A Practical Manual on Basic Techniques in
BIOTECHNOLOGY & NANOTECHNOLOGY

Edited by
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PREFACE

A practical manual on Basic Techniques in Biotechnology and Nanotechnology published by ISCA provides activities that are well standardized for large as well as small class groups of the Undergraduate and Post graduate biotechnology students. These techniques form the central building pillar in any biotechnological approach around which most of the recent research developments revolve. The manual emphasizes basic experimental, investigatory, observational and interpretational skills in students which is quintessential of acquiring laboratory skills. A comprehensive safety note and the principle/rationale behind each experiment is very well laid out. As reflected in the title, I am sure that this manual would bring out the fundamental principles in biotechnological and nanotechnological experiments and would certainly help the new generation biologists as the work would be available on line. I congratulate my staff Dr S R Madhan Shankar and Dr E M Rajesh from the Department of Biotechnology for taking up this task. I also accord my gratitude to International Science Congress Association for helping out with the publication process.
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1. GENERAL PROCEDURES, EQUIPMENT USAGE AND SAFETY CONSIDERATIONS IN THE LAB

(i) Safety Procedures

a. Chemicals:

A number of chemicals used in any molecular biology laboratory are hazardous. All manufacturers of hazardous materials are required by law to supply the user with pertinent information on any hazards associated with their chemicals. This information is supplied in the form of Material Safety Data Sheets or MSDS. This information contains the chemical name, CAS#, health hazard data, including first aid treatment, physical data, fire and explosion hazard data, reactivity data, spill or leak procedures, and any special precautions needed when handling this chemical. A file containing MSDS information on the hazardous substances should be kept in the lab. In addition, MSDS information can be accessed on World Wide Web. You are strongly urged to make use of this information prior to using a new chemical and certainly in the case of any accidental exposure or spill. The instructor/lab manager must be notified immediately in the case of an accident involving any potentially hazardous reagents.

The following chemicals are particularly noteworthy:

- Phenol - can cause severe burns
- Acrylamide - potential neurotoxin
- Ethidium bromide - carcinogen

These chemicals are not harmful if used properly: always wear gloves when using potentially hazardous chemicals and never mouth-pipet them. If you accidentally splash any of these chemicals on your skin, immediately rinse the area thoroughly with water and inform the instructor. Discard the waste in appropriate containers.

b. Ultraviolet Light

Exposure to ultraviolet light can cause acute eye irritation. Since the retina cannot detect UV light, you can have serious eye damage and not realize it until 30 min to 24 hours after exposure. Therefore, always wear appropriate eye protection when using UV lamps.

c. Electricity

The voltages used for electrophoresis are sufficient to cause electrocution. Cover the buffer reservoirs during electrophoresis. Always turn off the power supply and unplug the leads before removing a gel.

d. General Housekeeping

All common areas should be kept free of clutter and all dirty dishes, electrophoresis equipment, etc. should be dealt with appropriately. Since you have only a limited amount of space to call your own, it is to your advantage to keep your own area clean. Since you will use common facilities, all solutions and everything stored in an incubator, refrigerator, etc. must be labeled. In order to limit confusion, each person should use his initials or other unique designation for labeling plates, etc. Unlabeled material found in the refrigerators, incubators, or freezers may be
destroyed. Always mark the backs of the plates with your initials, the date, and relevant experimental data, e.g. strain numbers.

(ii) Preparation of Solutions

a. Calculation of Molar, % and "X" Solutions.

1. A molar solution is one in which 1 liter of solution contains the number of grams equal to its molecular weight. Ex. To make up 100 ml of a 5M NaCl solution = 58.456 (mw of NaCl) g/mol x 5 moles/liter x 0.1 liter = 29.29 g in 100 ml of solution

2. Percent solutions. Percentage (w/v) = weight (g) in 100 ml of solution; Percentage (v/v) = volume (ml) in 100 ml of solution. Eg. To make a 0.7% solution of agarose in TBE buffer, weight 0.7 of agarose and bring up volume to 100 ml with TBE buffer.

3. "X" Solutions. Many enzyme buffers are prepared as concentrated solutions, e.g. 5X or 10X (five or ten times the concentration of the working solution) and are then diluted such that the final concentration of the buffer in the reaction is 1X. Eg. To set up a restriction digestion in 25 µl, one would add 2.5 µl of a 10X buffer, the other reaction components, and water to a final volume of 25 µl.

b. Preparation of Working Solutions from Concentrated Stock Solutions.

Many buffers in molecular biology require the same components but often in varying concentrations. To avoid having to make every buffer from scratch, it is useful to prepare several concentrated stock solutions and dilute as needed. Eg. To make 100 ml of TE buffer (10 mM Tris, 1 mM EDTA), combine 1 ml of a 1 M Tris solution and 0.2 ml of 0.5 M EDTA and 98.8 ml sterile water. The following is useful for calculating amounts of stock solution needed: C i × V i = C f × V f , where C i = initial concentration, or conc of stock solution; V i = initial vol, or amount of stock solution needed C f = final concentration, or conc of desired solution; V f = final vol, or volume of desired solution.

c. Steps in Solution Preparation:

1. Refer to a laboratory reference manual for any specific instructions on preparation of the particular solution and the bottle label for any specific precautions in handling the chemical. Weigh out the desired amount of chemical(s). Use an analytical balance if the amount is less than 0.1 g. Place chemical(s) into appropriate size beaker with a stir bar. Add less than the required amount of water. Prepare all solutions with double distilled water when the chemical is dissolved, transfer to a graduated cylinder and add the required amount of distilled water to achieve the final volume. An exception is in preparing solutions containing agar or agarose. Weigh the agar or agarose directly into the final vessel. If the solution needs to be at a specific pH, check the pH meter with fresh buffer solutions and follow instructions for using a pH meter. Autoclave, if possible, at 121°C for 20 min. Some solutions cannot be autoclaved, for example, SDS. These should be filter sterilized through a 0.22 µm or 0.45 µm filter. Media for bacterial cultures must be autoclaved the same day it is prepared, preferably within an hour or two. Store at room temperature and check for contamination prior to use by holding the bottle at eye level and gently swirling it. Solid media for bacterial plates can be prepared in advance, autoclaved, and stored in a bottle. When needed, the agar can be melted in a microwave, any additional components, e.g. antibiotics, can be added and the plates can then be poured.
2. Concentrated solutions, e.g. 1M Tris-HCl pH=8.0, 5M NaCl, can be used to make working stocks by adding autoclaved double-distilled water in a sterile vessel to the appropriate amount of the concentrated solution.

d. Glassware and Plastic Ware.

Glass and plastic ware used for molecular biology must be scrupulously clean. Dirty test tubes, bacterial contamination and traces of detergent can inhibit reactions or degrade nucleic acid.

Glassware should be rinsed with distilled water and autoclaved or baked at 150°C for 1 hour. For experiments with RNA, glassware and solutions are treated with diethyl-pyrocarbonate to inhibit RNases which can be resistant to autoclaving. Plastic ware such as pipettes and culture tubes are often supplied sterile. Tubes made of polypropylene are turbid and are resistant to many chemicals, like phenol and chloroform; polycarbonate or polystyrene tubes are clear and not resistant to many chemicals. Make sure that the tubes you are using are resistant to the chemicals used in your experiment. Micro pipette tips and microfuge tubes should be autoclaved before use.

(iii) Disposal of Buffers and Chemicals

1. Any uncontaminated, solidified agar or agarose should be discarded in the trash, not in the sink, and the bottles rinsed well.

2. Any media that becomes contaminated should be promptly autoclaved before discarding it. Petri dishes and other biological waste should be discarded in Biohazard containers which will be autoclaved prior to disposal.

3. Organic reagents, e.g. phenol, should be used in a fume hood and all organic waste should be disposed of in a labeled container, not in the trash or the sink.

4. Ethidium bromide is a mutagenic substance that should be treated before disposal and should be handled only with gloves. Ethidium bromide should be disposed of in a labeled container.

5. Dirty glassware should be rinsed, all traces of agar or other substance that will not come clean in a dishwasher should be removed, all labels should be removed (if possible), and the glassware should be placed in the dirty dish bin. Bottle caps, stir bars and spatulas should not be placed in the bins but should be washed with hot soapy water, rinsed well with hot water, and rinsed three times with distilled water.

(iv) Equipment

It is to everyone's advantage to keep the equipment in good working condition. As a rule of thumb, don't use anything unless you have been instructed in the proper use. This is true not only for equipment in the lab but also departmental equipment. Report any malfunction immediately. Rinse out all centrifuge rotors after use and in particular if anything spills. Please do not waste supplies - use only what you need. If the supply is running low, please notify either the instructor/lab manager before the supply is completely exhausted. Occasionally, it is necessary to borrow a reagent or a piece of equipment from another lab. Except in an emergency, notify the instructor.
(v) Working with DNA

a. Storage

The following properties of reagents and conditions are important considerations in processing and storing DNA and RNA. Heavy metals promote phosphodiester breakage. EDTA is an excellent heavy metal chelator. Free radicals are formed from chemical breakdown and radiation and they cause phosphodiester breakage. UV light at 260 nm causes a variety of lesions, including thymine dimers and cross-link. Biological activity is rapidly lost. 320 nm irradiation can also cause cross-link, but less efficiently. Ethidium bromide causes photo oxidation of DNA with visible light and molecular oxygen. Oxidation products can cause phosphodiester breakage. If no heavy metals are present, ethanol does not damage DNA. Nucleases are found on human skin; therefore, avoid direct or indirect contact between nucleic acids and fingers. Most DNases are not very stable; however, many RNases are very stable and can adsorb to glass or plastic and remain active. 5 E C is one of the best and simplest conditions for storing DNA. -20 deg C: this temperature causes extensive single and double strand breaks. -70 E C is probable excellent for long-term storage. For long-term storage of DNA, it is best to store in high salt (>1M) in the presence of high EDTA (>10mM) at pH 8.5. Storage of DNA in buoyant CsCl with ethidium bromide in the dark at 5 E C is excellent. There is about one phosphodiester break per 200 kb of DNA per year. Storage of λ DNA in the phage is better than storing the pure DNA. [Ref: Davis, R.W., D. Botstein and J.R. Roth, A Manual for Genetic Engineering: Advanced Bacterial Genetics. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y. 1980.]

b. Purification

To remove protein from nucleic acid solutions:

1. Treat with proteolytic enzyme, e.g., pronase, papaın, proteinase K.
2. Purify on a silica-based column such as a Qiagen PCR Prep Column.
3. CsCl/Ethidium bromide density gradient.
4. Phenol Extract. The simplest method for purifying DNA is to extract with phenol or phenol:chloroform and then chloroform. The phenol denatures proteins and the final extraction with chloroform removes traces of phenol.
5. Purify on silica-based column such as Qiagen Brand columns.

c. Quantitation

1. **Spectrophotometric.** For pure solutions of DNA, the simplest method of quantitation is reading the absorbance at 260nm where an OD of 1 in a 1cm path length =50µg/ml for double-stranded DNA, 40 µg/ml for single-stranded DNA and RNA and 20-33µg/ml for oligonucleotides. An absorbance ratio of 260nm and 280nm gives an estimate of the purity of the solution. Pure DNA and RNA solutions have OD 260/OD 280 values of 1.8 and 2.0, respectively. This method is not useful for small quantities of DNA or RNA (<1 µ g/ml).

2. **Ethidium bromide fluorescence.** The amount of DNA is a solution is proportional to the fluorescence emitted by ethidium bromide in that solution. Dilutions of an unknown DNA in the presence of 2 µ g/ml ethidium bromide are compared to dilutions of a known amount of a standard DNA solutions spotted on an agarose gel or Saran Wrap or electrophoresed in an agarose gel.
d. Concentration

Precipitation with ethanol. DNA and RNA solutions are concentrated with ethanol as follows: The volume of DNA is measured and the monovalent cation concentration is adjusted. The final concentration should be 2-2.5M for ammonium acetate, 0.3M for sodium acetate, 0.2M for sodium chloride and 0.8M for lithium chloride. The ion used often depends on the volume of DNA and on the subsequent manipulations; for example, sodium acetate inhibits Klenow, ammonium ions inhibit T4 polynucleotide kinase, and chloride ions inhibit RNA-dependent DNA polymerases. The addition of MgCl$_2$ to a final concentration of 10mM assists in the precipitation of small DNA fragments and oligonucleotides. Following addition of the monovalent cations, 2-2.5 volumes of ethanol are added, mixed well, and stored on ice or at -20°C for 20 min to 1 hour. The DNA is recovered by centrifugation in a microfuge for 10 min (room temperature is okay). The supernatant is carefully decanted making certain that the DNA pellet, if visible, is not discarded (often the pellet is not visible until it is dry). To remove salts, the pellet is washed with 0.5-1.0 ml of 70% ethanol, spun again, the supernatant decanted, and the pellet dried. Ammonium acetate is very soluble in ethanol and is effectively removed by a 70% wash. Sodium acetate and sodium chloride are less effectively removed. For fast drying, the pellet can spin briefly in a Speedvac, although the method is not recommended for many DNA preparations as DNA that has been over dried is difficult to resuspend and also tends to denature small fragments of DNA. Isopropanol is also used to precipitate DNA but it tends to coprecipitate salts and is harder to evaporate since it is less volatile. However, less isopropanol is required than ethanol to precipitate DNA and it is sometimes used when volumes must be kept to a minimum, e.g., in large scale plasmid preps.

e. Restriction Enzymes

Restriction and DNA modifying enzymes are stored at -20°C in a non-frost free freezer, typically in 50% glycerol. The enzymes are stored in an insulated cooler which will keep the enzymes at -20°C for some period of time. The tubes should never be allowed to reach room temperature and gloves should be worn when handling as fingers contain nucleases. Always use a new, sterile pipet tip every time you use a restriction enzyme. Also, the volume of the enzyme should be less than 1/10 of the final volume of the reaction mixture.

(vi). Sterile Technique

1. All media, including plates, liquid media and top agar must be autoclaved immediately after it is prepared. It is best to prepare media in several small bottles, only opening one at a time. Check the bottle for contamination before you use it by gently swirling it and looking for cloudy material in the center. Always grow up a small amount of broth alone when growing cells overnight. A small amount of contamination is not always evident until the media is incubated at 37°C.

2. Use a flame on inoculating loops and on the lips of media bottles before and after pipetting from them. Never leave a media or agar bottle open on the bench and don't take an individually-wrapped pipet out of its protective wrapper until you are ready to use it (i.e., don't walk across the room with an unwrapped pipet). Always use a fresh, sterile pipet or pipet tip when pipetting culture media, and never go back into a media bottle or cell culture with a used pipet.
3. To prevent wide-scale, untraceable contamination, each person should have his own stock of liquid culture media, top agar, plates, 100% glycerol, glycerol stocks of cells, etc. and don't share.

4. Overnight cultures should be grown only from a single colony on a fresh plate or from a previously-tested glycerol stock that was grown from a single colony. To prepare an overnight culture from a glycerol stock, take an individually-wrapped 1-ml pipet and a culture tube of media to the -80°C freezer. Quickly remove the cap from the freezer vial containing the glycerol stock, scrap a small amount of ice from the surface of the culture, replace the cap on the freezer vial, and place the pipet into the culture tube. Sufficient numbers of bacteria are present in the ice in order for the culture to grow to saturation in 16 hours. Never let the glycerol stock thaw.
2. EXTRACTION AND PURIFICATION OF GENOMIC DNA FROM BACTERIA

The isolation of DNA is one of the more commonly used procedures in many areas of bacterial physiology, genetics, molecular biology and biochemistry. Purified DNA is required for many applications such as studying DNA structure and chemistry, examining DNA-protein interactions, carrying out DNA hybridizations, sequencing or PCR, performing various genetic studies or gene cloning. The isolation of DNA from bacteria is a relatively simple process. The organism to be used should be grown in a favorable medium at an optimal temperature, and should be harvested in late log to early stationary phase for maximum yield. The cells can then be lysed and the DNA isolated by one of several methods. The method of choice depends in part on the organism of interest and what the DNA will be used for after purification. Following lysis, other cellular constituents are selectively removed. Once this is accomplished, DNA can be precipitated from solution with alcohol and dissolved in an appropriated buffer.

Aim:
To isolate and purify genomic DNA from the given bacterial strain and to check on agarose gel

Principle:
The lysis of the bacteria is initiated by resuspending a bacterial pellet in a buffer containing lysozyme and EDTA. In addition to inhibiting DNases, the EDTA disrupts the outer membrane of the gram-negative envelope by removing the Mg++ from the lipopolysaccharide layer. This allows the lysozyme access to the peptidoglycan. After partial disruption of the peptidoglycan, a detergent such as SDS is added to lyse the cells. Most gram-negative cells will lyse after this treatment and many can even be lysed without lysozyme. Once the cells are lysed, the solution should be treated gently to prevent breakage of the DNA strands.

Subsequent steps involve the separation of the DNA from other macromolecules in the lysate. Both phenol (that has been equilibrated with Tris buffer) and chloroform (with isoamyl alcohol as a defoaming agent) are commonly used to dissociate protein from nucleic acids. These reagents also remove lipids and some polysaccharides. Proteolytic enzymes such as pronase or Proteinase K are often added to further remove protein. Proteinase K is a particularly useful enzyme in that it is not denatured by SDS and in fact works more effectively in the presence of SDS. The nucleic acids (including RNA) may then be precipitated in ice cold ethanol if the ionic strength of the solution is high. This is followed by RNase treatment to degrade the RNA. The solution may then be reprecipitated with ethanol. In this precipitation, the ribonucleotides from RNase treatment will remain in solution leaving purified DNA in the pellet. The pellet can then be dissolved in an appropriate buffer.

Alcohol precipitations of DNA and RNA are widely used in molecular biology and are valuable because they allow the nucleic acids to be concentrated by removing them from solution as an insoluble pellet. If concentrations of DNA are relatively high (>1ug/ml) DNA can be effectively precipitated in 10-15 min by shielding the negative charge with monovalent cations (0.3M Na or 2.5 M ammonium ions are commonly used) followed by the addition of 2 volumes of 95% ethanol. Factors affecting alcohol precipitations are given below.

A major consideration in any DNA isolation procedure is the inhibition or inactivation of DNases which can hydrolyze DNA. The buffer in which the cells are suspended should have a
high pH (8.0 or greater) which is above the optimum of most DNases. EDTA is also included in the resuspension buffer to chelate divalent cations (such as Mg++) which are required by DNases. The SDS also reduces DNase activity by denaturing these enzymes. DNase activity is further controlled by keeping cells and reagents cold, using proteolytic enzymes such as pronase or Proteinase K, and a heating step that will thermally denature DNase (but must not be hot enough to denature the DNA).

The procedure used here is useful for isolating DNA from a large variety of gram negative bacteria. It yields partially purified DNA of sufficient quality for most techniques, such as restriction digestion, ligation, and cloning.

Materials Required:

1 M Tris- Cl pH 8.0, 0.5 M EDTA pH 8.0, 5% NaCl, 20mg.ml Proteinase K, 10mg/ml Lysozyme, 20% SDS, 1X RNase, Buffer saturated Phenol, 70% ethanol, absolute ethanol, Phenol: chloroform(1:1), chloroform : IAA( 24:1), 3M Sodium acetate pH5.2 ( adjust pH with glacial acetic acid), TE buffer.

TES Buffer: 10 mM Tris –Cl pH8.0, 5mM EDTA pH8.0, 1.5% NaCl.

Strain: E.coli CSH 123 or CSH 125 or JM 109 or DH5α

Media: LB broth (Per Liter: Nacl- 10g, Yeast extract- 5 g, Tryptone- 5g, pH 7.2)

Protocol:

01. Inoculate a single colony of E.coli strain in 10 ml of L broth and allow it to grow at 37°C for 12 to 16 hours at 110 rpm.

02. Harvest the cells at 7000 rpm for 10minutes at 4°C. Decant the media as much as possible and gently tap the pellet.

03. Resuspend the pellet in 5 ml of ice cold TES buffer and add 50 µl of 10mg/ml lysozyme and incubate at RT for 5- 10minutes.

04. Add Proteinase K to a final concentration of 40 µg/ml and incubate at 55°C for 10minutes.

05. Add SDS to a final concentration of 1% and incubate in a water bath at 55°C for 45 minutes.

06. Add an equal volume of buffer saturated phenol and vortex well and centrifuge at 12,000 rpm for 10 minutes.

07. Transfer the aqueous layer to a fresh tube and add 2 volumes of ice cold 70% ethanol and incubate on ice 5 to 10 minutes and spool the precipitating DNA fibers to a fresh tube.

08. Resuspend in 5 ml of TE buffer completely.

09. Add 100 µg/ml of RNase and incubate at 45°C in a water bath for 15 minutes followed by the addition of an equal volume of Phenol: chloroform (1:1), vortex well and centrifuge at 12,000rpm for 10 minutes at RT.

10. Collect the top aqueous layer and extract once with chloroform: IAA (24:1).

11. Transfer the top aqueous layer to a sterile tube and add 1/10th volume of 3M sodium acetate pH5.2 and 2 volumes of ice cold ethanol to precipitate the DNA. Centrifuge at 10K to pellet the DNA.

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12. Air dry the DNA and redissolve in 200 µl of TE buffer.
13. Check 5 µl on a 0.8% agarose gel.

**Result:**

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**Inference/Discussion:**

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3. **EXTRACTION AND PURIFICATION OF TOTAL HUMAN DNA**

Many protocols are available for isolating DNA from tissues, cells and body fluids. The basic methodology involves the digestion of proteins followed by organic solvent extraction and precipitation of DNA. The degree of purity required will depend upon what the DNA will ultimately be used for. In most detection methodologies where DNA is the target, multiple samples need to be analyzed. This therefore requires procedures that are rapid and involve minimal handling of DNA.

**Aim:**
To extract and purify total human DNA from whole frozen blood

**Principle:**
This procedure dissolves the cytoplasmic membrane and pellets the nuclei. Therefore cytoplasmic DNA and RNA are lost. Only nuclear DNA is extracted. The DNA yield is about 40 µg/ml of starting blood.

**Materials Required:**
- 5% EDTA, TEN A buffer (10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0, 400mM NaCl),
- 20 X SSC Solution (3M NaCl, 0.3 M Sodium citrate pH 7.0),
- 1X SSC,
- 10% SDS,
- Proteinase K (20mg/ml),
- Saturated NaCl (~ 6M),
- 100% ethanol, 70% ethanol,
- TE buffer.

**Protocol:**
1. Collect 10 ml of venous blood in a sterile polypropylene tube containing 5% EDTA and mix it thoroughly and keep it at -20°C for overnight.
2. Thaw the frozen blood gently in a 37°C water bath with intermittent shaking.
3. To the thawed blood add 2 volumes (~ 20 ml) of 1X SSC solution, mix gently and centrifuge at 4000 rpm for 15 minutes.
4. Discard the red supernatant and add 20 ml of 1X SSC solution mix by inversion and centrifuge at 4000 rpm for 10 minutes.
5. Discard the supernatant leaving just enough solution to cover the pellet.
6. Dissolve the pellet in 18 ml of TEN A buffer and transfer the contents to a sterile 250ml conical flask.
7. Add 2 ml of 10% SDS and 25 µl of Proteinase K and incubate at 56°C for 2 hours.
8. After the incubation add 5 ml of saturated NaCl, mix the contents gently and centrifuge at 4000 rpm for 15 minutes.
9. Transfer the supernatant obtained to a sterile 250 ml beaker and precipitate the DNA by adding 2.5 volumes of ethanol.
10. Spool the DNA fibers using a glass rod and transfer it to a sterile tube and wash with 70% ethanol twice and air dry the pellet.
11. Dissolve the pellet in 1 ml of TE buffer and check on agarose gel.
Result:__________________________________________________________________________
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Inference/Discussion:_________________________________________________________________________________
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4. EXTRACTION AND PURIFICATION OF PLASMID DNA
   (BRINBOIM & DOLY, 1979)

Bacterial plasmids are double stranded closed circular DNA molecules ranging in size from 1 Kb to more than 200Kb. They are inherited independently of the bacterial chromosomes. Nevertheless, they rely on the enzymes and proteins coded by the host for their transcription and replication. Plasmids contain genes conferring resistance to antibiotics, production of antibiotics, degradation of complex organic compounds, enterotoxins, restriction enzymes and modification enzymes. Plasmids are always purified from cultures of bacteria that have been grown on a selective media. Also for many years it has been a normal practice to add chloramphenicol to the cultures to achieve complete inhibition of protein synthesis such that the yield of plasmid DNA would be sufficiently high enough for future cloning purposes.

Aim:
To extract and purify plasmid DNA from the given bacterial strain

Principle:
Bacteria can be recovered by centrifugation and lysed by any one of the methods like treatment with alkali, organic solvents or heat. The choice of a method depends on the size of the plasmid in question, the nature of the host and the purification strategy that is going to be employed for purification. Large plasmids of the range 15 Kb and more which are susceptible to damage should be released gently. The bacteria are suspended in an isotonic solution of sucrose and then treated with lysozyme and EDTA to break the cell wall and outer membrane. The resulting spheroplasts can be lysed by either heat or alkali or by detergents. For small plasmids after addition of EDTA and lysozyme the same method as above is followed. These (heat/alkali/detergents) disrupt the base pairing and cause the linear chromosomal DNA of the host to denature. However the closed strands of the plasmid DNA are unable to separate from one another because they are intertwined. When conditions are returned to normal, the strands of the plasmid DNA fall into perfect register and completely native superhelical molecules are reformed.

Materials Required:
Strain: E.coli DH5α with plasmid pUC18
Media: LBA, L broth
Antibiotic: Ampicillin (50 mg/ml)/ Working Concentration 50 µg/ml.
1 M Tris-Cl pH 8.0, 0.5 M EDTA pH 8.0, 1M glucose, 5 N NaOH, 10% SDS,
3 M Potassium acetate pH 5.2, 5M sodium acetate pH 5.2, Equilibrated Phenol, Phenol: chloroform (1:1), Chloroform: IAA (1:1), 70% ethanol, absolute ethanol, TE buffer
TEG buffer: glucose 50mM, EDTA 10mM, Tris-Cl 25mM
Lysis Buffer: NaOH 0.2N, 1% SDS
Protocol:

01. Take 1.5ml of the overnight grown culture and harvest the cells at 12,000 rpm for 1 minute at 4°C and remove the supernatant.

02. Tap the pellet well and resuspend in 100 µl of TEG buffer, vortex thoroughly.

03. Immediately add 200µl of Lysis buffer and mix well by inversion and incubate on ice for 5 min on ice.

04. Add 150 µl of 3 M Potassium acetate, mix by vortexing and incubate on ice for 5 minutes.

05. Centrifuge the contents at 12K for 5 minutes at 4°C and collect the supernatant and add equal volume of Equilibrated Phenol: chloroform (1:1), vortex well and centrifuge at at 12K, 5 min at 4°C.

06. Collect the top aqueous layer and precipitate with 0.1 volume of 5 M sodium acetate and double the volume of ice cold 70% ethanol on ice for 2 minutes.

07. Centrifuge the contents at 12K, 5 minutes at 4°C.

08. Wash once with 70% ethanol and absolute ethanol.

09. Air dry the pellet and redissolve in 20 µl of TE.

10. Check 5 µl on 0.8% agarose gel.

Result:__________________________________________________________________________  
______________________________________________________________________________  
______________________________________________________________________________  
______________________________________________________________________________  

Inference/Discussion:__________________________________________________________________________  
______________________________________________________________________________  
______________________________________________________________________________  
______________________________________________________________________________
AGAROSE GEL ELECTROPHORESIS

The standard method used to separate, identify, and purify DNA fragments are electrophoresis through agarose gels. The technique is simple, rapid to perform, and capable of resolving mixtures of DNA fragments that cannot be separated adequately by other sizing procedures. Furthermore, the location of DNA within the gel can be determined directly: Bands of DNA in the gel are stained with the intercalating dye ethidium bromide; as little as 1 ng of DNA can be detected by direct examination of the gel in ultraviolet light.

Structure of Agarose

Aim:
To separate DNA molecules on agarose gel

Principle:
Commercially available agarose is not completely pure, it is contaminated with other polysaccharides, salts, and proteins. These differences can affect both, the migration of the DNA and the ability of the DNA recovered from the gel to serve as a substrate to enzymatic reactions. Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode. The electrophoretic migration rate of DNA through agarose gels is dependent upon four main parameters given below.

The molecular size of the DNA. Molecules of linear, duplex DNA, which are believed to migrate in an end-on position travel through gel matrices at rates that are inversely proportional to the logarithm of their molecular weights.

The agarose concentration. A DNA fragment of given size migrates at different rates through gels containing different concentrations of agarose. There is a linear relationship between the logarithm of the electrophoretic mobility of DNA ($\mu$) and gel concentration ($\tau$), which is described by the equation:

$$\log \mu = \log \mu_0 - K_\tau \tau$$

where $\mu_0$ is the free electrophoretic mobility and $K_\tau$ is the retardation coefficient, a constant that is related to the properties of the gel and the size of the migrating molecules. Thus, by using gels of different concentrations, it is possible to resolve a wide-range of DNA fragments.
**The conformation of the DNA.** Closed circular, nicked circular and linear DNA of the same molecular weight migrate through agarose gels at different rates. The relative mobilities of the three forms are dependent primarily on the agarose concentration in the gel but are also influenced by the strength of the applied current, the ionic strength of the buffer, and the density of superhelical twists in the DNA.

**The applied current.** At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the electric field strength is raised, the mobility of high-molecular-weight fragments of DNA is increased differentially. Thus, the effective range of separation of agarose gels decreases as the voltage is increased. Gels should be run at no more than 5 V/cm.

**Base composition and temperature.** The electrophoretic behavior of DNA in agarose gels (by contrast to polyacrylamide gels) is not significantly affected either by the base composition of the DNA or the temperature at which the gel is run.

The agarose concentration is varied for different fragment ranges. For analyzing the complete 2ar codon (1239 bp), a 1% agarose gel is made by dissolving agarose in 1 XTAE buffer by heating in a microwave oven. After cooling to bearable warmth, ethidium bromide is added to a final concentration of 0.5 µg/ml. The agarose solution is poured into a taped gel former mold to make the gel. A well-forming comb (12 slots for mini gels) is placed near one edge of the gel. The gel is cooled to harden until it becomes milky and opaque (approximately one hour). The gel mold is placed horizontally into the electrophoresis tank, which is filled with 1 XTAE (0.5 µg/ml ethidium bromide).

The gel loading buffer is applied to the samples and they are carefully added to individual wells. The electrophoresis is run by 70-100 V/20-80 mA for about an hour or at 20 to 30 V overnight. The size of fragments can be determined by calibrating the gel, using known standards (e.g., λDNA EcoRI / HindIII digest, Boehringer Mannheim, or 100bp ladder,), and comparing the distance the unknown fragment has migrated.

The most convenient method of visualizing DNA in agarose gels is by use of the fluorescent dye ethidium bromide (2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium bromide). This substance contains a planar group that intercalates between stacked bases of DNA. The fixed position of this group and its close proximity to the bases causes dye bound to DNA to display an increased fluorescent yield compared to dye in free solution. UV-irradiation absorbed by the DNA at 260 nm and transmitted to the dye, or irradiation absorbed at 300 nm and 360 nm by the bound dye itself, is emitted at 590 nm in the red-orange region of the visible spectrum.

Structure of Ethidium Bromide
Materials Required:

Gel casting tray, gel platform, comb, power packs, gel tank, 1X TAE, Ethidium Bromide (50 mg/ml stock), 4X loading dye( 0.25% Bromophenol Blue in 40% sucrose), sterile water, agarose, DNA sample.

Protocol:

1. Determine the amount of Agarose (grams) required to make the desired Agarose gel concentration and volume. Example: For a 1% Agarose gel, add 1 gram of Agarose to 100 ml of 1x electrophoresis buffer.

   Table-1 Gel Concentration Required for DNA Separation

<table>
<thead>
<tr>
<th>Gel Concentration (%)</th>
<th>DNA Size (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>1-30</td>
</tr>
<tr>
<td>0.75</td>
<td>0.8-12</td>
</tr>
<tr>
<td>1.00</td>
<td>0.5-10</td>
</tr>
<tr>
<td>1.25</td>
<td>0.4-7</td>
</tr>
<tr>
<td>1.50</td>
<td>0.2-3</td>
</tr>
<tr>
<td>2-5*</td>
<td>0.01-0.5</td>
</tr>
</tbody>
</table>

* Sieving agarose such as AmpliSize® agarose

2. Add the Agarose to a suitable container. Add the appropriate amount of 1x electrophoresis buffer and swirl to suspend the Agarose powder in the buffer.

3. Place the gel solution into the microwave oven and boil and swirl the solution until all of the small translucent Agarose particles are dissolved. Stopping the microwave oven and swirling the flask every 30 seconds helps dissolve the Agarose faster.

4. Cool the molten Agarose to 60 °C before pouring an Agarose gel slab.

5. Level the gel caster on an even surface, seal the two ends of the platform with sealing tapes and pour the molten agarose without air bubbles getting trapped in the solution.

6. Let it stand until it hardens to a gel.

7. Gently remove the sealing tapes and the comb and place the gel in the tank containing 1X TAE buffer and pre run the gel for 2 to 5 minutes.

8. Load the DNA samples along with the loading dye.

9. Use Marker DNA on the first lane.

10. Apply a constant electric current of 50 V/cm² and track the mobility of the sample dye.

11. Stain the agarose gel for 2 to 5 minutes in 50 µg/ml ethidium bromide solution and destain briefly in sterile water.

12. Place the gel on an UV trans illuminator and observe the bands and document.
Result:_______________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________

Inference/Discussion:
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6. QUANTITATION OF DNA BY SPOT TEST ASSAY AND UV SPECTROSCOPY

Aim:
To quantify the given DNA sample by Spot test assay and UV spectroscopy and compare the results.

Principle:
1. Spot test assay: A fairly accurate, rapid assay of DNA concentration can be obtained by UV visualization of samples spotted onto ethidium bromide-containing agarose plates.

2. UV Spectroscopy: If sample is pure (i.e. without significant amounts of contaminantes such as proteins, phenol, agarose, or other nucleic acids), can use spec to measure amount of UV irradiation absorbed by the bases.

For quantitating DNA or RNA, readings should be taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample.

- 1 O.D. at 260 nm for double-stranded DNA = 50 ng/µl of dsDNA
- 1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/µl of ssDNA
- 1 O.D. at 260 nm for RNA molecules = 40 ng/µl of RNA

The reading at 280 nm gives the amount of protein in the sample.

Pure preparations of DNA and RNA have \( \frac{OD_{260}}{OD_{280}} \) values of 1.8 to 2.0, respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

So typically, dilute sample 1 ul in 100 µl so the dilution factor is 100. Put whole 100 µl in spectrophotometer cuvette. The DNA concentration read will then be:

\[ \text{OD}_{260} \times 50 \text{ ng/µl} \times \text{dilution factor} \]

For example, if have \( \text{OD}_{260} = 1.6 \). Then the concentration is:

\[ 1.6 \times 50 \text{ ng/µl} \times 100 = 8000 \text{ ng/µl or 8 µg/µl}. \]

Materials Required:
DNA sample to be quantified, Lambda DNA, Marker DNA, ethidium bromide, agarose, petri plates, quartz cuvettes, UV Spectrophotometer, UV trans illuminator, 50 X TAE buffer, TE buffer

Protocol:
1. Spot Test Assay:
   1) Prepare 0.8% (w/v) agarose/ethidium bromide plates by melting 0.8g agarose in 100ml 1 x TAE buffer (50 x TAE: 2M Tris-acetate, 50mM EDTA), allowing agarose to cool to approximately 50°C and adding 10µl of a 10mg/ml ethidium bromide stock solution (final concentration was 1µg/ml). Plates can be stored for up to 1 month at 4°C in the dark.
2) Prepare standard DNA solutions of known concentrations to cover the concentration range of 10ng/µl to 200ng/µl in distilled water or TE buffer.

3) Spot standards (1ul) carefully onto the surface of a plate in duplicate followed immediately afterwards by the DNA sample of unknown concentration (1µl).

4) Allow spots to absorb for 10 to 15 minutes.

5) Visualize DNA by illumination using a shortwave UV light box.

The concentration of DNA in the unknown sample can be approximated by comparison with the standards.

1. **UV Spectroscopy:**
   1. Turn on machine and wait for it to warm up
   2. Wash quartz cuvettes thoroughly before use and if 2 are used then make sure they are a ‘matching’ pair.
   3. Set wavelength to 260 and auto zero with water.
   4. Put 5µl of DNA concentration into a clean quartz cuvette, add 1ml of water and mix by covering with parafilm and inverting.
   5. Insert cuvette into machine and note reading
   6. Follow steps 3 again but this time set wavelength to 280, note reading.
   7. Repeat procedure 3 times with new samples of DNA noting readings at both A260 and A280.
   8. Take an average of the A260 readings and multiply the figure by 10 to give the concentration of DNA per ml.
   9. Take average of A280 readings and divide A260 average by the A280 average this should give a figure between 1.8-2.0 if it is out of the range then the DNA is not pure and sample should be purified using phenol/chloroform.

**Result:** ____________________________________________________________
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**Inference/Discussion:** ____________________________________________
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7. EXTRACTION OF PROTEINS

Extraction of proteins is perhaps the most crucial steps in proteomic analysis. This aims to prepare the protein in its biologically active conformation.

Aim:
To extract and quantify total protein from *E.coli*.

Principle:
Although there are exceptions, many nuclear and cytoplasmic proteins can be solubilized by lysis buffers that contain the non ionic detergent Nonident P 40 and either no salt at all or relatively high concentrations of salt. However the efficiency of extraction is often greatly influenced by the pH of the buffer and the presence or absence of chelating agents like EGTA or EDTA. Extraction of membrane bound proteins and hydrophobic proteins are less affected by the ionic strength of the lysis buffer but often requires a mixture of ionic and non ionic detergents. Many methods of solubilization, particularly the mechanical methods like sonication release intra cellular proteases that can digest the proteins. Hence it is essential to incorporate some degraded proteins in the extraction buffer which are more prone to be attacked by the proteases. It is also important to keep the extracts in colder conditions. In addition some protease inhibitors may also be incorporated in to the extraction buffer. Here in we are attempting to prepare total lysates from small scale cultures of *E.coli*.

Materials Required:
Overnight grown *E.coli* culture, 1M Tris Cl pH 7.4, 2X SDS gel loading buffer (100mM Tris-Cl pH6.8, 200 mM DTT, 4%SDS, 0.2% Bromophenol Blue, 20% glycerol)

Protocol:
1. Inoculate a single colony of *E.coli* in 10 ml LB and aerate overnight at 100 rpm at 37°C for about 8-12 hours.
2. Transfer 1 ml of the overnight grown culture to a micro centrifuge tube and harvest at 12000 rpm at 4°C.
3. Remove the supernatant by aspiration leaving the bacterial pellet as dry as possible.
4. Resuspend the pellet by vortexing in 25µl of sterile water. As soon as the pellet disperses add 25µl of 2X SDS gel loading buffer and vortex for 20 – 30 seconds.
5. Place the tube in a boiling water bath for 5 minutes.
6. Shear the chromosomal DNA by sonication, using a sonictor for 30 seconds to 2 minutes in a full pulse.
7. Centrifuge the sample at 12,000rpm at room temperature.
8. Dissolve the pellet in 25µl f 2X SDS loading buffer and store at 4°C.
Result:
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Inference/Discussion:
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________________________________________________________________________
8. **SEPARATION OF PROTEINS ON POLY ACRYLAMIDE GEL ELECTROPHORESIS (PAGE) AND DETERMINATION OF MOLECULAR WEIGHT**

Almost all analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of proteins into their individual polypeptide subunits.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecules, acquiring a high net negative charge that is proportional to the length of the polypeptide chain.

**Aim:**
To separate proteins on a denaturing Polyacrylamide gel and visualize the separated proteins by Coomassie Brilliant Blue stain.

**Principle:**
SDS PAGE uses an anionic detergent (SDS) to denature proteins. the protein molecules become linearized. One SDS molecule binds to 2 amino acids. Due to this, the charge to mass ratio of all the denatured proteins in the mixture becomes constant. These protein molecules move in the gel (towards the anode) on the basis of their molecular weights only & are separated.

The charge to mass ratio varies for each protein (in its native or partially denatured form). Estimation of molecular weight would then be complex. Hence, SDS denaturation is used.

The gel matrix is formed of polyacrylamide. The polyacrylamide chains are crosslinked by N,N-methylene bisacrylamide comonomers. Polymerisation is initiated by ammonium persulfate (radical source) and catalysed by TEMED (a free radical donor and acceptor).

The resolution & focus of the protein bands is increased by using discontinuous gels (Laemmli gels)- the stacking gel (pH 6.8, %T=3 to 5 %) & the resolving gel (pH 8.8, %T= 5 to 20 %). %T represents acrylamide percentage. These gels are usually run at constant current. At pH=6.8, most of the glycine in the population exist as zwitterions with no negative charge (pKa 1 =2.45; pKa 2 =9.6; pl=6.025). Only 0.0015% of the glycine is anionic at this pH (refer glycine titration curve & Henderson-Hasselbach equation). As such, bulk of the current is carried by the denatured, negatively charged, SDS-coated protein molecules. At this stage, the glycine ions lag behind the proteins. The order is as follows- chloride ions, denatured proteins, glycine ions.

Upon entering the resolving gel (pH=8.8), the glycine zwitterions deprotonate to the anionic form. The proportion of these ions increase from 0.0015% to 15.8%. The carrying of the current is now shared by the ions such that protein molecules have a greater freedom to separate on the basis of molecular weights. Due to their small size, the glycine anions also tend to overtake the protein band, thus providing a sandwiching effect & greater resolution in the gel.
**Materials Required:**

Protein sample, Molecular weight marker, gel apparatus, gel casting trays, staining trays, power packs,

Solutions:

A) 30% Acrylamide monomer solution:
   Acrylamide: 29g  
   N,N'- Methylene Bis Acrylamide: 1g  
   Distilled water to make: 100 ml

B) 10% stock solution of SDS

C) TEMED

D) Ammonium Persulphate 10% solution (Make Fresh)

E) Tris Buffer pH 6.8 (1.5M)

F) Tris Buffer pH 8.8 (1.5M)

G) Tris Glycine electrophoresis Buffer
   25 mM Tris base  
   250 mM Glycine  
   0.1% SDS  
   pH 8.3
   (Prepare a 5X stock by dissolving 15.1 g Tris base and 94 g glycine in 900 ml distilled water and then add 50 ml of 10% (w/v) SDS and adjust the volume to 1000 ml)

H) 1X gel loading buffer
   50 mM Tris .Cl (pH 6.8)  
   100 mM DTT  
   2% SDS  
   0.1% bromophenol Blue  
   10% glycerol  
   (Don’t add DTT and store. DTT must be added just before use from a 1M stock)

I) Coomassie Brilliant Blue staining solution
   Dissolve 0.25 g CBBR 250 in 90 m l of Methanol: water (1:1v/v) and 10 ml of glacial acetic acid. Filter the solution through Whatman No1 filter paper and store.

J) Destaining solution: Methanol: glacial acetic acid (30%: 10%).
### Table 1: Solutions for preparing Resolving gels (Separating gels) SDS PAGE

<table>
<thead>
<tr>
<th>Components</th>
<th>Component volume (ml) per gel mold volume of 5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6% gel</strong></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>2.6</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>1.0</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>1.3</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% Ammonium per sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.0004</td>
</tr>
<tr>
<td><strong>8% gel</strong></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>2.3</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>1.3</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>1.3</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% Ammonium per sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>10% gel</strong></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1.9</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>1.7</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>1.3</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% Ammonium per sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>12% gel</strong></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1.6</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>2.0</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>1.3</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% Ammonium per sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>15% gel</strong></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1.1</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>2.5</td>
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<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>1.3</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% Ammonium per sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Table 2 Solutions for preparing 5% Stacking gels for SDS PAGE

<table>
<thead>
<tr>
<th>Component</th>
<th>Component volume (ml) per gel mold volume of 2ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.4</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>0.33</td>
</tr>
<tr>
<td>1.5M Tris (pH 6.8)</td>
<td>0.25</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.02</td>
</tr>
<tr>
<td>10% Ammonium per sulphate</td>
<td>0.02</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Protocol:

1. Clean the glass plates with a few drops of SDS and wipe it dry with a clean soft tissue paper so that it is free from any sort of grease and dirt.

2. Assemble the plates as per the instructions given by the manufacturer.

3. Determine the volume of the mold by pouring water between the assembled plates. Also determine the stacking and separating gel volumes as per the requirement.

4. Prepare the Separating gel mix as per the Table 1 and pour it in between the plates and allow it to polymerize undisturbed (make sure that the solution is free from air bubbles before you pour). Overlay with iso butanol (overlaying prevents oxygen from diffusing into the gel and inhibiting the polymerization).

5. Clean the surface of the gel with Tris buffer pH6.8 once or twice to remove any traces of unpolymerized acrylic acid.

6. Prepare the Stacking gel as per Table 2 and pour on the polymerized separating gel.

7. Gently insert the comb into the space such that enough well size is obtained.

8. Leave it undisturbed to polymerize.

9. Clean the wells with the Tris glycine electrophoretic buffer or Tris buffer pH6.8 once or twice.

10. Assemble the gel on to the electrophoretic chamber and pour enough tris glycine electrophoretic buffer in both the tanks.

11. Denature the protein samples after adding the 1X gel loading buffer in a boiling water bath for 1 -2 minutes.

12. Cool the samples and load up to 15µl in to the wells along with Molecular weight marker using a microsyringe or a fine tip.

13. Separate the proteins by applying a constant current of 50 V/cm² till you see the glycine front down the separating gel.
**Staining the gel with CBBR 250:**

14. After the proteins have separated gently remove the spacers and the gel. Remove the stacking gel gently from the separating gel using a fine scalpel blade.

15. Immerse the gel in 5 gel volumes of Staining solution and rock it gently for 3-4 hours at room temperature.

16. Remove the stain and destain the gel several times in destining solution.

17. Store the gels in water and document the gel by photography for further record.

**Result:**

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________________________________________________________________________
________________________________________________________________________

**Inference/Discussion:**

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
9. **SCREENING FOR AUXOTROPHIC AND PROTOTROPHIC MARKERS**

**Aim:**

To isolate single colony and to check for genetic markers His, Arg, Met and lactose

**Principle:**

Auxotrophic markers present in the bacteria prevents its growth on Minimal media. Hence these must be supplemented in the media. These auxotrophic markers can be identified by plating the bacterial strain on minimal media along with all amino acids and glucose as the carbon source except the amino acid/sugar/nucleotide that is being screened for. For example if we are screening for Met, the minimal media would contain all amino acids except Methionine and glucose as the carbon source. Bacteria would not require the addition of the ingredients separately if they are prototrophic. Screening for markers is essential step in attributing the genetic trait to the strains and also helps the experimenter to use the right host strain for recombinant work.

**Materials Required:**

**Strain:** *E.coli* JM 109 (rec A1 supE44 end A1 hsdR17 gyrA96 relA1 thiΔ (lac-proAB) F’ (tra D36 proAB’ lacIq lacZΔ M15)

**Media:** LB agar, L broth, Minimal Media agar: Per liter (K2HPo4- 10.5 g, KH2Po4- 4.5g, (NH4)2 So4-1g, Sodium Citrate.dihydrate-0.5 g, pH 7.0), 20% stock solutions of glucose and lactose, 20% stock solutions of all required amino acids.

Other requirements: L rod, Lazy Susan Turn table, Petri plates, Sterile eppendorfs, microtips, micropipette etc….

**Protocol:**

1. Inoculate an overnight culture of *E.coli* JM 109 in 10 ml L broth.

2. Prepare the Minimal agar plates as follows:
   
   MMA+ Amino acids+ glucose (Control)
   
   MMA+ All amino acids except His+glucose
   
   MMA+ All amino acids except Arg+ glucose
   
   MMA+ All amino acids +lactose and not glucose

3. Maintain a control LB agar plate (Control)

4. Streak a loop full of the overnight grown culture on to the above plates and incubate at 37 ° C for 12-24 hours for the Minimal media plates and 12 hours for LB agar plates.

5. Compare the Minimal plates with the control plates and comment on the genetic makeup of the strain.
Result:

__________________________________________________________________________
__________________________________________________________________________
__________________________________________________________________________
__________________________________________________________________________
__________________________________________________________________________

Inference/Discussion:

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__________________________________________________________________________
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__________________________________________________________________________
10. **ANTIBIOTIC SENSITIVITY TESTS FOR BACTERIA (KIRBY-BAUER METHOD)**

Antibiotics are chemicals that are produced by living organisms which, even in minute amounts, inhibit the growth of or kill another organism. While thousands of them have been discovered since Sir Alexander Fleming observed the inhibitory activity of Penicillium on Staphylococcus in 1929, most are not useful medically because of undesirable toxicity or side effects. A simple method using paper discs saturated with the chemicals to be tested can be used to determine the resistance or sensitivity of an organism to different antibiotics. Following inoculation of an agar medium, discs are placed onto the surface of the medium so that the antibiotic will diffuse into the medium. Inhibition of the organism is evident following incubation as a clear region around the disc, called a "zone of inhibition", in which no growth has occurred.

**Aim:**

To perform the Kirby Bauer testing or the disc diffusion antibiotic sensitivity testing to assess the resistance and susceptibility status of the given bacteria to the given antibiotics

**Principle:**

A known quantity of bacteria is grown on agar plates in the presence of thin wafers containing relevant antibiotics. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the wafer where bacteria are not capable of growing (called a zone of inhibition).

The size of the zone and the rate of antibiotic diffusion are used to estimate the bacteria's sensitivity to that particular antibiotic. In general, larger zones correlate with smaller minimum inhibitory concentration (MIC) of antibiotic for those bacteria. This information can be used to choose appropriate antibiotics to combat a particular infection. Medical laboratory personnel select the antibiotic discs tested based upon the site and type of infection. Following incubation and measurement of the zone sizes, a chart is consulted which indicates whether the diameter of the zone of inhibition for an antibiotic indicates that it would be effective for use in treating the patient's infection.

**Materials Required:**

Fresh cultures of *E. coli*, Salmonella Sp, Staphylococcus, and Bacillus, Sterile swabs, Antibiotic discs, Disc dispenser, incubator and disinfectant bath

Media: Trypticase Soy Agar (TSA)- Per Liter (Tryptone-17g, Soytone- 3g, Dextrose-2.5g, Sodium Chloride-5 g, K2HPO4-2.5g, pH7.3, Agar 2%) or Nutrient Agar (NA): (Per Liter: Peptone-15g, yeast extract-3g, Sodium chloride -6 g, glucose -1g, pH7.2, Agar 2%)
Protocol:

1. Dip one swab into the broth of *E.coli*.
2. Swab the entire surface of one plate labeled *E.coli*. Go over the plate at least twice in each direction. Discard the swab in the beaker of disinfectant provided.
3. Repeat this procedure for the cultures.
4. Add the antibiotic discs to each plate using the dispenser. Remove the cover of the petri dish and set the dispenser over the plate.
5. Gently tap each antibiotic disc onto the surface of the agar with a sterile stick or toothpick to assure good contact. Discard toothpicks in the disinfectant.
6. Incubate plates at 37°C for 8-12 hours.
7. Examine each plate for the presence of zones of inhibition.
8. Measure the diameter of each zone in millimeters using the rulers provided.
9. Compare the diameter of each zone to the chart to determine if the organism is sensitive or resistant to the antibiotic.
10. Tabulate your results as follows:

<table>
<thead>
<tr>
<th>Antibiotic on the disc</th>
<th>Zone diameter</th>
<th>Resistant diameter</th>
<th>Intermediate diameter</th>
<th>Sensitive diameter</th>
<th>Sensitive/Resistant</th>
</tr>
</thead>
<tbody>
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Result:________________________________________________________________________________________
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Inference/Discussion:________________________________________________________________________
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11. **IN VITRO MUTAGENESIS OF BACTERIA**

**Aim:**

To induce mutations in the given bacterial strain by exposing it to UV rays and screen for mutants.

**Principle:**

UV irradiation causes numerous photo products such in DNA. Two types of photo dimmers occurring at adjacent pyrimidines have been correlated with mutagenesis. Both frame shifts and base substitutions are induced by UV, which also stimulates recombination and certain types of genetic rearrangements. Among base substitutions, transitions predominate, although their prevalence of four to five times over transversions is far less than for alkylating agents. In some systems, G:C→A:T transitions are favoured, whereas in other systems both transitions appear to occur with similar frequencies. UV mutagenesis requires the SOS system in bacteria. Since some of the lesions that trigger induction of the SOS system, as well as some premutagenic lesions, are photoreversions, precautions should be taken to carry out the mutagenesis in the absence of strong visible light.

**Materials Required:**

UV lamp, E.coli Strain CSH 100( F’ lac pro A+B+ ) araΔ (gpt-lac) 5), LBA, Rifampicin (50 mg/ml in methanol), Centrifuge, sterile test tubes, petri plates, 0.1 M MgSO₄

**Protocol:**

1. Inoculate a fresh overnight culture of *E.coli* CSH100 in 10 ml LB.
2. Subculture the overnight culture by diluting 1:20 in LB and incubate at 37°C for 3-4 hours.
3. When the cells have reached a density of 10⁸/ml, spin them down and resuspend the cells in an equal volume of 0.1 M MgSO₄. Pool the cells to one container and place on ice for 5 – 10 minutes.
4. For each time point (0 sec to 90 Sec), pipette 5 ml of the cells into a sterile petri dish, swirl the dish to evenly spread the cells. Place the dish on a flat surface under UV lamp1
5. Pipette the exposed cells into a sterile test tube and titer samples for viable cells by plating dilutions on LB plates. For the control (no exposure to UV) plate 10-5 and 10-6 dilutions and for all other time points plate 10-3, 10-4, 10-5 dilutions on LB and incubate at 37 °C overnight.
6. Inoculate LB medium with samples of mutagenized cells, diluting approximately 1:20 and aerate over night at 37 °C.
7. Place the overnight cultures both viable and to test for different mutants. To look for Rif⁵ mutants, plate neat, 1⁰¹ and 1⁰² dilutions of the mutagenized overnight grown cells on LBA+ 15 µg/ml rifampicin. Incubate the plates overnight at 37 °C.
8. Score for the number of Rifampicin resistant mutants and calculate the % survival rate and frequency of the mutants.

**Table 1: Survival after UV treatment**

<table>
<thead>
<tr>
<th>Time (UV) Sec</th>
<th>No of colonies on LBA</th>
<th>Titre Cfu/ml</th>
<th>Survival (%)</th>
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**Table 2: Frequency and total number of Rif\(^r\) mutants after UV irradiation**

<table>
<thead>
<tr>
<th>Time (UV) Sec</th>
<th>Survival (%)</th>
<th>Rif(^r) frequency</th>
<th>Total Rif(^r) mutants/ml mutagenized cells</th>
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</thead>
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**Result:**
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**Inference/Discussion:**
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PLANT BIOTECHNOLOGY
12. PLANT TISSUE CULTURE- AN INTRODUCTION

Plant tissue culture is the science or art of growing plant cells, tissues or organs isolated from the mother plant, on artificial media. The culture initiation and plant regeneration are still accomplished empirically by varying conditions until the desired response is obtained. Scheiden and Schwann’s suggestion that the totipotency of cells was the foundation of the plant cell and the tissue culture. However it was Haberlandt who took the first real steps in \textit{in vitro} cultures. Since then many investigators have studied plant regeneration under \textit{in vitro} conditions and have obtained further understanding of cell totipotency.

Tissue culture is a collective term commonly used to describe all kinds of \textit{in vitro} plant cultures. The tissue cultures can be cultures of an organized tissue, viz., callus cultures, suspension or cell cultures, proplast cultures, pollen cultures etc., or cultures of organized structure like meristem cultures, shoot and root cultures, embryo cultures, inflorescence cultures, ovule cultures, etc.. Plant tissue culture has direct commercial applications as well as value in basic research into cell biology, genetics and biochemistry.

Prepartion of glassware:

Cleaning:

It is important to use clean glassware for the growth of tissue \textit{in vitro}. In our laboratory these include:

1. Boil all glassware in washing soda solution (10%) for 1 hour.
2. Rinse thoroughly with tap water and leave in HCl (1N) for 2 hours.
3. Remove traces of acid by thorough washing with tap water.
4. Rinse glassware with double-distilled water.
5. Allow glassware to dry overnight at room temperature.

Sterilization:

The objective of sterilization is to make media, glassware and instruments free from microorganisms. It is accomplished by wet heat, dry heat or filtration.

1) Wet-heat sterilization:

1. Plug glassware such as conical flasks, test tubes, etc. with non-absorbent cotton.
2. Wrap petriplates dishes with wrapping paper or aluminium foil.
3. Place forceps and scalpels in test tubes. Plug the tubes with non-absorbent cotton and cover with brown paper.
4. Plug the mouth end of graduated pipettes (1, 2, 5 and 10ml) with cotton. wrap them individually in wrapping paper or aluminium foil.
5. Autoclave glassware and instruments at 121\(^{0}\)C for 1 hour.
2) Dry-heat sterilization:
Steps 1-4 are similar as wet-heat sterilization. But the final step involves placing the instruments in an oven at 140\(^0\)C-160\(^0\)C for 2 hours.

3) Filter sterilization:
Amino acids, vitamins, phytohormones, etc., may get destroyed during autoclaving. So such chemicals are therefore sterilized by filtration through a Seitz or Millipore filtration assembly using filter membranes of 0.45 or 0.22\(\mu\)m porosity.

1. Plug 500 or 1000 ml receiver flask with cotton.
2. Assemble Millipore filtration unit with bacteriological membrane filter (0.45 or 0.22 \(\mu\)m).
3. Wrap filtration until with wrapping paper.
4. Autoclave receiver flask and filtration unit at 121\(^0\)C for 1 hour (do not sterilize in dry heat as membrane filters get damaged).
5. Fix filtration unit to the receiver flask in a sterile cabinet.
6. Pour solution to be sterilized into the filtration.
7. Apply slight air pressure to commence filtration (do not exceed air pressure by 7.03 Kg/cm\(^2\)).
8. Transfer under aseptic conditions to sterile flasks.
9. Using a sterile pipette and filter sterilized solution to the autoclaved medium, shake well and dispense into sterile cultures tubes or flasks under aseptic conditions.

Preparation of media:
The appropriate composition of the medium largely determines the success of the cultures. Plant materials do vary in their nutritional requirements and therefore it is often necessary to modify the medium to suit a particular tissue. Initially tissues and organs from a wide variety of plant species were cultures on the nutrient salt solutions formulated by Gautheret and White. However, these were found inadequate for sustaining growth of many plant tissues. This led to the formation of several basal nutrient media (Hildebrandt et al., 1946; Burkholder & Nickel, 1949; Heller, 1953; Murashige and Skoog, 1962; Gamborg et al., 1968). In general, the medium contains i) inorganic salts, ii) vitamins, iii) growth regulators, iv) carbon source and v) organic supplements.

Inorganic salts:
These are divided into two groups: major and minor salts.

Major salts: The salts of potassium, nitrogen, calcium, magnesium, phosphorous and sulphur constitute the major salts. Nitrogen is generally used as nitrate or ammonium salts, sulphur as sulphates and phosphorus as phosphates.
**Minor salts:** The salts of iron, zinc, manganese, boron, copper, cobalt, molybdenum, iodine, etc., make up the minor salts. These salts are essential for the growth of tissues and are required in trace quantities.

**Vitamins:**

The B-vitamins play an important role in growth of tissues. Thiamine, nicotinic acid and pyridoxine are generally incorporated in all media, although pantothenic acid, folic acid, biotin, riboflavin, etc., have also been used.

**Growth regulators:**

Growth as well as differentiation of tissues in vitro is controlled by various growth regulators, auxins, cytokinins, gibberellins, ethylene, absciscic acid, etc..

**Auxins:**

Indole acetic acid, indole butyric acid, naphthalene acetic acid, 2,4-dichlorophenoxy acetic acid are the frequently used auxins. These are generally used at 0.1 to 10mg/l concentration in plant tissue culture media. Naphthalene acetic acid and 2,4-dichlorophenoxy acetic acid are thermostable and do not lose their activity on autoclaving. Whereas, Indole acetic acid is thermolabile and loses most of their activity on autoclaving. Hence, it is sterilized by filtration.

**Cytokinins:**

Cytokinins have a profound effect on cell division and cell differentiation. Kinetin, zeatin and 6-benzylamino purine, the commonly used cytokinins, are used in 0.1-10mg/l concentration.

**Others:**

Gibberellic acid, ethylene releasing compounds and absciscic acid are also incorporated in the media.

**Carbon sources:**

Plant tissues in culture can utilize a variety of carbohydrates-sucrose, glucose, fructose, starch and maltose. Sucrose, at 2-5% concentrations in the nutrient media, however, remains the most widely used carbohydrate source.

**Organic supplements:**

Complex substance such as yeast –extract, malt-extract and casein hydrolysates are also added (0.1-1% w/v).

Among various plant extracts, liquid endosperm of immature (coconut water) is widely used (5-20% v/v)

**General methodology for media preparation:**

Stock solutions of major salts, minor salts and vitamins are prepared to be used in the preparation of media are stored in a refrigerator. For preparing 1 litre of the medium:
1. Transfer appropriate amounts of stock solution of salts to 1 litre flasks.
2. Add vitamins, auxins, cytokinins and inorganic supplements.
3. Add a carbohydrate source such as sucrose (2-5%)
4. Adjust the pH to 5.6-5.8 using a pH meter.
5. Make up the volume to 1 litre with double distilled water.
6. Add powdered agar 8g/l for making the medium semisolid.
7. Cover the flask with paper of aluminium foil and keep in a steamer or water-bath at 100°C for dissolving the agar.
8. Shake the flask well for the uniform distribution of agar.
9. Dispense the medium into culture tubes or flasks as the case may be.
10. Autoclave tubes or flasks containing medium at 121°C for 20 minutes.
11. Again steam the medium for 20 minutes on the following day (i.e. the day after it is prepared).

**Surface sterilization of plant material:**

Before inoculating the medium with the explants, it is necessary to surface sterilize it. There are many sterilizing agents such as calcium hypochlorite, chlorine water, bleaching powder, mercuric chloride, hydrogen peroxide and ethylene oxide. The concentration of the sterilizing agent and the duration of treatment varies with the plant material. These should be no damage to the explants in the process of surface sterilization. The plant material should then be thoroughly washed with sterile distilled water before transferring it to the nutrient medium.

**a) Sterilization of seeds:**

1. About 15-20 healthy seeds were selected and wash them thoroughly with water.
2. Now added 2-3 drops of liquid detergent 100ml of water and shake well for 5 minutes.
3. Pour off the detergent solution and wash thoroughly to remove any traces of the detergent.
4. Rinse seeds in 50ml of 70% ethanol for 1 minutes.
5. Wash thoroughly 3-4 times with single distilled water.
6. Transfer seeds in a sterile 250ml flask and add mercuric chloride solution (0.1%).
7. Treat for 20 minutes, shake the flask occasionally. All the operations thereafter should be carried out under sterile conditions.
8. Transfer the flask to a sterile cabinet, decant the mercuric chloride solution and wash the seeds thoroughly with sterile water to remove any traces of mercuric chloride.
b) **Sterilization of leaves:**

1. Collect the apical portion about 20cm long from 6-8 month old plants.
2. Remove the outer green sheathing leaves with a scalpel till the inner thin and white leaves are exposed.
3. Dip 2-3 cm long segments of this portion in 70% alcohol for 1 minute. Then follow the steps 1-8 as in sterilization of seeds.
4. Using sterile forceps, place segments in a sterile petridish and remove 1-2 outer leaves with scalpel.
5. Cut the inner leaves into 5mm segments.
6. Transfer 1-2 leaf segments to each tube containing 20ml of semisolid medium.
13. MICROPROPAGATION

Tissue culture is particularly useful for multiplication of plants which are slow growing (turmeric, ginger, cardamom); cross-pollinated (coconut, teak, eucalyptus, cashew, mango and those which show wide variation in the progeny); male-sterile lines (cotton, sorghum, pearl-millet); newly produced varieties (normally vegetatively propagated); and for multiplication of virus free plants by meristem cultures (sugarcane, potatoes, tapioca, etc.).

Tissue culture is now being commonly used for clonal propagation of a large number of horticultural plants. Crop plants and also for forest trees (Murashige;1974; Conger,1981). The success of clonal multiplication in higher plants depends generally on 3 main stages:

**STAGE 1: ESTABLISHMENT OF AN ASEPTIC CULTURE**

The explants taken from the plant has first to be made free of microorganisms which would outgrow the plant tissue when placed on a nutrient medium. This would result in the death of the explants. These surface contaminants, e.g. bacteria, fungi and yeast are removed by surface sterilization prior to culture, but without killing the plant tissue.

**STAGE 2: MULTIPLICATION**

The surface sterilized material when inoculated on sterile nutrient media and incubated at 25±2°C with a definite photoperiod and light intensity grows to form large number of shoots.

**STAGE 3: ROOTING AND HARDENING OF PLANTS**

The shoots obtained are carefully excised and transferred to a rooting medium, preferably a liquid medium, containing an auxin and supported on a filterpaper platform in order to obtain rooting in these shoots.

These plants which have rooted and have developed secondary roots with root hairs can be transferred to pots containing soil:vermiculite mixture (1:1). This mixture is preautoclaved for 1 hour at 15 psi and steamed for 3 days successively and cooled. The potted plants can be transferred to the field where the first new leaf emerges.

**MULTIPLICATION BY SUBCULTURE AT STAGE 2**

However, excised shoot tips can be inoculated on the same medium used in stage 2 instead of the rooting media. By regular repetition of this subculture procedure, high rates of multiplication can be achieved.

Vegetative multiplication of plants depends on various factors as nutrient medium, agar concentration, photoperiod and light intensity, hydrogen ion concentration, size and source of the explants (Conger,1983).
Explants source:

Requirements:

a) Equipments

   Conical flasks (100ml capacity)
   Test tubes (25mm*150mm)
   Petridishes (80mm diameter)
   Pair of forceps and scalpel (15 cm long)

   Environmental growth cabinets adjusted to 25º±2ºC with 18hr photoperiod and 1500lux intensity and 15º±2º and 600 lux light intensity.
   Shaker with 120rpm and 1000 lux light intensity.

b) Culture media, washing solutions, sterilizing agents

   Glass distilled water
   Sterile glass distilled water
   0.5%HgCl solution
   Detergent
   Medium

b) Source tissue

Procedure

a. Sterilization of glassware

b. Preparation and sterilization of media

c. Explants collection:

   1. Select a twig (60-90 cm long, 10-15mm wide) from mature elite trees and cut, making sure that the twig contains many young axillary buds. The length is important in selecting twig that do not wither before being brought to the laboratory.

   2. Bring the twigs containing axillary bud to the laboratory, remove the leaves and cut them into small pieces of about 5-8 cm.

   3. Transfer the buds to a sterile 250ml conical flask and surface sterilize the explants.

d. Culture of buds:

   1. Keep sterile petridishes, scalpel, forceps and medium inside a sterile cabinet along with the flask containing surface-sterilized explants.
2. Transfer these explants into sterile petri dishes with the help of a pair of sterile forceps and cut these explants into small pieces of 10-15 mm each containing at least one axillary bud.

3. Inoculate 2 pieces to each tube containing medium.

4. Incubate the tubes in an environment growth cabinet at 15°C±2°C and 500 lux light intensity for 72 hours.

5. Transfer the cultures after 72 hr to another incubator maintained at 25°C±2°C with 16 hr photoperiod and 1500 lux intensity.

6. After 25 days, the young buds start sprouting.

7. When the sprouts are 10-15 mm long, transfer them to liquid medium in 100 ml Erlenmeyer flasks.

8. Incubate the flasks on a rotatory shaker at 120 rpm and 500 lux light intensity.

9. Observe the formation of multiple shoots after 10-15 days.

e. Multiplication by subculture:

1. Transfer the multiple shoots from the flask to a sterile petridish aseptically.

2. Incubate the cultures in an environmental growth cabinet at 25°C±2°C and at 1000 lux light intensity (12 hr photo periods) and observe the cultures regularly.

3. Observe the explants produce multiple shoots within 15 days.

4. Separate these shoots again aseptically and transfer the tubes containing medium for shoot formation.

f. Transfer of plants to pots:

1. Remove the rooted plantlet from the tube and wash the roots gently with tap water to remove any traces of medium.

2. Transfer the plantlets to soil: vermiculite (1:1) sterile mixture in a pot.

3. Irrigate with about 20 ml of tap water.

4. Keep the pots in a growth cabinet at 25°C±2°C and at 1000 lux light intensity and water them.

5. Transfer the plants to the field after 8 days of hardening in which 70-80% plants survive.
### Result:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Number of bottles inoculated</th>
<th>Number of explants inoculated per bottle</th>
<th>Number of explants developed per bottle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
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### Inference/Discussion:

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14. CALLUS INDUCTION AND PRODUCTION OF SECONDARY METABOLITES

Plant tissues in cultures can be in the form of callus cultures, suspension cultures or organ cultures.

Callus is basically a more or less non-organized tumor tissue which usually arises on wounds of differentiated tissues and organs. Thus, in principle, it is a non-organized and little differentiated tissue. The cells in callus are of a parenchymatous tissue.

Callus formation takes place under the influence of exogenously supplied growth regulators present in nutrient medium. The type of growth regulator requirement and its concentration in the medium depends strongly on the genotype and endogenous hormone extent of an explants.

Callus tissue induced from different plant species may be different in structure and growth habit: white or coloured, soft (watery) or hard, friable (easy to separate into cells) or compact. The callus growth within a plant species may also depend on factors such as the original position of the explants within the plant, and the growth conditions.

After callus induction, the callus is grown further on a new medium which is referred to as sub culturing. When sub cultured regularly on agar medium, callus cultures will exhibit an S-shaped or sigmoid pattern of growth during each passage. There are five phases of callus growth:

1. Lag phase, where cells prepare to divide
2. Exponential phase, where the rate of cell division is highest.
3. Linear phase, where cell division slows but the rate of cell expansion increases.
4. Deceleration phase, where the rates of cell division and elongation decreases.
5. Stationary phase, where the number and size of cells remain constant
6. Callus growth can be monitored by fresh weight measurements, which are convenient for observing the growth of cultures over time in a non-destructive manner.

Aim:

To induce callus from the leaves of _____ plant.

Requirements:

1. Equipments
   Conical flasks (100ml capacity)
   Test tubes (25mm*150mm)
   Petridishes (80mm diameter)
   Pair of forceps and scalpel (15 cm long)
   Environmental growth cabinets adjusted to 25°±2°C with 18hr photoperiod and 1500lux intensity and 15°±2° and 600 lux light intensity.
   Shaker with 120rpm and 1000 lux light intensity.
2. Culture media, washing solutions, sterilizing agents
   Glass distilled water
   Sterile glass distilled water
   0.5%HgCl solution
   Detergent
   Medium

3. Source tissue

Procedure:
1. Transfer 1-2 leaf segments to each tube containing 20 ml of semisolid medium.
2. Incubate tubes at 25°C in dark. Callus initiation starts after one week. Enough callus will be observed within one month.
3. Place 5mm*5mm pieces of callus on the surface of fresh medium.
4. Incubate cultures at 25°C in dark.
5. Repeat step 4 every month for maintaining the callus tissue.

Result:_______________________________________________________________________
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Inference/Discussion:_______________________________________________________________________
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15. ISOLATION OF TANNINS, ALKALOIDS FROM TISSUE CULTURE GROWN TISSUE & CHARACTERIZATION BY GCMS & FTIR

Introduction:
Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to particulate directly in growth and development. These substances, traditionally referred to as secondary metabolites, often are differentially distributed among limited taxonomic groups within the plant kingdom. Their functions, many of which remain unknown, are being elucidated with increasing frequency. The primary metabolites in contrast, such as phytosterols, acyl lipids, nucleoyides, amino acids, and organic acids, are found in all plants and perform metabolic roles that are evident. Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups: the terpenoids, the alkaloids, and the phenylpropanoids and allied phenolic compounds.

Alkaloids have a 3000 year history of human use. For much of human history, plant extract has been used as ingredient in potions and poisons. In the eastern Mediterranean, use of latex of the opium poppy (Papavae soniferum) can be traced back at least to 1400-1200BC. The Sarpagandha root (Rauwolfia serpentina) has been used in India since approximately 1000BC.

Tannins are naturally occur in plant polyphenols and widely distributed in plant Kingdom. Their main characteristic is that they bind and precipitate protein. They can have a large influence on the nutritive value of many foods eaten by humans and feed stuff eaten by animals.

Aim:
To isolate tannins and alkaloids from tissue culture grown tissues and characterization by GCMS and FTIR.

Principle:
Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer’s reagent to give a cream coloured precipitate. This is due to halogenations followed by complex reaction mechanism. Gas chromatography (GC) can separate volatile and semi volatile compounds with great resolution, but it cannot identify them. Therefore, it was not surprising that the combination of the two techniques was suggested shortly after development of GC. GC works on principle that a mixture will separate into individual substances when heated. The separated substances flow into the MS, which identifies compounds by the mass of the analyst molecule.

Materials required:
Callus, water bath, Mayers reagent, HCl, ethanol, bezene, chloroform, water, etc..

Procedure:
Isolation of alkaloids:
1. Weigh 50mg of plant material and add ethanol
2. Mix well and incubate for 48 hours.
3. Allow the above mixture to evaporate by using water bath at 60ºC for about 15 minutes.
4. Dispense the above extract in dilute HCl and mix thoroughly.
5. Filter the extract and add few drops of Mayer’s reagent.
6. Observe the change in color creamy precipitate.

a) Isolation of tannins:
   1. Weigh 50mg of plant material and add ethanol.
   2. Mix well and incubate for 48 hours.
   3. Allow the above mixture to evaporate by using water bath at 60ºC for about 20 minutes.
   4. Dispense the above extract in dilute HCl and mix thoroughly.
   5. Filter the extract and add few drops of ferric chloride.
   6. Observe the change in color green colour.
   7. 

b) Characterization by FTIR and GCMS
   Analyse the extract in GCMS and FTIR and observe the results.

Result:________________________________________________________________________
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Inference/Discussion:
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16. PREPARATION OF SYNTHETIC SEEDS

Introduction:
The concept of artificial seeds was first introduced in 1970 as a noble analogue to plant seeds. The production of artificial seeds is especially useful for plants which do not produce viable seeds. It represents a method to propagate these plants. Artificial seeds are small sized and this provides further advantage to storage, handling, shipping and planting.

Artificial seeds are the living seed like structures derived from somatic embryoids in vitro culture which is produced by encapsulating a plant propagule in a matrix and will allow it to grow into a plant. The preserved embryoids are also called synthetic seeds. The three major parts of a seeds are endosperm, embryo, testa. In the production of artificial seeds, an artificial endosperm can be created within the encapsulating matrix.

Aim:
To prepare synthetic seeds from _____ and test their viability.

Principle:
The major principle involved in the alginate encapsulation process is that the sodium alginate droplets containing the somatic embryo when dropped into the CaCl$_2$.2H$_2$O solution. The hardness or rigidity of the capsule mainly depends upon the number of sodium ions exchanged with calcium ions. Hence, the concentration of the two gelling i.e., sodium alginate and CaCl$_2$.2H$_2$O and complexing should be standardized for the formation of capsule.

Materials required:
Plant material, 2-4% sodium alginate solution in MS basal media, Calcium or sodium nitrate, sterile distilled water, pipette.

Procedure:
Dropping method:
1. Mix the seeds with 2%-4% sodium alginate prepared in MS-basal medium.
2. With the help of pipette (with desirable pore diameter) drop the embryos in the bath of calcium salt e.g., Ca(NO$_3$)$_2$ or CaCl$_2$.2H$_2$O solution (75-100mM) for 30 minutes. This results in very quick complex formation at the surface due to exchange of ions (i.e) Na$^+$ and Ca$^+$. Consequently, enclose individual seeds into clear and hardened beads of about 4mm diameter.
3. Rinse the artificial seeds with sterile water and then sieve the beads through a nylon mesh.
4. Store beads at 4°C or use as synthetic seeds for immediate sowing.
Result:

<table>
<thead>
<tr>
<th>S.No</th>
<th>No. of seeds inoculated</th>
<th>No. of seeds germinated</th>
<th>% viability</th>
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<tbody>
<tr>
<td>1</td>
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Inference/Discussion:

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17. PREPARATION OF PRIMARY CULTURE

Introduction to animal cell culture:

Animal cell cultures were successfully undertaken by Ross Harrison in 1907. Several developments occurred which made cell cultures a widely available tool. First there was the development of antibiotics that made it easier to avoid contamination problems. Second, was the development of techniques such as the use of Trypsin to remove cells from culture vessels. Third, development of standardized chemically defined culture media that made cells grows easier.

When the cells are surgically removed from an organism and placed into a suitable culture environment, they will attach, divide and grow. This is called primary cultures. When the cells in primary culture vessel have grown, and filled up all the available culture, substrate they must be sub cultured to give them space for continuous growth. This is usually done by enzyme treatment or by gently scrapping the cells off the bottom of the culture vessels.

Establishment of chick embryo fibroblast primary cultures:

Embryo is very good source for processing primary culture. A primary culture is a heterogeneous culture having definite life span and limited population doublings.

Aim:

To initiate the primary culture of fibroblast from 9th day old chick embryo.

Principle:

Embryo from an embryonated egg is a potent source of fibroblastic cells. The embryo is carefully isolated mechanically and enzymatically disaggregated. Trypsin is the most commonly used proteolytic enzyme to disaggregate the tissue. The action of trypsin is neutralized by the addition of media, containing serum which has anti-trypsin. The cells are then pelleted and seeded to the flask at 1x10^6 cells /ml per flask and incubated at 37°C, 5% CO_2 for the formation of confluent monolayer. This gives the primary culture. The primary culture of fibroblastic cell has typical spindle shape morphology. When viewed under a inverted microscope.

Materials required:

Embryonated chick eggs (9days old), 70% ethanol, sterile scissors, bent scissors, forceps, sterile petriplates, pipettes, sterile 1*PBS, culture flask, funnel with muslin cloth, centrifuge inverted microscope, canted neck flask.

Media: RPMI 1640, Penicillin-Streptomycin, trypsin

Procedure:

1. Examine 9th day embryonated egg under a bright light source for its viability (the live embryo can be seen as a shadow).
2. Place the egg in a beaker with the blunt end up and swab with 70% ethanol.
3. Puncture the top of the egg gently with a pair of sterile forceps and remove the shell thus exposing the underlining, chorioallantoic membrane (care should be taken that the shell should not fall into the egg).

4. Cut chorioallantoic membrane with a pair of sterile scissors such that the embryo gets exposed.

5. Remove the embryo gently by the neck using a sterile forceps and transfer to a petridish containing 1X PBS and wash twice or thrice to remove the yolk or blood.

6. Using forceps, remove the head, lymph and viscera. Transfer the remaining tissues to another petridish. With 1X PBS, mix the embryo thoroughly using a pair of scissors and allow pieces to settle.

7. Add 8ml of 1:125 trysin versene phosphate (TVP) to the minced tissue and incubate at 37ºC for 15 minutes.

8. Neutralize the trypsin by adding an equal volume of RPMI1640 medium with serum.

9. Filter the cells through a sterile muslin cloth (Prewetted with 1X PBS) to remove the debris and centrifuge the cell suspension at 1000rpm for 20 minutes.

10. Resuspend the pellet in fresh RPMI 1640 medium with serum and take 0.1ml to a haemocytometer and count for viability with Trypan Blue dye.

11. Then take the cells in 25cm² canted neck flask with 1X10⁶ cells/ml flask and incubate at 37ºC with 5% CO₂ in a CO₂ incubator.

12. Observe the following day under an inverted microscope.

**Result:**

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**Inference/Discussion:**

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18. PASSAGING & CELL VIABILITY CHECK BY TRYPHAN BLUE METHOD

Aim:

To passage the primary culture of chick embryo fibroblast and to check cell viability by using trypan blue method.

Principle:

First, subculture represents an important transition for a culture. The need to subculture implies that the primary culture has increased to occupy all of the available substrates. Hence cell proliferation has become an important features. Although the primary culture may have a variable growth fraction depending on the types of cells present in the culture after the first subculture, the growth fraction is usually high (80% or more) from a very heterogenous primary culture containing many of the cell types present in the original tissue, a more homogenous cell lines emerges.

Materials required:

1XPBS, Trypsin, RPMI 1640, culture vessel with primary culture.

Protocol:

1. Remove the spent cell culture medium and discard from the culture vessel.
2. Wash the cells using 1XPBS by adding it on the sides of the vessel opposite to the attached cell layer to avoid disturbing the cell layer. Rock the vessel back and forth several times.
3. Remove the wash solution and discard from culture vessel.
4. Add the pre-warmed dissociation reagent (Trypsin) to the side of the flask. Gently rock the container to get the complete coverage of the cell layer.
5. Incubate the culture vessel at room temperature for approximately 2 minutes.
6. Observe the cells under microscope for detachment.
7. Add equal volume of prewarmed growth medium with serum. Dispense the medium by pipette over the cell layer surface several times.
8. Transfer the cells to 15ml of conical tubes and spin at 1000rpm for 5-10minutes.
9. Resuspend the cell pellet in a minimal volume of prewarmed complete growth medium and remove a sample for counting.
10. Determine the total number of cells and % viability using the haemocytometer.
11. Dilute the cell suspension to the seeding density and pipette out the approximate volume into a new culture and incubate.

Tryphan Blue Count for Viability:

Quantitation in cell culture in required for the characteristics of the growth properties of different cell lines for experimental analysis and to establish reproducible culture conditions for the consistency of primary culture and maintenance of cell lines.
Materials required:
Haemocytometer chamber, microscope, coverslip, Trypan blue dye, alcohol for cleaning, buffered isotonic salt solution of pH 7.2-7.3 (i.e PBS) for preparation of 0.4% of tryphan blue.

Protocol:
1. Clean the chamber with alcohol and place coverslip on it.
2. Add 10µl of the harvested cells to the haemocytometer and it should not be overfilled.
3. Place the chamber in inverted microscope under 10X objective and under phase contrast to distinguish the cells.
4. Count the cells in large central gridded square (1mm square). Determine the cell density using haemocytometer.
5. Prepare 0.4% of Tryphan blue in PBS and add 0.1ml of it to 1ml of the cells.
6. Load on haemocytometer and examine immediately under the microscope at low magnification.
7. Count the number of blue stained cells and number of total cells and calculate the cell viability. It should be at least 95% for healthy log phased culture.

Result:
\[
\text{% Viability} = \frac{\text{Number of live cells} - \text{Number of dead cells}}{\text{Total number of cells}}
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Result:_______________________________________________________________________
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Inference/Discussion:
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19. MTT ASSAY

Introduction:
Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population’s response to extend factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric method.

Aim:
To check cell viability and cell proliferation by MTT assay.

Principle:
Cells in the exponential phase of growth are exposed to a cytotoxic drug. The duration of exposure is usually determined as the time required for maximal damage to occur, but is also influenced by the stability of the drug. After removal of the drug, the cells are allowed to proliferate for two to three population doubling times (PDTs) in order to distinguish between cells that remain viable and are capable of proliferation and those that remain viable but cannot proliferate. The number of surviving cells is then determined indirectly by MTT dye reduction. The spectrophotometrically once the MTT-formazan has been dissolved in a suitable solvent.

Materials required:
Growth medium, Trypsin, MTT, microtitre plate (flat-bottomed), microtitre plate reader with 650-570nm filters, inverted microscope, sterile tubes, multichannel pipette, serological pipette, 37ºC incubator, sterile pipette tips, Laminar air flow chamber.

Protocol:
1. Add 3ml of Ficol hypaque to the centrifuge tube.
2. Carefully layer the diluted blood sample (4ml) on Ficol hypaque. When layering, do not mix Ficol hypaque and diluted blood.
3. Centrifuge at 400Xg for 30-40 minutes at 18-20ºC.
4. Draw off the upper layer using a clean Pasteur pipette, leaving the lymphocyte layer undisturbed at interphase. Care should be taken not to disturb lymphocyte layer. The upper layer of plasma, which is essentially free of cells, may be saved for later use.
5. Carefully remove the lymphocyte, and suspend in balanced salt solution.
6. Prepare the cells for MTT assay.
7. Add the suspended cells for about 50µl onto the wells from 1-6 of the microtitre plate among which 1st well is the control.
8. Add 5 fold dilution of cytotoxic drug serially from the well 2-6
9. Make up the volume to 200µl with sterile RPMI1640 medium and allow the cells to grow.
10. Incubate the cells for 24 hours.
11. After incubation, discard the medium and add 50µl of MTT to all the wells and allow to incubate for 4 hours.
12. At the end of 4 hours, remove the medium and MTT from the wells and dissolve the remaining MTT formazan crystals by adding 200µl of DMSO to all the wells.
13. Record the absorbance at 570nm immediately using an ELISA plate reader.
14. Plot a graph of absorbance (Y axis) against drug concentration (X axis).
15. Determine the IC\textsubscript{50} concentration as the drug concentration, that is required to reduce the absorbance to half that of the control.

Result:__________________________________________________________________________
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Inference/Discussion:
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20. MICRONUCLEUS TEST

**Introduction:**

The micronucleus (MN) test is a part of the battery of tests that many new products must go through prior to bringing them to market.

The *in vitro* micronucleus assay is a mutagenic test system for the detection of chemicals which induce the formation of small membrane bound DNA fragmentation (i.e) micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes which are unable to migrate with the rest of the chromosomes during the anaphase of cell division.

**Aim:**

To perform micronucleus test with buccal cells.

**Principle:**

Cell cultures (V79TM) are exposed to the test substance both with and without metabolic activation. After exposure to a test substance, and addition of cytochalasin B for blocking cytokinesis cell cultures are grown for a period sufficient to allow chromosome damage to lead to the formation of micronuclei in bi- or multinucleated interphase cells. Harvested and stained interphase cells are then analysed microscopically for the presence of micronuclei. Micronuclei are scored in those cells that complete nuclear division following exposure to test them.

The purpose of the micronucleus assay is to detect those agents which modify chromosome structure and segregation in such a way as to lead to induction of micronuclei in interphase cells.

The following criteria for MN analysis were used in oral epithelial cells. A micronucleus

a) Must be less than one third the diameter of the main nucleus.
b) Must be on the same focal plane.
c) Must have the same color, texture and refraction as the main nucleus.
d) Must have a smooth oval or round shape; and
e) Must be clearly separated from the main nucleus.

**Material required:**

Buccal cells, soft tooth brush, eppendorf tubes, PBS at pH 7.0, cold methanol:acetic acid (3:1), 2% Giemsa stain, light microscope.

**Protocol:**

1. Collect buccal smear from the participant (who need to wash his mouth with water before).
2. Place it on slide.
3. Spread the sample with a drop of water and allow to dry.
4. Dip the slide in a mixture of Glacial acetic acid: methanol (3:1) for about 1 minute.
5. Dry the slides and stain with Giemsa stain for about 2-3 minutes.
6. Wash the slide with tap water and air dry it.
7. View the slide under microscope and count the number of micronucleus present in the sample.

Result:

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NANOTECHNOLOGY
21. SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLE

Introduction:

One key aspect of nanotechnology concerns the development of the rapid and reliable experiment protocols for the synthesis of nanomaterials over a range of chemical compositions, sizes, high monodispersity and large scale production. A variety of techniques have been developed to synthesis metal nanoparticles including chemical reduction using number of chemical reductants such as NaBH₄, N₂H₄, NH₂OH, ethanol, ethylene glycol and N,N-dimethyl formamide (DMF), aerosol technique, electrochemical or sonochemical deposition, photochemical reduction and laser irradiation technique.

Many method, a chemical reduction method (Chu et al., 2005), a polyol method (Lin & Yang, 2005) and radiolytic process (Shim et al., 2004) have been developed for the synthesis of silver nanoparticles. Among the methods, chemical reduction was widely studied, due to its advantages of yielding nanoparticles without aggregation, high yield and low preparation cost (Kim et al., 2004).

Aim:

To synthesis silver nanoparticle by chemical reduction method and biological method and to characterize synthesized silver nanoparticle by UV-Spectrophotometry, FTIR and SEM.

Principle:

The chemical reduction method involves the reduction of AgNO₃ in aqueous solution by a reducing agent in the presence of a suitable stabilizer, which is necessary in protecting the growth of the silver particles through aggregation. In the formation of silver nanoparticles by the chemical reduction method, the particle size and aggregation state of the silver nanoparticles are affected by various parameters such as initial AgNO₃ concentration, reducing agent, AgNO₃ molar ratios and stabilizer concentration (Song et al., 2009). The silver nanoparticles were prepared by using chemical method (Fang et al., 2005). All solutions of reacting materials were prepared in double distilled water. In typical experiment, 50ml of 1*10⁻³M AgNO₃ was boiled and to this solution 5ml of 1% trisodium citrate was added drop by drop and mixed vigorously until colour change is evident (pale brown). Then it was removed from boiling and stirred until cooled to room temperature

Mechanism of reaction could be expressed as follows:

$$4Ag + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4AgO + C_6H_5O_7H_3 + 3Na^+ + H^+ + O_2$$

The aqueous solution air dried up to 3 days and the dry powdered particles that were taken for further analysis.
Materials Required:

1 mM silver nitrate (50 ml)
15 Trisodium citrate (5 ml)
Ethanol
Double distilled water
Stirrer
Orbital shaker
UV-Spectroscopy, FTIR, SEM for characterization

Protocol:

a) Chemical Reduction Method:

1. Boil 50 ml of silver nitrate in boiling water bath.
2. To the solution, add 5 ml of tri sodium citrate drop by drop.
3. Stir vigorously and boil thoroughly until the change of color is evident.
4. Observe the pale brown color solution after boiling.
5. Cool down the solution to room temperature.
6. Centrifuge the solution at 8000 rpm for 10 minutes.
7. Wash the pellet thrice with ethanol.
8. Characterize the nanoparticles by UV-Vis, FTIR and SEM.

b) Biological method:

1. Weigh 10 g of biological material and add 100 ml of water
2. Boil the solution for 15 minutes at 80°C and filter it. Collect the filtrate in a beaker.
3. Add 90 ml of 1 mM silver nitrate to 10 ml of the filtrate.
4. Boil the solution for 20 minutes.
5. Observe color change from reddish brown.
6. Cool down the solution to room temperature.
7. Centrifuge the solution at 8000 rpm for 10 minutes.
8. Wash the pellet thrice with ethanol.
9. Characterize the nanoparticles by UV-Vis, FTIR and SEM.
c) **Characterization of silver nanoparticle:**

**UV-Visible absorption spectroscopy:**

1. With the resolution of 1nm, formation of metal nanoparticles was verified by recording the UV-Visible absorption spectrum provided surface plasmon resonance exists for the metal.

2. The synthesized silver nanoparticle reveals a strong band surface plasmon peak located at 390-420nm.

**Fourier Transform Infrared Spectroscopy:**

1. Prepare KBr pellets by excessive grinding with the sample.

2. Insert the pellet into IR sample holder and attach the scotch tape.

3. Run IR spectrum and observe the presence of silver nanoparticle.

**Scanning Electron Microscope:**

Completely dry the sample and analyze in SEM for observing its size & morphology

**Result:**

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Reproduce your result here.

**Inference/Discussion:**

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22. ATOMIC FORCE MICROSCOPY AND TRANSMISSION ELECTRON MICROSCOPE

Atomic Force Microscope

The Atomic Force Microscope was developed to overcome a basic drawback with STM - that it can only image conducting or semiconducting surfaces. The AFM, however, has the advantage of imaging almost any type of surface, including polymers, ceramics, composites, glass, and biological samples.

Binnig, Quate, and Gerber invented the Atomic Force Microscope in 1985. Their original AFM consisted of a diamond shard attached to a strip of gold foil. The diamond tip contacted the surface directly, with the interatomic van der Waals forces providing the interaction mechanism. Detection of the cantilever’s vertical movement was done with a second tip - an STM placed above the cantilever.

AFM probe deflection

Today, most AFMs use a laser beam deflection system, introduced by Meyer and Amer, where a laser is reflected from the back of the reflective AFM lever and onto a position-sensitive detector. AFM tips and cantilevers are microfabricated from Si or Si$_3$N$_4$. Typical tip radius is from a few to 10s of nm.

Measuring forces

Because the atomic force microscope relies on the forces between the tip and sample, knowing these forces is important for proper imaging. The force is not measured directly, but calculated by measuring the deflection of the lever, and knowing the stiffness of the cantilever. Hook’s law gives $F = -kz$, where $F$ is the force, $k$ is the stiffness of the lever, and $z$ is the distance the lever is bent.

![Graph showing force vs. probe distance](image)

Tip is in hard contact with the surface; repulsive regime
Tip is far from the surface; no deflection
Tip is pulled toward the surface - attractive regime

International Science Congress Association
AFM Modes of operation

Because of AFM’s versatility, it has been applied to a large number of research topics. The Atomic Force Microscope has also gone through many modifications for specific application requirements.

Contact Mode

The first and foremost mode of operation, contact mode is widely used. As the tip is raster-scanned across the surface, it is deflected as it moves over the surface corrugation. In constant force mode, the tip is constantly adjusted to maintain a constant deflection, and therefore constant height above the surface. It is this adjustment that is displayed as data. However, the ability to track the surface in this manner is limited by the feedback circuit. Sometimes the tip is allowed to scan without this adjustment, and one measures only the deflection. This is useful for small, high-speed atomic resolution scans, and is known as variable-deflection mode.

Because the tip is in hard contact with the surface, the stiffness of the lever needs to be less than the effective spring constant holding atoms together, which is on the order of 1 - 10 nN/nm. Most contact mode levers have a spring constant of < 1N/m.

Lateral Force Microscopy

LFM measures frictional forces on a surface. By measuring the “twist” of the cantilever, rather than merely its deflection, one can qualitatively determine areas of higher and lower friction.

Noncontact mode

Noncontact mode belongs to a family of AC modes, which refers to the use of an oscillating cantilever. A stiff cantilever is oscillated in the attractive regime, meaning that the tip is quite close to the sample, but not touching it (hence, “noncontact”). The forces between the tip and sample are quite low, on the order of pN (10⁻¹² N). The detection scheme is based on measuring changes to the resonant frequency or amplitude of the cantilever.

Dynamic Force / Intermittant-contact / “tapping mode” AFM

Commonly referred to as “tapping mode” it is also referred to as intermittent-contact or the more general term Dynamic Force Mode (DFM). A stiff cantilever is oscillated closer to the sample than in noncontact mode. Part of the oscillation extends into the repulsive regime, so the tip intermittently touches or “taps” the surface. Very stiff cantilevers are typically used, as tips can get “stuck” in the water contamination layer. The advantage of tapping the surface is improved lateral resolution on soft samples. Lateral forces such as drag, common in contact mode, are virtually eliminated. For poorly adsorbed specimens on a substrate surface the advantage is clearly seen.

Force Modulation

Force modulation refers to a method used to probe properties of materials through sample/tip interactions. The tip (or sample) is oscillated at a high frequency and pushed into the repulsive
regime. The slope of the force-distance curve is measured which is correlated to the sample's elasticity. The data can be acquired along with topography, which allows comparison of both height and material properties.

**Phase Imaging**

In Phase mode imaging, the phase shift of the oscillating cantilever relative to the driving signal is measured. This phase shift can be correlated with specific material properties that effect the tip/sample interaction. The phase shift can be used to differentiate areas on a sample with such differing properties as friction, adhesion, and viscoelasticity. The technique is used simultaneously with DFM mode, so topography can be measured as well.

**High Resolution TEM (HRTEM)**

After understanding that an accelerated electron beam can have a very high resolving power, we then move on to see how one can use this electron beam in imaging technology. There are 3 types of electron microscopes, namely the transmission electron microscope (TEM), scanning electron microscope (SEM), and scanning tunneling microscope (STM). A TEM contains four parts: electron source, electromagnetic lens system, sample holder, and imaging system

**Electron Source**

The electron source consists of a cathode and an anode.

The cathode is a tungsten filament which emits electrons when being heated. A negative cap confines the electrons into a loosely focused beam (Fig. 5). The beam is then accelerated towards the specimen by the positive anode. Electrons at the rim of the beam will fall onto the anode while the others at the center will pass through the small hole of the anode. The electron source works like a cathode ray tube.

**Electromagnetic lens system**

After leaving the electron source, the electron beam is tightly focused using electromagnetic lens and metal apertures. The system only allows electrons within a small energy range to pass through, so the electrons in the electron beam will have a well-defined energy.

1. Magnetic Lens: Circular electro-magnets capable of generating a precise circular magnetic field. The field acts like an optical lens to focus the electrons.

2. Aperture: A thin disk with a small (2-100 micrometers) circular through-hole. It is used to restrict the electron beam and filter out unwanted electrons before hitting the specimen.

**Sample holder**

The sample holder is a platform equipped with a mechanical arm for holding the specimen and controlling its position
**Imaging system**

The imaging system consists of another electromagnetic lens system and a screen. The electromagnetic lens system contains two lens systems, one for refocusing the electrons after they pass through the specimen, and the other for enlarging the image and projecting it onto the screen. The screen has a phosphorescent plate which glows when being hit by electrons. Image forms in a way similar to photography.

**Working principle**

TEM works like a slide projector. A projector shines a beam of light which transmits through the slide. The patterns painted on the slide only allow certain parts of the light beam to pass through. Thus the transmitted beam replicates the patterns on the slide, forming an enlarged image of the slide when falling on the screen.

TEMs work the same way except that they shine a beam of electrons (like the light in a slide projector) through the specimen (like the slide). However, in TEM, the transmission of electron beam is highly dependent on the properties of material being examined. Such properties include density, composition, etc. For example, porous material will allow more electrons to pass through while dense material will allow less. As a result, a specimen with a non-uniform density can be examined by this technique. Whatever part is transmitted is projected onto a phosphor screen for the user to see.
ABOUT THE AUTHORS

Dr S R Madhan Shankar had his M Sc in Biotechnology from Dr GRD College of Science in 2001, M.Phil-Biotechnology from Bharathidasan University in 2004 and was awarded with PhD in Systematic Botany by the Bharathiar University in 2010. He did his PhD at the Institute of Forest Genetics and Tree Breeding, Coimbatore. Presently he is working as Assistant Professor in Biotechnology at PSG College of Arts & Science, Coimbatore since November 2005. He has over 12 years of teaching experience spanning teaching UG & PG students of Biotechnology and handle projects for the individual students in the field of Plant Molecular Markers and Nanobiotechnology. He has guided around 50 students to obtain their Masters degree. He is currently guiding two PhD students. He is a life member of the Indian Society of Human Genetics, Indian Immunological Society, and Society for Applied Biotechnology and was selected to be the Senior Member of the Asia-Pacific Chemical, Biological& Environmental Engineering Society (APCBEES), Hongkong in the year 2012. He is an expert member of Board of studies in various colleges. He was awarded with Fellow Award 2010 for his outstanding contribution in the field of Forest Biotechnology by the Society of Applied Biotechnology. He has published 8 papers in Journals, 3 in magazines and 5 in proceedings. He has presented 12 papers in various national and international seminars/conferences. He has worked as resource person in 15 workshops/training programmes. He has organized 3 exhibitions for Popularization of Science sponsored by the Tamil Nadu State Council for Science & Technology, Chennai. He has attended several conferences, trainings and workshops and is also an online tutor. He is University First Rank and Gold Medalist in BSc Biotechnology and the recipient of Subbiah-Dharmanithi Gold Medal and Govt. of India PG Scholarship.
Dr. E. M. Rajesh, graduated from PEE GEE College of Arts & Science (Microbiology) 2001, did his Post Graduate (Microbiology) from Sengunthar Arts & Science College, Tiruchengode, INDIA. M. Phil and Ph. D both from the PG & Research Department of Microbiology, PSG College of Arts & Science, affiliated to Bharathiar University, Coimbatore, INDIA. He also has a Masters in Business Administration (Hospital Management). He has 5 years of industrial and research experience, presently he is working as Assistant Professor in Biotechnology at PSG College of Arts & Science, Coimbatore since February 2011. He has over 4 years of teaching experience spanning teaching UG & PG students of Biotechnology and handled projects for the individual students in the field of textile Biotechnology and nanoparticles in protective textiles. He is also a life member of the Indian Society of Human Genetics, Indian Immunological Society, and Society for Applied Biotechnology and was selected to be the Senior Member of the Asia-Pacific Chemical, Biological & Environmental Engineering Society (APCB EES), Hongkong in the year 2013. He has so far authored 14 research papers in national and international journals and 15 research papers in the proceedings of national and international conferences/seminars. His research interests are mainly focused on the surface modification of textile substrate using microbial extracellular combinatorial enzymes and enhancements of antimicrobial efficacy of cotton fabrics using enzymes.