RAMAIAH
College of Arts, Science
& Commerce - Autonomous

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DBT
Department of Biotechnology
Ministry of Science & Technology
Government of India

M S RAMAIAH COLLEGE OF ARTS, SCIENCE AND COMMERCE
- AUTONOMOUS

DEPARTMENT OF MICROBIOLOGY

ADDITIONAL EXPERIMENTS PROTOCOL

UNDER

DBT STAR COLLEGE SCHEME

DEPARTMENT OF MICROBIOLOGY

ADDITIONAL EXPERIMENTS CONDUCTED FOR I YEAR BSc

under DBT Star College Scheme

AIM

The aim of the experiment is to learn the wrapping of the glass wares before autoclaving so as to keep the glass wares in sterile condition after autoclaving for the use in microbiology experiments

INTRODUCTION

Sterilization is the complete removal or inhibition of all live forms from any object or place. Petri plates, flasks, beakers, pipettes, etc. are some of the generally required glassware in microbiological laboratories. Sterilization of glassware by moist heat using autoclaving is not very effective since moisture enters the glassware and may cause contamination problems. The most effective and used method of sterilization of glassware is dry heat treatment using hot air oven. On exposing the glassware to dry heat, the inhibition in growth occurs due to denaturation of proteins and oxidation of biomolecules. Duration of sterilization is temperature dependent: at higher temperature sterilization is achieved faster, i.e., at 170^0C, 30 min; 160^0C, 60 min; and 150^0C, 150 min or longer. Spores of Bacillus are used as biological indicator to test the efficacy of sterilization process. Since glass wares are sensitive to rapid changes in temperature, they are wrapped in paper and then only kept in hot air oven

PRINCIPLE

The glassware is wrapped with the newspaper before sterilization so that proper sterilization can be done and contamination can be avoided.

REQUIREMNTS

1. Petri plates
2. Pipettes
3. News-paper/ brown paper
4. Thread or rubber band

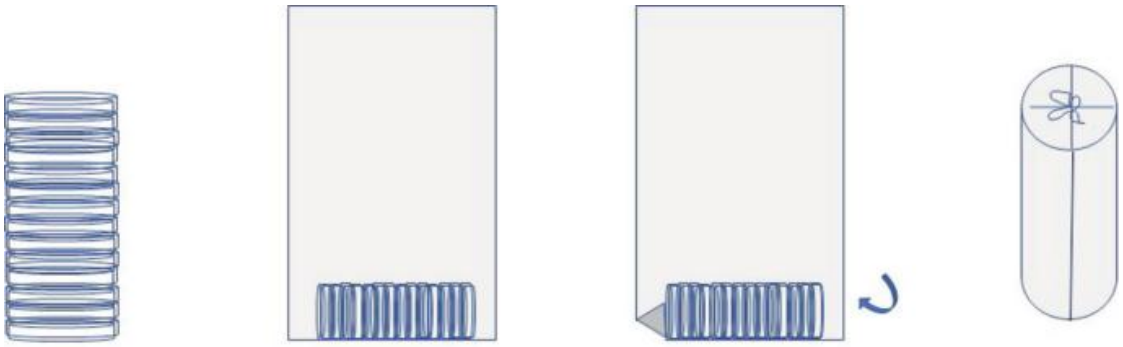
PROCEDURE

Wrapping of petri-plates

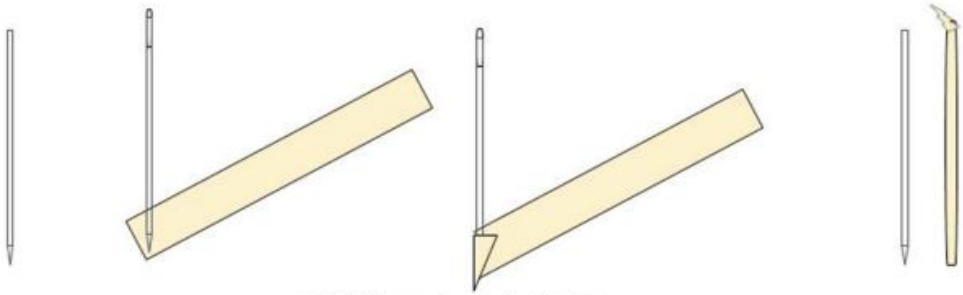
1. Take clean Petri plates and allow it to dry at room temperature.
2. Make a bundle of 5 Petri plates (upper lid and lower lid) by keeping the plates one above another; arrange the plate such that lower lid is toward base and upper lid covers it.
3. Tear newspaper/brown paper, to match the width of 5 plates, keep on the table to the width position, and keep the bundle of Petri plate at one edge.
4. The petri-plates are initially set properly to get a proper base and a fitting upper lid
5. With the help of the thumb, the newspaper is folded over the bundle of plate; the folding is continued with rotation to cover the circumference of the bundle. Check that the paper covers entire bundle.
6. Continue wrapping the petri-plates until the other end of the paper is reached
7. Gently press all sides of the brown paper and tie a thread or rubber band tightly

Wrapping of pipettes

1. Take clean glass pipette and put cotton beak on it's broader opening so that the mouth of the pipette is plugged with cotton before wrapping and sterilization.
2. Cut a strip of paper 15 cm 5 cm long.
3. Keep the narrower edge of pipette at an angle of 30^0 for the paper strip.
4. Screw and fold the paper at the other edge. Remove extra paper.
5. In the process of wrapping, fold both the ends of the paper strip so that it is enclosed.
6. Fold one edge tightly and then roll paper over pipette till its other edge.
7. Both the tips of the pipette are now completely wrapped and ready for sterilization.



(a) Wrapping of petri plates



(b) Wrapping of pipettes

EXPECTED RESULTS AND INTERPRETATION

Glass wares can be sterilized by wrapping which will keep the glassware in sterile condition. This process will avoid contamination during the microbiological work.

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Aseptic Transfer Technique

AIM

The main aim of the experiment is to avoid the contamination during microbiological media transfer.

INTRODUCTION

Louis Pasteur showed that microorganisms can be present in non- living matter – on solids, in liquids and in the air. In addition, he demonstrated conclusively in 1864 that microbial life can be destroyed by heat and that methods can be devised to block the access of airborne microbes to nutrient environments. In 1865, **Joseph Lister** who had read of Pasteur's work on pasteurization and Semmelweis's work on improving sanitation, initiated the use of diluted carbolic acid (phenol) on bandages and instruments to reduce infection in surgical wounds. These discoveries form the basis of **aseptic techniques**, techniques that prevent contamination by unwanted microorganisms, which are now the standard practice in microbiology laboratory, food processing industries, operation theatres and many other medical procedures. Modern aseptic techniques are among the first and the most important concepts that a beginning microbiologist learn.

PRINCIPLE

The aseptic transfer of culture means that no microbial contaminants are introduced into culture materials when the latter are inoculated or transferred from an agar slant culture to an agar slant, form broth culture to broth tube, from an agar plate to an agar slant, from and agar slant to an agar plate, and no contamination remains after you have worked with cultures. It also ensures that microbes that are being handled do not infect/contaminate the handler or others who may be present.

Prior to making any transfer, the work area is to be made microbes free by treating it with a disinfectant, transfer loops and needles need to be sterilized by inserting it into the flame of a Bunsen burner until it is red hot, growth medium is ascertained to be free from all kinds of living microbes. Microbes are usually transferred by inoculating loop/needle (straight wire), inoculating loop is used for surface inoculation and straight wire is used for stab culture alone or both stab and surface inoculation, and a special transfer needle with the curved (bent) end is used for transferring molds.

Aseptic transfer of bacterial cultures varies with different types of transfer or inoculation, which will be demonstrated here.

1. **TRANSFER (OR INOCULATION) OF BACTERIA FROM AN AGAR PLATE TO AN AGAR SLANT**

Transfer (or subculture) of a microbe growing on an agar plate is done for maintaining stock cultures. Subculturing of some of the cells from the colony is done with a sterilized loop (for bacteria and yeasts) and sterilized needle (for molds) under aseptic conditions as outlined in the procedure.

REQUIREMNTS

- Agar plate with bacterial colonies
- Sterile nutrient agar slant
- Inoculating loop
- Bunsen burner
- Glass marker

PROCEDURE

- With a glass marker, label a sterile nutrient agar slant with the organism to be transferred.
- Hold the inoculating loop in the right hand and flame it in the hottest portion of the flame until it is red hot.

- Allow the loop to cool for a few seconds in the air or cool it by dipping in a fresh agar plate.
- Hold the lid of the agar plate in the left hand and raise it just enough to access a colony ans pick up loopful of the organism.
- Pick up the loopful of the organism and remove the tube enclosure and grasp the cap with the little finger in the hand.
- Flame the mouth of agar slant and streak the agar surface in a zig0zag motion by moving the loop gently across the agar surface from bottom of slant to top.
- Remove the loop and flame the mouth of the tubes and recap the tube.
- Flame the mouth of agar slant and streak the agar surface in a zig-zag motion by moving the loo gently across the agar surface from bottom of slant to top.
- Remove the loop and flame the mouth of the tubes and recap the tube.
- Flame the inoculating loop and place it in the container.
- Incubate the inoculated slant for 24-48 hours at 37 C.
- Observe for the growth of the organism.

EXPECTED RESULTS

Appearance of growth of the organism at the area of the streak, similar to that on the agar plate represents a successful subculturing of the organism on the agar slant.

PRECAUTIONS

- Inoculating loop should never tear the agar surface.
- The lid (cover) of the petri plate should be only partially be opened at the time of removing of the organism form the agar plate to avoid air contamination.
- The inoculating loop should be moved gently across the agar surface.

2. TRANSFER OF BACTERIA FROM BROTH CULTURE TO NUTRIENT BROTH TUBE

Nutrient broth is a liquid medium (without agar) for culturing microorganisms and to study their biochemical characteristics and production of various industrial products. Organisms are also maintained in broth cultures as they are easy to carry similar to the agar slants.

Procedure for transferring a bacterium from a broth culture tube to a broth tube is described here.

REQUIREMENTS

- **Bacterial culture**: Broth culture of a bacterium (Escherichia coli or Bacillus subtilis)
- Sterile nutrient broth tubes
- Inoculating loop
- Bunsen burner
- Glass marker

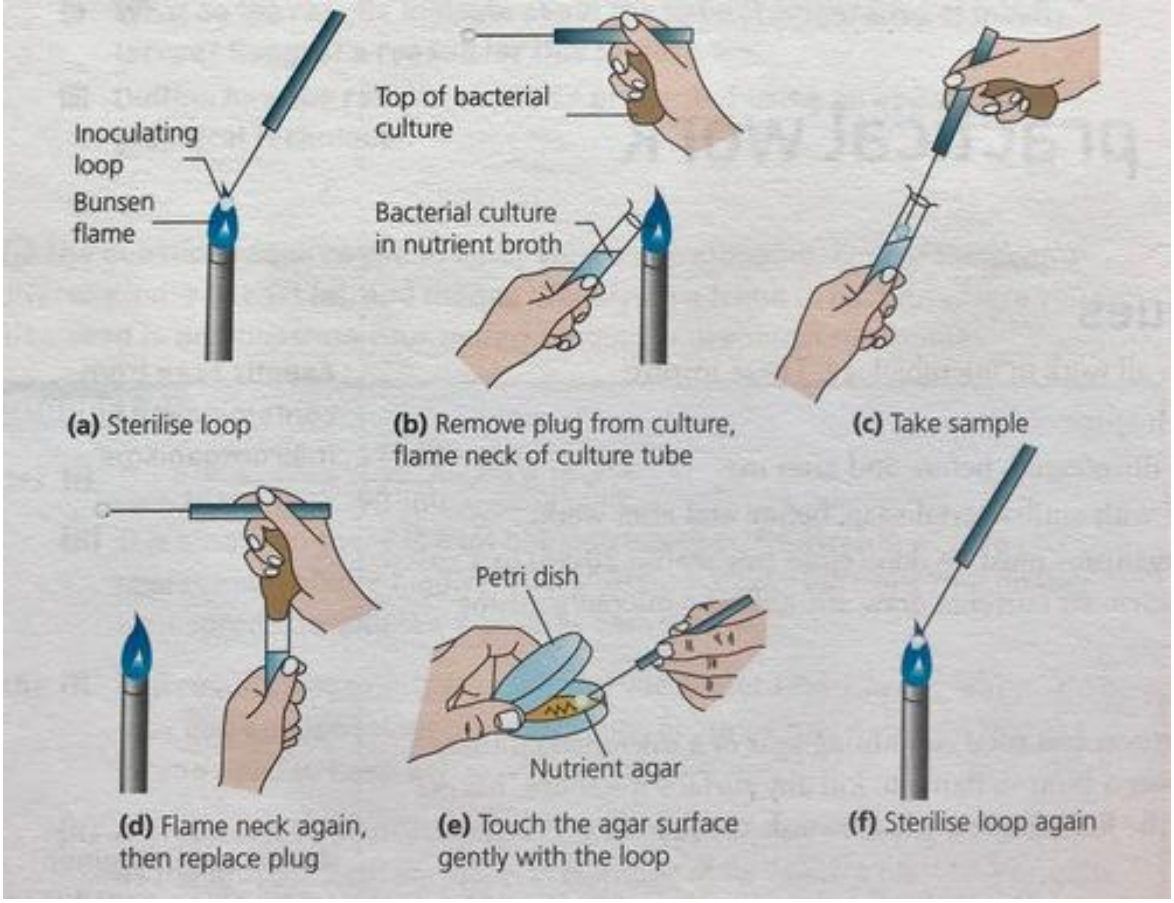
PROCEDURE

The procedure followed for transfer of culture from broth culture to sterile broth tube is same as for the transfer of slant culture to sterile agar slant except:

- For removing a culture (inoculum) from the broth culture, shake the broth culture well and the sterilised/flamed loop is dipped into the culture.
- While inoculating a nutrient broth, the loop having the inoculum is inserted into the sterile broth tube.
- Always disperse the organism in the broth culture before its removal by shaking the broth tube.
- Move the loop back and forth for the dispersal of the organism in the tube at the time of addition of inoculum onto the fresh nutrient broth tube.

EXPECTED RESULTS AND INTERPRETATION

Successful transfer of a culture from a broth culture to a fresh nutrient broth will result in the growth of a single culture of the same organism after 24-48 hours at 37 C. Prepare smears from the old and fresh broth cultures and compare the two organism is same in both tubes, demonstrating the successful transfer from a broth culture.


Inoculating loop
Bunsen flame
Top of bacterial culture
Bacterial culture in nutrient broth
Petri dish
Nutrient agar
(a) Sterilise loop
(b) Remove plug from culture, flame neck of culture tube
(c) Take sample
(d) Flame neck again, then replace plug
(e) Touch the agar surface gently with the loop
(f) Sterilise loop again

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Media Preparation

AIM:

PREPARATION OF NUTRIENT BROTH, MRBA, AGAR PLATES AND SLANTS

INTRODUCTION:

Nutrient agar, an example of complex medium (or chemically non defined medium), is a general-purpose medium used to culture non-fastidious bacteria. It is also used to isolate bacteria from the environment (air, soil, or floors). The constituents of the medium per litre are- peptone (50g), beef extract (3g) and sodium chloride(5g) with a pH of 7.0.

PRINCIPLE

The medium in liquid form (without sugar) is called a nutrient broth and when nutrient broth is added at a concentration of 2%, it is called nutrient agar. Agar used as a solidifying agent in the medium melts between 95^0C and 100^0C and solidifies at around 45^0C

Peptone (composed of shorter chains of amino acids, made by the partial digestion of proteins by acids or enzymes) and beef extract act as the sources of carbohydrates and amino acids.

REQUIREMENTS

- Constituents of the NA medium (Beef extract, peptone, sodium chloride and agar) or dehydrated NA medium
- Constituents for MRBA medium (Glucose 10g, Peptone 5g, K2HPO4 0.5g, KH2PO4 0.5g, MgSO4.7H2O 0.5g, Yeast extract 0.5g, Rose Bengal powder 0.03g, Agar 20g, Streptomycin sulfate 0.03g)

- Distilled water
- Flasks
- Culture tubes (20mm *15cm or 16mm *15cm)
- Measuring cylinders
- Glass stirring rods
- Hot plate/heater/Bunsen burner and tripod
- Cotton
- Magnetic stirrer
- pH meter /pH papers

PROCEDURE

Nutrient broth

- Weigh the constituents (beef extract 3g, peptone 5g, and sodium chloride 5g) and add these in distilled water (600 ml) and dissolve on a magnetic stirrer.
- Make the volume to 1 litre and check the pH with pH paper/ pH meter.
- In case of dehydrated medium, add the correct amount of the medium (as per the manufacturer specification mentioned on the label of the bottle) in a beaker containing 1000 ml distilled water and dissolve it by simple heating and check pH with pH test papers.
- Adjust its pH 7.2 with acid (1N or 0.1 HCL) or alkali (1N or 0.1 NaOH) using pH meter, as per recommendations or requirement.
- Dispense the medium 200 ml /250 ml Erlenmeyer flask or test tubes.
- Plug the flasks and tubes with the cotton plugs or plastic (polypropylene) caps, as the case be.
- Keep the stoppered flasks and tubes in the wire baskets.
- Sterilize the flasks/tube in an autoclave at 15psi steam pressure (121^0C) for 20 minutes.

Nutrient agar

- Take two flasks containing nutrient broth (200 ml/250 ml flask).
- Add 3 g agar powder to each flask and shake the contents vigorously.
- Keep the flask on hot plate and allow the agar to melt.
- Dispense the medium, as per requirement, in screw capped tubes/ tubes with cotton plugs, for making stabs and slants.
- When using a dehydrated agar medium, desired amount of the dehydrated medium is dissolved in a measured volume of water, and heated to dissolve the agar on a stirring hot plate/ or electric hot plate/heater/Bunsen burner, and dispense the melted constituents.
- Sterilize the flasks and tubes in an autoclave for 20 minutes at 15 psi pressure (121^0C)

Slants, Stabs and Nutrient agar plates

- Allow the autoclaved medium to cool in the autoclave to 55-65^0C
- For **agar stabs/deep tubes** preparation, arrange the agar tubes (6 ml medium) in a test tube rack in a vertical position, and be allowed to cool to room temperature.
- For **agar slants**, keep the agar tubes in a slanting position by resting these against glass rod or pipette or a piece of rubber tubing (1/2 diameter), the capped end of the tube facing upwards.
- For **agar plates**, arrange sterile petri plates on the LAF bench, hold the nutrient agar flask un the left hand, unplug the flask near the flame by holding the cotton plug in between fingers of the right hand.
- Transfer the flask to right hand and flame the mouth of the flask, open the sterile petri plate at an angle with left hand and pour the 12- 15 ml melted nutrient agar to each plate and immediately replace the lid and allow the agar to each plate and immediately replace the lid and allow the agar to solidify. After solidification, the nutrient agar plates can be used for culture work.
- In case of nutrient agar pours, aseptically transfer melted medium of one pour into a sterile Petri plate.

Preparation of MRBA (Martins Rose Bengal Agar)

- This media is used for fungal growth

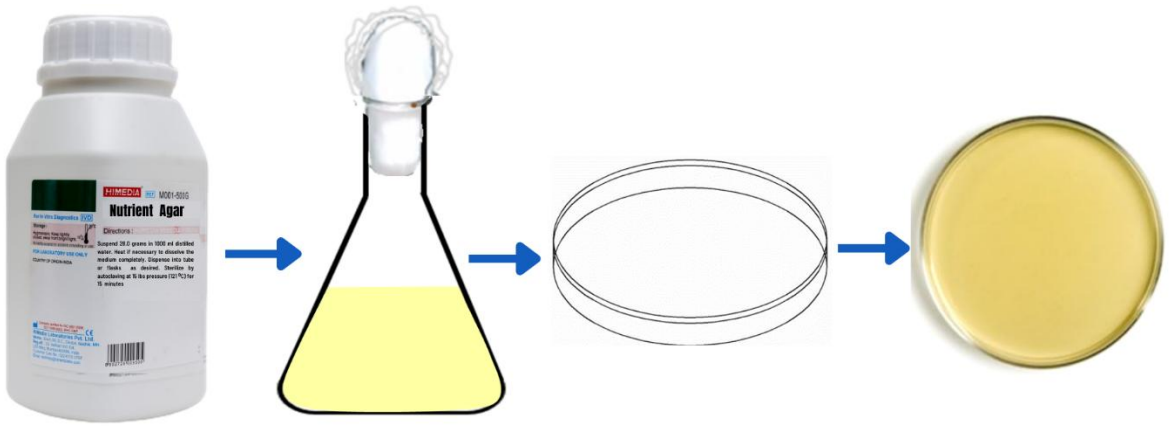
- Weigh the constituents (Glucose 10g, Peptone 5g, K2HPO4 0.5g, KH2PO4 0.5g, MgSO4.7H2O 0.5g, Yeast extract 0.5g, Rose Bengal powder 0.03g, Agar 20g, Streptomycin sulfate 0.03g) and add these in distilled water (600 ml) and dissolve on a magnetic stirrer.
- Make the volume to 1L and check the pH with pH test paper.
- In case of dehydrated medium, add the correct amount of the medium (as per the manufacturer specification mentioned on the label of the bottle) in a beaker containing 1000 ml distilled water and dissolve it by simple heating and check pH with pH test papers.
- Adjust its pH 4.5 with acid (1N or 0.1 HCL) or alkali (1N or 0.1 NaOH) using pH meter, as per recommendations or requirement.
- Dispense the medium 200 ml /250 ml Erlenmeyer flask
- Plug the flasks with the cotton plugs or plastic (polypropylene) caps, as the case be.
- Keep the stoppered flasks and tubes in the wire baskets
- Sterilize the flasks/tube in an autoclave at 15psi steam pressure (121^0C) for 20 minutes.
- Streptomycin is added in this media to avoid bacterial contamination which will be added after autoclaving when the temperature of the media is 45 to 50^0C
- Once the antibiotic is added, it is mixed and poured into sterile petri plates or test tubes to make slants

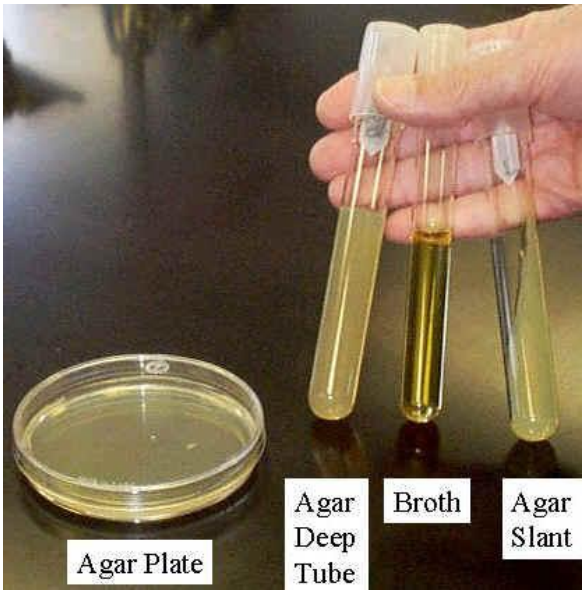
EXPECTED RESULTS AND INTERPRETATION

A semi solid medium in agar plates, agar slants, deep tubes and in broth tubes without any visible microbial growth 24-48 hours post storage at room temperature indicates a proper nutrient agar and MRBA media.

Learning Outcome:

Media for growth of Bacteria and Fungi are made in the liquid form or solid form either on plates or tubes (slants)


HIMEDIA
Nutrient Agar


Agar Plate
Agar Deep Tube
Broth
Agar Slant

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Aim:

The main purpose of simple staining is to determine the cell shape, size, and arrangement of bacterial cells.

Introduction:

The simple stain can be used as a quick and easy way to determine the cell shape, size, and arrangement of bacteria. True to its name, the simple stain is a very simple staining procedure involving a single stain solution. Any basic dye, such as methylene blue, safranin, or crystal violet, can be used to colour the bacterial cells.

These stains will readily give up a hydroxide ion or accept a hydrogen ion, which leaves the stain positively charged. Since most bacterial cells and cytoplasm surface is negatively charged, these positively charged stains adhere readily to the cell surface. After staining, bacterial cell morphology (shape and arrangement) can be appreciated.

Principle

In simple staining, the bacterial cells are first fixed on a clean, oil-free slide and then flooded with stain (safranin, methylene blue, carbol fuchsin, Crystal violet, etc). This stain will produce a distinctive contrast between the organism and its background so that we can easily distinguish them.

These stains will readily give up a hydroxide ion or accept a hydrogen ion, as result they become positively charged. Hence, the bacterial cell wall components and cytoplasm are negatively charged, which strongly attracts with the and binds to the cationic chromogen.

Materials Required

1. 24 hours old bacterial culture (*Bacillus subtilis, Escherichia coli,* and *Staphylococcus aureus.)*
2. Methylene blue or crystal violet or safranin
3. Staining tray
4. Glass slide
5. Inoculating loop
6. Bunsen burner/spirit lamp
7. Blotting paper

Procedure

Preparation of a smear

1. Using a sterilized inoculating loop, transfer a loopful of liquid suspension containing bacteria to a slide (clean grease-free microscopic slide) or transfer an isolated colony from a culture plate to a slide with a water drop.
2. Disperse the bacteria on the loop in the drop of water on the slide and spread the drop over an area the size of a dime. It should be a thin, even smear.
3. Allow the smear to dry thoroughly.
4. Heat-fix the smear cautiously by passing the underside of the slide through the burner flame two or three times. It fixes the cell in the slide. Do not overheat the slide as it will distort the bacterial cells.

Staining

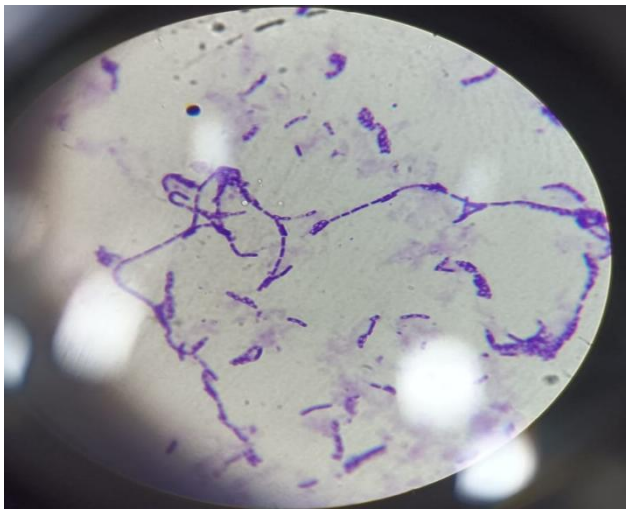
1. Cover the smear with methylene blue/Crystal Violet/ Safranin and allow the dye to remain in the smear for approximately one minute (Staining time is not critical here; somewhere between 30 seconds to 2 minutes should give you an acceptable stain, the longer you leave the dye in it, the darker will be the stain).

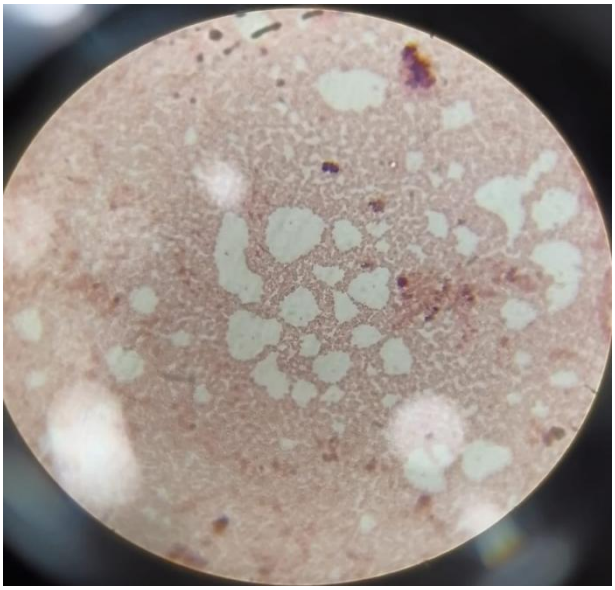
2. Using distilled water wash bottle, gently wash off the excess methylene blue from the slide by directing a gentle stream of water over the surface of the slide.
3. Wash off any stain that got on the bottom of the slide as well.
4. Place the stained smear on the microscope stage smear side up and focus the smear using the 10X objective.
5. Choose an area of the smear in which the cells are well spread in a monolayer. Center the area to be studied, apply immersion oil directly to the smear, and focus the smear under oil with the 100X objective.

Result and Discussion: The bacterial cells usually stain uniformly and the color of the cell depends on the type of dye used. If methylene blue is used, some granules in the interior of the cells of some bacteria may appear more deeply stained than the rest of the cell, which is due to the presence of different chemical substances.

Learning Outcome:

- Bacilli and diplobacilli will appear in rod-shape and in purple colour (crystal violet).
- Spirilla will appear in spiral-shaped and in purple colour (crystal violet).
- Cocci will appear in spherical-shaped and in purple colour (crystal violet).





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Agarose Gel Electrophoresis

Introduction: Agarose gel electrophoresis is a procedure used to separate DNA fragments based on their molecular weight and is an intrinsic part of almost all routine experiments carried out in molecular biology.

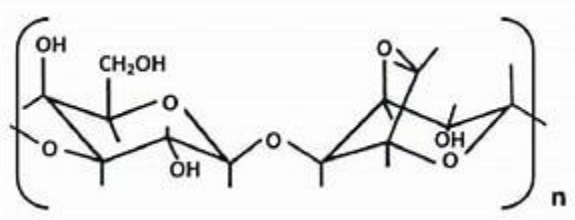
The technique consists of 3 basic steps:

1. Preparation of agarose gel
2. Electrophoresis of DNA
3. Visualization of DNA fragments

Principle

Preparation of agarose gel:

Agarose is a linear polymer extracted from sea weeds. Its basic structure is shown in the figure.



Purified agarose is powder insoluble in water or buffer at room temperature but dissolves on boiling. molten solution of agarose is then poured into a mould and allowed to solidify. As it cools agarose undergoes polymerization, sugar polymers cross links with each other and cause the solution to gel, the density or pore size of which is determined by agarose

Electrophoresis of DNA fragments

Electrophoresis is a technique used to separate charged molecules. DNA is negatively charged at neutral Ph and when electric field is applied across the gel, DNA migrates toward the anode. Migration of the DNA through the gel is dependent on

1. Molecular size of DNA
2. Agarose concentration
3. Conformation of DNA
4. Applied current

Matrix of agarose gel acts a molecular scene through which DNA fragments move on application of electric current. Higher concentrations of agarose give firmer gels, spaces between cross linked molecule are less and hence smaller DNA fragments easily crawl through these spaces. As the length of the DNA increases its becomes harder for the DNA to pass through these spaces. Lower concentration of agarose helps in movement of larger DNA fragments as the spaces between the cross link is more. The progress of gel electrophoresis is monitored by observing migration of the visible dye through the gel. Two commonly used dyes are xylene cynol and bromophenol blue that migrate at the same speed as double stranded DNA of size 5000bp and 300bp respectively. These tracking dyes are negatively charged. Low molecular weight compounds that are loaded along with each sample at the start of electrophoresis when the tracking dye reaches toward the anode, electrophoresis is terminated

Visualization of DNA fragments

Since DNA is not naturally coloured, it will not be visible on the gel. Hence, the gel after electrophoresis is stained with a dye specific to the DNA. Discrete bands are observed when there is enough DNA present to bind to the dye and make it visible, otherwise the band is not detected. The gel is observed against the light background wherein DNA appears as dark coloured bands.

Alternatively, an intercalating dye like ethidium bromide is added to agarose gel and bands are visualizes by examining the gel under UV light DNA appears fluorescent

Procedure

Preparation of 1% agarose gel

1. Prepare 1xTAE by diluting appropriate amount of 50xTAE buffer (for one experiment, approximately 200ml of 1xTAE is required dilute 4ml of 50x TAE to 200ml with distilled water)
2. Weigh 0.5g of agarose and add to 50ml of 1x TAE, this gives 1% agarose.
3. Boil till agarose dissolves completely and a clear solution is obtained.
4. Meanwhile place the comb of electrophoresis set such that it is approximately 2cm away from the cathode.
5. Add 2ml ethidium bromide to molten agarose (from a stock of 10mg/ml in water), when the temperature is around 55^0C mix and caste the gel

6. Pour the agarose solution in the central part of the tank when the temperature reaches approximately 55^0C. Do not generate air bubbles, the thickness of the gel should be around 0.5 to 0.9cm. keep the gel undisturbed at room temperature for the agarose to solidify.

Electrophoresis

7. Pour 1x TAE buffer into the gel tank till the buffer level stands at 0.5 to 0.8cm above the gel surface
8. Gently lift the combs ensuring that wells remain intact
9. Connect the power cord to the electrophoretic power supply according to the convention. Red = anode, black= cathode.
10. Load the samples in the wells in the desired order
11. Take 10ml of DNA sample+5ml of loading dye
12. Load the sample in the well
13. Set the voltage to 50V and switch on the power supply
14. Switch off the power when the tracking dye from the well reaches $3/4^{th}$ of the gel this takes approximately one hour.
15. After electrophoresis DNA samples can be visualized under UV light, they appear fluorescent, no destaining is required in this case.

Result:

The DNA present in the sample was successfully separated based on their shape and molecular weight.

Quantification of DNA:

There are mainly three methods used for measurement of DNA concentration. These methods are based on the spectrophotometric measurement of UV absorbance of binding of fluorescence dye.

The advantage of spectrophotometric measurement is that the amount of protein concentration cab also be determined by measuring the OD at 280nm.

The disadvantage of this method is that it is sensitive to contaminating RNA which can lead to overestimation of DNA concentration.

Spectrophotometric determination of DNA

Transfer of 10-100µl of DNA sample to 900-990µl of TEb (Ph 7.5) in a small cuvette. Mix the contents thoroughly and record OD from 250nm-320nm. A smooth peak should be observed within an absorption maximum at 260nm and no noticeable should be at 280nm. The 260:280nm absorbance rate should be more than 1.9. if the ratio is less than 1.9, then contamination with protein should be suspected.

Calculate the concentration of DNA as follows

$$\text{ds DNA conc. in µg/ml} = \frac{\text{measured OD 260 x 50mug/ml}}{\text{OD 260 x dilution factor}}$$

$$\text{ss DNA conc. in µg/ml} = \frac{\text{measured OD 260 X 36mug/ml}}{\text{OD 260 x dilution factor}}$$

Note:

1. An OD 260 of 1 corresponds to 50µg/ml ds DNA or 36µg/ml ss DNA
2. OD 260 of 1 corresponds to 40µg/ml solution of ss RNA

Result and Discussion:

DNA fragments were separated by Agarose Gel Electrophoresis and Visualized under UV transilluminator.

Learning Outcome:

Students were given hands on training for preparation of reagents and separation and visualization of DNA fragments using Agarose Gel Electrophoresis.

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Isolation of Plasmid DNA from Bacteria and Separation by Agarose Gel Electrophoresis

INTRODUCTION AND PRINCIPLE: -

Alkaline lysis: - The universal method for plasmid DNA extraction was invented by Bimboim and Doly in 1979. The lysis buffer contains NaOH and SDS. The purpose of which is to completely denaturation of the plasmid and genomic DNA that is separate the DNA into single stands. It is critical that this step is performed quickly because too long in the denaturing condition of this solution may result in irreversibly denatured plasmid at the end.

Next sample is neutralized in a potassium acetate solution to renature the plasmid and this is the key to the plasmid separation and genomic DNA.

ROLE OF DIFFERENT CHEMICALS IN PLASMID DNA: -

Cell growth and harvesting: - Bacteria will be grown in a media to achieve sufficient growth. Then cells are pelleted by centrifugation of removal from the growth medium.

Re – Suspension: - Pellet is then suspended in a solution containing Tris, EDTA, glucose and RNase A, divergent cat ions Mg 2+, Ca 2+ are essential for DNase activity and the integrity of the bacterial cell wall. EDTA chelates divalent cations in the solutions preventing DNase from damaging the plasmid and also helps by destabilizing the cell wall. Glucose maintenance the osmotic pressure so the cells don't burst and RNase A is included to degrade cellular RNA when the cells are lysed.

Lysis: - The lysis buffer solution -3 Contains NaOH and SDS detergent. SDS is there to solubilize cell membrane. NaOH helps to break down the cell wall also it disrupts the hydrogen bonding between double standard DNA and single standard DNA (genomic and plasmid both). This process is called alkaline lysis.

SDS also determines proteins in the cells which helps with the separation of proteins from the plasmid later in the process.

Do not carry out vigorous mixing because genomic DNA will be broken down.

Neutralization: - Addition of solution -3 potassium acetate written decreases the alkalinity of the mixture under these conditions the hydrogen bonding between bases of single standard DNA can be reestablished, to form double stranded DNA. It is easy for small circular plasmid DNA to renature it is impossible to properly anneal those huge genomic DNA stretches. While double stranded plasmid DNA can dissolve easily in the solution. The single stranded genomic DNA, SDS and denatured cellular proteins stick together through hydrophobic interactions to form white precipitate which can be separated by centrifugation.

Cleaning and concentration: - can be done by phenol chloroform step and precipitation by ethanol.

Phenol chloroform: - it will remove all proteins when phenol chloroform is added. It will not be mixed with water so two layers are formed upper aqueous phase and lower organic phase.

DNA is hydrophilic so it will remain in upper aqueous layer.

On the other hand, proteins which contain polar and nonpolar groups (hydrophobic and hydrophilic groups which are retained by folding) will be comfortable to be in organic phase so they will come in lower organic phase.

Role of salt and ethanol in precipitate: - role of salt and ethanol in precipitation is to neutralize the charges in the sugar phosphate backbone.

Sodium acetate – Na^+ and CH_3COO^-

Na^+ will neutralize PO_3^- groups on DNA and make molecules less hydrophilic and less solution in water.

Electrostatic attraction between Na^+ and PO_3^- is affected by dielectric constant of the solution.

Water has high dielectric constant which makes difficult for Na^+ and PO_3^- to come together.

Ethanol has much lower dielectric constant making it much easier for Na+ and PO3- to interact and shield its charge and make the NA less hydrophilic and causing it to drop out of the solution.

REAGENTS: -

1. Alkaline Lysis solution – 1
   50mM of glucose; 25mM of Tris; 10mM of EDTA
   pH of solution – 8.

Note: - autoclave the solution and then use.

2. Alkaline Lysis solution – 2
   0.2N of NaOH and 1% of SDS/SLS.
   (Freshly prepared and store that room temperature).
3. Alkaline Lysis solution – 3
   3M of potassium acetate
   pH of solution – 5.4

Note: - pH should be adjusted with glacial acetic acid.

4. Phenol chloroform (1:1)
5. T10E1 buffer: -
   10mM of Tris; 1mM of EDTA
   pH of buffer – 8

PROTOCOL: -

1. 1.5ml culture.
2. To get at 4^0C at 10000 RPM for 10 minutes discard the supernatant.
3. Add 100 µl of alkaline Lysis solution-I (ice cold) vortex and incubate the vials on the ice for 10-15 mins.
4. Add 200 µl of alkaline lysis solution-II.
5. Mix by inverting 5 times rapidly.
6. Keep in ice for 5 mins.
7. Add 250 µl of ice-cold alkaline lysis solution-III mix properly.

8. Keep on ice for 5-10 mins and centrifugate at 4^0C, at 10000 RPM for 5-10 mins.
9. Transfer supernatant to new tube and add equal volume of 1:1 (phenol chloroform).
10. Mix by vertexing and Centrifugate at 4 ^{0}C for 2 minutes.
11. Transfer the supernatant to new tube and add double volume of ice-cold ethanol or isopropanol.
12. Keep overnight at 4^0C centrifuge, at 10000 RPM at 4^0C for 5 to 10 minutes.
13. Discard the supernatant and dissolve the pallet in 20 µl of T10E1 buffer.
14. Vertex properly.
15. Add 10 µl of Gel loading buffer and load the samples
16. Detect using agarose gel electrophoresis.

RESULT:

The plasmid DNA is isolated and separated using agarose gel electrophoresis.

Learning Outcome:

Students were trained for preparation of different reagents and chemicals used for plasmid DNA isolation and given hands on experience on isolation of plasmid DNA and separation using Agarose Gel Electrophoresis

DEPARTMENT OF MICROBIOLOGY

ADDITIONAL EXPERIMENTS CONDUCTED FOR II YEAR BSc

under DBT Star College Scheme

Restriction Digestion of the Substrate DNA

Aim: To perform restriction digestion of lambda DNA using Eco RI and Hind III enzymes.

Introduction: In 1978, the noble prize for medicine was awarded to Warner Arber, Daniel Nathars and Hamilton Smith for their discovery of restriction endonucleases, which led to the development of recombinant DNA technologies. The restriction enzymes have been discovered in many different bacteria and other multicellular organisms. These restriction enzymes are able to scan along a length of DNA looking for a particular sequence of bases that they recognise.

Principle: Restriction digestion involves fragmenting DNA molecule into smaller pieces with special enzyme called as restrictions endonuclease commonly known as restriction enzyme (R.E). Because of this property the restriction enzymes are also known as molecular scissors. The restriction enzymes are named from the cellular strains from which they are isolated. Restriction enzymes recognise specific sequence in the double stranded DNA molecules [for ex: - GAATTC] and then cut the DNA to produce fragments called restriction fragments. The target site or sequence which the restriction enzyme recognise is generally from 4-6 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 enzyme will continue to do this along the full length of DNA molecule which will then break into fragments. The size of these fragments is measured in base pairs or kilo base pairs [1000 bases].

Materials Required:

- Lambda [λ] DNA
- DNA marker

- Agarose
- Restriction enzyme: Eco RI
- Restriction enzyme: Hind III
- 10x, assay buffer for EcoRI
- 10x, assay buffer for Hind Ill
- Molecular biology grade water
- 50x, TAE
- 6x, gel loading buffer
- Poly propylene tubes - 0.5 ml
- Ethidium Bromide - 10 mg/ml
- Electrophoresis apparatus and UV transilluminator

Procedure:

1. Place restriction enzymes (EcoRI, Hind III), substrate (lambda DNA) and assay buffers on ice.
2. Thaw the assay buffers vials completely.
3. Label fresh vials with the name of enzymes and keep them on ice.
4. Set up the restriction digestion reaction was follows:

Components	Volume to be added		
	Vial 1 (Eco RI)	Vial 2 (Hind III)	
Nuclease free water	30 µl	30 µl	To make up to 40 µl
10x assay buffer	4 µl	4 µl	Final conc. becomes1x
DNA	4 µl	4 µl	1µg
Enzyme	2 µl	2 µl	10 units
Total volume	40 µl	40 µl	

Note: - Gently mix the components by finger flicking after every addition. Maintain the storage temperatures for enzymes and assay buffers while using also.

5. Incubate for 1hour at 37°C in water bath/ drybath/ incubator.
6. Stop the reaction after one hour by adding 3µl of gel loading dye to the vials.
7. Prepare 1% agarose gel.
8. Add 10µl of distilled water into a fresh 1.5ml tube. Add 3µl of λ DNA and 2µl of gel loading dye into this vial.
   Label this vial as uncut [lane 2]
9. Load samples in separate wells in 1% agarose gel in the following order.

1	2	3	4
Marker 1kb ladder	Uncut λ DNA	Eco RI digest	Hind III digest

Note: Load 10µl of marker DNA.

Load the digested samples completely in lanes 3-4.

Agarose Gel Electrophoresis:

1.Preparation of Agarose Gel: To prepare 50ml of 1% agarose gel, measure 0.5g agarose in a glass beaker or flask and add 50ml TAE buffer. Heat the mixture on a microwave or burner, swirling the glass beaker/ flask occasionally until agarose dissolves completely/ (ensure that the lid of the flask is loose to avoid build-up of pressure). Allow the solution to cool to about 55-60°C. Add 0.5µl Ethidium bromide, mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 mins at room temperature.

2.Loading of DNA samples: Load 3µl of ready to use DNA marker into well-1. To prepare sample for electrophoresis, add 2 ul of 6x gel loading buffer to 10 µl of DNA samples. Mix well by pipetting and load the samples into the well.

3. Electrophoresis: Connect the power card to the electrophoresis power supply according to the conventions: Red - anode and Black-cathode. Electrophoresis at 100-120V and 90 mA until dye markers have migrated an appropriate distance depending on size of DNA to be visualized.

4.Result and Interpretations: Restriction Digestion patterns of λ DNA obtained upon treatment with Eco RI and Hind III are marked differently which demonstrates the fact that each restriction enzymes 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 run on the same gel.

5.Applications of Restriction Enzymes: Construction of recombinant DNA molecules and DNA libraries, mapping the locations of restriction sites, southern blot hybridisation.

Result:

The substrate DNA (λ DNA) is digested with restriction endonuclease enzyme viz. EcoRI and Hind III and fragments were separated and analysed using Agarose Gel Electrophoresis.

Learning outcome:

Students were given hands on training in restriction digestion of substrate DNA and analysis of fragments and molecular weight determination using Agarose Gel Electrophoresis.

DEPARTMENT OF MICROBIOLOGY

ADDITIONAL EXPERIMENTS CONDUCTED FOR II YEAR BSc

under DBT Star College Scheme

In Vitro DNA Ligation

Aim: To perform ligation reaction using T4 DNA ligase.

Introduction and Principle:

DNA ligation is the act of joining together DNA strands with covalent bonds with the aim of making new viable DNA or plasmids. DNA fragments with either sticky ends or blunt ends can be inserted into vector DNA with the aid of DNA ligases. Ends of DNA strands may be joined by the enzyme polynucleotide ligase, called 'glue' of the recombinant DNA molecule. The enzyme catalyses the formation of a phosphodiester bond between the 3'OH and 5'P terminals of two nucleotides. The enzyme is thus able to join unrelated DNA, repair nicks in single strand of DNA and join the sugar phosphate backbones of the newly repaired and resident region of a DNA strand.

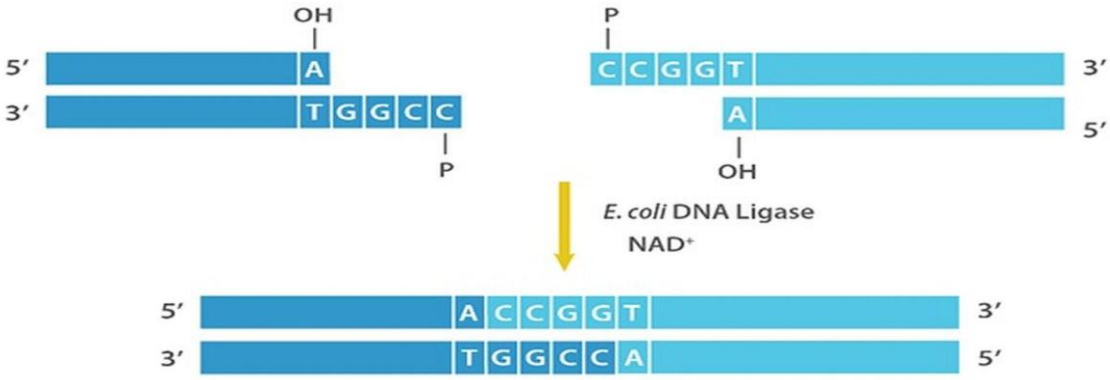
Bacteriophage T4 DNA ligase is a single polypeptide with a M.W of 68,000 Dalton requiring ATP as energy source. The maximal activity pH range is 7.5-8.0. The enzyme exhibits 40% of its activity at pH 6.9 and 65% at pH 8.3. The presence of Mg++ ion is required and the optimal concentration is 10mM. T4 DNA ligase has the unique ability to join sticky and blunt ended fragments. Cohesive end ligation is carried out at 12°C to 16°C to maintain a good balance between annealing of ends and activity of the enzyme. If reaction is set at higher temperatures annealing of the ends become difficult, while lower temperatures diminish the ligase activity. All T4 DNA ligase is inactivated by heating at 65°C for 10 minutes. Beside of these ligating complementary sticky ends, T4 ligase can ligate any two blunt DNA ends. Lack of cohesive termini makes blunt end ligation more complex and significantly slower. Since

annealing of ends is not a factor, the reaction is done at 24˚C. However, 10 - 100 times more enzyme is required to achieve similar ligation efficiency as that of cohesive end ligation. The enzyme has involved in the catalysis of the joining of RNA to either a RNA or DNA strand in a duplex molecule but this will not involve in the joining of single stranded nucleic acids.

The ligation reaction is controlled by several factors, such as pH, temperature, concentration and kinds of sticky ends, etc. As ligase uses the ends of DNA molecules as substrates rather than the entire DNA, the kinetics of joining depend on the number of ends (concentration) available for joining.

There are 4 types of DNA ligase

1. E. coli DNA ligase
2. T4 DNA ligase
3. Mammalian DNA ligase
4. Thermostable DNA ligase



A typical ligation reaction requires the following components:

- 2 or more fragments of DNA that has either blunt or cohesive ends
- a buffer which contains ATP
- T4 DNA ligase

Materials required:

- Lambda DNA-Hind III digest

- 10X ligase assay buffer
- T4 DNA ligase
- Molecular biological grade water
- Agarose
- 50X TAE buffer
- 6X gel loading buffer

Procedure:

1.Thaw the ligase assay buffer, Lambda\EcoR1 DNA digest completely place on ice .

2.set up flag is reaction as follows

Component	Quantity
Nucleus free water	15µl
Lambda DNA/EcoR1 digest	2µl
Ligase assay buffer	2µl
T4 DNA ligase	1µl
Total volume	20µl

3. Incubate at 16 degrees Celsius for 3 hours in a preset water for ligation

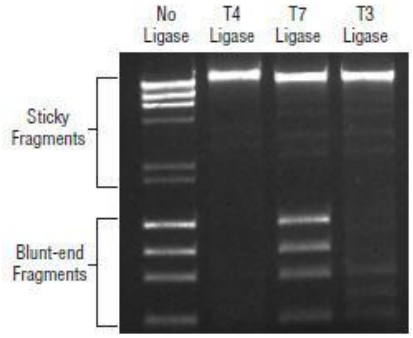
4.add 2 µl of gel loading buffer to the ligated sample

5.pipette out 2 µl of Lambda DNA digest in to fresh vials and add 10 µl water and 2µl of gel loading dye (digested control) close

6.meanwhile prepare 1% agarose gel

7.load ligated samples along with control digest in 1% agarose gel and subject to electrophoresis

Interpretation of the result:



Result and Discussion:

λ DNA digested with EcoRI enzyme was successfully ligated with the help of T4 DNA ligase and results were analysed using Agarose Gel Electrophoresis

Learning Outcome:

Students were trained to carry out molecular biology experiment of in vitro DNA ligation and also interpret the results obtained by agarose gel electrophoresis.