

I n d e x

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Experiment - 1

→ Aim:- Introduction to the lab equipments

→ Material:-

Pipettman, weighing balance, laminar hood, spectrophotometer, oven (hot air), autoclave, water bath

→ Observations:-

i. Pipette:-

• Applications:- measuring small amount of solvents and solutions, to collect and dispense liquid

• Working :- A plunger is depressed by the thumb and principle as it is released, the liquid is drawn into a disposable tip. When the plunger is pressed again, the liquid is dispensed

• Precautions:-

a. Never forcibly expel any hazardous material from the pipette.

b. Carefully eject the disposable pipette tips to minimise aerosol formation



2. Autoclave :-

- Applications:- sterilize media in the lab and ensure growth of desired microbes

- Working :- Machine that creates an environment principle of high temperature and pressure inside it. Operated at 121°C and 15 psi, uses principle of steam sterilization (for 20 minutes)

- Precaution:-

Steam valve should be tightened before use to ensure there is no leak, and released before opening after completion

3. Laminar hood :-

- Application:- For contamination sensitive processes and operation of particle sensitive electronic devices are performed inside the cabinet.

- Working :- Filtered air passes through the particulate filter which facilitates the removal of micro contaminants and achieves a sterile environment

- Precautions:-

a. UV light and air flow should not be used at the same time

b. Laminar flow cabinet should be sterilized with the UV light

4. Water bath:-

- It is an instrument used for indirect heating. As many lab substances cannot be heated directly, they are placed in a water reservoir and heat is provided through it.

5. Heating Oven:-

- Used for sterilization of lab apparatus and drying. It is based on the principle of heat sterilization, as high temp. causes the microbes to die.

6. UV Spectrophotometer:-

- Used to check the concentration of a given liquid and works on the principle of how much light is absorbed. Light is passed through the sample and a reference.

7. Weighing Balance:-

- A sensitive weigh scale that can be used to measure the weight of very small amounts of substance.

→ Experiment observed:-

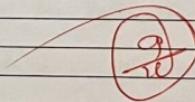
- To use the laminar hood, we would turn on the light & the blower, after cleaning the platform with 10% ethanol, we would pour the LB Agar solution in into the petri dish (the one with more thickness) and then cover with the lid. Before covering, streaking is done.

Expt. No. 1

- Streaking is a technique used to isolate a pure strain from a single species of microorganisms. It is done using a sterile tool such as ~~as~~ cotton swab or commonly an inoculation loop. Also to sterilize the equipment. Turn on the UV light and plate the equipment for 20 minutes.

→ Conclusion:-

We conclude that the e-coli is now cultured when in the petri-dish


Signature: *Hemanth*
Date: 01/01/22

Teacher's Signature

Experiment - 2

→ Aim:- To document the growth and characterization of E. coli in Luria-Bertani (LB) Agar

→ Material required :-

- LB Agar powder
- Sterile petri dish
- Starter culture for E. coli
- Sterile loop or spreader
- Incubator
- Personal Protective Equipments (PPE)
- Parafilm
- laminar flow hood

→ Theory :-

- E. coli is a commonly used model organism in molecular biology & genetic engineering research, and the ability to grow and maintain the bacteria in the laboratory setting is crucial for many experiments.
- The LB Agar medium is a medium rich growth medium that supports the growth of a wide variety of bacteria, including E. coli.

→ Procedure :-

- Prepare the LB Agar according to the manufacturer's instructions
- Pour the LB Agar into the petri dishes and allow to solidify
- Sterilize the loop or spreader by flaming it or dipping it in alcohol.

Expt. No. 2



E. coli culture in LB Agar

- Obtain a starter culture of E. coli & streak it onto the surface of the agar using the loop.
- The petri plate is now covered with a lid and sealed with parafilm.
- Place the petri plate upside down in the incubator for 24 hours. 16°C.
- Observe the plates after incubation and record the number and appearance of the colony.

→ Results :-

- Following incubation, numerous colonies of E. coli were visible on the surface of the agar.
- The colonies appeared as small, round and smooth.
- Average number of colonies were around 50 per plate.

→ Conclusion :-

In conclusion, E. coli can be easily grown in LB Agar, a rich growth medium that supports the growth of a wide variety of bacteria. The experiments demonstrate the ability to maintain and propagate E. coli in a lab setting.

→ Precautions :-

Use sterile techniques when handling the bacteria and the growth medium.

Always work in a laminar hood or a designated area with proper ventilation.

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Experiment - 3

→ Aim:- Isolation of pure microbial culture and quantification of viable cells

→ Material required :-

- Test tube ✓
- Laminar flow hood ✓
- Autoclave ✓
- Incubator ✓
- Petri plates ✓

→ Theory :-

Serial dilution are used to calculate the concentration of microorganisms. As it would usually be impossible to actually count the number of microorganism in a sample, the sample is diluted & plated to get a reasonable no. of colonies to count. Since each colony on an agar plate theoretically grew from a single microorganism, the no. of colonies or Colony Forming units is representative of the no. of viable microorganisms. Since the dilution factor is known, the no. of microorganisms per ml in the original sample can be calculated.

Expt. No. 3

→ Procedure :-

- Obtain a culture of *E.coli* and label it with the appropriate information
- Prepare a sterile saline solution and set it aside
- Using a sterile pipette, transfer 1 mL of the *E.coli* culture into a sterile, labeled test tube.
- Using a sterile pipette, add 9 mL of sterile saline solution to the test tube, resulting in a 1:10 dilution.
- Mix the contents of the test tube by gently flicking it.
- Using a sterile pipette, transfer 1 mL of the 1:10 dilution into a new labeled test tube.
- Add 9 mL of sterile saline solution to the new test tube, resulting in 1:100 dilution. Mix the contents of the test tube.
- Repeat the above steps for 1:1000 and 1:10000 dilutions.
- Using the spread-plate technique, transfer a small amount of each dilution to separate labeled petri plate.
- Incubate the plates for 24 hours.
- Count the no. of colonies on each plate and record the results in a table.
- Using dilution factor & the no. of colonies, calculate the concentration of bacteria in the original culture.

→ Observation Table and Calculations :-

$$\text{Colony Forming Units (CFUs)} = \frac{\text{Colonies Formed}}{\text{DF} \times \text{mL plated}} \quad \text{gml}^+$$

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S.No.	Vol. of Medium	Vol. of sample	Total Vol	Dilution Fold	Plates	No. of Colonies	CFU/mL
1.	9mL	1mL	10mL	10-Fold	10 ¹	13950714	1.39x10 ⁸
2.	9mL	1mL	10mL	10 ² -Fold	10 ²	1214358	1.21x10 ⁸
3.	9mL	1mL	10mL	10 ³ -Fold	10 ³	118869	1.18x10 ⁸
4.	9mL	1mL	10mL	10 ⁴ -Fold	10 ⁴	10997	1.09x10 ⁸

Mean = 1.21×10^8 CFU/mL

→ Conclusion :-

In conclusion, the serial dilution of E coli experiment is an important tool for quantifying bacterial cells. The number of colony forming unit per milliliter in the parent culture is roughly 1.21×10^8

→ Precautions :-

- It is important to maintain sterile techniques throughout the experiment to prevent contamination.
- It is important to inoculate the plates at the appropriate temperature.

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Expt. No. 4

Experiment - 4

→ Aim:- To perform gram staining on a bacterial sample and determine the gram-positive and gram-negative ~~nature~~ nature of the bacteria.

→ Material Required:-

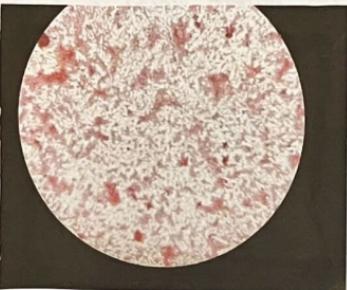
- Gram crystal violet (S_012) ($Sd^m A$) (Crystal violet + ethyl alcohol)
- Gram crystal violet (S_012) ($Sd^m B$) (Ammonium oxalate + distilled water)
- Gram's decolorizer (S_032) (Ethyl alcohol + acetone)
- Gram's iodine (S_013) (Iodine + potassium iodine + distilled water)
- Safranin, 0.5% w/v (S_021) (Safranin O + ethyl alcohol 95%)
- Bacterial culture (~~saliva~~ or saliva)
- Microscope slides
- Microscope
- Bunsen burner
- Loop or spreader
- Pipette or dropper

→ Theory:-

Gram staining is a widely used technique in microbiology for identifying and classifying bacteria based on their cell wall composition.

Gram-+ve bacteria have a thick mesh-like wall made of peptidoglycan (50-90%) and are stained purple by crystal violet and gram-negative bacteria have a thinner layer (10%), so do not retain the purple stain & are counter stained pink by safranin.

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(g). Pink stained gram-negative bacteria under microscope

→ Procedure :-

- Solution A and B are mixed and stored for 24 hours before use. The resulting stain is stable.
- Prepare a thin smear on clean, dry glass slide.
- Allow it to air dry and fix by gentle heat.
- Flood with Gram's Crystal violet (S_012) for 1 minute.
- Drain the stain.
- Flood the smear with Gram's Iodine (S_013). Allow it to remain for 1 minute.
- Decolorise with Gram's Decolourizer (S_032) until the blue dye no longer flows from the smear.
- Wash with tap water.
- Counter stain with 0.5% w/v Safranin (S_022). Allow it to remain for 1 minute.
- Wash with water.
- Allow the slide to air dry or blot dry by sheets of clean filter paper & examine under oil immersion objective.

→ Observation and result :-

- When the slide is observed under a microscope, we can observe that majority the bacteria is pink in colour.
- Hence, we can conclude that the bacteria in our sample are majority gram-negative.

→ Precautions:-

- Over staining results in improper decolorization of known gram-negative organisms. Use less crystal violet.
- Handle bacteria cultures and stains with care, and avoid spilling or splashing them.
- Use the microscope's diaphragm and aperture to adjust the lighting and focus.

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Experiment - 5

→ Aim: Preparation of fresh competent cells of *E. coli*.

→ Material required:-

- DHS_α host cells stock
- MgCl₂, 0.1 M (autoclaved)
- Luria Bertani medium - Bactotryptone : 10gm
 - + Yeast extract : 5 gm
 - Sodium chloride : 10 gm
- CaCl₂, 0.1 M (autoclaved)
- Ampicillin (100 mg/ml) filter sterilized

→ Principle:-

The ability of the taking the DNA by a bacterial cell is called competence. *E. coli* cells can be made competent chemically. These cells are able to take foreign DNA (recombinant plasmids or amplicons). The DNA is added to competent cells on ice. During a heat shock at 42°C, the cells are transformed. Once the *E. coli* cells are transformed, the DNA can be extracted easily.

→ Procedure:-

- Prepare LB-Agar medium for petri plate (100 ml for 5 plates). Let petri plates cool down to room temp and store them at 4-8°C in a refrigerator.
- Prepare LB agar liquid media (50 ml) in a 150/250 mL conical flask.

Expt. No. 5

- Prepare 0.1 M $MgCl_2$ (500 ml) and autoclave.
- Prepare 0.1 M $CaCl_2$ (500 ml) and autoclave
- * Inoculation ~~and growth preparation~~:
 - Inoculate 100 μl of cells from the frozen glycerol stock
 - into 5 mL of LB medium. Grow the cells overnight at 37°C at 200 rpm.
- ~~All harvest. Inoculation and growth~~
- * Inoculate 1 ml of inoculum to 50 mL LB in conical flask and allow the cells to grow at 37°C, 200 rpm.
- * Cell harvest:
 - Allow the cells to grow till $OD_{600\text{nm}} = 0.4-0.6$ for about 2 hours.
 - Transfer the flask to ice and cool the cells for 10 min.
- * Competent cells:
 - Centrifuge 40 mL culture from the flask in a 50 mL centrifuge tubes at 4000 rpm, at 4°C 10 min. Discard the supernatant.
 - Resuspend the cell pellet gently first in 1-2 mL and then, 20 mL (in each tube) of 0.1 M $MgCl_2$ (Ice cold).
 - Centrifuge at 4000 rpm, at 4°C 10 min. Discard the supernatant.
 - Resuspend cells gently in 2 mL (each tube) of 0.1 M $CaCl_2$ (Ice cold).
 - Leave the cells at 0°C (on ice) for 2 hrs.
 - The cells can be stored at 0-4°C for a week or use directly for transformation.

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→ Observation:-

- We observe that after the UV centrifugation, we get different set of values after different time slots.

$$\lambda = 600 \text{ nm}$$

Sample No.	Absorption	$K^* \text{ Abs}$
1.	0.0386	23494
2.	0.1453	95.898

- If we shake the sample, the solution gets mixed properly as denser liquid will settle down due to gravity and will not give accurate result

→ Precautions:-

- Calibrate the weighing machine to zero before measuring the powder weight.
- Mix the LB Agar powder completely in the distilled water.
- Don't touch the culture from the bottom as it can create error.

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Teacher's Signature

Expt. No. 6

Experiment - 6

→ Aim - Transformation of competent cells with plasmid DNA.

→ Material required -

- DH5 α host cells
- Plasmid DNA
- SOC medium - Bactotryptone : 10gsm 2%
- Bacto yeast extract : 0.5%
- Sodium chloride : 10mM
- KCl : 2.5mM
- MgCl₂ : 10 mM
- MgSO₄ : 10mM
- Glucose : 20 mM
- LB Agar Medium
- Ampicillin
- Petri plates
- Auto dome Autoclave
- Water Bath (42°C)
- 800 incubator shaker (37°C)
- Ice Shaker.

→ Theory -

The uptake of the DNA by the cells is called transformation. The transformation can be brought about by heat shock ~~treatment~~ treatment as well as by electroporation. The DNA is added to competent cells on ice.

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During a heat shock at 42°C the cells are transformed. Once the *E. coli* cells are transformed, the DNA can be extracted easily and amplified. The selection of the right colonies is done on LB agar medium containing ampicillin because only those transformed cells will grow which contain plasmid that has ampicillin resistance gene.

→ Procedure:-

- Take $200\text{ }\mu\text{l}$ of freshly prepared competent cells in 1.5 mL tube and keep on ice for 10 min.
- Take $10\text{ }\mu\text{l}$ ($2-3\text{ mg}$) of plasmid DNA & add to $200\text{ }\mu\text{l}$ of competent cells, gently mix by micro-pipette and incubate on ice for 30 min, undisturbed.
- Subject the mixture (DNA + ~~plasmid~~) to a heat shock treatment at 42°C , for exactly 40 sec and place back on ice for ≤ 5 min.
- Add $800\text{ }\mu\text{l}$ of SOC medium (42°C) to the tube containing cells and DNA.
- Incubate the mixture in incubator shaker at 37°C , 200 rpm for 1 hr.
- Centrifuge the ~~mix~~ cells at 1600 rpm at 25°C for 10 min, remove carefully $800\text{ }\mu\text{l}$ of the supernatant from the top.
- Resuspend the cells in the remaining $200\text{ }\mu\text{l}$ supernatant in the same micro tube.
- Take the cells in the laminar hood and pour the $200\text{ }\mu\text{l}$ cells using sterile micro tips on

Expt. No. 6

LB Agar plates (containing ampicillin, 100 µg/ml) and spread the cells with a sterile glass spreader.

- Dry the plates for 15 min in the laminar hood.
- Invert the plates and incubate the incubator at 37°C for overnight.
- Count the number of transformed cells and compare with control.
- Calculate the transformation efficiency.

→ Observations :-

$$\text{CFU} / \mu\text{g DNA} = x \cdot y \cdot z$$

x → no. of DNA plasmid used

y → No. of colony observed

z → Dilution factor of DNA

- A large number of colonies will be formed on the LB Agar plates containing ampicillin.
- The number of colonies on the LB agar plates containing ampicillin will be much higher compared to the negative control plate.
- The colony forming unit per μgm (CFU/ μg) comes out to be $\approx 10^7$ ^{How?}

→ Result :-

We obtained a bacterial culture of E. coli which contains ampicillin resistance plasmid, hence, the culture is ~~of~~ ampicillin resistance.

→ Precautions :-

- Use precise timing for heat shock which is 40 sec at 42°C to avoid over or under treatment.
- Centrifugation of the cell should be done properly done to avoid damaging the cells.
- Mixing of cells and DNA must be done very gently to avoid damaging the cells.

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Expt. No. 7

Experiment-7

→ Aim: Isolation of plasmid DNA from a microbial source

→ Material required -

- E. coli cells (DHS α)
- Plasmid DNA isolation kit
- Ethidium Bromide
- Luria Bertani medium
- Electrophoresis buffers: - TBE electrophoresis buffer (10x stock)

• Tris Base 10.8 gm

• Boric acid 5.5 gm

• EDTA 40 mL of 0.5M ($pH\ 8$)

• Adjust volume to 1L

- Nucleic acid sample buffer (5X)

• Tris HCl 50 mM

• Glycine 25%

• EDTA 5 mM

• Bromophenol Blue 0.2%

• Xylene Cyanol ($pH\ 8.0$) 0.2%

• Autoclave

• Micro-centrifuge

• Incubator shaker

• Eppendorf tubes (1.5 ml)

• Micropipette (P1000, P200, P20)

• Cell documentation / trans-illuminator

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Expt. No. 7

→ Theory:-

• Plasmids are extra chromosomal DNA that replicate independently of the bacterial chromosome. They are normally covalently closed, circular, super-coiled molecules. They carry genes encoding functions (such as antibiotic resistance) which may be useful to the cell but are not essential for normal cellular activities. In the recombinant DNA technology, plasmid DNA are used as vectors for carrying any foreign DNA. They can replicate within host cell and carries phenotypic traits by which they can be detected. This way large number of isolated genes and their products can be synthesized and used for industrial, therapeutic and agriculture purposes.

→ Procedure:-

* Day 0:-

• Inoculate 50 mL of bacterial cells (*E. coli*, DH5α) from frozen glycerol stock to 5 mL LB medium. Allow the cells to grow overnight at 37°C at ~200 rpm.

Day 1 Isolation of plasmid DNA

Transfer 1.5 mL of broth containing cells into 1.5 mL centrifuge tubes and centrifuge the cells at 1500 rpm for 1 min. Discard supernatant.

* Day 1 & 2: Plasmid isolation

- Grow bacterial culture in LB medium with appropriate antibiotics at 37°C overnight with shaking (180 rpm).
- For >10 copies plasmid, 3 mL cell culture is usually enough.
- Transfer culture to a 1.5 mL Eppendorf tube, and spin down cell culture (twice) at highest speed for 1 min at table-top centrifuge.
- Discard the supernatant. Remove the media completely either by pipetting or by upside down tube onto a piece of paper towel for a few seconds.
- Add 100 μ L of resuspension solution (P1 buffer ice cold) into each tube and vortex to completely resuspend all pellet.
- Add 200 μ L of lysis solution (P2 buffer, ice cold) into each tube (freshly prepared) 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.
- Add 150 μ L of neutralizing solution (P3 buffer, ice cold) and mix by inverting the tubes several times.
- At this point bacterial chromosomal DNA is usually seen as a white precipitate.
- Centrifuge the tubes at highest speed for 10 min at 4°C. Carefully transfer the supernatant to a new labelled 1.5 mL eppendorf tube with a 1 mL pipette.
- Add equal amount of phenol:chloroform:isoamyl-alcohol (P:C:I) to the collected supernatant, mix by inverting and then centrifuge at 10,000 rpm for 5 min.

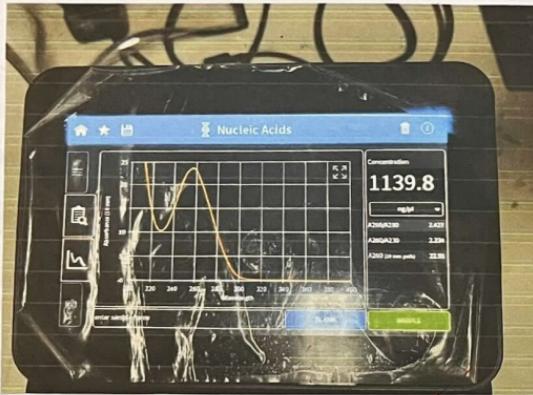


Fig: Reading of OD^{nm} in nanophotometer

- Collect the supernatant in 1.5mL eppendorf tube and add 1mL of chilled ethanol (absolute) to each tube and mix by inverting the tubes a few times and leave for 15 min.
- Spin down plasmid DNA precipitate at highest speed for 10 min.
- 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 tube in a tube holder and air dry for 10-20 min. To dry faster, keep tube at 37°C heat blocker. DNA precipitate turns white when dry.
- Rersuspend the DNA pellet with 30-50 μL TE buffer. Completely dissolve the pellet by ~~tip~~ pipetting solution several times.

→ Observations:-

- The bacterial culture grew overnight and showed turbidity in nutrient broth
- The bacterial cells were effectively lysed, as evidenced by the release of plasmid DNA into the lysis solution.
- After the addition of isopropanol, the plasmid DNA was observed as a white precipitate at the bottom of the tube.
- The purified plasmid DNA was a white pellet that was obtained after the final centrifugation step.
- After observing the purified plasmid DNA in a spectrophotometer, a ratio of A_{260}/A_{280} of 2.42 nice was observed.

Expt. No. 7

→ Conclusion :-

In conclusion, the plasmid DNA was successfully isolated from E. coli bacterial culture using alkaline lysis method.

→ Precaution :-

- All material and equipment should be sterilized before use to avoid contamination.
- The purified plasmid DNA samples should be stored in a refrigerated environment at 4°C to prevent degradation.
- All hazardous materials, such as lysis solution and isopropanol, should be disposed of properly according to local regulation and guidelines.

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Experiment - 8

→ Aim:- Analysis of the recombinant plasmid using restriction enzymes

→ Material required:-

- Plasmid DNA (isolated)
- Restriction enzymes
- Agarose and buffer for electrophoresis
- Sterile nuclease free water, sterile tips
- Electrophoresis buffers

- TBE Electrophoresis buffer (10x stock) ~~TAE was used~~

- 10.8 gm Tris base
- 5.5 gm boric acid
- 40 mL of 0.5M (pH 8.0) EDTA

- Adjust volume to 1L (~~use 1x for electroph.~~)
- Nucleic acid sample Buffer (5X)

- 50 mM Tris HCl
- 25% glycerol

• Water bath

- Agarose gel electrophoresis kit
- Gel documentation System
- Transilluminator
- Micropipettes

→ Theory :-

Recombinant plasmid containing the target gene are digested by restriction enzymes to confirm the ligation of the inserted gene and hence the positive clones. The digested fragment is run on agarose gel and its size can be determined by comparing the size obtained with the DNA ladder, the recombinant clones are confirmed.

→ Procedures:-

* Day 1:-

A. Restriction digestion of plasmid DNA

- Take $10\text{ }\mu\text{l}$ of plasmid DNA and add prescribed reaction buffer and then $1\text{ }\mu\text{l}$ of restriction enzyme (NheI, $1\text{U}/\text{mg}$) and make the volume to $25\text{ }\mu\text{l}$ and incubate at 37°C in a water bath for 2 hrs. Then add $1\text{ }\mu\text{l}$ of second restriction enzyme (XbaI, $1\text{U}/\text{mg}$) and the required reaction buffer and further incubate for 2 hrs at 37°C in a water bath.

* Day 2:-

B. Preparation of Agarose gel for electrophoresis:-

- Prepare 0.8% Agarose solution in 1X TBE
- Pour the agarose into gel casting trays pre set along with combs. Allow the agarose gel to polymerize & then remove combs without breaking the wells.
- Submerge the gel into the horizontal electrophoresis tank containing 1X TBE buffer.



Fig - Bands of DNA fragment in agarose gel electrophoresis

- c. Electrophoresis of the isolated plasmid DNA -
- Take about 5 μL of the (top eluate) plasmid DNA and add 2 μL of the sample buffer.
 - Load about 7 μL into each well along with a marker DNA or ladder.
 - Run electrophoresis at constant voltage and allow DNA to run in 1X TBE running buffer, keeping track of the dye front.
 - Remove the gel and place in a solution of Ethidium Bromide for staining DNA for 30 min. Remove the gel, and place the solution with gloves, rinse with water and place in the gel documentation system/Transilluminator and visualizing the DNA bands in the UV light.
 - Quantify the DNA concentration from the standard (ladder) used.

→ Observation :-

An agarose gel electrophoresis was performed on the plasmid DNA that was digested by the restriction enzymes NheI and XbaI. After staining with Ethidium Bromide, the gel was viewed under UV light to visualize the DNA fragments. The DNA fragments appear to be clear bands against the stain background. The size of the fragment was compared to a DNA ladder and the fragments were quantified based on the standard used.

Result :-

The restriction enzymes $NheI$ and $XbaI$ successfully digested the plasmid DNA, resulting in the formation of clear bands of DNA fragments on the agarose gel. The size of the fragments was confirmed by comparing them to the DNA ladder, and the concentration of the fragments was quantified based on the standard used. These results indicate that the recombinant plasmid has been successfully confirmed by the analysis of the restriction enzymes.

Precautions :-

- Use nucleic-acid-free water, tips and solutions when preparing the samples to prevent contamination of the DNA.
- Dispose of all waste materials in accordance with local regulation.
- Store the DNA samples in appropriate conditions to maintain their integrity.

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