the addition of appropriate reagent.

Examples: *Actinobacillus spp.*, *Aeromonassalmonicida*, *Alcaligenes sp.*, most *Bacillus sp.*, *Bordetella sp.*, *Enterobacter sp.*, *Lactobacillus spp.*, most *Haemophilus sp.*, most *Klebsiella sp.*, *Neisseria sp.*, *Pasteurellahaemolytica*, *Pasteurellaureae*, *Proteus mirabilis*, *P. penneri*, *Pseudomonas sp.*, *Salmonella sp.*, *Serratia sp.*, *Yersinia sp.*



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### Composition

#### **1. Indole Kovacs Reagent(1000ml):**

- p-Dimethylaminobenzaldehyde - 50.0 gm
- Hydrochloric Acid, 37% - 250.0 ml
- Amyl Alcohol - 750.0 ml

#### **2. 2. Tryptone broth (1000ml)**

- Tryptone                    10.0
- Sodium chloride        5.0
- Final pH ( at 25°C)  $7.5 \pm 0.2$

X-----X-----X-----X

## **2. METHYL RED TEST**

### **INTRODUCTION:**

The methyl red (MR) test detects the production of sufficient acid during the fermentation of glucose and the maintenance of conditions such that the pH of an old culture is sustained below a value of about 4.5, as shown by a change in the colour of the methyl red indicator which is added at the end of the period of incubation.

Clark and Lubs developed MR-VP Broth which allowed both the MR and VP tests to be performed from the same inoculated medium by aliquoting portions to different tubes.

### **PRINCIPLE:**

Some bacteria have the ability to utilize glucose and convert it to a stable acid like lactic acid, acetic acid or formic acid as the end product.

These bacteria initially metabolise glucose to pyruvic acid, which is further metabolized through the ‘mixed acid pathway’ to produce the stable acid. The type of acid produced differs from species to species and depends on the specific enzymatic pathways present in the bacteria. The acid so produced decreases the pH to 4.5 or below, which is indicated by a change in the colour of methyl red from yellow to red.

In the methyl red test (MR test), the test bacteria are grown in a broth medium containing glucose. If the bacteria have the ability to utilise glucose with production of a stable acid, the colour of the methyl red changes from yellow to red, when added into the broth culture.

The mixed acid pathway gives 4 mol of acidic products (mainly lactic and acetic acid), 1 mol of neutral fermentation product (ethanol), 1 mol of CO<sub>2</sub>, and 1 mol of H<sub>2</sub> per mol of glucose fermented. The large quantity of acids produced causes a significant decrease in the pH of the culture medium.

### **MATERIALS REQUIRED:**

MRVP broth, Methyl red solution, Incubator

### **PROCEDURE:**

1. Prior to inoculation, allow medium to equilibrate to room temperature.
2. Using organisms taken from an 18-24-hour pure culture, lightly inoculate the medium.
3. Incubate aerobically at 37 degrees C. for 24 hours.
4. Following 24 hours of incubation, aliquot 1ml of the broth to a clean test tube.
5. Reincubate the remaining broth for an additional 24 hours.
6. Add 2 to 3 drops of methyl red indicator to aliquot.
7. Observe for red color immediately.

**OBSERVATION AND RESULT:**

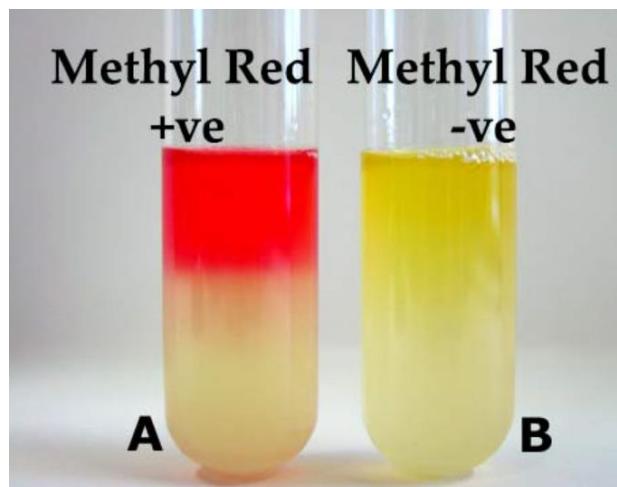
**Positive Reaction:** A distinct red color (A)

**Examples:** *E. coli, Yersinia sps*, etc.

**Negative Reaction:** A yellow color (B)

**Examples:** *Enterobacter aerogenes, Klebsiella pneumoniae*, etc.

**A weak positive** is red-orange. If an orange color is seen, incubate the remainder of the broth for up to 4 days and repeat the test after further incubation. In this case it may also be helpful to set up a duplicate broth at 25°C.

**1.MRVP broth (pH 6.9)(1000ml):**

- buffered peptone - 7.0 gm
- glucose - 5.0 gm
- dipotassium phosphate - 5.0 gm

**2.Methyl red solution, 0.02%:**

- Dissolve 0.1 g of methyl red in 300 ml of ethyl alcohol, 95%.
- Add sufficient distilled water to make 500 ml.
- Store at 4 to 8 degree C in a brown bottle. Solution is stable for 1 year.

X-----X-----X-----X

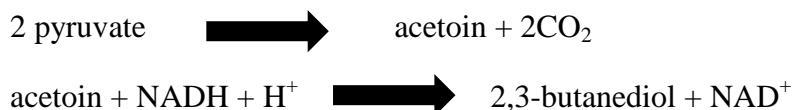
### **3. VOGES-PROSKAUER (VP) TEST**

#### **INTRODUCTION:**

Voges and Proskauer, in 1898, first observed the production of a red color after the addition of potassium hydroxide to cultures grown on specific media. Harden later revealed that the development of the red color was a result of acetyl-methyl carbinol production. In 1936 Barritt made the test more sensitive by adding alpha-naphthol to the medium before adding potassium hydroxide.

#### **PRINCIPLE:**

The Voges-Proskauer (VP) test is used to determine if an organism produces acetyl methyl carbinol from glucose fermentation. If present, acetyl methyl carbinol is converted to diacetyl in the presence of  $\alpha$ -naphthol, strong alkali (40% KOH), and atmospheric oxygen. The  $\alpha$ -naphthol was not part of the original procedure but was found to act as a color intensifier by Barritt and must be added first. The diacetyl and quanidine-containing compounds found in the peptones of the broth then condense to form a pinkish red polymer.



#### **MATERIALS REQUIRED:**

MRVP broth, Barrits reagent A & B, incubator

#### **PROCEDURE:**

1. Prior to inoculation, allow medium to equilibrate to room temperature.
2. Using organisms taken from an 18-24-hour pure culture, lightly inoculate the medium.
3. Incubate aerobically at 37 degrees C. for 24 hours.
4. Following 24 hours of incubation, aliquot 2 ml of the broth to a clean test tube.
5. Re-incubate the remaining broth for an additional 24 hours.
6. Add 6 drops of 5% alpha-naphthol, and mix well to aerate.
7. Add 2 drops of 40% potassium hydroxide, and mix well to aerate.
8. Observe for a pink-red color at the surface within 30 min. Shake the tube vigorously during the 30-min period.

#### **OBSERVATION AND RESULT:**

**Positive Reaction:** A pink-red color at the surface

**Examples:** *Viridans group streptococci* (except *Streptococcus vestibularis*), *Listeria*, *Enterobacter*, *Klebsiella*, *Serratiamarcescens*, *Hafniaalvei*, *Vibrio eltor*, *Vibrio alginolyticus*, etc.

**Negative Reaction:** A lack of a pink-red color

**Examples:** *Streptococcus mitis*, *Citrobacter sp.*, *Shigella*, *Yersinia*, *Edwardsiella*, *Salmonella*, *Vibrio furnissii*, *Vibrio fluvialis*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* etc.

A copper color should be considered negative. A rust color is a weak positive reaction.



### **1. MRVP broth (pH 6.9) (1000ml)**

buffered peptone - 7.0 gm

glucose - 5.0 gm

dipotassium phosphate - 5.0 gm

### **2. Voges-Proskauer Reagent A: Barritt's reagent A**

Alpha-Naphthol, 5% - 50 gm

Absolute Ethanol - 1000 ml

### **3. Voges-Proskauer Reagent B: Barritt's reagent B**

Potassium Hydroxide - 400 gm

Deionized Water - 1000 ml

X-----X-----X-----X

## 1. CITRATE UTILIZATION TEST

### INTRODUCTION:

This test is among a suite of IMViC Tests (Indole, Methyl-Red, Vogues-Proskauer, and Citrate) that are used to differentiate among the Gram-Negative bacilli in the family Enterobacteriaceae.

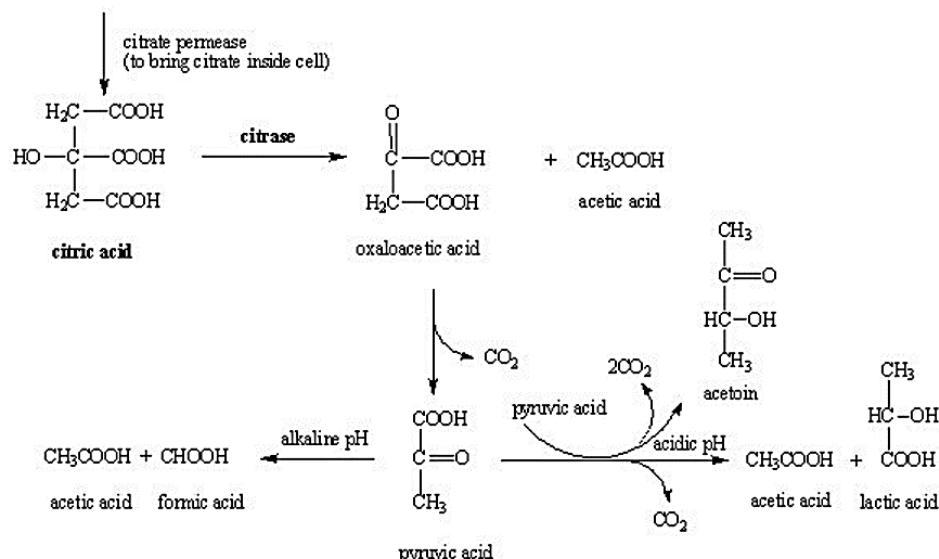
### PRINCIPLE:

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts ( $\text{NH}_4\text{H}_2\text{PO}_4$ ) as the sole source of nitrogen.

Bacteria that can grow on this medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy. Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle.

When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6.

Christensen developed an alternative citrate test medium that does not require the organism to use citrate as a sole carbon source. Christensen's medium contains both peptone and cysteine. Thus citrate-negative bacteria can also grow on this medium. A positive reaction shows that the organism can use citrate but not necessarily as the sole carbon source.



### MATERIALS REQUIRED:

Simmon's Citrate Agar slants, bacterial slants, incubator

## **PROCEDURE:**

1. Streak the slant back and forth with a light inoculum picked from the center of a well-isolated colony.
2. Incubate aerobically at 35 to 37°C for up to 4-7 days.
3. Observe a color change from green to blue along the slant.

## **OBSERVATION AND RESULT:**

**Positive Reaction:** Growth with color change from green to intense blue along the slant.

**Examples:** *Salmonella*, *Edwardsiella*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, *Providencia*, etc.

**Negative Reaction:** No growth and No color change; Slant remains green.

**Examples:** *Escherichia*, *Shigella*, *Morganella*, *Yersinia* etc.

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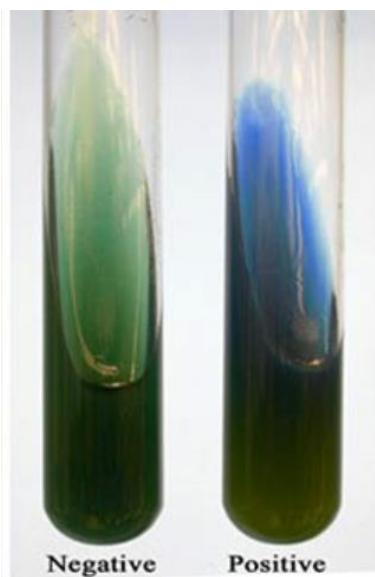
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### **1. Simmon's Citrate Agar**

Sodium Chloride - 5.0 gm  
Sodium Citrate (dehydrate) - 2.0 gm  
Ammonium Dihydrogen Phosphate - 1.0 gm  
Dipotassium Phosphate - 1.0 gm  
Magnesium Sulfate (heptahydrate) - 0.2 gm  
Bromothymol Blue - 0.08 gm  
Agar - 15.0 gm  
Deionized water = 1,000 ml  
Final pH 6.9 +/- 0.2 at 25 degrees C.

#### **Preparation**

- i. Dissolve above salts in deionized water.
- ii. Adjust pH to 6.9.
- iii. Add agar and Bromothymol blue.
- iv. Gently heat, with mixing, to boiling until agar is dissolved.
- v. Dispense 4.0 to 5.0 ml into 16-mm tubes.
- vi. Autoclave at 121 degree C under 15 psi pressure for 15 minutes.
- vii. Cool in slanted position (long slant, shallow butt).
- viii. Tubes should be stored in a refrigerator to ensure a shelf life of 6 to 8 weeks.
- ix. The uninoculated medium will be a deep forest green due to the pH of the sample and the bromothymol blue.



X-----X-----X-----X

## **15. GROWTH PHASES OF BACTERIA**

**Aim:**

To study different growth phases of bacterial population and plot a bacterial growth curve.

**Introduction:**

Most bacteria reproduce by an asexual process called binary fission, which results in doubling of the number of viable bacterial cells. In binary fission, events occur in sequences i.e. the chromosome duplicates, the cell elongates, and the plasma membrane pinches inward to the center of the cell. The time required for a cell population to divide and double in number is called the generation time or the doubling time. Generation time varies with organism and environment and can range from 20 minutes for a fast growing bacterium under

ideal conditions, to hours in not so ideal conditions. The standard bacterial growth curve describes various stages of growth of pure culture of bacteria, beginning with the addition of cells in sterile media to the death of the cells.

The phases of growth typically observed include:

- Lag phase
- Exponential (log, logarithmic) phase
- Stationary phase
- Death phase (exponential or logarithmic decline)

**Principle:**

Bacterial growth refers to an increase in the number of cells. When bacteria are inoculated into a liquid medium and the cell population is counted at intervals, it is possible to plot a typical bacterial growth curve that shows the growth of cells over time. It shows four distinct phases of growth.

- **Lag phase:** During the lag phase, the bacterial cells adapt themselves to the new environment as they are introduced into a new medium. The bacterial population increase in size but show no change in number as they prepare themselves for cell division. They synthesize new enzymes to utilize new nutrients. The length of the lag phase is determined by the characteristics of the bacterial species and by conditions in the media, both the medium from which the organisms are taken and the one to which they are transferred.
- **Log or exponential phase:** During this phase, the bacteria divide exponentially. The mass of each cell increases rapidly followed by reproduction. The number of bacteria doubles during each generation time. The growth curve is linear during this phase and the cells are metabolically most active. As the amount of nutrients decrease, the vigor of the population changes and the rate of reproduction and death equalizes. The bacterial population then enters a plateau called as stationary phase.
- **Stationary phase:** During the stationary phase, as the nutrients get exhausted, and there is a buildup of waste and secondary metabolic products, the growth rate decreases to a point where it almost equals to the death rate, hence viable cell

number remains same. If incubation is still continued the population then enters the death phase.

- **Decline or death phase:** During the death phase, viable cell population decreases exponentially as the amount of waste products increases. The death of cells in the population exceeds the formation of new cells. This continues until the population is diminished to a tiny fraction of the more resistant cells or the population might die out entirely.

**Materials required:** E coli, LB broth, conical flask, test tubes, spectrophotometer

#### **Protocol:**

- Pick up a single colony of E coli from the LB agar plate and inoculate it into a test tube containing 5 ml of autoclaved LB broth. Incubate the test tube overnight at 37°C.
- Take 250 ml of autoclaved LB Broth in a sterile 500 ml conical flask.
- Inoculate 2.5 ml of the overnight grown culture in above flask
- Take OD at zero hour. Incubate the flask at 37oC
- Aliquot 1 ml of the culture suspension at an interval of every 30 minutes and take the optical density
- At the end of experiment, plot a graph of time in minutes on X axis versus optical density at 600nm on Y axis to obtain a growth curve of Escherichia coli.

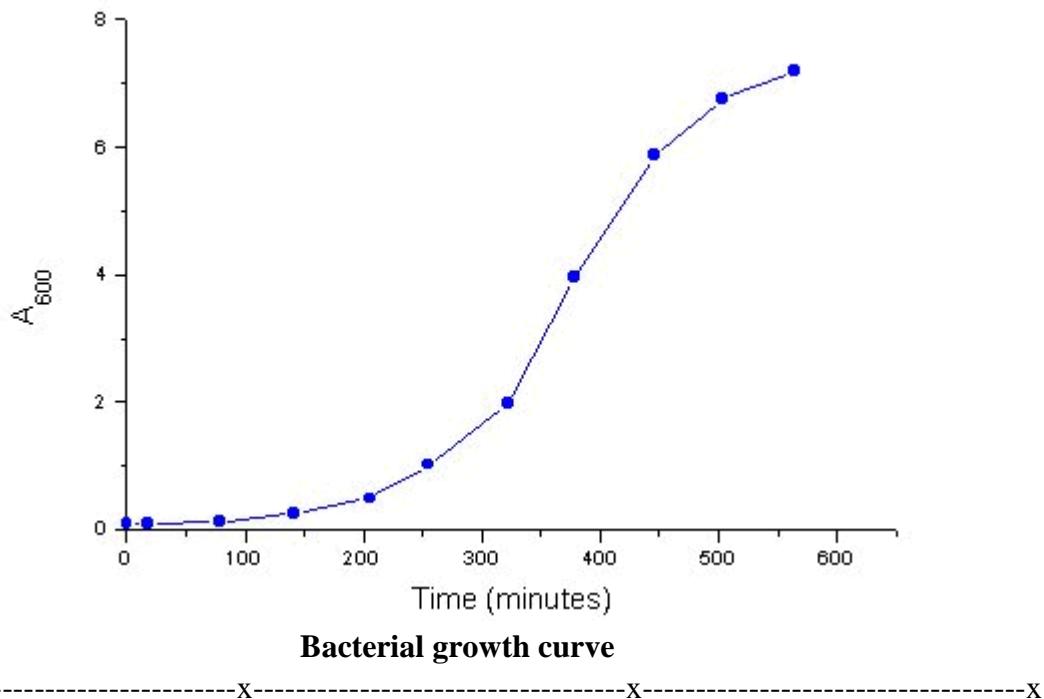
#### **Observation & Result:**

The OD at 600nm was taken every 30 minutes and graph was plotted with time on X axis and optical density at 600 nm.

Sr. No.	Time in minutes	Optical Density (OD) at 600 nm
1	00	
2	30	
3	60	
4	90	
5	120	
6	150	
7	180	
8	210	
9	240	
10	270	
11	300	
Read O.D. till absorbance becomes static		

By performing this experiment, the different stages of bacterial growth curve can be observed. In the lag phase the OD remains constant. With time the cells enter the log phase where the OD increases until the cells reach the stationary phase, where the OD remains

more or less constant



## 16.ISOLATION OF INDUSTRIALLY IMPORTANT MICROORGANISMS FOR MICROBIAL PROCESSES

**Aim:** To isolate microorganisms capable of producing one of the industrially important enzyme amylases.

**Introduction:** Fermentation process is widely used to produce commercially viable products. The success of the fermentation depends upon microorganism used. The organism to be used should be capable of producing the product in large amounts and grow in ambient temperature. They should also be capable of producing large amounts of the desired products using cheap substrates.

**Principle:** Screening and isolation of microorganism is an important prerequisite for a successful fermentation process. Serial dilution of soil samples followed by plating on screening media for amylase production. Microorganism identified by primary screening are further validated.

**Materials and Methods:** Soil, Test tubes, Autoclaved water, Starch agar plate, 1% iodine solution.

### **Procedure:**

- One gram of soil sample was collected, weighed and mixed to 9 ml of sterile distilled water in a boiling tube.
- Serial dilution was done up to  $10^{-5}$ .
- 100ul of  $10^{-4}$  and  $10^{-5}$  was plated onto starch agar and incubated at  $37^{\circ}\text{C}$ .
- The isolated colonies were then plated onto a starch agar plate and incubated for 48 hours
- After incubation 1% iodine solution was flooded with a dropper for 30 seconds on the starch agar plate.
- The isolates produced clear zones of hydrolysis were considered as amylase producers

### **Observation and Result:**

Colonies producing clear zone on starch agar plate following 1% iodine solution was isolated and subcultured.

X-----X-----X-----X

## 17. ISOLATION OF PESTICIDE-DEGRADING MICROORGANISMS FROM SOIL

**Aim:** To isolate pesticide-degrading microorganisms from soil

**Introduction:** The wide application of pesticides are employed for plant protection against insect pests. The pesticide is one of the major chemicals responsible for the contamination and deterioration of soil and groundwater, particularly in the close vicinities of agricultural fields. Owing to their high toxicity and persistence in the environments, most of them are banned all over the world. Insecticides and their degradation products generally get accumulated in the top soil and influence not only the population of various groups of soil microbes but also their biochemical activities like nitrification, ammonification, decomposition of organic matter and nitrogen fixation. Microorganisms play an important role in degrading synthetic chemicals in soil. They have the capacity to utilize virtually all naturally and synthetically occurring compounds as their sole carbon and energy source.

**Principle:** Microorganisms capable of degrading pesticides can be isolated using an enrichment technique where the major source of carbon for microbes is the pesticides. Organisms capable of degrading pesticides are preferentially allowed to multiply faster due to the presence of pesticides.

**Materials required:** Conical flask, MSM media, soil sample, Nutrient agar plates, pesticide.

**Procedure:**

- Soil samples are collected from agricultural lands. The roots and stones and larger soil fragments are broken down
- 1gm of each soil sample was inoculated into 100ml of mineral salt medium(MSM) in a conical flask.
- The flasks were incubated on a rotary shaker at 150 cycles per minute for 7 days at room temperature (25-30°C).
- At daily intervals, one loop full of enrichment culture from the flask was streaked on nutrient agar plates supplemented with pesticide (5g) and incubated at room temperature for 24-48hrs.
- Individual colonies of bacteria which varied in shape and color were picked up and were subcultured onto nutrient agar plates.

**Result:**

Colonies were observed on nutrient plates with pesticides indicating they are capable of degrading pesticides

## Composition of Minimal Salt Media

Chemical	Formula	Amount (g/L)
Dipotassium hydrogen phosphate	K <sub>2</sub> HPO <sub>4</sub>	1.73
Potassium dihydrogen phosphate	KH <sub>2</sub> PO <sub>4</sub>	0.68
Magnesium sulfate heptahydrate	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.1
Sodium chloride	NaCl	4
Ferrous sulfate heptahydrate	FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.03
Ammonium nitrate	NH <sub>4</sub> NO <sub>3</sub>	1
Calcium chloride	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.02
Glucose	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	5

Note: Add 0.5g of pesticide to 100ml of MSM after sterilization before addition of diluted bacterial culture.

X-----X-----X-----X

## 18.PREPARATION OF VERMICOMPOST

**Aim:** To prepare vermicompost

**Introduction:** Vermicompost is a method of making compost with the use of earthworm, which eats biomass and excreta in a digested form. The Red worms (*Eiseniafoetida*) and African earthworm (*Eudrillusengenae*) are promising worms used for vermicompost production. Vermicompost can be produced in any place with shade, high humidity and cool. Abandoned cattle shed or poultry shed or unused buildings can be used. If it is to be produced in an open area, the shady place is selected. Cattle dung (except the pig, poultry and goat), farm wastes, crop residues, vegetable waste, agro-industrial waste, fruit market waste, and all other bios degradable waste are suitable for vermicompost production. The cattle dung should be decomposed before used for vermicompost production. All other waste should be pre-digested with cow dung for twenty days before put into vermibed for composting. Heavy spices and metallic products are not used in this process.

**Materials required:** Vermi bin/cemented tank, Thatch roof, Polythene sheet (black), Waste materials, Cow dung, Water, Gunny bags. Plastic net, Vermi worm

**Procedure:**

1. The compost can be prepared in a concrete tank (size is depending upon the availability of raw materials) could be used.
2. Collect and heap the weed biomass under the sun for about 7-10 days or until well decomposed. Chop the hard materials required.
3. Sprinkle cow dung slurry on the heap for quick decompose
4. Place a thin layer of surface soil/sand (1-2 inch) at the bottom of the tank.
5. Place fine bedding material such as partially decomposed cow dung/dried leaves etc. over the soil or sand layer
6. Place the chopped bio-waste and partially decomposed cow dung layer-wise in the tank up to a depth of 0.5-1.0 ft.
7. Release about 1000-2000 worms/m<sup>2</sup> of any of the above earthworm species over the mixture.
8. Cover the compost mixture with dry straw or thatch or gunny bag.
9. Sprinkle water as and when necessary to maintain 70-80% moisture content.
10. Provide shade over the compost mixture to protect from rainwater and direct sunshine
11. Stop sprinkling of water when 80-98% ofbiowaste is decomposed. Maturity could be judged visually by observing the formation of the granular structure of the compost at the surface of the tank
12. Collect the vermicompost by scrapping layer-wise from the top of the tank and keep it under shade.

**Result:**

Vermicompost was prepared



Vermicomposting

X-----X-----X-----X

## 19. Determination of Nitrate Ion in Water

### **Aim:**

To determine the amount of nitrate present in water.

### **Introduction:**

Nitrate nitrogen may be present in small amounts in fresh domestic wastewater. However, it is seldom found in influents to treatment plants because the nitrates serve as an oxygen source in the biologically unstable wastewater. On the other hand, nitrate is often found in the effluents of biological treatment plants because it represents the final form of nitrogen from the oxidation of organic nitrogen compounds.

Nitrate may be found in river water, lake water, and most importantly in ground water. The U.S. Public Health Service has designated the safe limit for nitrogen in nitrates to be 10mg/L. Nitrates in drinking water are particularly dangerous to small children, infants, and fetuses.

In this experiment, nitrate will be reduced to nitrite with zinc. The nitrite reacts with sulfanilic acid and N-1-naphthylethylenediamine to produce a red compound. The intensity of the red color is analyzed spectrophotometrically. The amount of zinc and the contact period are important.

### **Special Apparatus:**

Spectrophotometer set at 550nm

Filter paper and vacuum filtration apparatus

### **Reagents:**

Stock potassium nitrate solution 50mg/L

Hydrochloric acid

Sulfanilic acid

Zinc/NaCl

N-1-naphthylethylenediamine dihydrochloride reagent

Sodium acetate solution

### **Procedure:**

1. To a 50mL sample in a 250mL Erlenmeyer flask (lake water, blank, or standard), add 1.0mL of hydrochloric acid, and 1.0mL of sulfanilic acid reagent and mix thoroughly.
2. In a dry 10mL graduated cylinder, measure 1mL of 1 M  $\text{ZnCl}_2$  and add it to the Erlenmeyer flask.
3. Swirl the flask for seven minutes. Filter with a vacuum flask after seven minutes.
4. Rinse the Erlenmeyer flask well with distilled water and pour the water sample back

- into the flask. Add 1.0mL of the naphthylethylenediamine reagent to the filtered sample and mix.
5. Add 1.0mL of the 2M sodium acetate solution and mix. Allow 5 minutes (or more) for color development. Measure the color intensity with the spectrophotometer set at 550nm.
  6. Determine the concentration of nitrate from the standard graph prepared from stock potassium nitrate solution.

**Result:**

The nitrate concentration in water was determined.

---

**Preparation of standard**

Prepare a 5 point calibration curve using the stock  $\text{KNO}_3$  solution. Make your concentrations in the range of 0 – 15mg/L.

## 1.MITOSIS

**AIM:** To visualize and understand the different stages of mitosis.

### INTRODUCTION

All organisms are made of cells. For an organism to grow, mature and maintain tissue, new cells must be made. All cells are produced by the division of pre-existing cells. Continuity of life depends on cell division. There are two main methods of cell division: mitosis and meiosis.

Mitosis is very important to life because it provides new cells for growth and replaces dead cells. Mitosis is the process in which a eukaryotic cell nucleus splits in two, followed by division of the parent cell into two daughter cells. Each cell division consists of two events: cytokinesis and karyokinesis. Karyokinesis is the process of division of the nucleus and cytokinesis is the process of division of cytoplasm.

#### Events during Mitosis

1. Prophase:
  1. Mitosis begins at prophase with the thickening and coiling of the chromosomes.
  2. The nuclear membrane and nucleolus shrink and disappears.
  3. The end of prophase is marked by the beginning of the organization of a group of fibres to form a spindle.
2. Metaphase
  1. The chromosome becomes thick and two chromatids of each chromosome become clear.
  2. Each chromosome attaches to spindle fibres at its centromere.
  3. The chromosomes are arranged at the midline of the cell.
3. Anaphase
  1. In anaphase, each chromatid pair separates from the centromere and move towards the opposite ends of the cell by the spindle fibres.
  2. The cell membrane begins to pinch at the centre.
4. Telophase
  1. Chromatids arrive at opposite poles of the cell.
  2. The spindle disappears and the daughter chromosome uncoils to form chromatin fibres.
  3. The nuclear membranes and nucleolus re-form and two daughter nuclei appear at opposite poles.
  4. Cytokinesis or the partitioning of the cell may also begin during this stage.

**Principle**

Somatic growth in plants and animals take place by increase in the number of cells. A cell divides mitotically to form two daughter cells wherein the number of chromosomes remains the same (i.e., unchanged) as in the mother cell. In plants, such divisions rapidly take place in meristematic tissue of root and apices, where the stages of mitosis can be easily observed. In animals, mitotically dividing cells can be easily viewed in the bone marrow tissue of vertebrate, epithelial cells from gills in fishes and the tail of growing tadpole larvae of frog.

**MATERIALS REQUIRED**

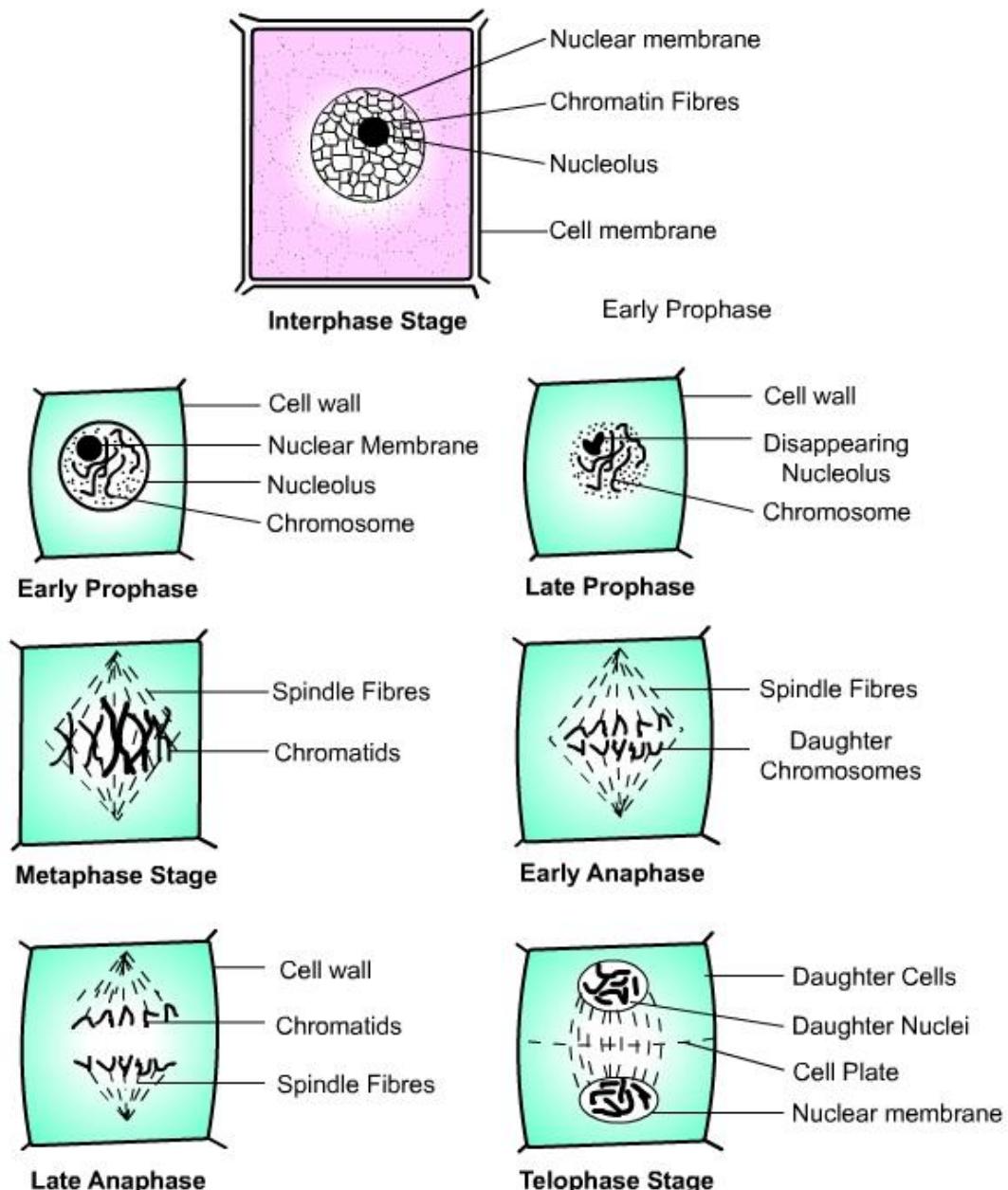
Onion root, Distilled water, Slides and coverslips, razor blade, needles, paper tissue, light microscope, 1 N HCl, Acetocarmine dye.

**PROCEDURE**

1. Place onions to root in a test tube by filling the tube with tap water until the root area is covered.
2. After two or three days the roots should be sufficiently long cut approximately 5mm off the tips of a couple of roots put them in 1N hydrochloric acid for 2-3 minutes.
3. With a pipette and some distilled water, rinse away the acid.
4. Then place the root tip in acetocarmine dye for 5 minutes. Then transfer the root tips to a microscope slide.
5. Gently placed the cover slip on the slide with root tip and firmly press the upper portion of the slip using thumb.
6. Observe under Light microscope at 10 X, 45X and 100X respectively.

**RESULT**

All the distinct phases were observed.



## CALCULATION

### 1. Preparation of 1 N, 100ml HCl

Generally, the HCl that we get from manufacturer is 37%. Its concentration is 12N. So, Using the formula  $N_1V_1 = N_2V_2$ , we can make the 1N solution of desired quantity

$$12 \times X = 1 \times 100$$

$$X = 100/12 ; \quad x = 8.3\text{ml}$$

So, you can mix 8.3ml of con.,HCl and 91.7ml of distilled water to prepare 100ml of 1 N HCl.

## 2. Acetocarmine stain

### Reagents

- Carmine -0.5g
- Glacial acetic acid- 100ml
- Ferric chloride (optional) -5ml

Add 0.5g carmine in 100ml boiling 45% glacial acetic acid for 1 to 2 minutes or until there is a sudden change to a darker colour, cool the room temperature, then filtered and stored for future use.

X-----X-----X-----X

## 2. OBSERVATION OF BARR BODIES

**Aim:** To identify the Barr body in a female cheek cell.

### **Introduction**

The Barr body, also sometimes called the sex chromatin, is the inactive X chromosome in female somatic cells. Human females have two X chromosomes, while males have one X and one Y. In all of the female somatic cells, which don't take part in sexual reproduction, one of the X chromosomes is active, and the other is inactivated in a process called lyonization, becoming the Barr body. The reason for shutting off one X chromosome is so that only the necessary amount of genetic information is expressed, rather than double or even more.

The mechanism of inactivation is due to the presence of X-inactivation center (XIC) found near the centromere on the X chromosomes, containing a gene called X-inactive specific transcript (Xist), and another called Tsix. Xist leads to gene inactivation on the X chromosome, and so we find that the XIC is coated with Xist RNA. This then causes the DNA to be condensed (heterochromatin) leading to the formation of the Barr body in heterochromatin.

### **Materials required:**

Light microscope, Beaker, Paper towel, Glass slide, Dropper pipette, Coverslip, Water, Flat toothpick, Methylene blue (0.3%)

### **Procedure**

1. Obtained a clean glass microscope slide.
2. Placed a drop of water in the centre of the slide.
3. Gently scrape the inside of your cheek with the flat edge of a toothpick.
4. Spread cells in the drop of water.
5. Applied one drop of methylene blue on the specimen, Put coverslip after 5 minutes.
6. Along the opposite edge of the coverslip, gently touch with a piece of paper towel. This will draw the methylene blue under the coverslip to stain the cells.
7. Observed under microscope and draw diagram, labelled, and recorded magnification.

### **Result**

Barr body was observed as a dark dot inside the nucleus of the cheek cells

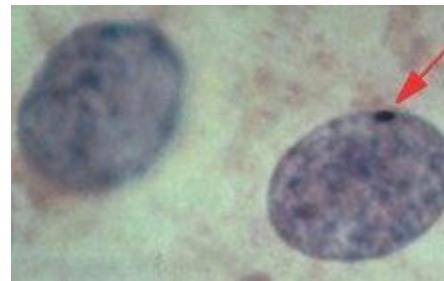
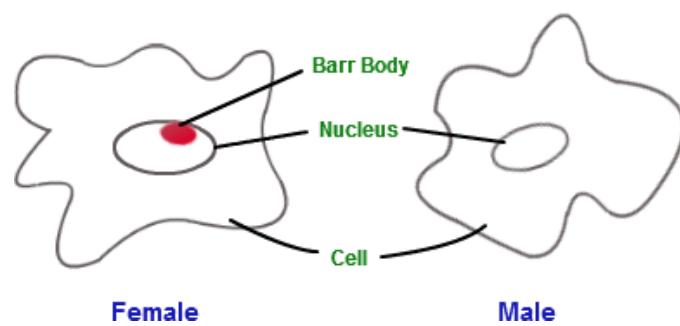


Figure: Cheek cell with a red arrow showing Barr body

X-----X-----X-----X

### 3. SQUASH PREPARATION- POLYTENE CHROMOSOME

#### Aim

To observe polytene chromosome from drosophila salivary gland

#### Introduction

The fly, *Drosophila melanogaster*, is a holometabolous insect with four main stages to its lifecycle: embryo, larva, pupa, adult. As a larva, the organism is primarily concerned with obtaining food for the rapid increase in size characteristic of this stage of development. During this time, the salivary glands must be large and well-developed so that a sufficient supply of salivary enzymes is available for digestion. The salivary glands of Drosophila and some other insects achieve their growth through an increase in cell mass and volume rather than an increase in the number of cells. After an initial population of salivary gland cells is established during early larval development, cell division ceases. While the cells increase in size, the nuclei grow too, as the chromosomes duplicate repeatedly without accompanying cell division. Although the exact reason for this unusual process is unknown, it is apparently a more efficient way of producing salivary enzymes need for rapid larval development.

The chromosomes are in an extended interphase of the cell cycle and, as such, are stretched out to their full length. Because each chromosome actually consists of many strands, they are called polytene (“many threaded”) chromosomes. Because polytene chromosomes are extended and consist of so much DNA, they are easily visible under the light microscope. A useful feature of these chromosomes is that they have a pattern of dark and light bands, like a bar code, which is unique for each chromosome. The dark bands represent regions where the DNA is most densely packed, and the light bands (interbands) are regions where the DNA is less densely packed. These bands provide visible landmarks that can be used to identify the location of a specific gene on the chromosome or the sites of chromosomal rearrangements. Both bands and interbands contain genes and when a gene is being actively transcribed, puffs appear in the chromosomal region containing that gene. The puffs represent areas where the DNA is unwound from the normal densely-packed state so that it is accessible to the transcriptional machinery.

#### Materials required

Dissecting scope, Bottle of wild type Drosophila stock, 10 ml saline (0.7% NaCl), 1 ml 45% Acetic Acid, 0.5 ml Orcein stain, blotting paper, H<sub>2</sub>O wash bottle, Microscope slides, Coverslips, 2 pairs of Forceps.

#### Procedure

1. Place a drop of 0.7% saline on a clean microscope slide. Transfer an appropriate larva (see above) to the slide and place it on the stage of a dissecting microscope.
2. Using the enhanced detail afforded by the microscope, firmly grasp the posterior end of the larva with forceps and use a dissecting needle, or another pair of forceps

to pierce through the head, just behind the darkly pigmented mouthparts. Using a continuous motion, pull the needle (and attached head) away from the body.

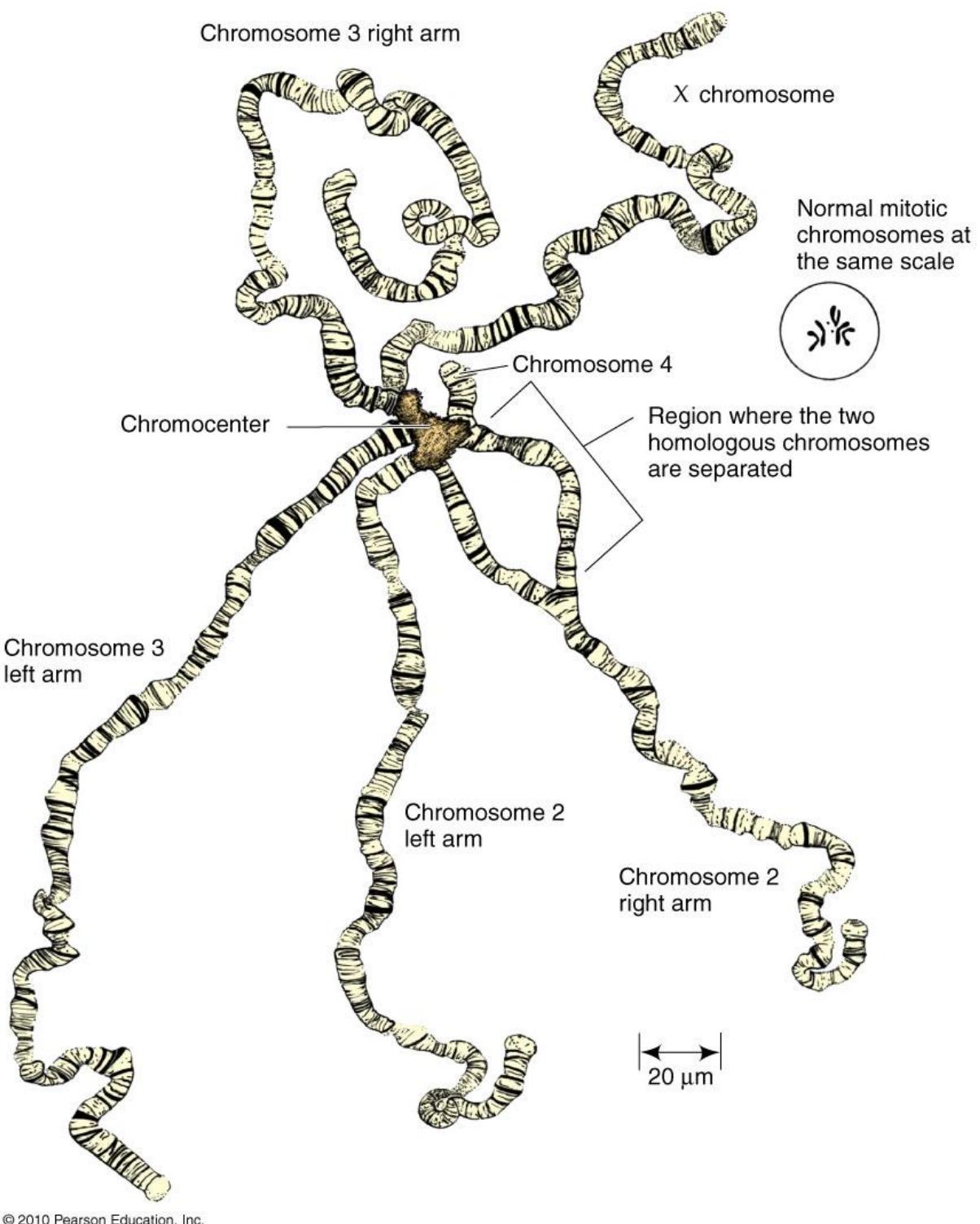
3. While continuing to observe under the dissecting microscope, separate the salivary glands from any extraneous material, such as the fat bodies or parts of the digestive tract.
4. Once cleaned, remove the used saline and debris from the slide. Add fresh saline and allow the glands to soak for ten minutes.
5. Using a laboratory tissue, blot the saline from the slide containing the salivary glands in saline. Be careful not to touch the glands with the tissue as they will stick and it will not be possible to recover them. Place two drops of aceto-orcein stain directly on the glands.
6. Place the slides in a petri dish containing a moist filter paper (this prevents the stain from evaporating during incubation). Incubate for fifteen minutes.
7. Carefully blot excess stain away from the salivary gland. Be careful not to touch the glands with the tissue as they will stick. Add one drop of fresh stain and incubate for two minutes
8. Apply a coverslip to the stained salivary gland and place a paper towel or folded laboratory tissue over the coverslip. Using steady, moderate pressure, press down on the coverslip in a vertical direction. Do not twist the coverslip or allow it to move laterally; this will shear the chromosomes.
9. Observe the salivary gland squash at high-dry magnification with a compound microscope.

## **Result**

Elongated chromosomes with distinct banding patterns were observed.



Figure: Polytene chromosome as when observed under 100X oil immersion



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X-----X-----X-----X

## 4. SUBCELLULAR FRACTIONATION

**Aim:** To isolate different cellular fractions by differential centrifugation

### **Introduction:**

Separation of cellular compartments from one another is an important step for studying a specific intracellular structure or organelle or protein, or to assess possible associations between these macromolecular structures. Subcellular fractionation uses one or more of the properties of each compartment, such as buoyant density, surface charge density, size and shape, and is mainly based on differential centrifugation in media of high viscosity at 4°C. Media used for differential centrifugation are mainly sucrose, mannitol, glycerol, Ficoll 400 (a polymer of sucrose), Percoll (a type of colloidal silica) and iodixanol. Sucrose is widely used because it is inexpensive.

The simplest form of separation by centrifugation is differential centrifugation, sometimes called differential pelleting. Particles of different densities or sizes in a suspension will sediment at different rates, with the larger and denser particles sedimenting faster. These sedimentation rates can be increased by using centrifugal force. A suspension of cells subjected to a series of increasing centrifugal force cycles will yield a series of pellets containing cells of decreasing sedimentation rate.

Differential pelleting is commonly used for harvesting cells or producing crude subcellular fractions from tissue homogenate. For example, a rat liver homogenate containing nuclei, mitochondria, lysosomes, and membrane vesicles that is centrifuged at low speed for a short time will pellet mainly the larger and more dense nuclei. Subsequent centrifugation at a higher centrifugal force will pellet particles of the next lower order of size (e.g., mitochondria) and so on.

### **Materials:**

Fractionation Buffer, Cooling centrifuge, animal culture cells, DTT, microfuge tubes, syringe.

### **Procedure:**

1. Transfer animal culture cells from 10 cm plates into 500 µL fractionation buffer, eg by scraping. Incubate 15 min on ice.
2. Using 1 mL syringe pass cell suspension through a 27 gauge needle 10 times (or until all cells are lysed).
3. Leave on ice for 20 min.
4. Centrifuge sample at 720 xg (3,000 rpm) for 5 min. The pellet will contain nuclei and the supernatant will contain cytoplasm, membrane and mitochondria.
5. Transfer supernatant into a fresh tube and keep on ice. This will be dealt with in Steps 8–11.
6. Wash nuclear pellet from Step 4 with 500 µL fractionation buffer. Disperse the pellet with a pipette and pass through a 25 gauge needle 10 times. Centrifuge again at 3,000 rpm for 10 min. Discard the supernatant and keep the pellet that contains nuclei.

7. Resuspend the pellet from Step 6 in TBS with 0.1% SDS. Sonicate the suspension briefly to shear genomic DNA and homogenize the lysate (3 s on ice at a power setting of 2-continuous).
8. Centrifuge the supernatant recovered in Step 5 at 8,000 rpm (10,000 x g) for 5 min. The pellet contains mitochondria. Transfer the supernatant into a fresh tube and keep on ice: this is the cytoplasm and membrane fraction.
9. Process the mitochondrial pellet from Step 8, as described for the nuclear pellet in Step 7, to obtain mitochondrial lysate in TBS/0.1% SDS.
10. For a membrane fraction, centrifuge the supernatant from Step 8 in an ultracentrifuge at 40,000 rpm (100,000 x g) for 1 h. Wash pellet by adding 400 µL of fractionation buffer. Resuspend by pipetting and pass through a 25 gauge needle. Re-centrifuge for 45 min. Resuspend the membrane pellet in the same buffer as used for the nuclei.

**Result:**

Fractions containing mitochondria, cytoplasm, nuclear extract were prepared.

**Subcellular fractionation buffer**

	MW	mM	Add per L
HEPES (pH 7.4)	238.30	20	4.77 g
KCl	74.55	10	0.75 g
MgCl <sub>2</sub>	95.21	2	0.19 g
EDTA	292.24	1	0.29 g
EGTA	380.35	1	0.38 g

X-----X-----X-----X

## 1. PREPARATION OF BUFFERS

**Aim:** To prepare different buffers at the required pH

**Principle:** The primary purpose of a buffer is to control the pH of the solution. Buffers can also play secondary roles in a system, such as controlling ionic strength or solvating species, perhaps even affecting protein or nucleic acid structure or activity. Buffers are used to stabilize nucleic acids, nucleic acid-protein complexes, proteins, and biochemical reactions (whose products might be used in subsequent biochemical reactions). Buffers are solutions that contain mixtures of weak acids and bases that make them relatively resistant to pH change. Conceptually buffers provide a ready source of both acid and base to either provide additional H<sup>+</sup> if a reaction (process) consumes H<sup>+</sup>, or combine with excess H<sup>+</sup> if a reaction generates acid. The most common types of buffers are mixtures of weak acids and salts of their conjugate bases, for example, acetic acid/sodium acetate. Buffers are most effective within  $\pm 1$  pH unit of their pKa. Outside of that range the concentration of either the acid or its salt is so low as to provide little or no capacity for pH control. The Henderson-Hasselbalch Equation

$$\text{pH} = \text{pK}_a + \log \left( \frac{[\text{A}^-]}{[\text{HA}]} \right)$$

The species " HA " and " A<sup>-</sup> " represent the conjugate acid and conjugate base, respectively, of a buffer system.



Here, the "acid" is that which donates H<sup>+</sup> to yield a deprotonated species, and the "base" is that which accepts H<sup>+</sup> to yield the protonated species.

Procedure:

### 1. Preparation of Phosphate Buffer

Phosphate buffers, consist of a mixture of monobasic dihydrogen phosphate and dibasic monohydrogen phosphate. By varying the amount of each salt, a range of buffers can be prepared that buffer well between pH 5.8 and pH 8.0. Phosphates have a very high buffering capacity and are highly soluble in water.

- (a) 0.1 M Sodium phosphate monobasic; 13.8 g/l (monohydrate, M.W. 138.0)
- (b) 0.1 M Sodium phosphate dibasic; 26.8 g/l (heptahydrate, M.W. 268.0)

Mix Sodium phosphate monobasic and dibasic solutions in the proportions indicated and adjust the final volume to 200 ml with deionized water. Adjust the final pH using a sensitive pH meter.

ml of Sodium phosphate, Monobasic	92.0	81.5	73.5	62.5	51.0	39.0	28.0	19.0	13.0	8.5	5.3
ml of Sodium phosphate, Dibasic	8.0	18.5	26.5	37.5	49.0	61.0	72.0	81.0	87.0	91.5	94.7
pH	5.8	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8	8.0

## **2.Preparation of Acetate Buffer**

- (a) 0.1 M Acetic acid (5.8 ml made to 1000 ml)
- (b) 0.1 M Sodium acetate; 8.2 g/l (anhydrous; M.W. 82.0) or 13.6 g/l (trihydrate; M.W. 136.0)

Mix acetic acid and sodium acetate solutions in the proportions indicated and adjust the final volume to 100 ml with deionized water. Adjust the final pH using a sensitive pH meter.

ml of Acetic acid	46.3	41.0	30.5	20.0	14.8	10.5	4.8
ml of Sodium acetate	3.7	9.0	19.5	30.0	35.2	39.5	45.2
pH	3.6	4.0	4.4	4.8	5.0	5.2	5.6

## **3.Preparation of Tris HCl Buffer**

- (a) 0.1 M Tris(hydroxymethyl)aminomethane; 12.1 g/l (M.W.: 121.0)
- (b) 0.1 M Hydrochloric acid

Mix 50 ml of Tris(hydroxymethyl)aminomethane and indicated volume of hydrochloric acid and adjust the final volume to 200 ml with deionized water. Adjust the final pH using a sensitive pH meter.

ml of HCl	44.2	41.4	38.4	32.5	21.9	12.2	5.0
pH	7.2	7.4	7.6	7.8	8.2	8.6	9.0

## **4.Preparation of Borate buffer**

- A: 0.2 (M) solution of boric acid (12.4 g in 1000 ml distilled water).  
 B: 0.05 (M) solution of borax (19.05 g in 1000 ml of distilled water; 0.2 (M) in terms of sodium borate).  
 Mix A and B and dilute to 200 ml.

Vol of Boric acid	50	50	50	50	50	50
Vol of borax	2	4.9	8.4	30	59	115
pH	7.6	8.0	8.4	8.8	9	9.2

**Result:** Buffers of different pH were prepared and pH was checked.

X-----X-----X-----X

## 2. AGAROSE GEL ELECTROPHORESIS

**Aim:** To perform agarose gel electrophoresis.

**Introduction:** Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying 0.5- to 25-kb DNA fragments. The protocol can be divided into three stages: (1) a gel is prepared with an agarose concentration appropriate for the size of DNA fragments to be separated; (2) the DNA samples are loaded into the sample wells and the gel is run at a voltage and for a time period that will achieve optimal separation; and (3) the gel is stained or, if ethidium bromide has been incorporated into the gel and electrophoresis buffer, visualized directly upon illumination with UV light.

### Materials

- Electrophoresis buffer (TAE)
- Ethidium bromide solution
- Electrophoresis-grade agarose
- 10× loading buffer (see recipe)
- DNA molecular weight markers
- 55°C water bath
- Horizontal gel electrophoresis apparatus
- Gel casting platform
- Gel combs (slot formers)
- DC power supply

### **Procedure:**

#### **Preparing the gel**

1. Prepare an adequate volume of electrophoresis buffer to fill the electrophoresis tank and prepare the gel.
2. Add the desired amount of electrophoresis-grade agarose to a volume of electrophoresis buffer sufficient for constructing the gel. Melt the agarose in a microwave oven or autoclave and swirl to ensure even mixing. Gels typically contain 0.8 to 1.5% agarose.
3. Seal the gel casting platform if it is open at the ends. Pour in the melted agarose and insert the gel comb, making sure that no bubbles are trapped underneath the combs and all bubbles on the surface of the agarose are removed before the gel sets.

#### **Loading and running the gel**

4. After the gel has hardened, remove the tape from the open ends of the gel platform and withdraw the gel comb, taking care not to tear the sample wells.
5. Place the gel casting platform containing the set gel in the electrophoresis tank. Add sufficient electrophoresis buffer to cover the gel to a depth of about 1 mm (or just until the tops of the wells are submerged). Make sure no air pockets are trapped within the wells.
6. DNA samples should be prepared in a volume that will not overflow the gel wells by addition of the appropriate amount of 10x loading buffer. Samples are typically loaded into the wells with a pipettor or micropipet. Care should be taken to prevent mixing of the samples between wells.

7. Be sure that the leads are attached so that the DNA will migrate into the gel toward the anode or positive lead. Set the voltage to the desired level, typically 1 to 10 V/cm of gel, to begin electrophoresis. The progress of the separation can be monitored by the migration of the dyes in the loading buffer.
8. Turn off the power supply when the bromphenol blue dye from the loading buffer has migrated a distance judged sufficient for separation of the DNA fragments. If ethidium bromide has been incorporated into the gel, the DNA can be visualized by placing on a UV light source and can be photographed directly

**Result:**

DNA was visualized on the agarose gel stained with ethidium bromide.

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**Reagents:**

***Ethidium bromide, 10 mg/ml***

Dissolve 0.2 g ethidium bromide in 20 ml H<sub>2</sub>O

Mix well and store at 4°C in dark

***TAE (Tris/acetate/EDTA) electrophoresis buffer***

*50× stock solution:*

242 g Tris base

57.1 ml glacial acetic acid

37.2 g Na<sub>2</sub>EDTA.2H<sub>2</sub>O

H<sub>2</sub>O to 1 liter

***TE (Working solution), pH - 8.5:***

40 mM Tris.acetate

2 mM Na<sub>2</sub>EDTA.2H<sub>2</sub>O

X-----X-----X-----X

### 3. ISOLATION OF GENOMIC DNA FROM BACTERIA

**Aim:** to isolate genomic DNA from *E coli*.

**Principle:** The purification of genomic DNA from bacterial cultures provides the basis for downstream molecular analysis. The major steps involved in genomic isolation from bacteria are a) cell disruption by enzyme-detergent lysis; b) extractions with organic solvents, and c) recovery of the DNA by alcohol precipitation. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl sulfate) is used to disrupt the cell membrane. Once cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. DNA can be protected from endogenous nucleases by chelating Mg<sup>2+</sup> ions using EDTA. Mg<sup>2+</sup> ion is considered as a necessary cofactor for action of most of the nucleases. Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup. Phenol and chloroform are used to denature and separate proteins from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol.

#### **Material & methods:**

Bacterial culture, T.E buffer, 10% SDS, Phenol chloroform(1:1), Proteinase K, Ethanol, RNase, LB broth

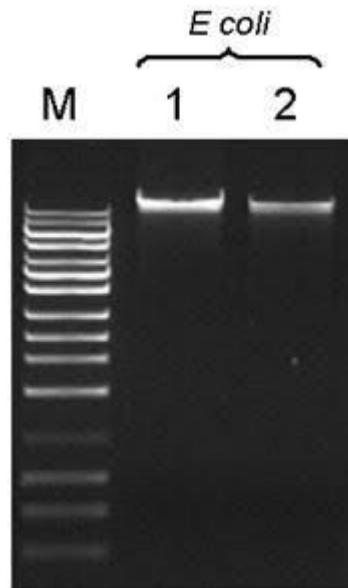
#### **Procedure:**

1. Transfer 1.5 ml of the overnight *E. coli* culture (grown in LB medium) to a 1.5 ml Eppendorf tube and centrifuge at max speed for 1min to pellet the cells.
2. Discard the supernatant. Resuspend the cell pellet in 600 µl lysis buffer and vortex to completely resuspend the cell pellet. Incubate 1 h at 37 °C.
3. Add an equal volume of phenol/chloroform and mix well by inverting the tube until the phases are completely mixed.  
*Note: Do not vertex the tube—it can shear the DNA.*
4. Spin at max speed for 5 min at RT (all spins are performed at RT unless indicated otherwise). There is a white layer (protein layer) in the aqueous: phenol/chloroform interface.
5. Carefully transfer the upper aqueous phase to a new tube by using 1 ml pipetman (to avoid sucking the interface, use 1 ml tip with wider mouth-cut 1 ml tip-mouth about ~2 mm shorter).
6. Steps 4-6 can be repeated until the white protein layer disappears.
7. To remove phenol, add an equal volume of chloroform to the aqueous layer. Again, mix

- well by inverting the tube. Spin at max speed for 5 min.
8. Remove aqueous layer to a new tube. Add 100ul of 5M potassium acetate
  9. To precipitate the DNA, add 2.5 or 3 volumes of cold 200 proof ethanol (store ethanol at -20 °C freezer) and mix gently (DNA precipitation can be visible). Incubate the tube at -20 °C for 30 min or more.
  10. Spin at max speed for 15 min at 4 °C.
  11. Discard the supernatant and rinse the DNA pellet with 1 ml 70% ethanol (stored at RT).
  12. Spin at max speed for 2 min. Carefully discard the supernatant and air-dry the DNA pellet (tilt the tube a little bit on paper towel). To be faster, dry the tube at 37 °C incubator.
  13. Resuspend DNA in TE buffer. Check isolated Genomic DNA on an agarose gel and quantify the amount of DNA present using a spectrophotometer at 260nm.

### **Result:**

DNA was visualized as orange bands in 1% agarose stained with ethidium bromide.




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### Reagent Preparation:

#### **TE buffer**

10 mM Tris-Cl (pH 8.0)  
1 mM EDTA (pH 8.0)

For 100 ml TE buffer, Take 1 ml of 1M Tris Buffer stock solution + 200µl of 0.5M EDTA and make up to 100 ml with distilled water

**Lysis buffer (30 ml)** ( To be mixed on the day of the experiment)

28.02 ml TE buffer

1.8ml of 10% SDS

180  $\mu$ l of proteinase K (20 mg  $ml^{-1}$ )

**5M potassium acetate ( M.W 98.15)**

4.91g of potassium acetate is 10ml of distilled water. Store at -20°C

**70% Ethanol**

35ml of ethanol +15 ml of distilled water. Store at -20°C

**100% Ethanol ( 50ml). Store at -20°C**

**Phenol chloroform(1:1)**

15ml of Tris saturated phenol (Kept at 4C, dark bottle in fridge stick rack)+15ml of chloroform. Store at 4°C.

**1X TAE Buffer ( For running)**

20ml of 50X TAE is made up to 1000ml with distilled water. Store at room temperature

**1XTAE Buffer ( for gel preparation)**

4ml of 50X TAE is made up to 200ml with distilled water. Store at room temperature.

To this 2g of agarose can be added, boiled and 2ul of Ethidium bromide added and poured into the tray.

X-----X-----X-----X

## 4. ISOLATION OF PLANT GENOMIC DNA

**Aim:** To isolate DNA from plant sources

**Principle:** Isolating DNA from plant tissues can be very challenging as the biochemistry between divergent plant species can be extreme. Plants have variable levels of metabolites and structural biomolecules. Polysaccharides and polyphenols are two classes of plant biomolecules that vary widely between species and are very problematic when isolating DNA. The isolation procedure utilizes detergent such as cetyl trimethyl ammonium bromide(CTAB) or SDS which disrupts the membranes, a reducing agent such as  $\beta$  mercaptoethanol which helps in denaturing proteins by breaking the disulfide bonds between the cysteine residues and for removing the tannins and polyphenols present in the crude extract, a chelating agent such as EDTA which chelates the magnesium ions required for DNase activity , a buffer which is almost always Tris at pH 8 and a salt such as sodium chloride which aids in precipitation by neutralizing the negative charges on the DNA so that the molecules can come together. Phenol –chloroform extraction helps to extract the nucleic acid solution by successively washing with a volume of phenol(pH 8.0); a volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) and chloroform: isoamyl alcohol ( 24:1). Precipitation of DNA is achieved by alcohol in the presence of monovalent salt, sodium acetate pH 5.2(final volume 0.3M), sodium chloride (final concentration 0.2M), ammonium acetate (2- 2.5M), lithium chloride (0.8M) and potassium chloride

**Materials required:**

CTAB, Tris Buffer pH 8.0, EDTA, NaCl, PVP, Ascorbic acid, mercaptomethanol 20% (w/v) sodium dodecyl sulphate (SDS),5 M potassium acetate (Stored at -20°C), 3 M sodium acetate (pH 5.2), 70% ethanol (stored at -20°C), Absolute isopropanol (stored at -20°C)

**Procedure:**

1. Weigh out 0.3 g of plant tissue
2. Place tissue on a clean glass slide. Chop the tissue into a paste using a clean single edge razor blade.
3. Immediately transfer tissue to a 1.5 mL microcentrifuge tube Once the sample is prepared, add 300  $\mu$ L EBA, 900 $\mu$ L EBB, and 100  $\mu$ L SDS.
4. Vortex and incubate at 65° C for 10 min. 6. Place the tube on ice and add 410  $\mu$ L cold potassium acetate. Mix by inversion and place the tube back on ice for 3 min.
5. Centrifuge at 13,200 rpm for 15 min at 4°C.
6. Transfer 1 mL of the supernatant to a new 1.5 mL microcentrifuge tube, add 540  $\mu$ L of ice-cold absolute isopropanol and incubate in ice for 20 min.
7. Centrifuge at 10,200 rpm for 10 min. Discard the supernatant. Wash the pellet once in 500  $\mu$ L 70% ethanol and let dry.
8. Resuspend the dry pellet in 600  $\mu$ L of TE. Add 60  $\mu$ L 3M sodium acetate (pH 5.2) and 360  $\mu$ L ice-cold absolute isopropanol. Incubate on ice for 20 min.

9. Repeat Steps 7–8 twice.
10. Resuspend the pellet in 50 µL TE and verify the presence of DNA using 0.8% agarose gel.

**Result:**

DNA was visualized as orange bands in 0.8% agarose stained with ethidium bromide.

**Reagents:**

<b><u>Extraction Buffer A (EBA)</u></b>	<b><u>Per 100 mL</u></b>
2% (w/v) hexadecyltrimethylammonium bromide (CTAB)	2.0 g
100 mM Tris (pH 8.0) (Use 1 M stock)	10 mL
20 mM EDTA (Use 0.5 M stock)	1 mL
1.4 M NaCl	8.2 g
4% (w/v) polyvinylpyrrolidone (PVP)	4.0 g
0.1% (w/v) ascorbic acid	0.1 g
10 mM β-mercaptoethanol (BME)* (Use 14.3 M stock)	70 µL

<b><u>Extraction Buffer B (EBB)</u></b>	<b><u>Per 100 mL</u></b>
100 mM Tris-HCl (pH 8.0) (Use 1 M stock)	10 mL
50 mM EDTA (Use 0.5 M stock)	2.5 mL
100 mM NaCl	0.6 g
10 mM β-mercaptoethanol (BME)* (Use 14.3 M stock)	70 µL

<b><u>TE Buffer</u></b>	<b><u>Per 100 mL</u></b>
10 mM Tris (pH 8.0) (Use 1 M stock)	1.0 mL
1 mM EDTA (Use 0.5 M stock)	50 µL

X-----X-----X-----X

## 5. ISOLATION OF PLASMID DNA

**Aim:** To isolate plasmid DNA

**Principle:** Purification of plasmid DNA from bacterial DNA using is based on the differential denaturation of chromosomal and plasmid DNA using alkaline lysis in order to separate the two. The basic steps of plasmid isolation are disruption of the cellular structure to create a lysate, separation of the plasmid from the chromosomal DNA, cell debris and other insoluble material. P1 buffer contains glucose, Tris, and EDTA. Glucose provides osmotic shock leading to the disruption of the cell membrane, Tris is a buffering agent used to maintain a constant pH8. The plasmid can be protected from endogenous nucleases by chelating Mg<sup>2+</sup> ions using EDTA. Mg<sup>2+</sup> ion is considered as a necessary cofactor for most nucleases. P2 buffer contains NaOH and SDS and this alkaline solution is used to disrupt the cell membrane and NaOH also denatures the DNA into single strands. P3 buffer contains acetic acid to neutralize the pH and potassium acetate to precipitate the chromosomal DNA, proteins, along with the cellular debris. Phenol /chloroform is used to denature and separate proteins from the plasmid. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. Once the plasmid DNA is released, it must be precipitated in alcohol. The plasmid DNA in the aqueous phase is precipitated with cold (0°C) ethanol or isopropanol. The precipitate is usually redissolved in the buffer and treated with phenol or organic solvent to remove the last traces of protein, followed by reprecipitation with cold ethanol.

**Materials required:**

Glucose, EDTA, Tris Buffer, Sodium hydroxide, SDS, Potassium acetate, Glacial acetic acid, ethanol

**Procedure:**

1. Grow bacterial (*E. coli*) culture in LB medium with appropriate antibiotics at 37 °C overnight with shaking. For >10 copies plasmid, 3 ml cell culture is usually enough.
2. Transfer overnight culture to a 1.5-ml eppendorf tube, and spin down cell culture (twice) at high speed for 1 min at table-top centrifuge.
3. Discard the supernatant. To remove the liquid completely by upside-down tube onto a piece of paper towel for a few seconds.
4. Add 100 µl of resuspension solution (P1 buffer) into each tube, and vortex to completely resuspend the cell pellet.
5. Add 100 µl of lysis solution (P2 buffer) and mix by gently inverting the tube 5-6 times. The solution should quickly turn transparent and become more viscous indicating bacterial lysis has taken place.
6. Add 150 µl of neutralizing solution (P3 buffer) and mix by inverting the tubes several times. At this point bacterial chromosomal DNA is usually seen as a white precipitate.
7. Centrifuge the tubes at high speed for 10 min. Carefully transfer the supernatant (try to not

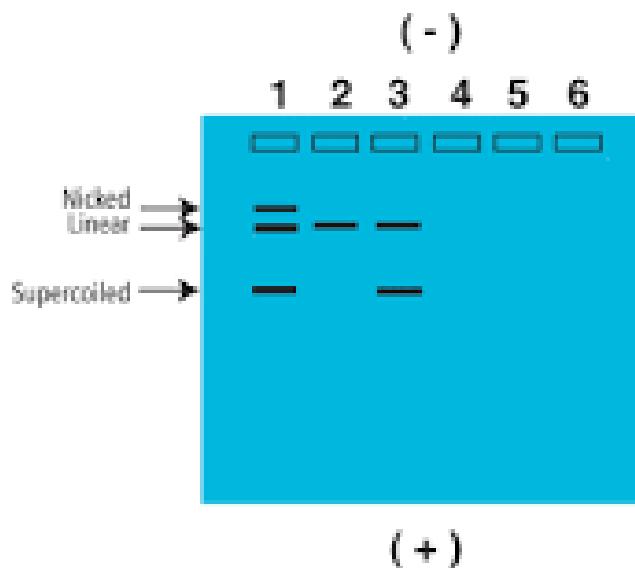
- disturb the white precipitate) to a new labeled 1.5-ml eppendorf tube with a 1 ml pipette.
8. Add 2.5-3 volume of 200-proof cold ethanol (stores at -20 °C) to each tube and mix by inverting the tubes a few times.
  9. Spin down plasmid DNA precipitate (transparency pellet) at high speed for 10 min.
  10. Discard the supernatant and remove the remaining liquid as much as possible by leaving the tube upside-down on a piece of paper towel, then keep the tubes in a tube holder and air dry for 10-20 min. To dry faster, keep tubes at 37 °C heat blocker. DNA precipitate turns white when dry.
  11. Resuspend the DNA pellet with 50 µl TE. Completely dissolve the pellet by pipetting the solution several times.

### **Result:**

Multiple bands of plasmid DNA was observed on 1% agarose gel visualized using ethidium bromide.

### **Discussion:**

Plasmid DNA can exist in three conformations namely, the supercoiled, Open-circular and linear DNA. Upon purification of plasmid DNA and verification of the purified plasmid DNA on agarose gel, the lowest band observed will be super coiled as it suffers the least friction, followed by linear DNA and lastly the open circular DNA. More often, the linear conformation is not found in all purification.



- Lane 1: Uncut Plasmid DNA
- Lane 2: DNA cut with Restriction Enzyme 1
- Lane 3: DNA cut with Restriction Enzymes 1 & 2

## **Reagents**

### **1. Resuspension solution (P1 buffer)**

50 mM glucose

10 mM EDTA

25 mM Tris (pH 8.0)

Store at 4 °C

### **2. Lysis solution (P2 buffer)**

0.2 N NaOH

1% SDS

Store at room temperature

### **3. Neutralizing solution (P3 buffer)**

3 M Potassium acetate (pH 6.0)

For 100 ml solution, 60 ml 5 M potassium acetate (49.07 g potassium acetate in 100 ml H<sub>2</sub>O)

11.5 ml glacial acetate and 28.5 ml H<sub>2</sub>O, store at room temperature.

### **4. TE**

1 mM EDTA

10 mM Tris-HCl (pH 8.0)

X-----X-----X-----X

## 6. SPECTROPHOTOMETRIC DETERMINATION OF NUCLEIC ACID (DNA) PURITY AND CONCENTRATION

**Aim:**

To determine the purity and concentration of the given DNA sample.

**Principle:**

The most common technique to determine DNA yield and purity is measurement of absorbance. UV absorption is a property of the bases, and each base absorbs differently. The actual peak absorbance of a particular DNA, then, depends on its base composition. Single-stranded DNA absorbs more UV than double stranded DNA. This is due to interactions between the stacked bases in double-stranded DNA. The difference can be also demonstrated directly by comparing the OD's of double-stranded DNA and DNA that has been denatured by boiling. The change in OD is referred to as the hyperchromic shift. However, DNA is not the only molecule that can absorb UV light at 260nm. Since RNA also has a great absorbance at 260nm, and the aromatic amino acids present in protein absorb at 280nm, both contaminants, if present in the DNA solution, will contribute to the total measurement at 260nm.

In order to check the purity of DNA ratio of the absorbance  $A_{260}/A_{280}$  has to be measured. Good quality DNA will have an  $A_{260}/A_{280}$  ratio of 1.7–2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present.

The DNA concentration can be determined by the following equation:

$$1 \text{ OD}_{260} \text{ unit} = 50 \mu\text{g/ml}$$

DNA concentration is estimated by measuring the absorbance at 260nm, adjusting the  $A_{260}$  measurement for turbidity (measured by absorbance at 320nm), multiplying by the dilution factor, and using the relationship that an  $A_{260}$  of 1.0 = 50 $\mu\text{g/ml}$  pure dsDNA.

$$\text{Concentration } (\mu\text{g/ml}) = (A_{260} \text{ reading} - A_{320} \text{ reading}) \times \text{dilution factor} \times 50\mu\text{g/ml}$$

**Materials Required:**

DNA, TE Buffer, Micropipettes, UV-Spectrophotometer, Quartz Cuvettes.

**Procedure:**

1. DNA sample was taken (10  $\mu\text{l}$ ) in TE buffer.
2. The sample was diluted by the factor of 100 *i.e.*, by taking 10 $\mu\text{l}$  of the sample in 990 $\mu\text{l}$  of TE buffer.
3. The optical density value was measured at  $A_{260}$ ,  $A_{280}$  and  $A_{320}$  for both purity and

concentration respectively.

4. The Purity and Concentration of the DNA was calculated using the following formula.

**Calculations:**

**Purity**

$$\text{DNA purity} = (A_{260} / A_{320})$$

**Concentration**

$$\text{Concentration } (\mu\text{g/ml}) = (A_{260} \text{ reading} - A_{320} \text{ reading}) \times \text{dilution factor} \times 50\mu\text{g/ml}$$

**Results:**

The purity and concentration of the given DNA sample was determined spectrophotometrically.

X-----X-----X-----X

## 7. QUALITATIVE ANALYSIS OF DNA

**Aim:** To separate and visualize DNA bands by Agarose gel electrophoresis.

**Introduction:** Agarose gel electrophoresis is a powerful and widely used method that separates molecules on the basis of electrical charge, size, and shape. The method is particularly useful in separating charged biologically important molecules such as DNA (deoxyribonucleic acids), RNA (ribonucleic acids), and proteins. Agarose forms a gel like consistency when boiled and cooled in a suitable buffer.

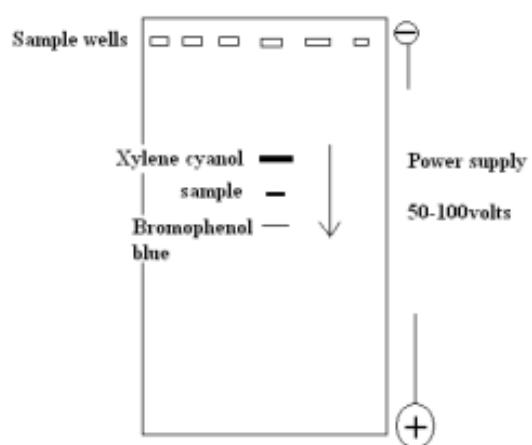
**Principle:** The agarose gel contains molecule-sized pores, acting like molecular sieves. The pores in the gel control the speed that molecules can move. DNA molecules possess a negative charge in their backbone structure due to the presence of  $\text{PO}_4^{4-}$  groups thus this principle is exploited for its separation. Smaller molecules move through the pores more easily than larger ones. Conditions of charge, size, and shape interact with one another depending on the structure and composition of the molecules, buffer conditions, gel thickness, and voltage. Agarose gels are made with between 0.7% (provides good resolution of large 5–10 kb DNA fragments) and 2% (good resolution for small 0.2–1 kb fragments).

The gel setup provides wells for loading DNA into it. The loaded DNA molecules move towards the positively charged electrode (anode) and get separated along the length of the gel. Ethidium bromide (EtBr), a chromogen is added to the gel to visualize the separated DNA under UV transillumination. EtBr intercalates between the bases and glows when UV radiation is passed through the gel.

### Purpose of gel loading buffer

The loading buffer gives colour and density to the sample to make it easy to load into the wells. Also, the dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis. This allows you to monitor the progress of the gel. The gel loading dye possesses bromophenol blue and xylene cyanol. Density is provided by glycerol or sucrose.

Xylene cyanol gives a greenish blue colour while bromophenol blue provides bluish coloured zone. The successful DNA run is determined by the presence of both the coloured dye in the gel.



### Materials Required:

- Electrophoresis buffer: 1x TAE buffer
- Agarose ultra-pure (DNA graded)
- Electrophoresis tank, gel tray, sample comb

- and power supply
- Plastic or insulation tape
  - Ethidium bromide: 10 mg /ml stock solution
  - 5x Gel loading dye
  - DNA marker solution, DNA sample and gloves.

PROCEDURE:

**1. Making a 1% Agarose Gel**

- 0.5 g of agarose was weighed and dissolved in 50 mL of 1x TAE Buffer. (Note: Use 250 ml conical flask for preparing 50 ml solution to avoid overflow of gel solution while heating and to avoid its loss.)
- The solution was heated over a hot plate for boiling constituency.
- The solution was allowed to cool and 2 $\mu$ l of EtBr solution was added to it, mixed it well by gentle swirling.
- The solution was poured on the gel tray-comb set up. (Also be sure the gel plates have been taped securely and contain the well combs prior to pouring)
- The solution was allowed to cool and hardened to form a gel.

**2. Loading of Samples**

- The gel was carefully transferred to the electrophoresis tank filled with 1x TAE buffer.
- Samples were prepared [8  $\mu$ l of DNA sample (0. 1 ug to 1 ug) and 2  $\mu$ l of 5x gel loading dye]
- The comb was removed and the samples were loaded into the well.
- Appropriate electrodes were connected to the power pack and operated at 50- 100volts for 20 minutes.
- The progress of the gel was monitored with reference to the tracking dye (Bromophenol blue). And the operation was when the marker has run 3/4<sup>th</sup> of the gel.

**3. Examining the gel**

- The gel was placed on the UV-transilluminator and checked for the orange coloured bands.

**Results:**

DNA was observed as orange coloured bands when visualized under an UV-transilluminator

X-----X-----X-----X

## 8. SDS-PAGE

**Aim:** To separate the protein sample using SDS PAGE

**Principle:** Electrophoresis is a method used to separate charged particles from one another based on differences in their migration speed. Polyacrylamide gel electrophoresis (PAGE) is a method used for the separation of proteins. The presence of SDS makes the proteins lose their secondary structures and attain net negative charge. Thus the proteins separate now on the basis of molecular weight under electric current. In case of PAGE gels, they are divided into two layers: the upper one is a macroporous gel (4-5%) with low concentration, called stacking gel with a pH 6.7; the lower one is composed of higher concentrations (6-18%), called separating gel or electrophoresis gel with a pH 8.9. Electrode buffer in the electrophoresis tank is Tris-glycine, pH 8.3. Once electrophoresis unit is supplied with power, glycine, proteins, chloride ions and bromophenol in HCl would be dissociated into anion, forming an ion flow and moving to the anode. Its mobility depends on the number of electric charges of the ion, molecular size, and shape. However, when the glycine ions of electrophoresis buffer (pH 8.3) entered into the stacking gel and encountered lower pH (6.7), which lowered down by nearly two units, almost close to the isoelectric point (5.97) of glycine, the dissociation degree of glycine suddenly drop, the amount of charge reduced significantly and then the mobility became slower. The protein sample entering into the stacking gel also gets affected by the pH change, but the impact is much smaller than on glycine. Thus it has larger mobility than glycine. Thus the proteins get stacked between the fact moving chloride ions and slower-moving glycine ions thereby causing the stacking effect. Once the proteins enter the resolving gels prepared pH 8.9 buffer, the protein molecule encountered resistance. At pH 8.9, glycine would fully dissociate and its mobility increases faster than that of proteins. Proteins begin to migrate at different rates, because of the sieving properties of the gel. Smaller protein-SDS complexes migrate more quickly than larger protein SDS complexes. Within a certain range determined by the porosity of the gel, the migration rate of a protein in the running gel is inversely proportional to the logarithm of its molecular weight.

**Materials Required:**

Acrylamide, Bisacrylamide, 10% SDS, Tris, 10% Ammonium sulphate, TEMED, Glycine, 5X Sample Buffer, Commassive staining solution

**Procedure:**

**1. Preparation of separating gel (resolving gel):**

- Set the casting frames (clamp two glass plates in the casting frames) on the casting stands.
- Prepare the gel solution as described in a separate small beaker.
- Swirl the solution gently but thoroughly.
- Pipet appropriate amount of separating gel solution into the gap between the glass

plates.

- To make the top of the separating gel horizontal, fill in water or isopropanol into the gap until a overflow.
- Wait for 20-30min to let it gelate.

Acrylamide percentage	6%	8%	10%	12%	15%
H <sub>2</sub> O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

## 2. Prepare the stacking gel:

- Discard the water and dry using a filter paper.
- Prepare the stacking gel components
- Pipet in stacking gel untill a overflow.
- Insert the well-forming comb without trapping air under the teeth. Wait for 20-30min to let it gelate.

H <sub>2</sub> O	2.975 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (AP)	0.05 ml
TEMED	0.005 ml

3. Make sure a complete gelation of the stacking gel and take out the comb. Take the glass plates out of the casting frame and set them in the cell buffer dam. Pour the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow until the buffer surface reaches the required level in the outer chamber.

## 4. Prepare the samples:

Mix your samples with sample buffer (loading buffer).

Heat them in boiling water for 5-10 min. Cool and keep in ice

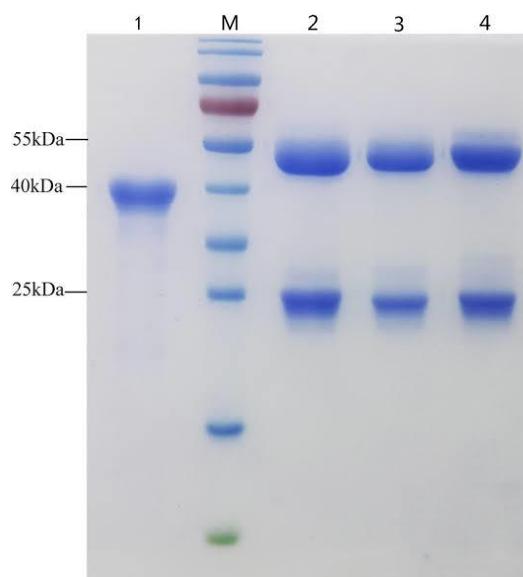
5. Load prepared samples into wells along with protein marker into the first lane. Then

cover the top and connect the anodes.

6. Run the PAGE at constant voltage of 80 till the sample enters then separating gel and shift it to 100V. Run the gel until the dye front reaches the bottom of the plate.
7. Take out the gel from the apparatus, remove the glass plates. Cut the stacking gel out and put the separating gel in a tray containing fixing solution for 1 hr. Stain gel in Staining solution for 20 min with gentle agitation. Destain gel in Destaining solution. Replenish the solution several times until background of the gel is fully destained.

### **Result:**

The protein was separated and visualized as blue bands after running SDS PAGE.



### **Reagents:**

**1. 30% acrylamide-bisacrylamide:** weigh 29g acrylamide, 1g N, N – methylene bis-acrylamide. Add 60 ml warmed deionized water and heat to 37 °C. Add deionized water to make a final volume of 100ml; filter. Store at 4°C

**2. 10% sodium dodecyl sulfate (SDS)**

**3. Stacking gel buffer (1mol / L Tris-HCl pH 6.8):** dissolve 12.12g Tris in 80ml deionized water. Adjust the pH to 6.8 with concentrated hydrochloric acid; add deionized water to 100ml and store at 4°C.

**4. Resolving gel buffer (1.5mol / L Tris-HCl pH 8.8):** dissolve 18.16g Tris in 80ml deionized water; adjust the pH to 8.8 with concentrated hydrochloric acid; add deionized

water to 100ml; store at 4 °C.

### 5. 10% ammonium persulfate (APS)

### 6. TEMED (N, N, N, N – tetramethylethylenediamine

7. **Tris-glycine electrophoresis buffer:** weigh 15.1g Tris and 94g glycine; Dissolve in 900ml deionized water; then add 50ml 10% (w/v) SDS and deionized water to 1000ml. Dilute 5-fold when using. The final concentration would be: Tris, 25mmol/L; glycine, 250mmol/L; SDS, 0.1% and the pH of the buffer is 8.3.

### 8. 5X SDS sample buffer

#### 5X SDS sample buffer composition (10ml) -

1. 0.5 M Tris-HCl pH 8.0	4.0ml
2. 20% SDS	2.5ml
3. DTT	386.0mg
4. Glycerol	2.5ml
5. BPB	Pinch
6. dH <sub>2</sub> O	1.0ml

Store this buffer at 4°C.

### 9. Staining solutions

- Fixing solution (50% methanol and 10% glacial acetic acid)
- Staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid)
- Destaining solution (40% methanol and 10% glacial acetic acid) Storage solution (5% glacial acetic acid)

X-----X-----X-----X

## 9. ESTIMATION OF DNA BY DIPHENYLAMINE METHOD

**Aim:** To estimate the concentration of DNA by diphenylamine reaction.

**Principle:** This is a general reaction given by deoxypentoses. The 2-deoxyribose of DNA, in the presence of an acid, is converted to  $\omega$ -hydroxylevulinic aldehyde, which reacts with diphenylamine to form a blue colored complex, which can be read at 595 nm.

**Requirements:**

1. Standard DNA solution- Dissolve calf thymus DNA (200 $\mu$ g/ml) in 1N perchloric acid/buffered saline.
2. Diphenylamine solution- Dissolve 1g of diphenylamine in 100 ml of glacial acetic acid and 2.5 ml of concentrated H<sub>2</sub>SO<sub>4</sub>. This solution must be prepared fresh
3. Buffered Saline- 0.5 mol/litre NaCl; 0.015 mol/litre sodium citrate, pH 7.

**Procedure:**

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labeled test tubes.
2. Pipette out 1 ml of the given sample in another test tube.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as the blank.
4. Now add 2 ml of DPA reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and incubate on a boiling water bath for 10 min.
6. Then cool the contents and record the absorbance at 595 nm against blank.
7. Then plot the standard curve by taking concentration of DNA along X-axis and absorbance at 595 nm along Y-axis.
8. Then from this standard curve calculate the concentration of DNA in the given sample.

**Result:** The given unknown sample contains ---- $\mu$ g DNA/ml.

Vol of STD DNA(ml)	Vol of Water(ml)	Conc of DNA(µg)	Vol of DPA		OD 595nm
0	1	0			
0.2	0.8	40			
4	0.6	80			
0.6	0.4	120			
0.8	0.2	160			
1	0	200			
Unknown	upto 1				

X-----X-----X-----X

## 10. ESTIMATION OF RNA BY ORCINOL REACTION

**Aim:** To estimate the concentration of RNA by orcinol reaction.

**Principle:** This is a general reaction for pentoses and depends on the formation of furfural when the pentose is heated with concentrated hydrochloric acid. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green colour, which can be measured at 665 nm.

**Requirements:**

1. Standard RNA solution- 200 $\mu$ g/ml in 1 N perchloric acid/buffered saline.
2. Orcinol Reagent- Dissolve 0.1g of ferric chloride in 100 ml of concentrated HCl and add 3.5 ml of 6% w/v orcinol in alcohol.
3. Buffered Saline- 0.5 mol/litre NaCl; 0.015 mol/litre sodium citrate, pH 7.

**Procedure:**

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard into the series of labeled test tubes.
2. Pipette out 1 ml of the given sample in another test tube.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as the blank.
4. Now add 2 ml of orcinol reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and heat on a boiling water bath for 20 min.
6. Then cool the contents and record the absorbance at 665 nm against blank.
7. Then plot the standard curve by taking concentration of RNA along X-axis and absorbance at 665 nm along Y-axis.
8. Then from this standard curve calculate the concentration of RNA in the given sample.

**Result:** The given unknown sample contains ---- $\mu$ g RNA/ml.

Vol of STD RNA(ml)	Vol of Water(ml)	Conc of RNA(µg)	Vol of Orcinol		OD 665nm
0	1	0	2 ml	Incubate in waterbath for 20 mins	
0.2	0.8	40			
4	0.6	80			
0.6	0.4	120			
0.8	0.2	160			
1	0	200			
Unknown	upto 1				

X-----X-----X-----X

## 11. ISOLATION OF TOTAL RNA

**Aim:** To isolate total RNA from bacteria/yeast

**Principle:** TRIZol (or TRI Reagent) is a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilizes biological material and denatures protein. After solubilization, the addition of chloroform causes phase separation (much like extraction with phenol:chloroform: isoamyl alcohol), where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase. Therefore, RNA, DNA, and protein can be purified from a single sample (hence, the name TRIZol). TRIZol extraction is also an effective method for isolating small RNAs, such as microRNAs, piwi-associated RNAs, or endogenous, small interfering RNAs.

**Materials required:** Nuclease free water, TRIZol Reagent, Ice cold PBS, 70% ethanol, Isopropyl alcohol, IX TBE buffer.

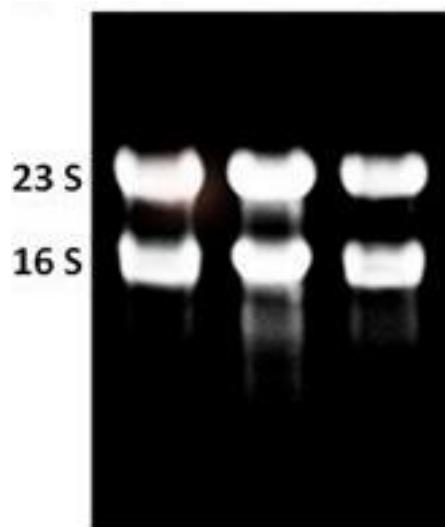
**Protocol:**

1. Centrifuge bacterial/yeast cells for 5 min at 5000rpm. Remove media and resuspend cells in ice-cold PBS. Pellet cells by spinning at 5000rpm for 5 min.
2. Add 1 ml of TRIZOL reagent and mix thoroughly, incubate the sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Centrifuge to remove cell debris. Transfer the supernatant to a new tube.
3. Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 x g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.
4. Transfer the upper aqueous phase carefully without disturbing the interphase into fresh tube.
5. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization.
6. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at not more than 12,000 x g for 10 minutes at 2 to 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.
7. Remove the supernatant completely. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the samples by vortexing and centrifuge at no more than 7,500 x g for 5 minutes at 2 to 8 °C. Repeat the above washing procedure once. Remove all leftover ethanol.
8. Air-dry or vacuum dry RNA pellet for 5-10 minutes. Do not dry the RNA pellet by centrifuge under vacuum. It is important not to let the RNA pellet dry completely as

- this will greatly decrease its solubility.
9. Dissolve RNA in nuclease-free water bypassing solution a few times through a pipette tip.
  10. Visualize the RNA by running on 2% agarose gel prepared in 0.5X TBE buffer.

**Result:**

Two bands of RNA was observed in 2% agarose gel stained with the Ethidium bromide. The bands represent the 23S and 16S rRNA. These are observed as they are the most abundant RNA in a cell.



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**Reagents**

1. 5X stock solution in 1 L of H<sub>2</sub>O:  
54 g of Tris base  
27.5 g of boric acid  
20 mL of 0.5 M EDTA (pH 8.0)

For preparation of agarose gel and for running buffer dilute 5X to 0.5X concentration.

X-----X-----X-----X

## 12. RESTRICTION DIGESTION OF LAMBDA DNA

### **AIM**

To perform restriction digestion of Lambda DNA using EcoRI and HindIII enzymes

### **PRINCIPLE**

Restriction involves fragmenting DNA molecules into smaller pieces with special enzymes called **Restriction Endonucleases** which are commonly known as **restriction enzymes**(RE). Because of this property the restriction enzymes are also known as molecular scissors. Restriction enzymes recognize specific sequences in double-stranded DNA and then cut the DNA to produce fragments, called restriction fragments. The target site or sequence which the restriction enzyme recognizes is generally from 4 to 6 base pairs and arranged in a palindromic sequence. Once it is located, the enzyme will attach to the DNA molecule and cut each strand of the double helix. The restriction will continue to do this along the full length of the DNA molecule which will then break into fragments. The size of these fragments is measured in base pairs or Kilobase pairs (1000 bases).

Every restriction enzyme has unique target sites for digestion. Lambda DNA has multiple restriction sites for both EcoRI and HindIII which results in several fragments of varying sizes.

### **MATERIALS REQUIRED**

Glasswares: measuring cylinder, beaker

Reagents: ethidium bromide(10mg/mL)

Electrophoresis apparatus, UV Transilluminator, Water bath, Vortex mixer, Micropipettes & tips, Adhesive tape, Crushed ice, Microwave

Lambda DNA, EcoRI, HindIII, buffer for restriction enzyme, nuclease-free water.

### **PROCEDURE**

1. The vials were placed on crushed ice
2. 5.0  $\mu$ I Lambda DNA, 2.5 $\mu$ I 10 X assay buffer, 16.5 $\mu$ I milli Q water, and 1.0  $\mu$ I E.coRI (or HindIII) were added to this prechilled vials
3. After preparing the reaction tubes, the contents were mixed by gentle pipetting and tapping
4. The tubes were incubated at 37°C for 1 hour
5. After 1 hour incubation, the vials were immediately placed at room temperature (15-25°C) for 10 minutes
6. Load 10 $\mu$ l of the sample on 1% agarose gel.

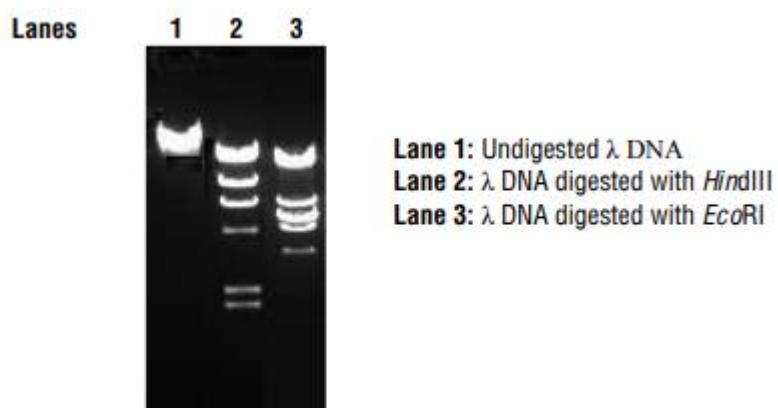
**Result:**

Multiple bands were observed in restriction digested samples of lambda DNA when visualized on a UV transilluminator.

**INTERPRETATION**

Restriction digestion pattern of lambda DNA obtained upon treatment with EcoRI and HindIII were markedly different which demonstrates the fact that each restriction enzyme recognizes and cleaves only a specific base sequence unique to it.

The size of the fragments can be determined by comparing with that of the DNA marker ran on the same gel.



**Fig 1:  $\lambda$  DNA digested with EcoRI & HindIII**

**Agarose gel Electrophoresis****Preparation of 1X TAE:**

To prepare 500 mL of 1X TAE buffer, add 10 mL of 50X buffer to 490 ml of sterile distilled water, mix well before use

**Preparation of agarose gel:**

To prepare 50 mL of 1% agarose gel, measure 0.5g agarose in a glass beaker and add 50 mL 1X TAE buffer.

Heat the mixture on a microwave, swirling the glass beaker occasionally until agarose dissolves completely

Allow the solution to cool to about 50-60°C. Add 0.5  $\mu$ l ethidium bromide , mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 minutes at room temperature

**Electrophoresis:**

Electrophoresed at 100-120 V and 90 mA until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized

X-----X-----X-----X

## 1. TRANSFORMATION

### **AIM:**

To prepare competent cells and transform plasmid DNA.

### **INTRODUCTION:**

Bacterial transformation is a process that involves the genetic alteration of bacteria by incorporation and stable expression and stable expression of foreign genetic material from the environment or surrounding medium. Since DNA is a very hydrophobic molecule it will not normally pass through a bacterial cell membrane. In order to uptake foreign DNA, the bacterial cell must first be made competent. Competence is the ability of a cell to take up extracellular DNA from its environment. There are different methods of carrying out transformation, example: chemical transformation, electroporation, gene gun, liposome-mediated transfer, and microinjection. Chemical transformation include the usage of calcium chloride. This mode of transformation is easy to perform and requires a minimum number of equipment.

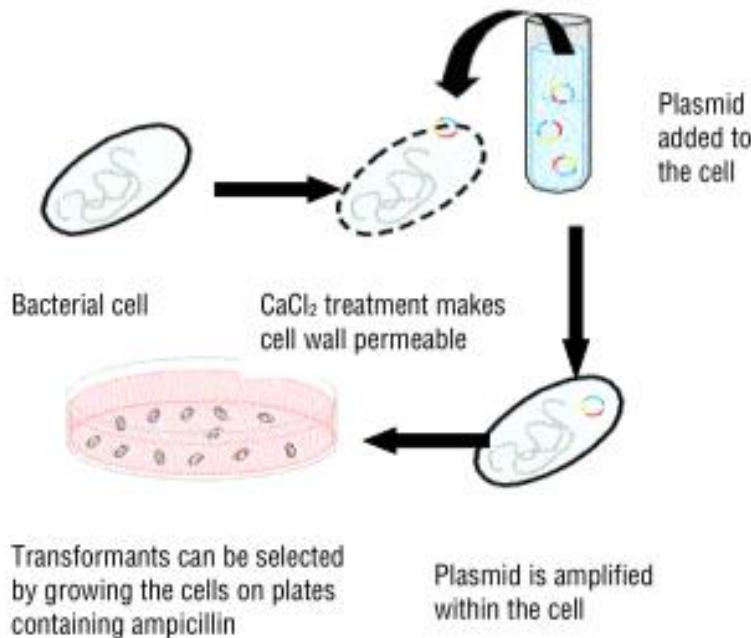
### **PRINCIPLE:**

For the incorporation of the plasmid into a cell, bacteria must first be made "competent". This process includes the treatment of cells with bivalent calcium ion in the ice-cold condition. As a result small pores are formed on the cell membrane, which makes it permeable. The plasmid DNA may adhere to the surface of the cell and uptake is mediated by a pulsed heat shock at 42 degrees Celsius. A rapid chilling step on ice ensures the closure of the pores. The cell is allowed to propagate and selection of transformants can be done by growing the cells on a selective media which will allow only the plasmid containing cells to grow.

Plasmids are extrachromosomal DNA elements capable of independent replication inside a suitable host. Plasmids encode a wide variety of genes, including those required for antimicrobial resistance. These genes act as a selective marker when a transformation experiment is carried out.

The *E. coli* plasmid pUC19 encodes a gene that can be used as a selectable marker during a transformation experiment. pUC19 has an ampicillin resistance marker that enables only transformed cells to grow on LB-ampicillin plates. Transformants, thus having the ability to grow on ampicillin plates can be selected. This process of direct selection recombinants is called insertion-inactivation. Puc19 also carries the N-terminal coding sequence for beta-galactosidase of the lac operon. The *E. coli* host strain has a deletion at the amino-terminal end of the LacZ gene, which codes for beta-galactosidase. When pUC19 is transformed into the competent host cells, the truncated products from both complement each other and as a result enzymatically active beta-galactosidase is produced. This is called alpha complementation. The transformants turn blue on X-gal and IPTG containing plates due to the production of beta-galactosidase. X-gal is the chromogenic substrate of beta-

galactosidase and IPTG acts as the inducer for the expression of these enzymes.



**1: The process of bacterial transformation includes treatment of cells with  $\text{CaCl}_2$ , which makes cells permeable, and plasmid DNA can enter the cell.**

### **MATERIALS REQUIRED:**

- Glasswares: conical flask.
- Other requirements: micropipettes, tips, 50ml centrifuge tubes, water bath ( $42^\circ\text{C}$ ),  $37^\circ\text{C}$  shaker, centrifuge, UV transilluminator, crushed ice, sterile double distilled water, sterile loop, and spreader.

### **REAGENTS AND MEDIA:**

- Ampicillin
- $\text{CaCl}_2$
- Host(*E.coli*)
- IPTG
- Plasmid DNA
- X-gal
- LB broth
- Agar plates

### **PROCEDURE:**

#### DAY1

- Inoculate a single colony from the revived E coli host cell (DH5 $\alpha$ ) plate in 1ml LB broth.
- Incubate at 37 °C overnight.

#### DAY2

- Take 50ml LB broth in a sterile flask. Transfer 1ml of overnight grown culture into this flask.
- Incubate at 37 °C shaker at 300 rpm for 3-4 hours till the O D reaches -0.6.

#### PREPARATION OF COMPETENT CELLS:

- Transfer the above culture into a prechilled 50ml polypropylene tube.
- Allow the culture to cool down to 4 °C by storing on ice for 10minutes.
- Centrifuge at 5000 rpm for 10 minutes at 4 °C.
- Decant the medium completely. No traces of medium should be left.
- Resuspend the cell pellet in 30 ml prechilled sterile 0.1M CaCl<sub>2</sub> solution.
- Incubate on ice for 30 minutes.
- Centrifuge at 5000 rpm for 10 minutes at 4 °C.
- Decant the CaCl<sub>2</sub> solution completely. No trace of the solution should be left.
- Resuspend the pellet in 2ml prechilled sterile 0.1M CaCl<sub>2</sub> solution.
- This cell suspension contains competent cells and can be used for transformation.

#### TRANSFORMATION OF CELLS:

- Take 200ml of the above cell suspension in two 2.0 ml collection tubes and label them as “control” and “transformed”. Add 2 $\mu$ l of plasmid DNA to the tube labeled as transformed and mix well.
- Incubate both the tubes on ice for 30minutes.
- Transfer them to a preheated water bath set at a temperature of 42 °C for 2 minutes (heat shock).
- Rapidly transfer the tubes on ice bath.allow the cell to chill for 5minutes.
- Add 800ML of LB broth to both the tubes.incubate the tubes for 1 hour at 37 °C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
- The 4 LB agar plates containing ampicillin, X-gal, IPTG and label them as control,A,B and C. Plate 200 $\mu$ l of culture from the “control”tube and plate it on the corresponding plate with a sterile spreader. Plate 50  $\mu$ l, 100  $\mu$ l and 200  $\mu$ l of cell cultures from the ‘transformed’ to on the plates labelled as A,Band C respectively.
- Store at room temperature till the plates are dry.
- Incubate the plates overnight at 37 °C.

**RESULT:**

The presence of blue-coloured colonies on the list transformation culture plate clearly indicates the occurrence of transformation.

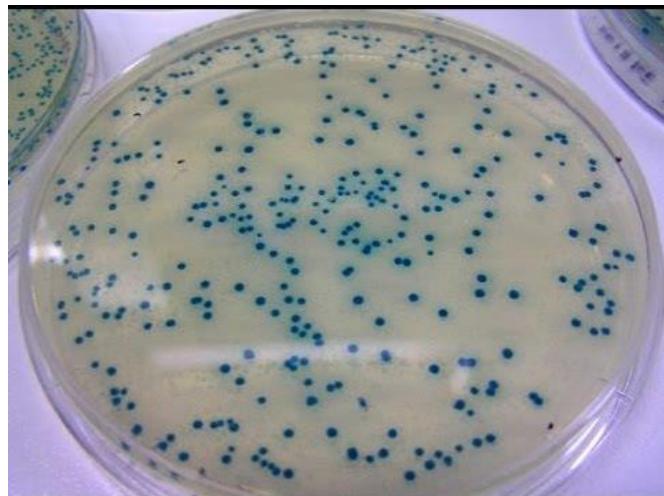


Figure showing Blue white colony selection for transformants

Sr. No.	Plate	Growth	Number of colonies	Transformation Efficiency
1	Control plate			
2	Transformed plate (A)			
3	Transformed plate (B)			
4	Transformed plate (C)			

Denote +ve when you observe bacterial growth, -ve when there is no growth

**Calculation of transformation efficiency:**

Transformation efficiency is defined as the number of cells transformed per microgram of supercoiled plasmid DNA in a transformation reaction.

**Transformation Efficiency** = Number of colonies  $\times$  1000 ng / Amount of DNA plated (ng) =      / $\mu$ g

**Calculation and preparation:**
**1)0.1M CaCl<sub>2</sub>(400ml)**

Take 40ml of 1M sterile CaCl<sub>2</sub> and add 360 ml of sterile distilled water. Store at 2-8°C.

**2)LB broth(55ml):**

Dissolve 1.38mg of LB media in 55ml of distilled water. Sterilize by autoclaving.

**3)LB agar plates(20ml):**

Dissolve 0.5g of LB media and 0.3g of agar in 20ml of sterile distilled water.

**4)Ampicillin:**

Dissolve 50mg of ampicillin powder in 1ml of sterile double distilled water to give a concentration of 50 mg/ml.

**5)LB agar plates containing ampicillin, X-gal, IPTG(100ml):**

Dissolve 2.5g of LB media and 1.5g of agar in 100ml of distilled water. sterilized by autoclaving and allow the media to cool down to 40-45°C. Add 100µl of ampicillin,200 µl of x-gal and 100 µl of IPTG to 100ml of autoclaved LB agar media mix well and pour on sterile petriplates.

X-----X-----X-----X

## 2. Reporter Gene Assay

**Aim:** To carry out qualitative analysis of lac Z gene.

**Principle:** *Escherichia coli* (*E. coli*) can produce the enzyme  $\beta$ -galactosidase which breaks lactose into galactose and glucose. However, the gene for  $\beta$ -galactosidase is normally switched off, except in the presence of lactose. The reporter gene assay is based on  $\beta$ -galactosidase, an enzyme from the bacterium *Escherichia coli*; this enzyme, when incubated with some specific colorless or non-fluorescent substrates, can transform them into coloured or fluorescent products. In this procedure, a sample of *E. coli* is treated with lactose, and then the  $\beta$ -galactosidase activity of this sample and an untreated sample are compared. ONPG (ortho-nitrophenyl- $\beta$ -D-galactoside) is used as a substrate for the enzyme action which produces galactose and ONP (ortho nitro phenol) a compound which is yellow in alkaline conditions. The intensity (or optical density) of the yellow colour produced is a qualitative indicator or quantitative measure (with a colorimeter) of the  $\beta$ -galactosidase activity. The procedure indicates that the gene which produces  $\beta$ -galactosidase in *E. coli* is induced or ‘switched on’ by the presence of lactose.

**Materials Required:**

*E. coli* strain with  $\beta$  galactosidase, test tubes, shaking incubator, spectrophotometer, LB medium with lactose, LB medium without lactose, ONPG, phosphate buffer, lysis buffer

**Procedure:**

1. LB medium (with and without lactose) were inoculated with two loopful of *E. coli* strain that was maintained on agar slant and incubated at 37°C overnight in shaking incubator.
2. Test tubes were labeled 1 to 4. To test tube 1, 2-5 ml of *E. coli* in LB broth that has not been induced with lactose was added; 2-5 ml of *E. coli* in LB broth that has been induced with lactose was added to the test tube 2.
3. Centrifuged at 5000 rpm for 5 min at 4°C.
4. To maintain a blank, 2 ml of lysis buffer alone was added to the test tube labeled as 3.
5. To the pellets in test tubes 1 and 2, 2 ml of lysis buffer was added and incubated for 1hour at 37°C.
6. Cellular debris was removed by centrifugation at 10,000 rpm for 10minutes at 4°C.
7. To all the test tubes, 2ml of ONPG was added and transferred to a water bath at 37°C for 10-minute intervals until there is no further change.
8. The presence of yellow colour in the test samples was measured at 420nm (or 440nm).

**Result:**

The intensity of yellow colour obtained after incubation of cell extract following cell lysis was determined. Cell extract from *E. coli* cells grown in LB medium containing lactose showed an intense yellow colour. This was mainly because of the induction of lac Z gene and the synthesis of  $\beta$ -galactosidase enzyme.  $\beta$ -galactosidase converted colourless substrate

ONPG to galactose and o-nitrophenol which gives an intense yellow colour.

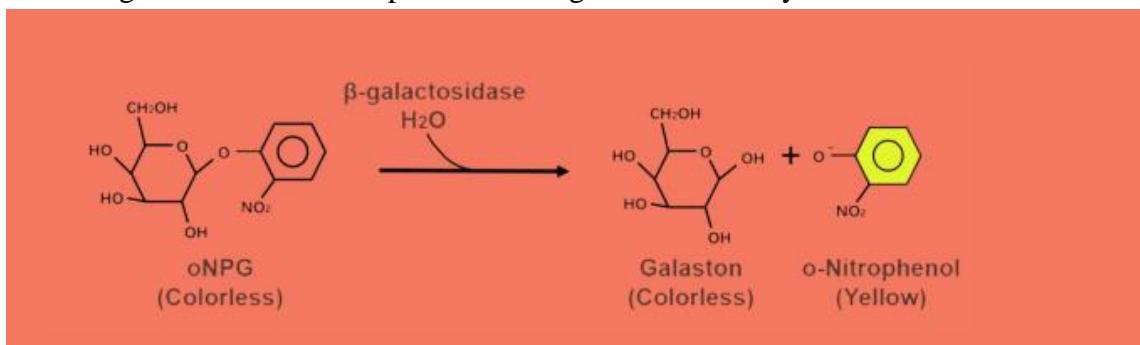


Figure showing the mechanism of O NPG conversion into coloured nitrophenol

## Reagent Composition

### 1. Composition of LB:

Tryptone -1 g  
 Yeast extract - 0.5 g  
 Sodium chloride- 1g  
 Distilled water – 100 ml

The different component of LB medium was added to 80 ml distilled water, pH was adjusted to 7.5 with 1.0 M sodium hydroxide. The volume is made up to 100ml with distilled water. LB broth of 50 ml was taken into two 250 ml flask. To one of the flask add lactose at a concentration of 0.01g/ml, label the two kinds of LB broth clearly ('with lactose' or 'without lactose') and sterilize by autoclaving.

**2. Phosphate buffer (pH7.0):** Weigh 1.60 g Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, 0.55 g NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O, 0.075g KCl and 0.012g MgSO<sub>4</sub> in 80 ml distilled water. Salts are then dissolved and pH was adjusted to 7.0 with 1 M or 2 M NaOH. The volume is made upto100 ml.

**3. Lysis buffer:** Lysis buffer is prepared by dissolving 1g of SDS and 300mM of sodium chloride in 50 ml of phosphate buffer. Add 0.01g of lysozyme to that.

**4. ONPG solution:** It is prepared freshly by adding 0.55g ONPG and dissolved in 100ml phosphate buffer and the bottle is wrapped in foil.

X-----X-----X-----X

### 3. POLYMERASE CHAIN REACTION (PCR)

**Aim:** To amplify a specific DNA fragment by Polymerase Chain Reaction.

**Introduction:** Polymerase Chain Reaction (PCR) is an in vitro method of enzymatic synthesis of specific DNA fragment developed by Kary Mullis in 1983. It is a very simple technique for characterizing, analyzing and synthesizing DNA from virtually any living organism (plant, animal, virus, bacteria). PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material called as template DNA.

A basic PCR requires the following components:

- DNA template that contains the region to be amplified
- Two primers complementary to the 3' ends of each of the sense and anti-sense strand of the DNA
- Thermostable DNA polymerase like Taq, Vent, Pfu etc.
- Deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), the building blocks from which the DNA polymerase synthesizes a new DNA strand.
- Buffer solution which provides a suitable chemical environment for optimal activity and stability of DNA polymerase.
- Bivalent magnesium/manganese ions, which are necessary for maximum Taq polymerase activity and influences the efficiency of primer to template annealing.

**Principle:** The purpose of a PCR is to amplify a specific DNA or RNA fragment. PCR comprises of basic steps:

**Initialization step:** This step consists of heating the reaction mixture to 94–96°C for 1–9 minutes to initiate the breaking of the hydrogen bonds in DNA strands.

**Denaturation step:** This step is the first regular cycling event and consists of heating the reaction mixture to 94–98° C for 20–30 seconds. As a result the template DNA denatures due to disruption of the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

**Annealing step:** In this step the reaction temperature is lowered to 50–65° C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is 3–5° C below the Tm (melting temperature) of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

**Extension/Elongation step:** In this step, the temperature depends on the DNA polymerase used. Taq polymerase has its optimum activity at 75–80° C. Commonly a temperature of 68–72° C is used with this enzyme. The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by incorporating dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand.

The extension time depends both upon the DNA polymerase used and on the length of the DNA fragment to be amplified. The DNA polymerase will polymerize a thousand bases per minute at its optimum temperature. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential amplification of the specific DNA fragment.

Final elongation: This single step is occasionally performed at a temperature of 70–74° C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. Denaturation, annealing and extension steps are repeated 20-30 times in an automated thermocycler that can heat and cool the reaction mixture in tubes within a very short time. This results in exponential accumulation of specific DNA fragments, ends of which are defined by 5' ends of the primers. The doubling of the number of DNA strands corresponding to the target sequences allows us to estimate the amplification associated with each cycle using the formula; Amplification =  $2^n$ , where n = No. of cycles.

Final hold: This step may be employed for short-term storage of the reaction mixture at 4°C for an indefinite time.

### **Materials Required:**

Glasswares: Measuring cylinder, Beaker  
Reagents: Ethidium bromide (10 mg/ml), Distilled water

Other requirements: Thermocycler, Electrophoresis apparatus, UV Transilluminator, Vortex Mixer, Micropipettes, Tips, Adhesive tape, Microwave/ Hotplate/ Burner, Crushed ice.

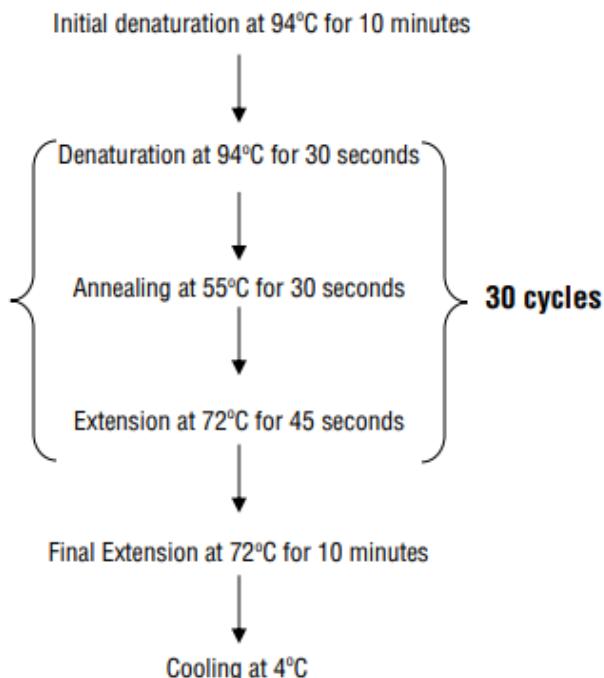
### **Procedure:**

- 1) Prepare a master mix in a PCR tube by adding the following components in order. Keep the PCR tube in ice at all times.

Sr. No.	Ingredients for PCR	Volume in $\mu$ l
1	Molecular Biology Grade Water	30.5 $\mu$ l
2	10X Assay Buffer	5 $\mu$ l
3	Template DNA	2 $\mu$ l
4	Forward Primer (10 nM)	1 $\mu$ l
5	Reverse Primer (10 nM)	1 $\mu$ l
6	25 mM MgCl <sub>2</sub>	5 $\mu$ l
7	2.5 mM dNTP Mix	5 $\mu$ l
8	Taq DNA Polymerase	0.5 $\mu$ l
	<b>Total volume</b>	<b>50 <math>\mu</math>l</b>

- 2) Tap the tube for 1–2 seconds to mix the contents thoroughly.

- 3) Add 25  $\mu$ l of mineral oil in the tube to avoid evaporation of the contents (optional).
- 4) Place the tube in the thermocycler block and set the program to get DNA amplification.



### Program for PCR

- 5) Run the PCR product in 1% agarose gel and visualize under UV transilluminator to observe DNA amplification.

#### **Result:**

DNA bands were observed in both control and test samples indicating DNA amplification by PCR.

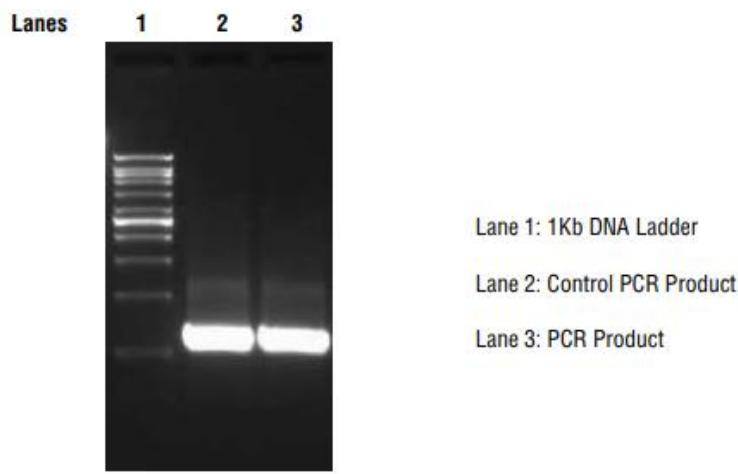


Fig 3: Gel image of PCR Product

X-----X-----X-----X

## 4.SOUTHERN BLOTTING

**Aim:** To learn the technique of Southern Blotting for the detection of a specific DNA fragment

**Introduction:** Southern blotting or Southern hybridization is a widely used technique in molecular biology for transfer of DNA molecules; usually restriction fragments, from an electrophoresis gel to a nitrocellulose or nylon membrane, and is carried out prior to detection of specific molecules by hybridization probing. In this method a DNA mixture is separated by agarose gel electrophoresis according to their size followed by transfer of the DNA bands to nitrocellulose/nylon membrane. Finally, the DNA of interest is probed for a specific sequence.

**Principle:** Southern hybridization, also called Southern blotting, is a commonly used method for the identification of DNA fragments that are complementary to a known DNA sequence. It allows a comparison between the genome of a particular organism and that of an available gene or gene fragment. This technique also tells us whether an organism contains a particular gene, and provides information about the organism and restriction map of that gene. Southern hybridization was named after its inventor, Edward M. Southern, who developed the technique in 1975. As a result subsequent blotting techniques have used similar nomenclature, for example Northern blotting, the transfer of RNA; Western blotting, the transfer of proteins; and Southwestern blotting, for the characterization of proteins that bind DNA. In Southern Blotting the chromosomal DNA is isolated from an organism of interest, and digested with restriction enzyme. The restriction digested fragments are electrophoresed on an agarose gel, which separates the fragments on the basis of size. The next step is to transfer fragments from the gel onto nitrocellulose filter or nylon membrane. This can be performed either by electrotransfer i.e. electrophoresing the DNA out of the gel and onto a membrane or by the simple capillary method. The transfer or a subsequent treatment results in immobilization of the DNA fragments, so the membrane carries a semi-permanent reproduction of the banding pattern of the gel. The DNA is bound irreversibly to the membrane by baking at high temperature (80°C) or by UV crosslinking. For the detection of a specific DNA sequence, a hybridization probe is used. A hybridization probe is a short (100-500bp), single-stranded nucleic acid that will bind to a complementary piece of DNA. Hybridization probes are labeled with a marker (radioactive or non-radioactive) so that they can be detected after hybridization. In non-radioactive detection the probe is labeled with biotin or digoxigenin. The membrane is washed to remove non-specifically bound probe and the hybridized probe is detected by treating the membrane with a conjugated enzyme, followed by incubation with the chromogenic substrate solution. As a result a visible band can be seen on the membrane where the probe is bound to the DNA sample.

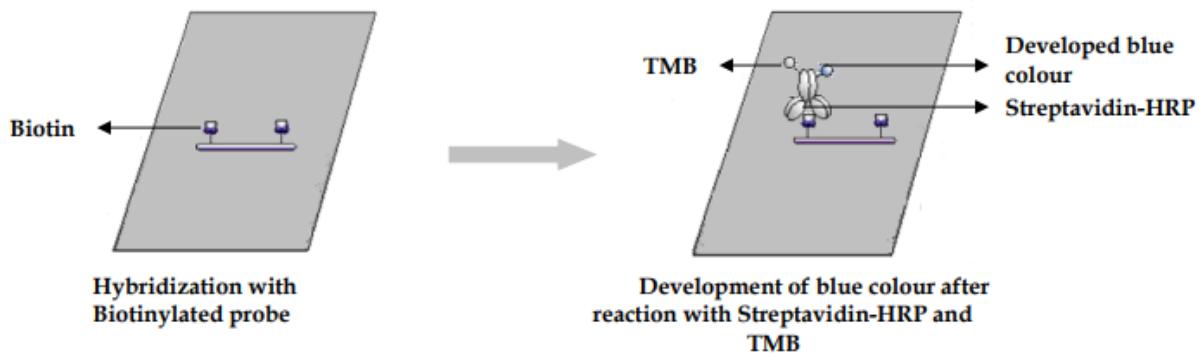
The entire procedure can be divided into following steps:

**I. Agarose Gel Electrophoresis:** Agarose gel electrophoresis is a technique for the separation of DNA molecules according to their molecular size. This is achieved when

negatively charged nucleic acids migrate through an agarose gel matrix under the influence of an electric field (electrophoresis). Shorter molecules move faster and migrate farther than the larger ones. The position of DNA in the agarose gel is visualized by staining with low concentration of fluorescent intercalating dyes, such as Ethidium bromide.

**II. Southern Blotting:** Southern blotting is the electrotransfer/capillary transfer of resolved DNA fragments from the agarose gel to the nitrocellulose/nylon membrane. For this transfer procedure, the gel is placed on the membrane and both of them are sandwiched between two filter papers. During electrotransfer the DNA bands are transferred to positively charged nylon membrane in the presence of a specific buffer. First transfer the set between two sponge pads and then place it in a plastic cassette. The entire set is then placed inside a gel tank filled with transfer buffer. The resolved DNA fragments are transferred to the corresponding positions on the nylon membrane after the electrotransfer. The DNA of interest is detected on the membrane.

**III. Detection:** After electrotransfer, DNA bands bound to the membrane are detected chromogenically. A suitable blocking reagent is used to block the unoccupied sites on the membrane. Then the DNA of interest is hybridized with a biotinylated probe specific to it. The membrane is washed to remove the excess unbound probe. It is then treated with Horseradish peroxidase (HRP)-conjugated streptavidin which attaches to the hybridized DNA. Finally, the membrane is incubated in a substrate solution containing TMB/ H<sub>2</sub>O<sub>2</sub> (Tetramethyl benzidine H<sub>2</sub>O<sub>2</sub> substrate) that reacts with HRP and as a result a blue coloured DNA band develops on the nylon membrane.



### **Materials Required**

Glassware: Test tubes  
 Reagents: Distilled water, Ethidium bromide (10 mg/ml), deionized water  
 Other requirements: Gel transfer apparatus, Gel rocker, Micropipettes, Tips, Microwave/Burner/Hotplate, Hot Air Oven, Incubator Shaker (45o C), Forceps. Himedia Southern blotting kit.

### **Procedure:**

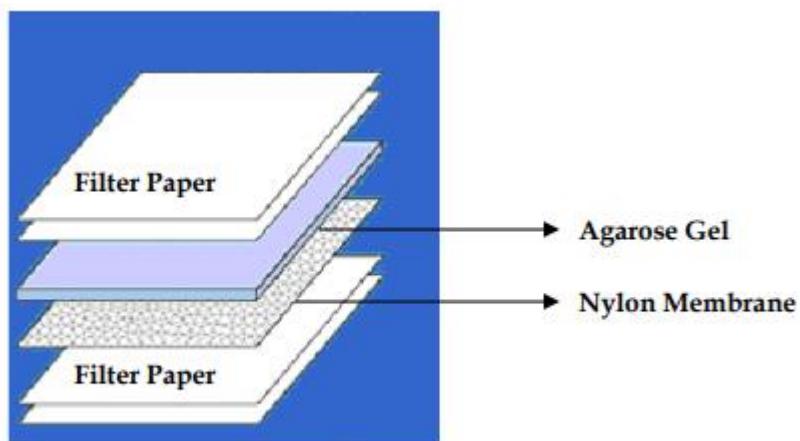
#### **Day 1: Agarose Gel Electrophoresis**

1. Prepare 1% agarose gel in 0.5X TBE buffer.
2. Run 10 µl of DNA sample at 100-120 volts until the dye marker have migrated to

- the end of the gel.
3. Using UV transilluminator, cut and lanes containing the DNA bands.
  4. Take out the nylon membrane with the filter paper (provided). Ensure there is no protrusion of the filter paper and membrane from the gel.
  5. Wet the cut gel, nylon membrane, filter papers and the electrotransfer cassette in 1X Electrotransfer buffer.

### **Electroblotting:**

1. Assemble the gel with nylon membrane and filter papers as shown below.



**Fig 1: Arrangement of the gel and membrane for transfer**

2. This blotting sandwich is placed within the sponge and blotting cassette. Try to avoid air bubble between the gel and nylon membrane by rolling a glass tube on the membrane.
3. Insert this cassette into the gel transfer apparatus filled with cold transfer buffer and then connect the transfer unit to the power supply as per conventions.
4. Electrophoreses the sample at 100V, 90 mA for 2 hours for blotting.

### **Immobilization of DNA on the membrane:**

1. Remove the nylon membrane after transfer from the blotting cassette and place the membrane in petri plate on a UV transilluminator (expose the membrane containing transferred DNA to UV light) for 20 minutes. This helps in fixing the DNA on the membrane.
2. Turn off the UV transilluminator and incubate the plate containing membrane in a hot air oven at 70 oC for 30 minutes. This ensures complete immobilization onto the membrane.

### **Hybridization:**

1. Bring the petri plate containing membrane to room temperature after incubation. Add 10 ml of prehybridization buffer into it and incubate at 45°C incubator shaker with mild shaking at 70-90 rpm for 45 minutes.
2. After incubation, discard the prehybridization buffer. Care should be taken not to

- discard the membrane.
3. Add 10 ml of hybridization buffer to the petri plate containing membrane.
  4. Keep 1 vial of biotinylated probe for 10 minutes in boiling water bath and immediately chill by placing it on ice for 5-10 minutes. Add 15 µl of this probe to the hybridization buffer in the petri plate.
  5. Incubate the petri plate at 45°C incubator shaker with mild shaking at about 70-90 rpm for 16 hours.

**Day 2: Blocking and Detection:**

1. Decant the hybridization buffer, add 10 ml of 1X Wash Buffer I and gently swirl the Petri plate for 5 minutes at room temperature. Repeat the washes twice (each wash for 5 minutes). Discard the buffer after each wash.
2. Add 10 ml of prewarmed 1X Wash Buffer II (70 °C) and gently swirl the Petri plate. Incubate at 70°C for 5 minutes in a hot air oven and gently swirl. Repeat the washes for another 2 times. Discard the buffer after each wash.
3. Add 10 ml of blocking buffer to the petri plate and incubate at room temperature for 1 hour with gentle rocking. Discard the blocking buffer.
4. Add 9 ml of diluted Streptavidin-HRP conjugate buffer to the petri plate and incubate at room temperature for 20 minutes with gentle rocking. Discard the conjugate buffer.
5. Add 10 ml of 1X Wash Buffer III to the petri plate and incubate at room temperature for 5 minutes each with gentle rocking. Repeat the washes two more times. Discard the buffer after each wash.
6. Add 10 ml of 1X Wash Buffer IV to the petri plate and incubate at room temperature for 5 minutes each with gentle rocking. Repeat the washes two more times. Discard the buffer after each wash.
7. Add 5 ml of TMB/H<sub>2</sub>O<sub>2</sub> and gently swirl at room temperature for 15-20 minutes until a blue colour band develops.
8. After the blue colour band is seen stop the reaction by placing the membrane in distilled water.

**Result:**

Blu band observed in the membrane indicates hybridization of probe with the specific DNA band.

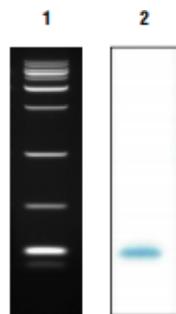
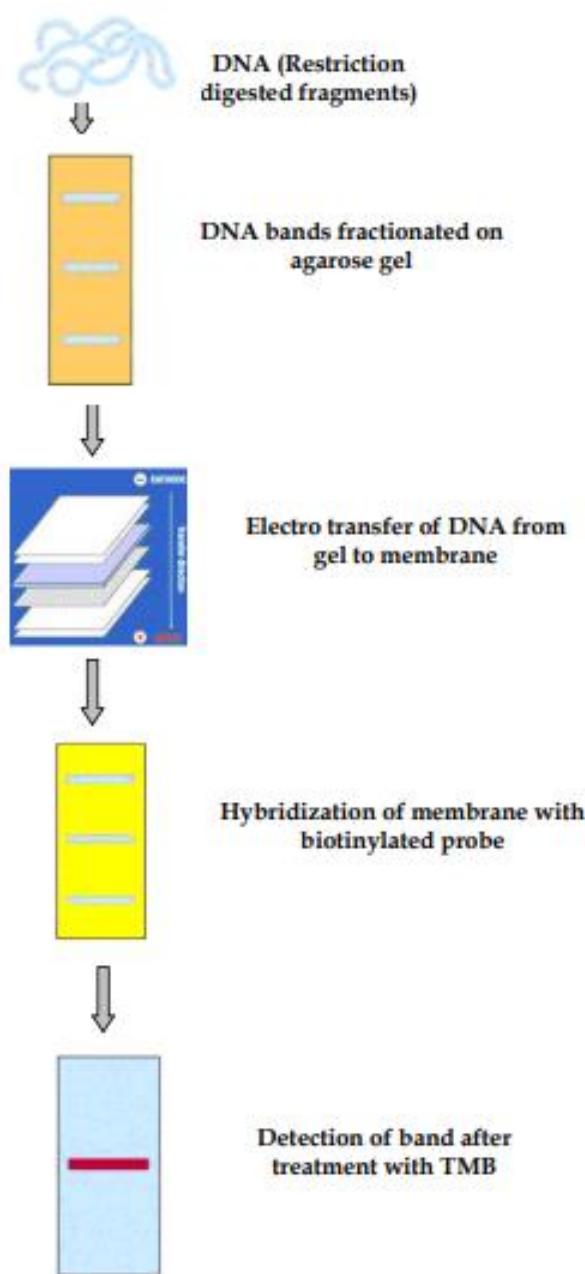


Fig 3: Gel image and immunoblot of the DNA sample after Agarose Gel Electrophoresis and Southern blotting



Flow chart showing steps in southern blotting

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### Composition & Preparation of buffers:

**Preparation of Prehybridisation Buffer:** Add 0.1g of blocking powder to 10 ml of prehybridisation buffer. Mix well before use.

**Preparation of Hybridization Buffer:** Add 0.1g of blocking powder to 10 ml of hybridization buffer. Mix well before use.

**Preparation of 1X Transfer Buffer:** To prepare 1 litre of 1X Transfer Buffer, take 100 ml of 10X transfer buffer and add 900 ml sterile distilled water\*. Store at 2-8°C. Mix well before use. The 1X Transfer Buffer can be reused 2-3 times.

**Preparation of 1X Wash Buffer I:** Dilute 15 ml of 2X Wash Buffer I with 15 ml of autoclaved deionized water. Mix well.

**Preparation of 1X Wash Buffer II:** Dilute 15 ml of 2X Wash Buffer II with 15 ml of autoclaved deionized water. Mix well.

**Preparation of 1X Wash Buffer III:** Dilute 15 ml of 2X Wash Buffer III with 15 ml of autoclaved deionized water. Mix well.

**Preparation of 1X Wash Buffer IV:** Dilute 15 ml of 2X Wash Buffer IV with 15 ml of autoclaved deionized water. Mix well.

**Preparation of Blocking Buffer:** Add 0.1g of blocking powder to 10 ml of blocking buffer

**Preparation of Streptavidin HRP-Conjugate Buffer:** Add 9 µl of Tween 20 in 9 ml of conjugate dilution buffer. Add 6.0 µl of Streptavidin- HRP Conjugate in 9 ml of conjugate dilution buffer for each experiment just prior to use.

X-----X-----X-----X

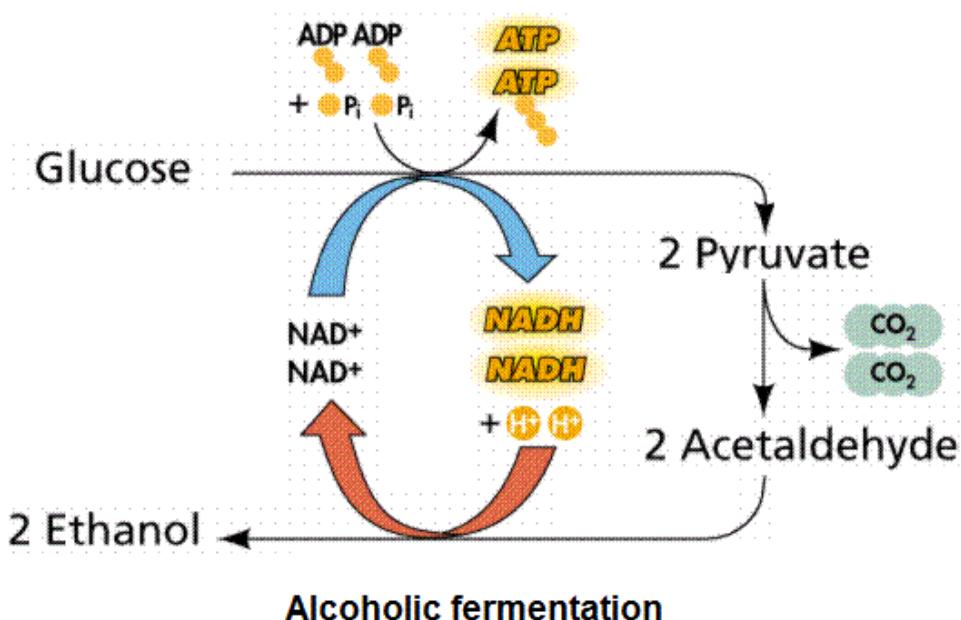
## 1. FERMENTATION OF GRAPE JUICE

### AIM

To conduct an experiment for the fermentation of grape juice.

### PRINCIPLE

Natural fermentation of grape juice and the other fruits by the addition of yeast cells. The biochemical conversion of juices to wine occurs when the yeast enzymatically degrades the fruit sugar fructose and glucose to aldehyde and then to alcohol.



### MATERIALS REQUIRED

Black grape juice, 1L Erylen mayor flask, weighing balance, glasswares, yeast.

### PROCEDURE

- 1) 1L of black grape juice was poured into 2L Erylen mayor flask.
- 2) 1Kg sucrose and 10ml of inoculums were added (10% starter culture).
- 3) Flask mouth was closed with a stopper containing a cotton plug.
- 4) The fermenting wine was incubated for 45 days at 25 °C.

## **RESULT**

Wine was produced by batch fermentation.



Wine produced by batch fermentation

X-----X-----X-----X

## 2. IMMOBILIZATION OF YEAST CELL BY SODIUM ALGINATE

### AIM

To learn the technique of immobilizing yeast cells in alginate beads.

### INTRODUCTION

Immobilization is a technique for the combination of biocatalyst in an insoluble support matrix. The matrix is usually a high molecular weight Polymer such as polyacrylamide, starch, cellulose, etc. The advantage of immobilizing enzymes or cells over free cells is to increase their stability and efficiency. The immobilized cells or enzymes can also be recovered at the end of the reaction can be used repeatedly

### PRINCIPLE

In 1996, scientists named Nelson & Griffin discovered that invertase (an enzyme) shows the same activity when absorbed to solid surface as when uniformly distributed throughout the solution. This was the first discovery of enzyme immobilization technique. An enzyme is usually immobilized on to an inert insoluble material. Eg: calcium alginate. This is produced by the reaction of a mixture of sodium alginate solution with calcium chloride; these beads provide increased resistance to changes in conditions such as pH or temperature. They also allow enzyme to be held in place throughout the reaction following which they are easily separated from the products & may be used again. A far more efficient process & so are widely used in industry for an enzyme-catalyzed reaction.

Whole-cell immobilization is an alternative to enzyme immobilization. Basically immobilization of live cells is very similar to the enzyme counterpart. In past, various cells have been immobilized bacteria, yeast, fungi, plant tissues, and mammalian tissues or insect tissues. Once the cells are immobilized the variability of the cell must be concomitantly sustained over a long period of time. The lower microorganisms (bacteria, yeast & fungi) can be easily immobilized with a number of methods: entrapment, ion exchange, adsorption, porous ceramics, and even covalent bondings. Most of the principles involved in enzyme immobilization are directly applicable to cell immobilization. There are five methods for immobilization of enzymes of cells.

#### 1. Adsorption

It is a method that involves electrostatic interaction such as van der Waal's force, ionic and hydrogen bonding between the enzyme or cell and the support of matrix.

#### 2. Covalent bonding

This method involves the formation of covalent bonds between the enzyme or cells and the support of matrix. The bond is normally formed between the functional group present in the surface of support & functional groups belonging to amino acid residues on the surface of the enzyme

#### 3. Entrapment

In this method, the enzyme molecules are mixed with a polyionic polymer material then

cross-linking of the polymer with multivalent cations in an ion-exchange reaction to form a lattice structure that traps the enzymes or cells.

#### 4. Encapsulation

This can be achieved by enveloping the enzyme or cells within various forms of semi-permeable membrane.

#### 5. Cross-Linking

This involves joining of enzymes or cells with each other to form large three-dimensional complex structures and can be achieved by physical or chemicals without any support system.

The enzyme may be entrapped within polymeric mesh such as agar, polyacrylamide gel or calcium alginate by carrying out the polymerization reaction and /or cross-linking reaction in the presence of enzyme. Alginate, the major structural polysaccharide of marine brown algae, contains  $\beta$ -D-mannopyranosyl uronate and  $\alpha$ -L-gulopyranosyl uronate in regular (1-4)-linked sequences. In the presence of monovalent cations, the polysaccharide forms a viscous solution even at high concentrations, whereas in the presence of divalent cations, especially  $\text{Ca}^{2+}$ , gelation occurs.



Gel formation can take place under mild conditions, entrapment in this matrix is very suitable for immobilization of enzymes, drugs, cells, proteins

### **MATERIALS REQUIRED**

1. Yeast slurry – 2%
2. Sodium Alginate -4%
3. Glucose solution-3%
4. Calcium chloride-0.2M

### **PROCEDURE**

1. Dry yeast cells were mixed with 25 ml of distilled water in a small beaker. It was covered and left for 30 minutes at room temperature
2. 25 ml of sodium alginate was added to yeast suspension and stirred well.
3. Some of the yeast alginate mixture was drawn into a clean syringe.
4. The solution was poured drop wise into a calcium chloride solution
5. The beads were left in the solution for about one hour for stable bead formation.
6. The beads were separated from the calcium chloride solution and the beads were placed on glucose solution. The flask mouth was closed with Aluminum foil
7. The next day the changes were observed.

## **RESULT**

The presence of gas and alcohol smell indicated the fermentation of sugar by yeast through immobilization.



Figure showing sodium alginate beads of yeast

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## **Composition**

### **1. Yeast slurry – 2%**

2g of yeast in 100 ml of distilled water.

### **2. Sodium Alginate -4%**

4g sodium alginate in 100ml of distilled water.

### **3. Glucose solution-3%**

3g glucose in 100ml distilled water.

### **4. Calcium chloride-0.2M**

2.21g of Calcium chloride(M.W.110.98) in 100 ml of distilled water.

X-----X-----X-----X

### **3. ISOLATION OF ANTIBIOTIC PRODUCING MICROBES FROM SOIL BY CROWDED PLATE TECHNIQUE AND DEMONSTRATION OF ANTIBIOTIC SENSITIVITY GIANT COLONY INHIBITION SPECTRUM**

#### **AIM**

To isolate the antibiotic-producing microbes from soil by crowded plate technique and giant colony inhibition spectrum.

#### **PRINCIPLE**

Antibiotics are produced by fermentation pathways that occur in many spore forming bacteria and fungi. The role of antibiotics produced by these microbes must be important. Since the genes for antibiotic production are preserved in evolution. Antibiotics secreted by microbes are toxic for other species. It related or prevented their growth and presumably functions as defence mechanism by the phenomenon of antibiotics.

Eg: Penicillin, bacitracin, Gentamycin, etc. By screening samples from soil, dust, muddy like sediments, rivers, estuaries oceans, plant surfaces, etc. Antibiotic producing bacteria and fungi can be isolated.

#### **MATERIALS REQUIRED**

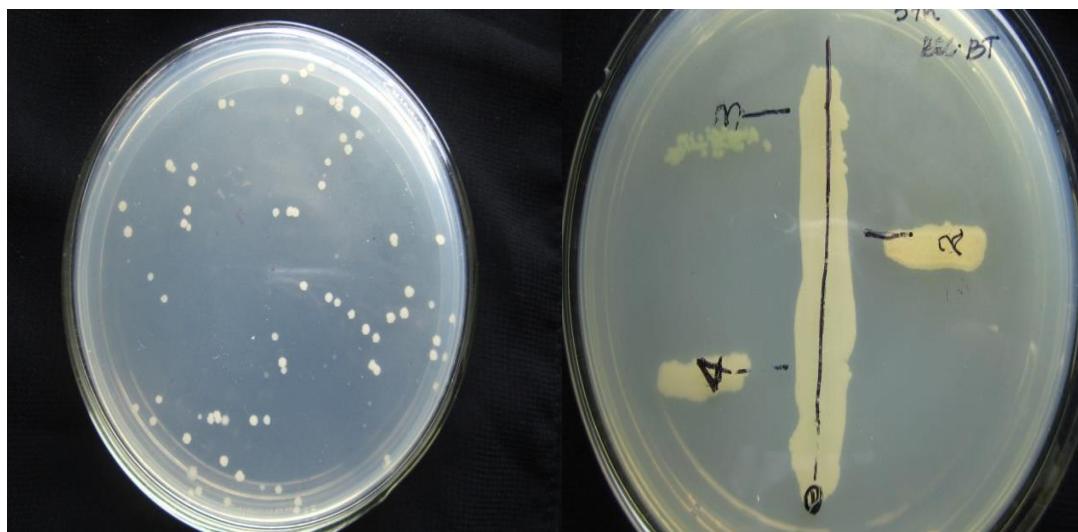
Glycerol yeast extract agar, glucose agar medium, glass wares, etc.

#### **PROCEDURE**

1. Soil sample was collected and serially diluted.
2. The diluted samples were plated on glycerol yeast extract medium using the spread plate method and the plates were incubated for 2 days.
3. After 2 days of incubation, zones of inhibition surrounding different colonies were checked.
4. The species that produced a zone of inhibition was selected [ie., species that produce antibiotics] and streaked on modified glucose agar medium and incubate at room temperature for 2-4 days.
5. After 2 days the different antibiotic-producing colonies were sufficiently grown, the multiple antibiotic-resistant or sensitive microbes were streaked at right angle to the fully grown antibiotic-producing organisms and incubated at  $37^{\circ}\text{C}$ .
6. The antimicrobial activity in terms of zone of inhibition of pathogen was determined.

#### **RESULT**

The isolated *Bacilli* have the ability to inhibit the growth of *Pseudomonas* and *Klebsilla*.



Crowded plate

Giant colony inhibition

### OBSERVATION

ORGANISM	INHIBITION
<u>E-coli</u>	
<u>Pseudomonas</u>	
<u>klebsiella</u>	

### Composition of Glycerol yeast extract agar

Glycerol	5 ml
yeast extract	2 gm
Dipottassium phosphate	1gm
Agar	15 gm
Distilled water	1000 ml

### **Composition of Glucose agar medium**

Glucose	10 gm
peptone	25 gm
Nacl	5 gm
Beef extract	3 gm
Agar	15 gm
Distilled water	1000 ml

X-----X-----X-----X

## **4. PRODUCTION OF MICROBIAL ENZYME (AMYLASE) AND DETECTION OF AMYLASE ACTIVITY BY ANTHRONE METHOD**

### **AIM**

To study the hydrolyze of starch to glucose by the production of microbial enzyme and to detect the formed glucose by anthrone method

### **PRINCIPLE**

Starch is a branched polymer of glucose molecule linked by glycosidic bonds. Starch as such cannot be transported to the cell for energy production, because of its high molecular weight. To assimilate starch for energy and catabolic reaction, it must be degraded to basic glucose units by the starch hydrolyzing enzyme.

Thus formed glucose was detected using the anthrone method. In the anthrone method, carbohydrates are dehydrated by using concentrated  $H_2SO_4$  to form furfural, which can be measured calorimetrically at 620nm by using spectrometer.

### **MATERIALS REQUIRED**

Starch broth, Gram's iodine stain, sample, Anthrone reagent, calorimeter, other routine microbiological laboratory facilities.

### **PROCEDURE**

#### **STEP 1**

1. Starch media was prepared. A single streak line of the organism was made across the center of the starch agar plate.
2. It was incubated at  $37^0c$  for 18-24 hours
3. After incubation, the plate was flooded with grams iodine solution,
4. Then the plate was analyzed for a clear zone around the streaked area.

#### **STEP 2**

1. 100ml of starch broth was prepared.
2. Inoculated the amylase producing microbes.
3. Incubated at  $37^0c$  for 24 hours.
4. Prepared the Anthrone reagent by dissolving 2g anthrone in 100ml concentrated  $H_2SO_4$  and also prepared a stock solution of glucose of concentration of  $100\mu g/mL$ .
5. 1ml of the incubated sample was collected.
6. Set up a range of serial dilution of glucose of  $20-100\mu g/ml$  in a volume of 1ml each as described in the observation table, similarly set up a blank with 1ml distilled water only.
7. Transfer 1ml of the unknown sample into separate test tube. Added 4ml of anthrone

- reagent to each tube and mixed.
8. Incubated them in a boiling water bath for 10 minutes. Cool the tubes at room temperature and measured the absorbance at 620nm.
  9. Draw a standard curve by using absorbance value of standard glass tubes that is by taking the sugar concentration on x-axis and absorbance value on y-axis. Calculate the amount of glucose present in the unknown sample from this curve and record our absorbance.

## **RESULT**

Starch was hydrolyzed and the estimated amount of glucose in the given sample is ..... $\mu\text{g}/\text{ml}$

---



Figure showing screening of bacteria for amylase production

Vol of Glucose(ml)	Vol of Water(ml)	Conc of RNA( $\mu\text{g}$ )	Vol of Anthrone	Incubate in boiling waterbath for 10 mins	OD 620nm
0	1	0			
0.2	0.8	20			
4	0.6	40			
0.6	0.4	60			
0.8	0.2	80			
1	0	100			
Unknown	upto 1				

## 5. EFFECT OF pH ON BIOMASS PRODUCTION [BAKER'S YEAST]

### **AIM**

To study the effect of pH on biomass production [Baker's Yeast] using wet weight as yardstick

### **PRINCIPLE**

Yeast is a microscopic unicellular organism that exists naturally on the surface of the earth. Yeast growth is affected by several factors like temperature, pH and nutrient content. Yeast grow well at acidic pH, they grow best between pH-4 to pH-6. Yeast can survive at very low pH, as low as pH-2. Baker's Yeast is a single cell microorganism it thrives when pH is slightly acidic, pH-4 is best. If the pH gets too acidic or too basic, they do not grow

### **MATERIALS REQUIRED**

- a) Sabouraud Dextrose Broth
- b) Baker's Yeast
- c) Centrifuge Tube
- d) Weighing Balance
- e) pH Meter

### **PROCEDURE**

1. 100 microliter of Sabouraud Dextrose Broth was prepared in 3 conical flasks at pH-4, pH-5.6, and pH-7.
2. The medium was sterilized by autoclaving.
3. The media was transferred into LAF and a loop full of yeast cells were inoculated into each conical flask under aseptic condition.
4. All the 3 flasks were incubated with different pH at room temperature for 42-70hrs.
5. Centrifuge tubes were labeled as pH-4, pH-5.6, and pH-7, and weighed on digital balance. The reading was noted.
6. 1microliter of inoculum was evenly transferred into a centrifuge tube using micropipette.
7. The centrifuge tubes were then placed in a centrifuge rotor in a way such that they are arranged in opposite beds.
8. These tubes were then placed in a centrifuge rotor in a way such that they are arranged in opposite positions and run at 5000 rpm for 15mins.
9. After taking out the tubes the supernatant [liquid portion] were decanted ensuring the biomass pellet retained in the tube was undisturbed
10. The droplet from the tubes with caps was weighed using digital balance and the corresponding reading at different pH was noted.

## **RESULT**

Maximum growth was obtained at \_\_\_\_\_ pH.

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### **Composition of SDA**

<b>Ingredients</b>	<b>In gm/L</b>
Dextrose (Glucose)	40 gm
Peptone	10 gm
Agar	15 gm
Distilled Water	1000 ml

X-----X-----X-----X

## 6. SEPARATION OF CELLS BY FLOCCULATION USING ALUM AS A FLOCCULATING AGENT TO SEPARATE YEAST FROM FERMENTATION BROTH

### **AIM**

To study separate cells by fermentation using ALUM as a flocculating agent to separate yeast from fermentation broth.

### **PRINCIPLE**

Flocculation is the agglomeration of used particles into micro floc and after that into bulky floccules that can be settled called flocs. The addition of another reagent called flocculent or flocculent aid may promote the formation of the floc. Some of the commonly used flocculants are ferric sulphate, aluminum sulphate (ALUM), etc.

The flocculation of biological cells is important in the biotechnology industry. Yeast flocculation typically refers to the clumping together of braving yeast. Yeast is capable of forming three aggregates matting aggregates for DNA exchange, chain formation, for development and differentiation of flocs as a survival strategy in adverse conditions.

### **MATERIAL REQUIRED**

Sabouraud Dextrose Broth, Yeast cells, petriplates, conical flask, etc.,

### **PROCEDURE:**

1. 500ml of Saboured dextrose broth was prepared and the media was sterilized by autoclaving.
2. The Sterile media was transferred into the LAF and a loop full of yeast was inoculated aseptically into the sterilized medium.
3. The culture medium was incubated at 37° c for 48-72 hours.
4. Then the fermentation broth was added with 0.4g of ALUM.
5. The broth was allowed to flocculate at room temperature and the changes were observed.

### **RESULT**

The yeast cells were separated from the fermentation broth by the flocculation method.

X-----X-----X-----X

## 7. CITRIC ACID PRODUCTION

### **AIM**

To produce and estimate citric acid using *Aspergillus niger*

### **PRINCIPLE**

Citric acid is an important organic acid and it was initially extracted from citrus fruits, nowadays it is largely produced by microbial fermentation. Citric acid is commercially used in food, soft drinks, pharmaceuticals, leather tanning, electroplating, etc.

*Aspergillus niger* is the most commonly used species for the production of citric acid. Most strains of *Aspergillus niger* which are mutants, cannot oxidize citric acid and accumulate in culture media. The composition of culture media is critical for obtaining the high yield of citric acid. It is essential to limit the growth of fungus so that high yield of citric acid is obtained.

This can be accomplished by keeping trace metal deficiency in the media. Acid is added to achieve low pH of 3.5. Sucrose serves as a carbon source for the production of citric acid. Ammonium nitrate is used to prevent the formation of Oxalic acid and Glutamic acid fermentation aerobic and can be carried out by submerged culture method.

The citric acid produced can be estimated by titrating against an alkali of a known strength using Phenolphthalein as indicator. The endpoint is the formation of pale pink colour. The volume of the alkali is used to find the normality and the percentage of acid in the sample.

### **MATERIALS REQUIRED**

1. *Aspergillus niger* culture
2. Citric acid production media
3. 250ml conical flask

### **PROCEDURE**

#### Production of citric acid

1. Citric acid production medium was prepared and sterilized by autoclaving.
2. Inoculate the medium with species of *Aspergillus niger* and incubate at some room temperature for 3-5 days on a rotatory shaker at 200 rpm.
3. After 5 days, the flask is taken inside a laminar airflow hood and using filter paper, the mycelium is separation from the medium.
4. The medium contains the citric acid produced which can be estimated using titrimetric method.

#### Titration Procedure

1. 10 ml of the filtrate was pipette into a conical flask and 2-3 drops of Phenolphthalein indicator was added to it.
2. This was titrated against 0.1 N NaOH taken in the burette till a pale pink colour

was formed. The titration was repeated till concordant values were obtained.  
 3. The percentage of citric acid was calculated using the given formula.

## **RESULT**

Percentage of citric acid found to be .....%  
 -----

## **OBSERVATION AND CALCULATION**

<b>Initial Burette reading</b>	<b>Final burette reading</b>	<b>Volume of NaOH (ml)</b>

$$\text{Normality of Citric acid} = \frac{\text{Normality of NaOH} * \text{Volume of NaOH}}{\text{Volume of filtrate}}$$

$$\text{Percentage of filtrate} = \frac{\text{Normality of citric acid} * \text{equivalent weight of citric acid} * 100}{\text{Volume of filtrate}}$$

## **Composition**

### Citric acid production media

Sucrose – 150 g  
 Ammonium nitrate – 2.5 g  
 Potassium dihydrogen orthophosphate – 1 g  
 Magnesium sulphate heptahydrate  
 Distilled water – 1 L  
 pH – 3.5

X-----X-----X-----X

## 8. PRODUCTION AND ESTIMATION OF PROTEASE

### AIM

To produce and estimate protease using protease producing bacteria.

### Introduction

Protease enzyme breaks down proteins. It conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases are essential constituents of all forms of life on earth. Microbial proteases are among the most important, extensively studied groups since the development of enzymology. Buffered skimmed milk agar was used for the detection of protease activity. This plate assay can also be used to distinguish both neutral and alkaline proteases by manipulating the pH of the buffer system.

Proteases exhibited large types of functions and have many important biotechnological applications in detergents, leather industry, food industry, pharmaceutical industry as well as in the bioremediation process. The use of proteases in laundry detergents is considered the more dominant application of proteases as they help in removing protein-based stains from clothing. Proteases may also be used in textile industry for removing the stiff and dull gum layer of sericine from the raw silk fiber leading to increase in its brightness and softness. Proteases have been also used in the hide dehairing process. Alkaline protease showed an interesting application in the decomposition of gelatinous coating of X-ray films (Ishikawa *et al.*, 1993), from which silver was recovered. Proteases also play a significant role in pharmaceutical applications in contact lens eye cleaners.

Enrichment method can be used for isolating protease producing microorganisms as well as suppressing the growth of other non-protease producing microorganisms by adding the protein source as the only enriching agent.

### Principle

Non-specific protease activity assay may be used as a standardized procedure to determine the activity of proteases. In this assay, casein acts as a substrate. When the protease digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin & Ciocalteus Phenol, or Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease. Absorbance values generated by the activity of the protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the F-C reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. From the standard curve the activity of protease samples can be determined in terms of Units, which is the amount in micromoles of tyrosine equivalents released from casein per minute.

**Materials required:**

Enrichment culture medium, sterile water, soil, skimmed milk plates, Potassium Phosphate Dibasic Trihydrate, Casein, Trichloroacetic Acid, Folin & Ciocalteu's Phenol Reagent, Sodium Carbonate, Anhydrous Sodium Acetate Trihydrate, Calcium Acetate , L-Tyrosine, Spectrophotometer, waterbath.

**Procedure:****Isolation of protease producing bacteria**

1. Adequate quantity of soil sample was suspended in sterile distilled water. (1gm soil was suspended in 4 ml sterile distilled water).Mixed well.
2. From this, 1 ml of supernatant was taken and inoculated in to conical flasks containing 20 ml of the enrichment medium.
3. Kept for incubation at room temperature for 48 hours in a rotary shaker at 200 rpm.
4. After 48 hours, 1 ml of the culture was transferred to 20 ml fresh sterile enrichment medium and incubated for 48 hours. This procedure was repeated for one more time.
5. After completing the third cycle of incubation, the broth cultures were taken for isolating microorganisms grown in it.
6. Sample from enrichment broth was spread plated onto nutrient agar plates and incubated overnight at 37 °C.
7. Isolated colonies grown on plates were selected and taken for confirming their protease production.
8. Selected colonies from the nutrient agar plates were spotted on selected points on the skim milk agar.
9. The plates were incubated for 24 hours at 37° C.
10. Colonies were checked for zone production around it due to proteolysis.
11. Colony showing maximum activity was selected based on zone diameter and was taken for further studies.

**Extraction of crude enzyme**

1. 20 ml of culture media (Minimal medium with skim milk powder as substrate) in triplicates were inoculated with the selected organism and incubated for 48 hours at room temperature in a rotary shaker (REMI Rotary Shaker) at 200 rpm.
2. The media were centrifuged at 5000rpm for 20 minutes at 4°C to obtain cell free supernatant (CFS), which was used as crude enzyme.

**Protease activity estimation**

1. 5ml of the substrate(casein) was incubated with 1ml of the crude protease and incubated at 37°C for 10min.
2. After incubation 5ml of Trichloroacetic acid(TCA) was added and incubated for 20 minutes.
3. To 1 ml of the supernatant, 5 mL of sodium carbonate is added followed by addition of 1 mL of Folin's reagent.Mixed by swirling and incubated at 37 °C for 30 minutes.
4. Measure the absorbance using spectrophotometer at 660nm.

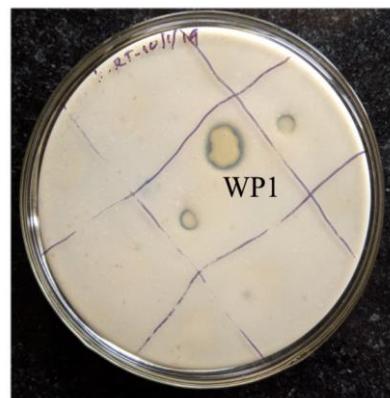
### Tyrosine standard

1. 1.1mM tyrosine standard stock solution was prepared.
2. Different volumes ranging from 0.05, 0.10, 0.20, 0.40, 0.50 was added into test tube. Blank didn't contain any tyrosine standard.
3. Once the tyrosine standard solution has been added, add an appropriate volume of purified water to each of the standards to bring the volume to 2 mL.
4. 5 mL of sodium carbonate is added followed by the addition of 1 mL of Folin's reagent.Mixed by swirling and incubated at 37 °C for 30 minutes.
5. Measure the absorbance using a spectrophotometer at 660nm.
6. Plot graph and derive the slope of the graph as well as a regression equation using excel.

Once unit of protease activity was defined as the amount of enzyme that liberated 1 $\mu$ mole of tyrosine per min per ml of protease broth under standard assay condition. The amount of tyrosine so released was calculated from tyrosine standard curve.

### Result:

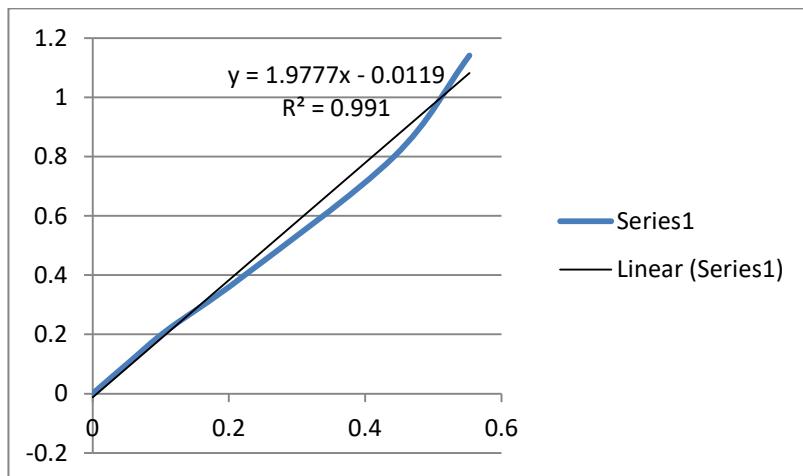
Protease producing bacteria was isolated using skimmed milk agar plates following enrichment culture. The protease activity of the enzyme was determined to be \_\_\_\_\_ $\mu$ moles/ml/min.



**Colony showing protease activity on skimmed milk agar plate**

Conc of Tyrosine	Absorbance
0	0
0.055	0.109
0.111	0.217
0.221	0.396
0.442	0.799
0.553	1.141

**Standard tyrosine curve**



- Substitute the OD value from protease activity in place of 'y' and determine the value of 'x'. This value so generated gives the number of  $\mu$ moles of tyrosine released.

**Calculations:**

$$\text{Units/mL Enzyme} = (\mu\text{mol tyrosine equivalents released}) \times (11)$$

$$(1) \times (10) \times (2)$$

Where;

11= Total volume (in milliliters) of assay

10= Time of assay (in minutes) as per the Unit definition

1= Volume of Enzyme (in milliliters) of enzyme used

2= Volume (in milliliters) used in Colorimetric Determination

The table shows details of reactions in plotting Tyrosine standard graph

Sample	S1	S2	S3	S4	S5	S6
<b>Volume of Standard taken (ml)</b>	0	0.05	0.10	0.20	0.40	0.50
<b>Volume of distilled water added (ml)</b>	2	1.95	1.90	1.80	1.60	1.50
<b>Volume of Alkaline solution (ml)</b>	5	5	5	5	5	5
<b>After incubating for 10-15 minutes at room temperature,</b>						
<b>Volume of Folin's reagent (ml)</b>	1	1	1	1	1	1
<b>After incubating at room temperature for 30 minutes, OD is measured at 660nm</b>						

**Reagent composition:****1. Enrichment media composition (100ml)**

NaH <sub>2</sub> PO <sub>4</sub> / KH <sub>2</sub> PO <sub>4</sub>	: 0.0496g
K <sub>2</sub> HPO <sub>4</sub>	: 0.2486g
FeCl <sub>3</sub> .6H <sub>2</sub> O	: 0.0016g
ZnCl <sub>2</sub>	: 0.0013g
MgCl <sub>2</sub>	: 0.0010g
CaCl <sub>2</sub>	: 0.0110g
Substrate	: 0.25g
pH	: 7

**2. 50 mM Potassium Phosphate Buffer, pH 7.5.**

Prepare using 11.4 mg/mL of potassium phosphate dibasic, trihydrate in purified water and adjusting pH with 1 M HCl. This solution is placed at 37°C before use.

**3. 0.65% weight/volume casein solution**

prepared by mixing 6.5 mg/mL of the 50 mM potassium phosphate buffer. The solution temperature is gradually increased with gentle stirring to 80-85 °C for about 10 minutes

until a homogenous dispersion is achieved. Do not boil the solution. The pH is then adjusted if necessary with NaOH and HCl.

**4.110 mM Trichloroacetic acid solution**

prepared by diluting a 6.1 N stock 1:55 with purified water. Trichloroacetic acid is a strong acid and should be handled with care.

**5. 0.5 M Folin & Ciocalteu's, or Folin's Phenol Reagent**

5ml of FC reagent mixed with 5ml of distilled water.

**6. 500 mM Sodium Carbonate solution**

prepared using 53 mg/mL of anhydrous sodium carbonate in purified water.

**7. L-tyrosine Standard stock solution.**

Prepared using 0.2 mg/mL L-tyrosine in purified water and heated gently until the tyrosine dissolves. As with the casein, do not boil this solution. Allow the L-tyrosine standard to cool to room temperature. This solution will be diluted further to make the standard curve.

X-----X-----X-----X

## 1. LABORATORY ORGANIZATION, STERILIZATION OF GLASSWARE & EXPLANT

A laboratory designed for in-vitro cultures with plant tissues should have sufficient space for performing several functions maintaining sterile conditions.

A plant tissue culture facility should be provided with adequate electrical power operation in case of power failure. Failures of power supply and controls can be disastrous. Safety cut-off devices, warning lights, and electronic warning systems are needed to safeguard against disasters.

### **Plan**

The general laboratory section includes the area for media preparation and often for autoclaving as well as many of the activities that relate to handling of the tissue-cultured materials. Sources of deionized and distilled water as well as lines of electricity and possibly natural gas should be installed. Preparation of field-grown and greenhouse plant materials for tissue culture initiation is best performed in an isolated space.

The area to be set aside for media preparation should have ample storage and bench space for chemicals, glassware, culture vessels, closures, and other items required to prepare media. The laboratory tables and boards must be equipped with taps and wash-basins.

An instrumentation room is required for the operation of analytical balances, research microscopes, freezer, gel documentation system, hot plate, laminar air flow hood, magnetic stirrer, micropipettes, oven with temperature control, pH meter, shaker, refrigerators, water distillation unit, water purification unit, etc.

To ensure the sterility required for the transfer operations sterile transfer cabinets are needed. Temperature control is essential because heat is produced continuously from the flames of burners or electric sterilizers used in the hoods. The room should be constructed to minimize the collection of dust and for easy cleaning.

A separate room for cleaning and dishwashing is essential. The storage area for culture vessels and glassware should be provided for so that they are readily accessible to the medium preparation area.

The following equipments are required in a plant tissue culture laboratory:

- Autoclave or Pressure Cooker
- Laminar Flow Cabinet
- Electronic Balance
- Centrifuge
- Refrigerator
- Water demineralizer
- Freezer
- Water distillation unit
- Dissecting Microscope
- Oven for dry sterilization

- Hot plate / Gas plate/Magnetic stirrer
- Parafilm strips
- pH meter digital
- Shaker

### **Autoclave**

Either a horizontal or vertical model is preferred based on the requirement. It is desirable to have two, one for media autoclaving and other for decontamination. The conditions maintained are 15lbs pressure, 121 C temperature and 15 minutes of duration.

### **Laminar airflow hood**

The laminar flow hood is the most commonly used equipment for sterile experimentation as it provides ample sterile working area. For plant tissue cultures, the horizontal laminar hood is essential, it is designed with horizontal airflow from the back to the front with gas burners. Air is forced into the cabinet, through a bacterial HEPA (high-efficiency particulate air) filter. It then flows outwards over the working bench at a uniform rate. The roof of the cabinet has UV germicidal light which is often used to sterilize the interior of the chamber. These lights are turned on about half an hour prior to using the chamber.

### **Small apparatus**

Small apparatus such as forceps of different sizes, scalpels with surgical blades, dissection needles, scissors, etc., are indispensable. Parafilm strips and glass markers are used during sterile operations.

### **Culturing Facilities**

Plant tissue cultures are maintained under controlled conditions like temperature, illumination, air circulation, humidity, photoperiod. Walk-in chambers, plant growth chambers, incubator cabinets, etc are required. Culture conditions are provided with proper air-conditioning, perforated shelves to support sterile glassware, timers, fluorescent tubes, and standard accessories. This type of culture room can also be used to house a variety of liquid culture equipment like gyratory and reciprocating shakers or batch and continuous bioreactors.

### **Physical factors**

The principal factors in the culture environment are light and temperature. Lighting requirements for the cultures grown under in-vitro conditions can be classified into various parameters of light intensity, duration of light and quality of light. In tissue culture cabins, light intensities usually vary from 5 to 25 W / cm<sup>2</sup> (1000 to 5000 lux), with the very common use of 10 to 15 W/cm<sup>2</sup>.

Culture rooms should have a light duration of 16 to 18 hr/day. Spectral quality of light received by invitro cultures is very important. In *Morus indica* calluses, blue light (around 467nm) induces bud formation and that red light (around 660nm) induces rhizogenesis. Earlier studies reveals that morphogenetic processes were found to be regulated by

photoreceptor pigments, phytochromes and others. Later commercial white fluorescent tubes were tried for optimization.

### **Greenhouses**

Plants for greenhouse facilities are available from many sources. The greenhouses should be equipped with cooling and heating systems to control the temperature, artificial lighting system which includes a mixture of fluorescent and incandescent lights for plant growth and photosynthesis. This facility is necessary as a transitional step of taking plant materials from culture containers present in the controlled room to the field. Thus in the green house, plants are acclimatized and hardened before being transferred to the field conditions. In greenhouses, plants develop adequate root systems and leaf structures so as to resist the field environment.

### **Operations**

1. Plan, design and organize experiments to implement tissue culture techniques in accordance with the program objectives.
2. Protocol development, stocks, media preparation and effective use of facilities.
3. Collection and maintenance of data
4. Record experimental observations
5. Data Analysis and preparation of reports.

### **Measures**

1. A laboratory should have up-to-date record of equipment, operating manuals, chemicals with manufacturer and grade.
2. Strong acids and bases should be stored separately. Chloroform, alcohol, phenol and other volatile compounds are stored in fume hood. Chemical compounds that are hygroscopic should be stored in dessicators. Few chemicals are kept in refrigerators or freezers based on the chemical behaviour of the compound.

### **Laboratory and Personal Safety**

Prior knowledge is required before operating instruments like laminar airflow, microscope, centrifuges, PCR thermocyclers, pH meters, gas burners etc.

The following regulations must be followed

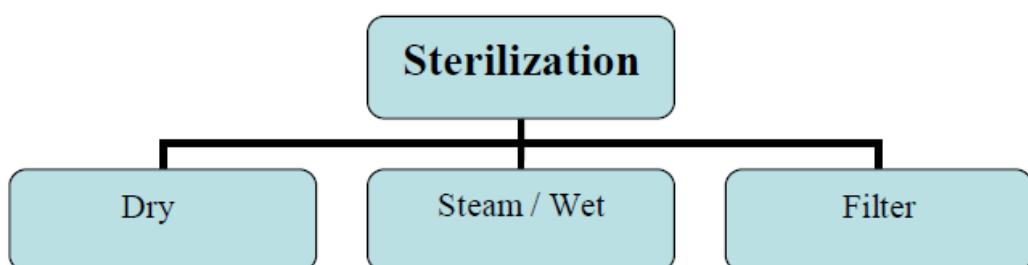
1. Contaminated vessels (e.g. pipettes, tips Pasteur pipettes and other things used for genetically – modified organisms or used for pathogen work) should be autoclaved before they are opened and the contents are eliminated.
2. Toxic chemicals must be handled with appropriate precautions e.g Mercuric Chloride, HCL etc.,
3. Beakers or flasks used for media should be rinsed quickly after dispensing the media into culture vessels to prevent drying of the residual agar in the beaker prior to washing.
4. First-aid kits and Fire extinguishers should be placed in every laboratory to avoid injuries.

## **Washing and Storage Facilities**

A tissue culture facility requires good quality running cold and hot water as well as provision for waste water disposal and ready access to a demineralized, distilled and double-distilled water apparatus. Space should be provided to set up drying ovens, washing machines, plastic or steel buckets for soaking glassware, acid or detergent baths, pipette washers, driers and cleaning brushes. It is mandatory that maximum cleanliness is maintained in all areas within the organization of a tissue culture plant.

## **STERILIZATION METHODS**

Sterilization is defined as the maintenance of aseptic environment for successful experimentation. To maintain sterile environment all culture vessels, media and instruments used in handling tissues as well as the explant itself must be sterilized. All operations are carried out in biosafety hood / laminar air flow cabinet as UV light penetration helps in elimination of pathogenic organisms.



### **Dry sterilization**

The sterilization of glassware and metallic instruments can be carried out in dry heat for 3 hr. at 160-180 C. Moist heat sterilization can be done at 121 C, 15 psi for 15 minutes duration. Glass ware, metal instruments like forceps, scalpels surgical blades etc., should be wrapped by aluminium foil and autoclaved followed by heating in an oven. Domestic pressure cookers are very useful in sterilizing a small amount of media.

### **Steam / Wet sterilization**

Nutrient media are sterilized by using either autoclave or pressure cookers. For the glass containers with a capacity of 20-40 ml of nutrient media autoclave with 121 C, 15 lbs pressure and 15- 20 minutes conditions are used. An autoclave has a normal temperature range of 115-130 C. Proper sterilization relies on time, pressure, temperature and volume of the object to be sterilized.

The uses of an autoclave are speed, easiness to handle and destruction of viruses and microbes, while the demerits are change in pH by 0.2 -0.5 units components becoming isolated and occurrence of chemical reactions, resulting in a loss of activity of media constituents.

## **Filter Sterilization**

Vitamins, aminoacids, growth regulators and toxins are heat labile and get destroyed during autoclaving with the other nutrients compounds. Those compounds can be sterilized by filtration through a sieve or filter membranes of 0.45 to 0.22 µm. Other membrane filters (Sartorius, Labgene, Millipore etc.,) and related equipment are available for sterilization of different volumes of the liquid in the range of 1 – 200 ml. Most filters are of cellulose acetate, cellulose nitrate and /or nitrocellulose are available. During filter sterilization, all the particles, microbes and viral particles which are bigger than the pore diameter of the filter used are eliminated.

## **Procedure for Aseptic Transfer**

Switch on the UV lamp for half an hour and close the door, afterwards switch off the light, open the door and switch on the air flow. The person who is working should spray 70% ethanol on his hands and rub with cotton and be sterile. Rub on all sides of the laminar flow cabinet with 70% ethanol. Use head cap, nasal covering in order to avoid contamination by air. It is always advisable to keep a sterile towel soaked with 70% ethanol in the working area.

The glassware may be tubes, petridishes, bottles with prepared and autoclaved media within are carefully transferred to the laminar flow cabinet by washing the surface of the glassware with 70% ethanol so that we can avoid transmission of surface contaminants. Set up all the glassware aside and then switch on the Bunsen burner / spirit lamp and flame sterilize the filter papers and be ready the explant sterilization.

## **Explant sterilization**

Explants (e.g: nodal segment, axillary bud, shoot tip, hairy root, anther, ovary etc.,) taken from the main plant are thoroughly washed in tap water then rinsed in teepol or tween 20 for three times then wash in distilled and autoclaved distilled water thrice. Now the explants are rinsed in any one of the following chemical sterilants viz.,

1. Mercuric Chloride 0.01-1% for 2-5 min
  2. Sodium hypochlorite 1-1.4% for 5-30 min
  3. Hydrogen peroxide 10-12% for 5-15 min
  4. Calcium hypochlorite 4-10% for 5-30 min
  5. Bromine water 1-2 % for 2-10 min
  6. Silver nitrate 1% for 5- 30 min
  7. Antibiotics 4-50 mg/l for 30-60 min

Explants after treatment with sterilants, must be thoroughly rinsed with several changes of sterile distilled water because retention of such noxious chemicals will seriously affect the establishment of culture

## 2. PREPARATION OF TISSUE CULTURE MEDIUM

### **INTRODUCTION:**

The basic nutritional requirements of cultured plant cells as well as plants are very similar. However, the nutritional composition varies according to the cells, tissues, organs and protoplasts and also with respect to particular plant species. The appropriate composition of the medium largely determines the success of the culture. A wide variety of salt mixtures have been reported in various media. A nutrient medium is defined by its mineral salt composition, carbon source, vitamins, growth regulators and other organic supplements. When referring to a particular medium, the intention is to identify only the salt composition unless otherwise specified. Any number and concentration of amino acids, vitamins, growth regulators and organic supplements can be added in an infinite variety of compositions to a given salt composition in order to achieve the desired results.

### **REQUIREMENTS**

#### **Glassware / plasticware / minor items**

- Aluminium foil
- Beakers of different sizes from 50ml to 2000ml
- Chemicals of Analar grade, depending upon the medium
- Conical flasks (with wide mouth) of different capacities
- (100ml, 150ml, 250ml, 1l, 2.5l)
- Culture tubes (25mm x 150 mm)
- Funnels
- Glass markers
- Graduated cylinders of various capacities
- Measuring cylinders of various capacities
- Non-absorbent cotton and muslin/cheese cloth for cotton plugs
- Petri dishes of different sizes (glass or sterilized plastic)
- Pipettes (different capacities from 1ml to 10ml)
- Sterile filtration assembly
- Wrapping paper (brown sheet)

### **MEDIA COMPOSITION**

The salt composition of Murashige and Skoog (1962) nutrient medium, referred to as MS medium, is very widely used in different culture systems as it gives satisfactory results. But it must be remembered that it is not always the best medium. Generally, in all the media, the nutritional milieu consists of inorganic nutrients, carbon and energy sources, vitamins, growth regulators, and complex organic supplements. It is desirable to choose a composition according to the knowledge of the physiology of species vis-a-vis mineral nutrition.

## **INORGANIC NUTRIENTS**

Mineral elements are very important in the life of a plant. Besides, C,H,N, and O, 12 other elements are known to be essential for plant growth. According to the recommendations of the International Association for Plant Physiology, the elements required by plants in concentration greater than 0.5 mmol/l are referred to as macroelements or major elements and those required in concentration less than the prescribed amount are microelements or minor elements. A variety of salts supply the needed macro and micronutrients that are the same as those required by the normal plant.

**Major salts :** The salts of potassium (K), nitrogen (N), calcium (Ca), magnesium (Mg), phosphorus (P) and sulphur (S) are required in macro or millimole quantities. Nitrogen is generally used as nitrate or ammonium salts, sulphur as sulphates and phosphorus as phosphates.

**Minor salts :** The salts of iron (Fe), manganese (Mn), boron (B), copper (Cu), zinc (Zn), iodine (I), molybdenum (Mo) and cobalt (Co) are required in micromolar concentrations and are considered to be minor salts. These salts are essential for the growth of tissues and are required in trace quantities. To achieve the maximum growth rate, the optimum concentration of each nutrient can vary considerably. The mineral composition of a culture medium is defined precisely by the equilibrium of the concentrations of different ions in a solution. When mineral salts are dissolved in water, they undergo dissociation and ionization. The active factor in the mediums is the ions of different types rather than the compounds. Therefore, a useful comparison between the two media can be made by looking into the total concentrations of different types of ions in them. To choose a mineral composition and then compare their different ionic balances, one uses ionic concentrations expressed in milli equivalents per litre. Any success with a medium is in all probability due to the fact that the ratios as well as concentrations most nearly match the optimum requirements for the cells or tissues for growth and/or differentiation.

Cultured tissues have specific needs vis-a-vis the following ions: K+, NO<sub>3</sub> -, NH<sub>4</sub> +, Ca++, Mg++. Phosphorus is often carried in low concentrations. The ions K+, NO<sub>3</sub> -, and NH<sub>4</sub> + have a profound influence on the growth of tissues.

**Nitrogen:** Nitrogen is the major component supplied in the form of nitrates or ammonium salts. Nitrogen is an important part of amino acids, proteins, nucleic acids. Inorganic nitrogen is utilized in order to synthesize organic molecules. For most purposes, a nutrient medium should contain from 25 to 60 mM inorganic nitrogen. The cells may grow on nitrate alone, but often there is a distinct beneficial effect and requirement for ammonium or another source of reduced nitrogen. Besides, nitrate alone in the medium drifts the pH towards alkalinity. Adding a small amount of an ammonium salt in the range of 25-40 mM and ammonium in the range of 2-20 mM. Nitrate cannot be simply used to synthesize organic molecules but has to be reduced to ammonia first. The response to ammonium varies from inhibitory to essential, depending upon the tissue and the purpose of culture.

**Potassium:** Potassium is required at concentrations of 2 to 26 mM. This element is

generally supplied as the nitrate or as the chloride form and cannot be substituted by sodium. It is a monovalent cation with high mobility in the plant. Potassium salts have an important function in the osmotic regulation of the cell. Potassium ion is essential for the activation of many enzymes. In photosynthesis, K<sup>+</sup> regulates the ion balance and pH of chloroplasts.

**Calcium:** Calcium is essential for cation-anion balance by counteracting organic and inorganic anions. A concentration of 1-3 mM of calcium is usually adequate. Calcium is also important for cell and root multiplication. Calcium, a component of the cell wall, is largely bound to the cell wall and membrane. This is because of the large number of Ca<sup>++</sup> binding places on the cell wall and limited mobility of calcium through the membrane into the cytoplasm. The stability of cell membrane is highly influenced by Ca<sup>++</sup>.

**Phosphorus:** Phosphorus is present in the plant in the form of inorganic phosphate (iP). A concentration of 1-3 mM phosphate is usual adequate. The high-energy pyrophosphate bond of phosphorus, when bound to another P atom as in ATP, is very important for the energy metabolism in the cell. Phosphorous is an essential element in DNA and RNA nucleic acids. In phospholipids, this element is very important for the energy metabolism of the plant in form energy-rich phosphate esters.

**Magnesium:** A concentration of 1-3 magnesium is usually adequate. This element is an essential component for many enzymes reactions and is very important in photosynthesis. Magnesium is indispensable for the energy metabolism of the plant because of its importance in the synthesis of ATP.

**Sulphur:** A concentration of 1-3 mM sulphate is usually adequate. These have to be reduced first for the synthesis of sulphur containing compounds such as amino acids, proteins and enzymes. Sulphur in its non-reduced form is incorporated in sulfolipids and polysaccharides.

**Boron:** Boron is required for the synthesis of cell wall as well as in the stabilization of the constituents of cell wall and cell membrane.

**Chlorine:** Chlorine is taken up as a chloride and is very mobile in the plant. The main functions of the ions are in osmoregulation. Chlorides play a role in photosystem II during the Hill reaction. Chlorine also regulates the opening and closing of stomata and is thus very important in the regulation of the osmotic potential of vacuoles as also to turgor-related processes.

**Copper:** Copper is taken up by the plant as Cu<sup>++</sup> or as a copper chelate complex. Within the cell, copper is mostly part of the enzyme complexes and important in redox reactions executed by these enzymes. It is useful in photosynthesis.

**Cobalt:** Cobalt is assumed to be important in nitrogen fixation. In higher plants the function of this element is not very clear.

**Manganese:** Manganese is taken up by the plant as bivalent unbound Mn<sup>++</sup> ions. The

element is strongly bound to several metalloproteins. The ion is involved in the Hill reaction of photosystem II in which water is split into oxygen and protons.

**Molybdenum:** Molybdenum is used as a cofactor in many enzymes, including nitrogenase and nitrate reductase. It is also directly involved in the reduction of N<sub>2</sub>.

**Zinc:** Zinc is taken up by the roots as Zn<sup>++</sup>. It is neither oxidized nor reduced in the plants. It is an important component of a number of enzymes, e.g. alcohol dehydrogenase in the meristem zone of the plant. Zinc is also very important for protein synthesis.

**Iron:** Iron is generally added as a chelate with ethylene diamine tetra acetic acid (EDTA). In this form, iron remains available up to a pH of 8.0. It is mainly bound to chelators and complex compounds in plants. Most plants absorb only ferric ions (Fe<sup>3+</sup>). The main function of iron is to form iron chelates and two kinds of proteins: haeme proteins and iron sulphur proteins. The most well-known haeme proteins are the cytochromes, functioning as intermediates for electrons required for the reduction of nitrate to nitrite by the enzyme nitrate reductase in nitrogen assimilation. The second group of iron-binding proteins are the iron sulphur proteins. The iron is bound to a thiol group (-SH) of cystine and/or inorganic sulphur. Ferridoxin is the most common iron sulphur protein. It functions as a carrier in the electron transport reaction catalyzed by nitrate reductase, sulphate reductase, the synthesis of NADP<sup>+</sup> during photosynthesis and nitrogen reduction by nitrogenase complex. Iron is also important in the biosynthesis of chlorophyll.

### **Carbon and energy source**

The standard carbon source without exception is sucrose but plant tissues can utilize a variety of carbohydrates such as glucose, fructose, lactose, maltose, galactose and starch. In the cultured tissues or cells, photosynthesis is inhibited and thus carbohydrates are needed for tissue growth in the medium. Sucrose, at a concentration of 2-5% in the medium, is widely used. The autoclaving process does cause an alteration in the sugars by hydrolysis but presents no drawbacks to the growth plan. Most media contain myoinositol at a concentration of 100-mg per litre, which improves cell growth.

### **VITAMINS**

Normal plants synthesize the vitamins required for growth and development, but plant cells in culture have an absolute requirement for vitamin B1 (thiamine), vitamin B (nicotinic acid) and vitamin B6 (pyridoxine). Some media contain pantothenic acid, biotin, folic acid, p-amino benzoic acid, choline chloride, riboflavin and ascorbic acid. The concentrations are in the order of one mg/l. Myo-inositol is another vitamin used in the nutrient medium with a concentration of the order of 10-100 mg/l.

### **GROWTH REGULATORS**

Hormones now referred to as growth regulators are organic compounds that have been naturally synthesized in higher plants, which influence growth and development. These are

usually active at different sites from where they are produced and are only present and active in very small quantities. Two main classes of growth regulators of special importance in plant tissue culture are the auxins and cytokinins, while others are of minor importance, viz., gibberellins, abscisic acid, ethylene, etc. Some of the naturally-occurring growth regulators are indole acetic acid (IAA), an auxin and zeatin and isopentenyl adenine (2 iP) as cytokinins, while others are synthetic growth regulators.

Characteristics of growth regulators and vitamins

Name	Chemical formula	Molecular weight	Solubility
p-Chlorophenoxy acetic acid	C <sub>8</sub> H <sub>7</sub> O <sub>3</sub> Cl	186.6	Alcohol
2,4-Dichlorophenoxy acetic acid	C <sub>8</sub> H <sub>6</sub> O <sub>3</sub> Cl <sub>2</sub>	221.0	Alcohol
Indole-3 acetic acid	C <sub>10</sub> H <sub>9</sub> NO <sub>2</sub>	175.2	1N NaOH
Indole-3 butyric acid	C <sub>12</sub> H <sub>13</sub> NO <sub>2</sub>	203.2	1N NaOH
α-Naphthalene acetic acid	C <sub>12</sub> H <sub>10</sub> O <sub>2</sub>	186.2	1N NaOH
β-Naphthoxy acetic acid	C <sub>12</sub> H <sub>10</sub> O <sub>3</sub>	202.3	1N NaOH
Adenine	C <sub>5</sub> H <sub>5</sub> N <sub>5</sub> ·3H <sub>2</sub> O	189.1	H <sub>2</sub> O
Adenine sulphate	(C <sub>5</sub> H <sub>5</sub> N <sub>5</sub> ) <sub>2</sub> ·H <sub>2</sub> SO <sub>4</sub> ·2H <sub>2</sub> O	404.4	H <sub>2</sub> O
Benzyl adenine	C <sub>12</sub> H <sub>11</sub> N <sub>5</sub>	225.2	1N NaOH
N-isopentenylamino purine	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub>	203.3	1N NaOH
Kinetin	C <sub>10</sub> H <sub>9</sub> N <sub>5</sub> O	215.2	1N NaOH
Zeatin	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O	219.2	1N NaOH
Gibberellic acid	C <sub>19</sub> H <sub>22</sub> O <sub>6</sub>	346.4	Alcohol
Abscisic acid	C <sub>15</sub> H <sub>20</sub> O <sub>4</sub>	264.3	1N NaOH
Folic acid	C <sub>19</sub> H <sub>19</sub> N <sub>7</sub> O <sub>6</sub>	441.4	1N NaOH
Colchicine	C <sub>22</sub> H <sub>25</sub> NO <sub>6</sub>	399.4	H <sub>2</sub> O

## Media preparation

For the preparation of media only analytical reagents of 'Hi Media' grade chemical and Borosil glasswares were used. De-ionised water was used for preparing the media. The nutrient media basically consists of inorganic nutrients, carbon source and vitamins. Stock solutions were prepared separately for macronutrients, micronutrients, iron EDTA, potassium iodide and vitamins. All the chemicals were weighed accurately in electronic weighing balance. All stock solutions were preserved in refrigerator at 4°C. Murashige and Skoog (1962) basal media was prepared (Table 1).

The final volume was made up with distilled water and the pH of the medium was adjusted to 5.6 with either 1N NaOH or 1N HCl. To the above said media, 0.8 to 0.9% agar (extra pure gelling point 32-35°C Hi Media) was added and melted in a water bath. Around 10-15 ml of the medium was dispensed into 250 mm × 150 mm culture tubes (Borosil). The mouth of the tubes was covered with aluminium foil and was autoclaved at 1.06 kg pressure for about 20 minutes at 121°C.

**Table1: Chemical composition of Murashige and Skoog (1962) medium**

S. No.	Components	Quantity (g/l)	
<b>1. Macronutrients</b>			<b>1000 ml</b>
NH <sub>4</sub> NO <sub>3</sub>	1.64		
KNO <sub>3</sub>	1.94		
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.44		
KH <sub>2</sub> PO <sub>4</sub>	0.17		
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.37		
Meso inositol	0.1		
<b>2. Micronutrients</b>	<b>g/100 ml</b>		<b>1 ml (from stock)</b>
H <sub>3</sub> BO <sub>3</sub>	0.620		
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.025		
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.0025		
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.0025		
ZnSO <sub>4</sub> . 7H <sub>2</sub> O	0.860		
MnSO <sub>4</sub> .4H <sub>2</sub> O	2.230		
<b>3.</b>	<b>KI</b>	<b>0.839</b>	<b>1 ml (from stock)</b>
<b>4.</b>	<b>Fe EDTA</b>	<b>0.73</b>	<b>5 ml (from stock)</b>
<b>5. Vitamins</b>	<b>g/50 ml</b>		<b>1 ml (from stock)</b>
Thiamine-HCl	0.005		
Nicotinic acid	0.025		
Pyridoxine-HCl	0.025		
<b>6.</b>	<b>Glycine</b>	<b>0.04 (g/100 ml)</b>	<b>5 ml (from stock)</b>
<b>7.</b>	<b>Sucrose</b>	<b>30 g/L</b>	
	<b>pH</b>	<b>5.5 to 5.8</b>	
<b>8.</b>	<b>Agar</b>	<b>8 g/L</b>	

### **UNITS FOR SOLUTION PREPARATION**

The concentration of a particular substance in the media can be expressed in various units that are as follows :

#### **Units in weight**

It is represented as milligram per litre (mg/l)

$10^{-6}$  = 1.0 mg/l or 1 part per million (ppm)

$10^{-7}$  = 0.1 mg/l.

$10^{-8}$  = 0.001 mg/l or 1  $\mu$ g/l.

#### **Molar concentration**

A molar solution (M) contains the same number of grams of substance as is given by molecular weight in total volume of one litre.

1 molar (M) = the molecular weight in g/l

1 mM = the molecular weight in mg/l or  $10^{-3}$  M

1  $\mu$ M = the molecular weight in  $\mu$ g/l or  $10^{-6}$  M or  $10^{-3}$  mM.

**Conversion from milli molar (mM) to mg/l**

For example, molecular weight of auxin 2,4-D = 221.0

1M 2,4-D solution consists of 221.0 g per litre

1 mM 2,4-D solution consists of 0.221 g per litre = 221.0 mg per litre

1  $\mu$ M 2,4-D solution consists of 0.000221 g/l = 0.221 mg/l

X-----X-----X-----X

### 3.CALLUS INDUCTION

#### **AIM:**

To induce callus from the explants of ***Daucus carota* (Carrot)**

#### **Introduction**

A callus is an actively-dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury (Wounding) or in tissue culture in the presence of growth regulators. Explants from both mature and immature organs can be induced to form callus. However, explants with mitotically active cells (young, juvenile cells) are generally good for callus initiation. Callus is produced on explants *in vitro* from peripheral layers as a result of wounding and in response to growth regulators, either endogenous or exogenously supplied in the medium. The season of the year, donor conditions of the plant, the age and physiological state of the parent plant contribute to the success of organogenesis in cell cultures.

Growth regulator concentration in the culture medium is critical for morphogenesis. Auxin, at a moderate to high concentration, is the primary hormone used to produce callus. In some species, a high concentration of auxin and a low concentration of cytokinin in the medium promotes abundant cell proliferation with the formation of callus. Callus may be serially subcultured and grown for extended periods, but its composition and structure may change with time as certain cells are favored by the medium and come to dominate the culture. Callus tissue from different plant species may be different in structure and growth habit: white or colored, soft (watery) or hard, friable (easy to separate into cells) or compact. The callus growth within a plant species is dependent on various factors such as the original position of the explant within the plant, and the growth conditions. Although the callus remains unorganized, with increasing growth, some kinds of specialized cells may be formed again. Such differentiation can appear to take place at random but may be associated with centers of morphogenesis, which can give rise to organs such as roots, shoots, and embryos.

#### **MATERIALS REQUIRED**

1. Culture tubes
2. Sterile Petri dishes
3. Scalpel, blades, forceps, and coupling jars
4. Sterile distilled water
5. Alcohol (70 %), HgCl<sub>2</sub> (0.1 %)
6. Nutrition medium reagents – MS basic salts and vitamins

Growth regulators – 2, 4-D

**Plant material** – Carrot

**Culture medium-** MS + 2, 4-D (2mg/L)

### **PROCEDURE**

1. A fresh taproot of the carrot was taken and washed thoroughly under running tap water to remove all surface specks of dirt.
2. The taproot was then dipped into 5% “Teepol” for 10 minutes and then the root was washed. The carrot root, sterilized forceps, scalpels, other instruments, autoclaved nutrient medium Petri dishes were then transferred to laminar airflow or inoculation chamber. Throughout the manipulation sequences forceps, scalpels must be kept in 95% ethanol and flamed thoroughly before use.
3. The taproot surface sterilized by immersing in 70% v/v ethanol for 40 seconds, followed by 2-3 minutes in Mercuric Chloride (0.1 %).
4. The root was washed three times with sterilized distilled water to remove the sterilizing agents.
5. The carrot was then transferred to a sterilized petri dish containing a filter paper. A series of transverse slice 1mm in thickness was cut from the taproot using a sharp scalpel.
6. Each piece was transferred to another sterile petri dish. Each piece contains a whitish circular ring of cambium around the pith. An area of 4mm across the cambium was cut from each piece so that each piece contains part of phloem. Cambium and xylem size and thickness of explant should be uniform.
7. Always the lid of the petri dish was replaced after each manipulation.
8. The closure from a culture tube was removed and flamed the uppermost 20 mm of the open end. While holding the tube at an angle of 45<sup>0</sup>, an explant was transferred using forceps onto the surface of the solid nutrient medium.
9. The closure was immediately placed on the open mouth of each tube, Date, medium, and name of the plant were written on the culture tube by a glass marking pen.
10. Culture tubes after inoculation were taken to the culture room where they were placed in the racks. Cultures were incubated in dark at 25<sup>0</sup>C.

### **RESULT**

The undifferentiated mass of cells (Callus) appeared after 5 weeks.



Figure: Callus induction from carrot

X-----X-----X-----X

## 4. ORGANOGENESIS FROM CALLUS

**Aim :**

To perform organogenesis from the callus of *Boerhavia diffusa* L.

**Introduction**

Plant production through organogenesis can be achieved by two modes: (i) Organogenesis through callus formation and (ii) Emergence of adventitious organs directly from the explant. By varying the growth regulator levels, i.e. lowering the auxin and increasing the cytokinin concentration is traditionally performed to induce shoots from the explant. The next phase involves the induction of roots from the shoots developed. IAA or IBA auxins, either alone or in combination with a low concentration of cytokinin, are important in the induction of root primordia. Thus organ formation is determined by quantitative interaction, i.e. ratios rather than absolute concentrations of substances participating in growth and development.

**Material Required :**

1. *B. diffusa* callus
2. MS Medium with BAP (2mg/L)
3. Culture tubes, Sterile Petri dishes, Scalpel, blades, forceps and coupling jars

**Procedure:**

1. MS medium with BAP (2mg/L) was prepared and sterilized
2. Callus formed from the leaf explants of *B. diffusa* separated from the culture tubes
3. Separated callus was cut into small pieces and transferred to the new cytokinin (BAP 2mg/L) containing medium for differentiation of callus or organ formation.
4. Then the culture tubes were incubated at 25°C with 8 hrs photo period.

**Result:**

Shoot proliferation was observed after 20 days from the explants.



Figure: Organogenesis from callus

X-----X-----X-----X

## 5.ISOLATION OF PROTOPLASTS

### **Introduction:**

Protoplast is the living material of the cell whereas an isolated protoplast is a cell from which the cell wall is removed. In-plant breeding programs many desirable combinations of characters could not be transmitted through the conventional method of genetic manipulation. higher plants that could lead to the genetic process involving fusion between the subsequent developments of a product to a hybrid plant is known as somatic hybridization. Plant protoplasts can be isolated from cells by two methods:

1. Mechanical method
2. Enzymatic method.

### **MECHANICAL METHOD**

#### **Aim:**

To isolate protoplast by mechanical method

#### **Principle:**

Protoplast can be isolated from almost all plant parts: roots, leaves, fruits, tuber, root nodules, pollen mother cell, etc. Protoplast isolated by mechanical is a crude and tedious procedure. Cells are plasmolyzed causing the protoplast to shrink from the cell wall. The protoplast obtained from this method is then cultured in suitable culture medium. The principal deficiency of this approach is that the protoplast released is few in number. Mechanical isolation was that of only historical events now.

#### **Materials Required:**

1. Plant leaves –
2. Mortar and pestle
3. Phosphate buffer pH-7.0
4. Glass slides
5. Microscope.

#### **Procedure:**

1. Young leaves were obtained from plants grown outdoors and initially washed with tap water to remove any dust particles.
2. The leaves were washed with phosphate buffer and homogenized gently with the mortar and pestle.
3. The crude protoplast suspension was centrifuged at a very low 50-100 rpm for 10 minutes.
4. The supernatant containing intact protoplast was carefully pipetted out and the pellet containing cell debris and other cell organelles were discarded.
5. A small volume of supernatant was placed in the slides and covered with coverslip.

6. The slide was observed in a light microscope to find out viable protoplast

**Result:**

The spherical shaped protoplasts were observed using the microscope.

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Figure: Isolated protoplast

X-----X-----X-----X

## 6.ISOLATION OF PROTOPLAST BY ENZYMATIC METHOD

### **AIM:**

To isolate protoplasts by the enzymatic method

### **PRINCIPLE:**

Protoplasts are isolated by treating tissues with a mixture of cell wall degrading enzyme in solution, which contains an osmotic stabilizer. A most suitable source of protoplasts is mesophyll tissue from fully expanded leaves of young plants or new shoots. The release of protoplast is very much dependent on the nature and composition of enzymes used to digest the cell wall. There are three primary components of the cell wall which have been identified as cellulose, hemicellulase and pectin substance. Pectinase (macrozyme) mainly degrades the middle lamella while cellulase and hemicellulase degrade the cellulose and hemicellulosic components of the cell wall. During this enzymatic treatment, the protoplast obtained should be stabilized because the mechanical barrier of the cell wall which offered support has been broken. For this reason an osmoticm is added which prevents the protoplast from bursting.

### **MATERIALS REQUIRED:**

1. Young leaves
2. 70% ethanol
3. 2% cellulose
4. 13% mannitol
5. 0.5% macrozyme
6. CPW salt solution

### **PROCEDURE:**

1. The young leaves were collected and washed in sterile distilled water thrice.
2. The leaves were cut into small bits.
3. Then the leaves were kept immersed in 13% mannitol for 1 h for pre-plasmolysis.
4. Mannitol was removed after incubation ant sterilized enzyme mixture (Cellulase + macrozyme) was added and incubated at 25°C in a shaker for 12 h
5. The filtrate was centrifuged at 100g for 5 min to sediment the protoplast.
6. The supernatant was removed and the protoplast pellet was suspended in 10ml of CPW +21% sucrose solution.
7. The mixture was centrifuged at 100g for 5 min. The viable protoplast will float to the surface of the sucrose solution.
8. The supernatant containing protoplast was collected and viewed under the microscope.

## **RESULT:**

Protoplasts were isolated by the enzymatic method and viewed under the microscope



Figure: Isolated protoplast

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## **COMPOSITIONS**

### **CPW salt solution**

KH<sub>2</sub>PO<sub>4</sub> - 27.2mg/l

KNO<sub>3</sub> - 101mg/l

CaCl<sub>2</sub> - 1480mg/l

MgSO<sub>4</sub> - 246mg/l

KI - 0.16mg/l

CaSo<sub>4</sub> - 0,026mg/l

pH - 5.8.

X-----X-----X-----X

## 7. ANTER CULTURE

### **AIM:**

To isolate and inoculate anthers for haploid production.

### **PRINCIPLE:**

Haploids refer to those plants which possess a gametophytic number of chromosomes in their sporophytes. Haploids may be grouped into two broad categories:

- (a) monoploids which possess half the number of chromosomes from a diploid species.
- (b) Polyhaploids which possess half the number of chromosomes from a polyploid species.

Haploid production through anther culture has been referred to as androgenesis while gynogenesis is the production of haploid plants from ovary or ovule culture where the female gamete or gametophyte is triggered to sporophytic development.

### **MATERIALS REQUIRED:-**

1. Anthers from *Hibiscus*
2. MS medium
3. Growth factors- 2,4,D (1 mg/L) and BAP (1 mg/L)
4. 70% ethanol
5. 0.1% mercuric chloride
6. Scissors
7. Scalpels
8. Petri plates
9. Forceps.

### **PROCEDURE:**

1. Flower buds of *Hibiscus* were collected.
2. The flower buds are surface sterilized by immersing in 70% ethanol for 60 sec followed by immersing in 0.1% Mercuric Chloride solution for 1 min.
3. The buds were washed four or five times with sterile distilled water.
4. The buds were transferred to a sterile Petri dish.
5. The buds were split open using a blade and the anthers were removed without damage and the filaments were removed.
6. The anthers were placed horizontally on the MS medium supplemented with different concentrations of plant growth regulators.
7. The Petri plates were sealed and incubated in dark at 28°C.
8. The Petri plates were examined for the germination of anthers.

**RESULT:**

The anther underwent germination leading to the formation of haploid plantlets.

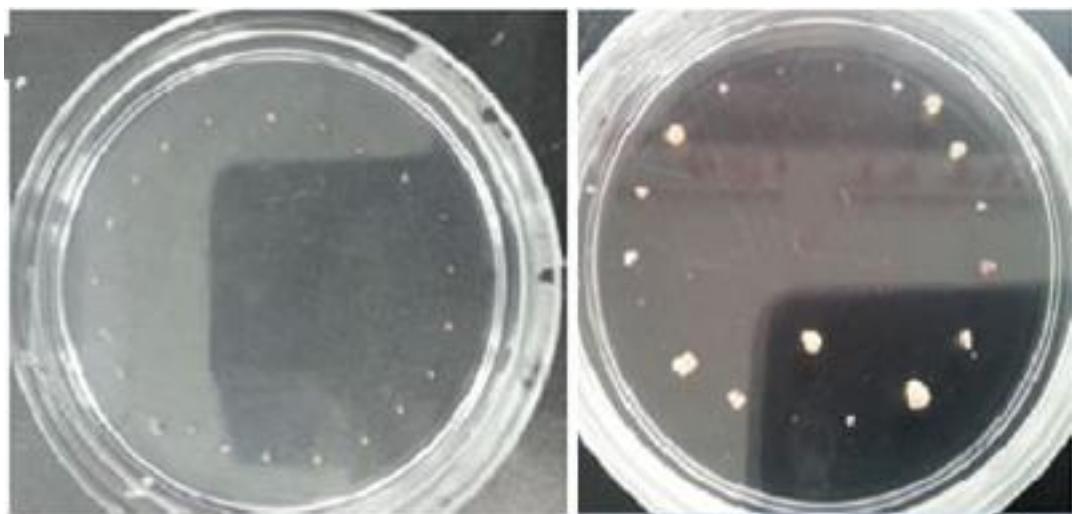


Figure: Anther culture

X-----X-----X-----X

## 1. ABO BLOOD GROUPING

**Aim:** To determine the ABO grouping of the blood sample.

**Principle:** All people belong to one of four inherited blood groups: A, B, AB and O. The letters A and B refer to the kind of antigen found on an individual's red blood cells. An antigen is a protein on the cell which triggers an immune response, such as the formation of antibodies, against the antigens which the red cell lacks. Most people also have an inherited condition of the red blood cells known as the Rh factor, or antigen D. When the D antigen is present, a person's blood type is designated Rh positive. When antigen D is missing, the blood type is classified Rh negative.

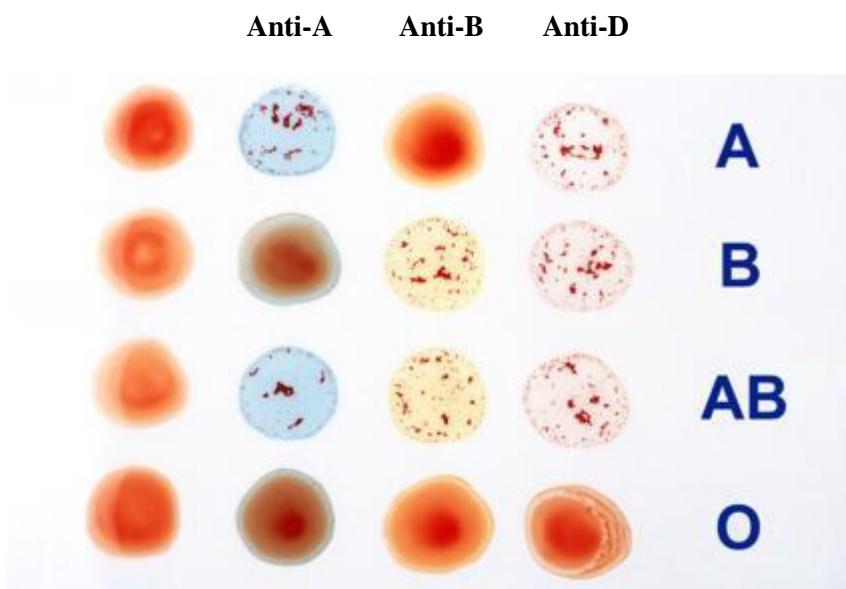
**Materials:** Glass slide, Anti-A, Anti-B, Anti-D, Blood sample, Applicator sticks, Tissue paper, Needle, Spirit, and Cotton.

**Procedure:**

1. Clean the glass slide and wipe it free of water.
2. Place a drop of blood by pricking the finger with needle in three different area.
3. Name the area as A, B and D.
4. Place anti-A in area A, anti-B in area B and anti-D in area D.
5. Mix the contents of each circle with separate wooden applicator stick and spread to fill the whole area of the individual circle.
6. Rock the slide for few seconds and observe for agglutination.

**Result:**

The blood groups of different individuals are identified.



## 2.DIFFERENTIAL WHITE BLOOD CELL COUNT

*(Differential leucocyte count)*

**Aim:**

To identify the different types of White Blood Cells (WBC)

**Principle:**

Differential staining is used to estimate the percentage of different WBC's percent in a given deciliter of the blood sample. There are different types of WBC's present within a human blood sample which can be differentiated on the basis of nuclear structure, shapes and presence of granules in the cytoplasm. WBC's are the most important cells within the human system and their estimation is very crucial for the diagnosis of various diseases like viral infections as well as hemolytic disorders. Leishman stain is a type of Romanowsky stain and is made up of eosin and methylene blue which selectively stains the nucleus, granules and cytoplasm of a cell. Eosin and methylene complex is dissolved in acetone-free methanol which acts as a chemical fixative for the blood smear. The stain dissociates in the presence of water and differentially stains different compounds of the cells.

**Material required:**

Glass slide, lancet, Leishman's stain

**Procedure:**

1. Place a drop of blood from the finger, about 2 mm in diameter in the central line of a slide about 1- 2 cm from one end.
2. The spreader is placed at an angle of 45 degrees to the slide and then moved back to make contact with the drop.
3. The drop should spread out quickly along the line of contact of the spreader with the slide.
4. The drop should be of such size that the film is 3- 4 cm in length.
5. The film should be dried rapidly. A good blood film preparation will be thick at the drop end and thin film at the opposite end.
6. The thickness of the spread when pulling the smear is determined by the :
  - Angle of the spreader slide.
  - Size of the blood drop.
  - Speed of spreading.

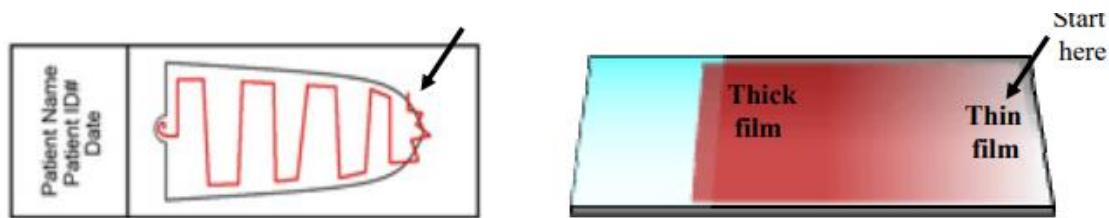
**Fixing of Blood Films**

Before staining, the blood films need to be fixed with acetone free methyl alcohol for 0.5 to 1 minute in order to prevent hemolysis when they come in contact with water when water has to be added subsequently. Alcohol denature the proteins and hardness the cell contents.

### Examination of a blood film

There are several necessary steps in the examination of a peripheral blood smear:

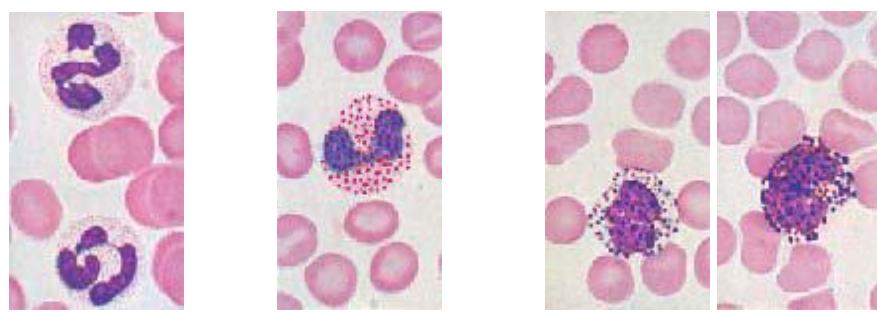
- 1- Under the low- power (10 X), determine the overall staining quality of the blood smear.
- 2- Determine if there is a good distribution of cells on the smear.
- 3- Find an optimal area for the detailed examination and enumeration of cells.



The high power evaluation (100x lens) is ideal for doing WBC differential counts in the monolayer of the smear. The monolayer is defined as the area of the smear where approximately half of the erythrocytes touch each other. Scan the blood smear in a meandering way, as shown in this figure, starting from the thinner edge of the blood film (arrow).

### The count

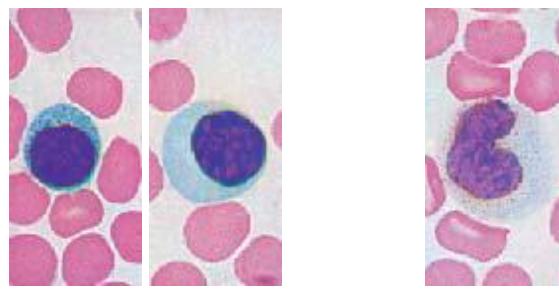
The dry and stained film is examined without a coverslip under the oil immersion objective. For differential leucocyte counts choose an area where the morphology of the cells is clearly visible. Do differential count by moving the slide in areas including the central and peripheral of the smear. A total of 100 cells should be counted in which every white cell seen must be recorded in a table under the following heading: *Neutrophil, Basophil, Eosinophil, Monocyte, and Lymphocyte*.



Neutrophil

Eosinophil

Basophil



Lymphocyte

Monocyte

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### **Stain preparation and staining**

#### **A- Leishman's stain**

Composition

Powdered Leishman's stain 0.15 gm

Methyl alcohol 133 ml

Method of staining

- 1- Pour a few drops (about 8) on the slide, wait for two minutes.
- 2- Add double the amount (16 drops) of buffered water. Mix by rocking and wait for 7- 10 minutes.
- 3- The stain is flooded off with distilled water and this should be completed in 2- 3 seconds, and allow it to dry.

X-----X-----X-----X

### 3. IMMUNOELECTROPHORESIS

**Aim:**

To learn the technique of Immunoelectrophoresis.

**Introduction:**

Immunoelectrophoresis is a powerful qualitative technique for the characterization of any antibody. In this method one antigen mixture is electrophoresed in an agarose gel that allows the separation of its different components based on their charge along the gel slide, followed by the lateral diffusion of the serum or monoclonal antibody within the gel. Antibodies specific to the antigens form white precipitation arcs which can be seen against a dark background. This technique is useful in determining the blood levels of three major immunoglobulins: IgM, IgG and IgA. The process combines the antigen separation technique of electrophoresis and immunodiffusion of the separated antigen against an antibody. It is used extensively to check the presence, specificity and homogeneity of the antibodies and can detect relatively high antibody concentrations.

**Principle:**

In immunoelectrophoresis, the antigen mixture is first electrophoresed to separate its constituents by charge. The antiserum containing the antibodies added into the troughs diffuses with a plane front to react with the antigens. Due to diffusion, density gradient of the antigen and antibody are obtained and at a specific antigen/antibody ratio (equivalence point) huge macromolecules are formed. They form a visible white complex which precipitates as arcs in the gel. The arc is closer to the trough at the point where the antigen is in highest concentration. The method is very specific and highly sensitive because distinct zones are formed. In this method, it is important that the ratio between the quantities of antigen and antibody be proper (Antibody titre).

**Materials Required:**

**Glasswares:** Conical flask, Measuring cylinder, Beaker

**Reagents:** Distilled water, alcohol

**Other requirements:** Incubator (37°C), Microwave or Bunsen burner, Electrophoresis unit, Vortex mixer, spatula, Micropipettes, Tips, Gel cutter, Moist chamber (box with wet cotton).

**Procedure:**

1. 10 ml of 1.5% agarose was prepared.
2. Side of the glass plate was marked and it was faced towards negative electrode during electrophoresis.
3. The solution was cooled to 55-60°C and poured 6 ml/plate on to grease-free glass plate placed on a horizontal surface. The gel was allowed to set for 30 minutes.
4. The glass plate was placed on the template provided.
5. A well was punched with the help of the gel puncture corresponding to the markings on

the template at the negative end. A gentle suction was used to avoid forming rugged wells.

6. Two troughs were cut with the help of the gel cutter, but the gel was not removed from the troughs.

7. 10 ml of the antigen was added to the well and the glass plate was placed in the electrophoresis tank such that the antigen well was at the cathode/negative electrode.

8. 1X Electrophoresis buffer was poured into the electrophoresis tank so that it just covers the gel.

9. Electrophoresis was performed at 80-120 volts and 60-70 mA, until the blue dye travels 3-4 centimetres from the well. (*Don't do electrophoresis beyond 3 hours, as it is likely to generate heat*)

10. After electrophoresis, the gel was removed from both the troughs and the plate was kept at room temperature for 15 minutes. 80 ml of antiserum A in one of the trough and antiserum B in the other.

11. The glass plate was placed in a moist chamber and incubated overnight at 37°C.

### **Observation and Result:**

Precipitin lines were observed between antiserum troughs and the antigen well. The formation of precipitin line indicates the presence of antibody specific to the antigen. Homogeneity of the antiserum to the antigen is denoted by presence of a single continuous precipitin line.



Figure showing precipitin line formation after immunoelectrophoresis

X-----X-----X-----X

## 4. OUCHTERLONY DOUBLE DIFFUSION (*Antibody Titration*)

### **Aim**

To learn the technique of Ouchterlony double diffusion for antibody titration.

### **Introduction**

Interaction between antigen and antibody at the molecular level forms the basis for several techniques that are useful in modern day scientific studies and in routine clinical diagnosis. These techniques are either based on the use of labeled reagents, a tracer or immunoprecipitation. Ouchterlony double diffusion (ODD) or double immunodiffusion technique is one of the simplest techniques extensively used to check antisera for the presence of antibodies for a particular Ag and to determine its titre.

### **Principle**

Precipitation of soluble antigen and antibody complexes as visible lattices in agarose can be utilized to find the ‘Titre’ value’ of the given antiserum. In Ouchterlony Double Diffusion technique a standard, uniform concentration of the antigen is subjected to diffusion with serially diluted antiserum samples. This is achieved when antigen sample is applied to a single central well, surrounded with suitable placed wells with serially diluted antiserum samples. Also, the same can be done in parallel rows of wells with constant antigen samples in all the wells of a row with corresponding rows of wells being loaded with the serially diluted antiserum samples. This later arrangement has the advantage with three rows of wells in a same agarose slide where, two sets of antisera samples can be tested against a common antigen. The highest dilution where the reaction between antigen and antibody stops is termed as the ‘Titre Value’ of the antiserum.

### **Materials Required**

**Glasswares:** Conical flask, measuring cylinder,

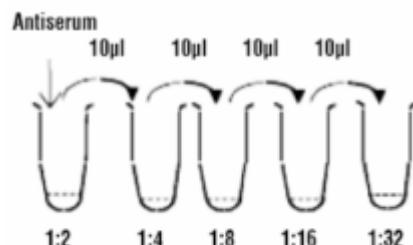
**Beaker Reagents:** Sterile distilled water, alcohol

**Other requirements:** Incubator (37 °C), Microwave or Bunsen burner, Vortex mixer, spatula, Micropipettes, Tips, Moist chamber (box with wet cotton)

### **Procedure**

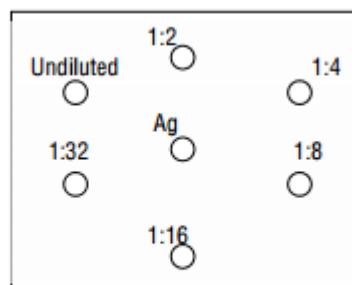
1. Prepare 10 ml of 1% agarose.
2. Cool the solution to 55-60 °C and pour 6 ml of the agarose solution on to grease free glass plate placed on a horizontal surface. Allow the gel to set for 30 minutes.
3. Place the glass plate on the template provided.
4. Punch wells with the help of gel puncher corresponding to the markings on the template. Use gentle suction to avoid forming rugged wells.
5. Serially dilute the test antiserum up to 1:32 dilution as described below:
  - a. Take 10µl of 1X assay buffer in each of the five vials.

- b. Add 10 $\mu$ l of test antiserum into the first vial and mix well. The dilution of antiserum in this vial is 1:2.
- c. Transfer 10 $\mu$ l of 1:2 diluted antiserum from the first vial into the second vial. The dilution is this is 1:4.
- d. Repeat the dilutions up to fifth vial as shown in figure 1



**Fig 1: Serial dilutions of antiserum**

6. Add 10 $\mu$ l of the antigen to the center well and 10  $\mu$ l each of undiluted, 1:2, 1:4, 1:8, 1:16, 1:32 dilutions of antiserum into the surrounding wells as shown in figure 2.



**Fig 2: Template for addition of antigen and antiserum to the wells.**

7. Keep the glass plate in a moist chamber overnight at 37°C.
8. After incubation, observe for opaque precipitin lines between the antigen and antisera wells.
9. Note down the highest dilution at which the precipitin line is formed, which is the titre value.

#### **Observation and Result:**

Observe for presence of precipitin lines between the center antigen well and the corresponding serially diluted antisera wells and note the titre value of the test antiserum

The titre value of antibody against the antigen was found to be \_\_\_\_\_.

**Interpretation:**

Precipitin lines will be observed between the center antigen and corresponding antiserum wells. The dilution of the antiserum where the precipitin line is no longer observed is the titre value of the antiserum.

X-----X-----X-----X

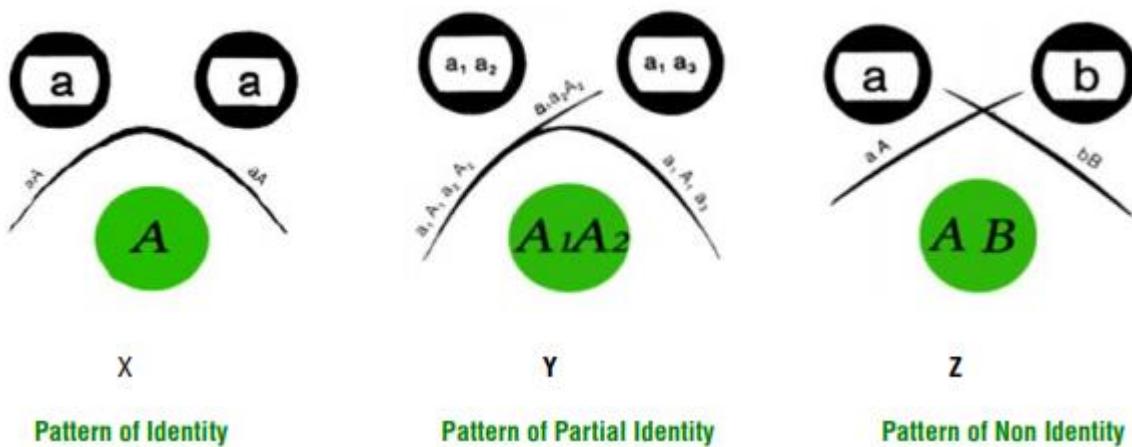
## 5. OUCHTERLONY DOUBLE DIFFUSION

### (Antigen Antibody pattern)

**Aim:** To study the reaction pattern of an antigen with a set of antibodies by Ouchterlony Double Diffusion method.

**Introduction:** Immunodiffusion in gels encompasses a variety of techniques, which are useful for the analysis of antigens and antibodies. Gel immunodiffusion can be classified into two groups: 1. Single Immunodiffusion 2. Double Immunodiffusion In the Ouchterlony double diffusion, both the antigen and the antibody diffuse toward each other in a semisolid medium to a point till their optimum concentration is reached. A band of precipitation occurs at this point. The qualitative Ouchterlony Test can simultaneously monitor multiple Antibody-Antigen system and can be used to identify particular antigens in a preparation. This procedure was developed by Örjan Ouchterlony in 1948.

**Principle:** When soluble antigen and antibody samples are placed in adjacent wells in agarose gel, they diffuse radially into the agarose gel and set up two opposing concentration gradients between the wells. Once the gradients reach to an optimal proportion, interactions of the corresponding molecules occur and a line of precipitation will form. Using such a technique, the antigenic relationship between two antigens can be analyzed. Distinct precipitation line patterns are formed against the same anti-sera depending on whether two antigens share all antigenic epitopes or partially share their antigenic epitopes or do not share their antigenic epitopes at all. The Ouchterlony test also can be used to estimate the relative concentration of antigens. When an antigen has a relatively higher concentration, the equivalent zone will be formed a little bit away from the antigen well. When an antigen has a relatively lower concentration, the equivalent zone will be formed a little bit closer the antigen well.



**Pattern of Identity:** X Pattern of identity occurs when the antigens in the two wells are identical and specific for the antibody in the antiserum present in the third well. The concentration of the two antigens being the same, they will diffuse at the same rate resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two

antigens i.e. the two antigens are immunologically identical.

**Pattern of Partial Identity:** Y Pattern of partial identity occurs when the antigens in the two wells share some epitopes which are same for both, yet each of the two antigens also have unique epitopes. In this case antiserum contains polyclonal antibodies specific for each epitope. When one of the antigen has some of the same epitopes compared to other, the polyclonal antibody population will respond differently to the two antigens and the precipitin line formed for each antigen will be different. The ‘spur’ is thought to result from the determinants present in one antigen but lacking in the other antigen. A similar pattern of partial identity is observed if the antibodies are cross reactive with an epitope on one of the antigen that is similar, but not identical to that present on the other antigen.

**Pattern of Non-Identity:** Z Pattern of non-identity occurs when the antigens in the two wells are totally different. They are neither cross reactive, nor do they have any epitopes which are same. In this case the antiserum containing the antibodies is heterogeneous as some of the antibodies react with antigen in one well while some react with antigen present in the other well. So the two antigens are immunologically unrelated as far as that antiserum is concerned

### **Materials Required:**

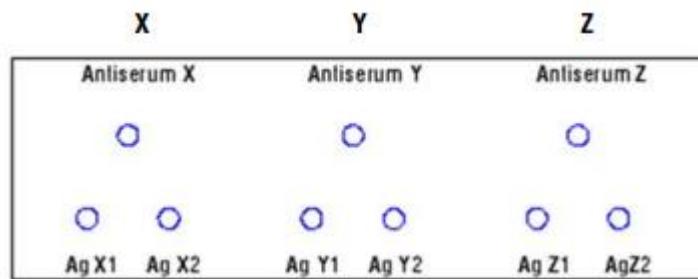
Glass wares: Measuring cylinder, Beaker

Reagents: Alcohol, Distilled Water

Other requirements: Incubator (37°C), Microwave or Bunsen burner, Vortex mixer, Spatula, Micropipettes, Tips, Moist chamber (box with wet cotton)

### **Procedure:**

1. Prepare 10 ml of 1% agarose in 1 X assay buffer.
2. Cool the solution to 55-60° C and pour 5 ml/plate on to grease free glass plates placed on a horizontal surface. Allow the gel to set for 30 minutes.
3. Place the glass plate on the template provided.
4. Punch wells with the help of the gel puncher corresponding to the markings on the template. Use gentle suction to avoid forming of rugged wells.
5. Add 10 µl each of the antiserum and the corresponding antigens to the wells
6. Keep the glass plate in a moist chamber overnight at 37° C.
7. After incubation, observe for opaque precipitin lines between the antigen and antiserum wells.



Template for the addition of antisera and antigen to their respective wells

### **Observation and Result:**

The presence of the precipitin line was observed between antigen and antisera wells.

### **Interpretation**

When antigen and antibody meet in optimal proportions a precipitation line is formed. In Ouchterlony Double Diffusion (Antigen Antibody Pattern), three patterns of precipitin lines can be observed. 1. If pattern X or pattern of identity is observed between the antigens and the antiserum, it indicates that the antigens are immunologically identical. 2. If pattern Y or pattern of partial identity is observed, it indicates that the antigens are partially similar or cross-reactive. 3. If pattern Z or pattern of non-identity is observed, it indicates that there is no cross-reaction between the antigens. i.e. the two antigens are immunologically unrelated.



**Fig 3: Diagram showing pattern of precipitin lines**

## 6.RADIAL IMMUNODIFFUSION

**Aim:** To study the immunodiffusion technique by Single Radial Immunodiffusion.

**Introduction:** Single Radial Immunodiffusion, also known as Mancini technique, is a quantitative immunodiffusion technique used to detect the concentration of antigen by measuring the diameter of the precipitin ring formed by the interaction of the antigen and the antibody at optimal concentration. In this method the antibody is incorporated into the agarose gel whereas the antigen diffuses into it in a radial pattern.

**Principle:** Single Radial Immunodiffusion is used extensively for the quantitative estimation of antigen. Here the antigen-antibody reaction is made more sensitive by the addition of antiserum into the agarose gel and loading the antigen sample in the well. As the antigen diffuses into the agarose radially in all directions, its concentration continuously falls until the equivalence point is reached at which the antigen concentration is in equal proportion to that of the antibody present in the agarose gel. At this point ring of precipitation ('precipitin ring') is formed around the well. The diameter of the precipitin ring is proportional to the concentration of antigen. With increasing concentration of antigen, precipitin rings with larger diameter are formed.

The size of the precipitin rings depend on

- Antigen concentration in the sample well
- Antibody concentration in the agarose gel
- Size of the sample well
- Volume of the sample

This test is commonly used in the clinical laboratory for the determination of immunoglobulin levels in patient samples.

### **Materials Required:**

**Glass wares:** Conical flask, Measuring cylinder, Beaker

**Reagents:** Distilled water, alcohol

**Other requirements:** Incubator (37°C), Microwave or Bunsen burner, Vortex mixer, spatula, Micropipettes, Tips, Moist chamber (box with wet cotton).

### **Procedure:**

1. 10 ml of 1% agarose gel was prepared. (6 ml of this gel solution was taken in a clean test tube).
2. The solution was allowed to cool down 55-60°C and 80 µl of antiserum was added to  
6 ml of agarose solution. Mixed well for uniform distribution of the antibody.
3. Agarose solution containing the antiserum was poured on to a grease free glass plate and placed on a horizontal surface. The gel was allowed to set for 30 minutes.
4. The glass plate was placed on the template provided.

5. Wells were made with the help of gel puncher corresponding to the markings on the template. Gentle suction was used to avoid forming rugged wells.
6. 10 µl of the given standard antigen and test antigen samples were added to the wells.
  - A. Standard Antigen A (3.75 mg/ml)
  - B. Standard Antigen B (7.5 mg/ml)
  - C. Standard Antigen C (15 mg/ml)
  - D. Standard Antigen D (30 mg/ml)
  - E. Test Antigen 1
  - F. Test Antigen 2
7. Incubate the glass plate in a moist chamber overnight at 37°C.

### **Observation & Results:**

The antigen wells surroundings were observed for the precipitin rings. The edges of the precipitin rings were marked and the diameter of the rings was measured.

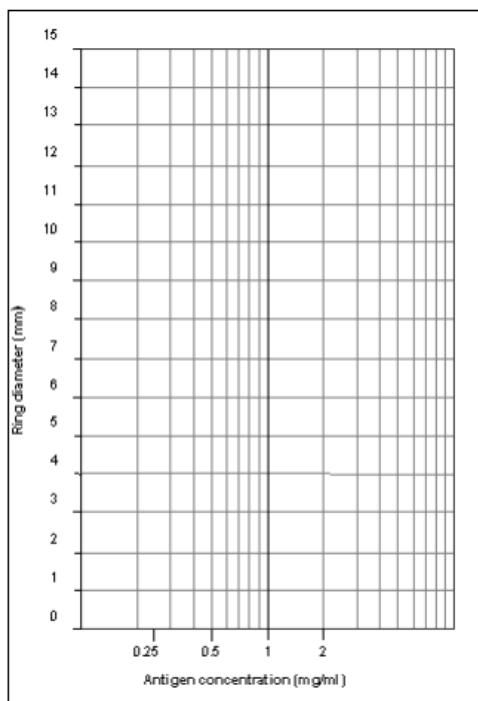
#### **Results of Single Radial Immunodiffusion**

<b>Sample</b>	<b>Standard Antigen Concentration (in mg/ml)</b>	<b>Ring Diameter (in mm)</b>
A	3.75	
B	7.5	
C	15.0	
D	30.0	
E	Test Antigen 1	
F	Test Antigen 2	

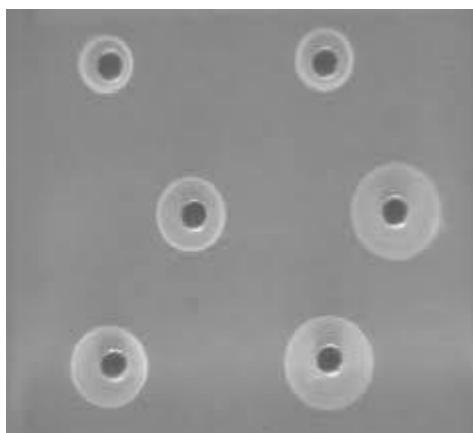
### **Interpretation:**

The diameter of the precipitin ring depends upon the concentration of antigens loaded in the wells. By plotting the graph of concentration of antigens versus diameter of the corresponding precipitin ring one can calculate the concentration of any test antigen.

Plot a graph of the diameter of the precipitin ring (on Y-axis) versus the concentration of antigen (on X-axis) on a standard graph sheet. Determine the concentration of the unknown antigen from the graph by finding the concentration against the ring diameter.



Standard Curve for RID Assay



Radial immunodiffusion

X-----X-----X-----X

## 7.WIDAL TEST

**Aim:** To perform Widal test, a serological method for the detection of *Salmonella* sp.,

**Introduction:** Widal test is a presumptive serological test for enteric fever or undulant fever whereby bacteria causing typhoid fever are mixed with serum containing specific antibodies obtained from an infected individual. The test is named after *Georges Fernand Widal*, a French physician and bacteriologist.

**Principle:** Patients' suffering from enteric fever would possess antibodies in their sera which can react and agglutinate serial doubling dilutions of killed coloured *Salmonella* antigens.

**Requirements:** Widal rack, round-bottomed Felix tubes, water bath, Test serum, killed coloured suspensions of *S.typhi* O antigen, *S.typhi* H antigen, *S.paratyphi* AH antigen and *S.paratyphi* BH antigen.

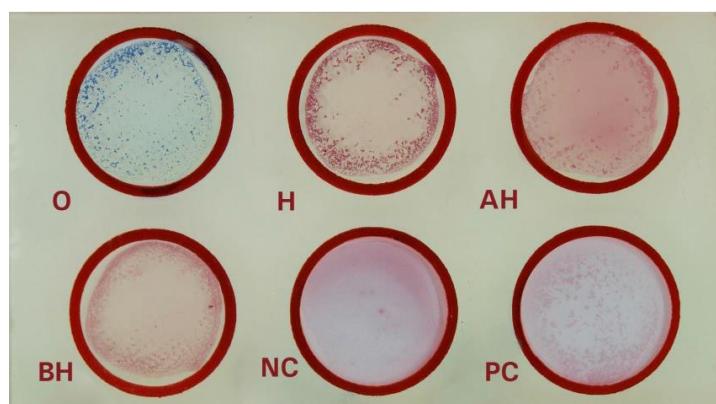
**Slide widal test:** A slide Widal test is more popular among diagnostic laboratories as it gives rapid results.

**Qualitative test:**

- One drop each of patients' serum samples for the four antigens was placed on the circled card.
- One drop of each of the four *Salmonella* antigens namely O, H, AH and BH were added separately and gently rotated for one minute.
- Appearance of agglutination was observed for qualitative results.

**Result:**

Visible clumps indicated positive agglutination and the serum is reactive to \_\_\_\_\_ except for \_\_\_\_\_.



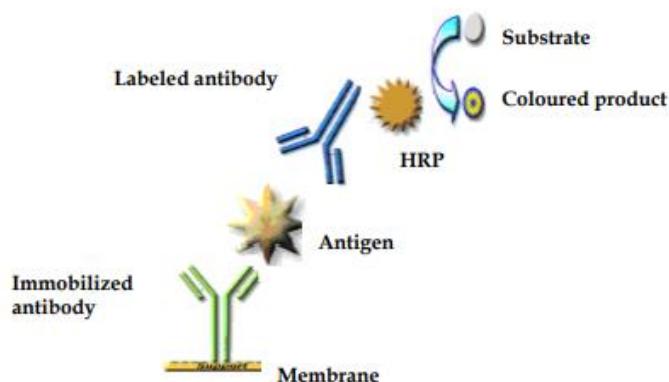
X-----X-----X

## 8.DOT ELISA

**Aim:** To learn the technique of Dot ELISA for the detection of an antigen.

**Introduction:** Enzyme linked immunosorbent assay or ELISA is a sensitive immunological technique to detect the presence of a specific antigen (Ag) or antibody (Ab) in a biological sample. It utilizes the dual properties of antibody molecules being specific in reactivity and their ability to be conjugated to active molecules such as enzymes. An enzyme conjugated with an antibody reacts with a chromogenic colourless substrate to generate a coloured reaction product. ELISA is extensively used for diagnostic purpose which utilizes the dual properties. It requires an immobilized antigen/antibody bound to a solid support (e.g. microtitre plate or membrane). There are different types of ELISAs for the detection of a protein of interest in a given sample. One of the most common ELISA is dot ELISA which can visually detect the presence of an antigen very quickly. The nitrocellulose dot technique was first developed for screening large number of hybridoma antibodies in 1983.

**Principle:** There are various forms of ELISA for the detection of antigen or antibody-based on antibody-antigen interactions. Dot ELISA, a qualitative ELISA test, can be performed very quickly with the end detection done visually. Because of its relative speed and simplicity, the dot ELISA is an attractive alternative to standard ELISA. In Dot-ELISA, small volumes of antibodies are immobilized on a protein binding membrane (Nitrocellulose) and the other antibody is linked to an enzyme Horseradish peroxidase (HRP). The test antigen at first reacts with the immobilized antibody and later with the enzyme-linked antibody. The amount of enzyme-linked antibody bound is determined by incubating the strip with an appropriate substrate (Hydrogen peroxide,  $H_2O_2$ ) and a chromogen [Tetramethylbenzidine (TMB)]. HRP acts on  $H_2O_2$  to release nascent oxygen, which oxidizes TMB to TMB oxide, which gives, a blue coloured product. The latter precipitates onto the strip in the area of enzyme activity and appears as a coloured dot, hence the name Dot-ELISA. The results can be visualized in naked eye. The enzyme activity is indicated by intensity of the dot, which is directly proportional to the antigen concentration.



### **Materials required**

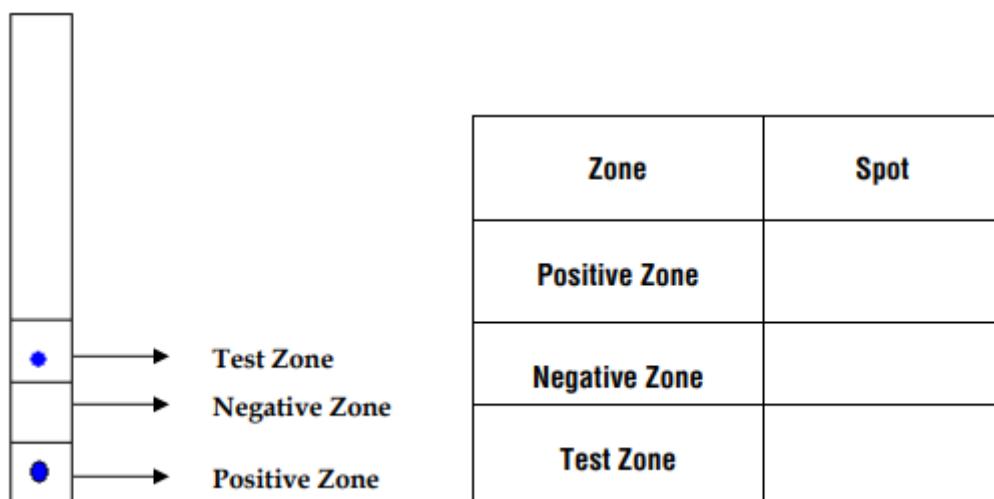
Test tubes , Distilled water, Micropipette, Tips, Himedia Dot Blot ELISA kit

### **Procedure:**

1. Take 2 ml of 1X Assay Buffer in a test tube and add 2  $\mu$ l of the test serum sample. Mix thoroughly by pipetting. Insert a Dot-ELISA strip into the tube.
2. Incubate the tube at room temperature for 20 minutes. Discard the solution.
3. Wash the strip two times by dipping it in 2 ml of 1X Assay Buffer for about 5 minutes each. Replace the buffer each time.
4. Take 2 ml of 1X Assay Buffer in a fresh test tube, add 2  $\mu$ l of HRP conjugated antibody to it. Mix thoroughly by pipetting. Dip the ELISA strip into it and allow the reaction to take place for 20 minutes.
5. Wash the strip as in step # 3 for two times.
6. In a collection, tube take 1.3 ml of TMB/H<sub>2</sub>O<sub>2</sub> and dip the ELISA strip into this substrate solution.
7. Observe the strip after 5 - 10 minutes for the appearance of a blue spot.
8. Rinse the strip with distilled water.

### **Observation and Result:**

Look for the appearance of the blue dot as shown.



**Interpretation:** Spot in the positive control zone and no spot in the negative control zone indicates proper performance of test. In the negative control zone the immobilized antibody is not present and the region is blocked with an inert protein. Therefore, there is no reaction when the reagents are added and no spot can be seen. In the test zone an antibody (specific to the test antigen, serum) is immobilized on it and then blocked with an inert protein. The

test serum binds to this region and the HRP-labeled antibody binds to serum which when reacts with substrate develops blue dot. In the positive control zone, the test serum binds to the immobilized antibody and the HRP-labeled antibody binds to serum which when reacts with substrate develops blue dot.

X-----X-----X-----X