MICROBIAL PHYSIOLOGY AND METABOLISM AND MICROBIAL GENETICS AND MOLECULAR BIOLOGY

M.Sc. MICROBIOLOGY

SEMESTER-II, PAPER-V

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FOREWORD

Since its establishment in 1976, Acharya Nagarjuna University has been forging ahead in the path of progress and dynamism, offering a variety of courses and research contributions. I am extremely happy that by gaining 'A+' grade from the NAAC in the year 2024, Acharya Nagarjuna University is offering educational opportunities at the UG, PG levels apart from research degrees to students from over 221 affiliated colleges spread over the two districts of Guntur and Prakasam.

The University has also started the Centre for Distance Education in 2003-04 with the aim of taking higher education to the door step of all the sectors of the society. The centre will be a great help to those who cannot join in colleges, those who cannot afford the exorbitant fees as regular students, and even to housewives desirous of pursuing higher studies. Acharya Nagarjuna University has started offering B.Sc., B.A., B.B.A., and B.Com courses at the Degree level and M.A., M.Com., M.Sc., M.B.A., and L.L.M., courses at the PG level from the academic year 2003-2004 onwards.

To facilitate easier understanding by students studying through the distance mode, these self-instruction materials have been prepared by eminent and experienced teachers. The lessons have been drafted with great care and expertise in the stipulated time by these teachers. Constructive ideas and scholarly suggestions are welcome from students and teachers involved respectively. Such ideas will be incorporated for the greater efficacy of this distance mode of education. For clarification of doubts and feedback, weekly classes and contact classes will be arranged at the UG and PG levels respectively.

It is my aim that students getting higher education through the Centre for Distance Education should improve their qualification, have better employment opportunities and in turn be part of country's progress. It is my fond desire that in the years to come, the Centre for Distance Education will go from strength to strength in the form of new courses and by catering to larger number of people. My congratulations to all the Directors, Academic Coordinators, Editors and Lesson-writers of the Centre who have helped in these endeavors.

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M.Sc. MICROBIOLOGY

SEMESTER-II, PAPER-V

205MB24 - MICROBIAL PHYSIOLOGY AND METABOLISM AND MICROBIAL GENETICS AND MOLECULAR BIOLOGY

SYLLABUS

Microbial Physiology

1) Spectrophotometric Estimation of Adenosine Triphosphate (ATP) From Fermented Broth
2) Volumetric Estimation of Microbial Ethyl Alcohol in Fermented Broth, Using the Potassium Dichromate Oxidation Method
3) Volumetric Estimation of Microbial Lactic Acid, By Titrable Acidity Method
4) Estimation of Microbial Polyunsaturated Fatty Acids (PUFA) By Volumetric Analysis

Molecular Biology

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2) Quantitative Analysis of Genomic DNA
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4) Qualitative Analysis of RNA
5) Quantitative Analysis of RNA

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MICROBIAL PHYSIOLOGY

EXPERIMENT 1

SPECTROPHOTOMETRIC ESTIMATION OF ADENOSINE TRIPHOSPHATE (ATP) FROM FERMENTED BROTH

Aim:

Spectrophotometric estimate ATP Production in fermented broth using enzymatic coupled assay

Principle:

Spectrophotometric estimation of Adenosine Triphosphate (ATP) from fermented broth generally involves extracting the intracellular ATP and measuring its absorbance, typically using UV spectrophotometry or coupled enzymatic assays. ATP in pure form exhibits a characteristic absorbance maximum at **259 nm** or **260 nm**.

1. Preparation and Extraction:

To estimate ATP from fermented broth, the intracellular ATP must first be released from the microbial cells, as free ATP in the broth is rapidly degraded.

- **Sample Pretreatment:** Centrifuge the fermentation broth to separate the biomass (cells) from the supernatant.
- **Extraction Method:** The most effective method for extracting intracellular ATP is using trichloroacetic acid (TCA) (e.g., 0.03 M to 0.5 M) or perchloric acid, which rapidly inactivates enzymes that degrade ATP.
- **Neutralization:** If using acid extraction, neutralize the extract with a buffer (e.g., 0.16 M potassium bicarbonate, 0.72 M KOH) to a pH suitable for enzymatic reactions.

2. Spectrophotometric Measurement:

- **Enzymatic Coupled Assay (More Specific):** For complex broth samples, a coupled enzymatic assay is preferred to avoid interference from other nucleotides. This method measures the formation of NADPH or NADH at **340 nm**, which is proportional to the ATP concentration.
  - *Reaction Example:* ATP + Glucose $\xrightarrow{\text{Hexokinase}}$ ADP + Glucose-6-phosphate.

    Glucose-6-phosphate + NADP+ $\xrightarrow{\text{G6PDH}}$ 6-phosphogluconolactone + NADPH.

M) and is often used to measure ATP as an indicator of cell viability.

EXPERIMENT 2

VOLUMETRIC ESTIMATION OF MICROBIAL ETHYL ALCOHOL IN FERMENTED BROTH, USING THE POTASSIUM DICHROMATE OXIDATION METHOD

Aim:

The volumetric estimation of microbial ethyl alcohol, particularly in fermented broth, is commonly performed using the **Potassium Dichromate Oxidation method**. Because fermentation broth contains interfering compounds (sugars, yeast extract), a preliminary distillation step is required to separate pure ethanol from the mixture.

Principle:

Ethanol is oxidized to acetic acid by potassium dichromate (K2Cr2O7) in an acidic medium (H2SO4) The orange-colored hexavalent chromium (Cr6+) is reduced to green-colored trivalent chromium (Cr3+). The amount of reduced dichromate is proportional to the concentration of ethanol, which can be determined either by titration or spectrophotometry.

$$\mathbf{2Cr_2O_7^{2-}+3C_2H_5OH+16H^+\rightarrow 4Cr3^++3CH_3COOH+11H_2O_2}$$

Reagents

- **Potassium Dichromate Reagent:** Dissolve 34 g of K2Cr2O7 in 500 mL of distilled water in a 1-liter volumetric flask. Slowly add 325 mL of concentrated H2SO4 while keeping the flask in an ice bath to avoid overheating. Cool and make up the volume to 1 liter with distilled water.
- **Standard Ethanol Solutions:** For calibration (e.g., 0% - 10% v/v).
- **Bromothymol blue indicator** (for neutralization).
- **NaOH solution** (0.1 N).

Experimental Procedure:

1. Sample Preparation and Distillation:

- **Pre-treatment:** If the sample is fermented broth, centrifuge it at 14,000 rpm for 10 min, or filter it to remove microbial cells. (Microbial cells growth was carried out in Yeast extract Peptone Dextrose (YPD) liquid medium. At first, one loopful fresh microbial colony was inoculated from a fresh YPD plate into the test tube containing 3 ml of YPD broth and incubated at 30˚C for 24 hours in a shaking water bath (100 rpm). Freshly grown cultures were inoculated 100-fold dilution into the conical flask containing 30 ml of YPD medium, incubated further at either 30˚C or 37˚C for 72 hours. The fermented sample was centrifuged at 14,000 rpm for 10 minutes. After centrifugation, the supernatant was collected, and filtered using syringe filter (0.22 - 0.45 μm).

- **Neutralization:** Add a few drops of bromothymol blue and titrate with 0.1 N NaOH to neutralize any volatile acids present.
- **Distillation:** Take 10-50 mL of the sample in a distillation flask. Distill and collect the distillate, ensuring the condenser tip dips into 10 mL of distilled water in the receiving flask (volumetric flask).
- **Final Volume:** Make up the volume of the distillate to a fixed volume (e.g., 100 mL) with distilled water.

2. Titration Method:

1) Pipette 1 mL of the distilled alcohol sample into a conical flask.
2) Add 10 mL of the potassium dichromate reagent.
3) Incubate the mixture at 60°C for 20 minutes in a water bath to ensure complete oxidation.
4) Cool the mixture to room temperature.
5) Titrate the remaining unreacted dichromate with a reducing agent (e.g., Ferrous Ammonium Sulfate or similar standard) until a color change is observed.
6) Perform a blank titration using distilled water instead of the sample.

3. Calculations:

Based on the dichromate consumed:

Experiment	**Vol of K2Cr2O7 ($V_{K2Cr2O7}$)**	**Vol of (Sample) Broth**	**Normality of K2Cr2O7 ($N_{K2Cr2O7}$)**	
1		5.0ml	0.1	
2			0.1	
3			0.1	

Note: 1.15is a conversion factor for ethanol oxidation.

$$\textit{Alcohol (\%w/V)} = \frac{V_{K2Cr2O7} \times N_{K2Cr2O7} \times 1.15}{\textit{Sample volumen}}$$

4.Observation and Result:

EXPERIMENT 3

VOLUMETRIC ESTIMATION OF MICROBIAL LACTIC ACID, BY TITRABLE ACIDITY METHOD

Aim:

Quantitative estimation of Lactic acid produced by Lactobacillus by volumetric analysis

Principle:

It is a common technique for quantifying the amount of acid produced during fermentation by lactic acid bacteria (LAB). This method is based on the neutralization of the carboxylic acid group in lactic acid with a strong base. **Titrimetric Analysis (Acid-Base Titration):** The most common method involves titrating a sample of the fermented broth against a standardized sodium hydroxide (NaOH) solution (e.g., 0.1 N) using phenolphthalein as an indicator

1. Materials and Reagents:

- **Sample:** Fermentation broth (e.g., MRS broth or fermented food supernatant).
- **Titrant:** 0.1 N or 0.1 M Sodium Hydroxide (NaOH) standard solution.
- **Indicator:** 1% Phenolphthalein solution (dissolved in ethanol).
- **Apparatus:** Burette, pipette (10 mL), Erlenmeyer flasks, distilled water, magnetic stirrer (optional).

2. Experimental Procedure:

1) **Sample Preparation:** Take 10 mL of the fermentation broth (supernatant, if centrifuged) and dilute it with 10 mL of distilled water.
    - *Note:* If the broth is dark, further dilution may be necessary to visualize the color change.
    - *Alternative:* For viscous samples, take 5 mL of the sample and dilute with 5 mL of distilled water.
2) **Indicator Addition:** Add 3-4 drops of 1% phenolphthalein indicator to the diluted sample.
3) **Titration:** Titrate the sample against 0.1 N NaOH solution from a burette.
4) **Endpoint Determination:** Continue adding NaOH dropwise with constant swirling until a faint, persistent pink color appears (lasting at least 15-20 seconds).
5) **Record Volume:** Record the volume of NaOH ($VNaOH$) in milliliters (mL).
6) **Replication:** Perform the titration in triplicate to ensure accuracy and calculate the mean volume.

3. Calculation:

Experiment	Vol of NaOH (V_{NaOH})	Vol of (Sample) Broth	Normality of NaOH (N_{NaOH})	
1		5.0ml	0.1	
2			0.1	
3			0.1	

The percentage of lactic acid (%w/V) in the broth is calculated using the following formula:

$$\text{Lactic Acid (\% w/v)} = \frac{V_{NaOH} \times N_{NaOH} \times \text{Equivalent Weight of Lactic Acid}}{\text{Sample Volume (mL)} \times 10}$$

Simplified formula for 0.1 N NaOH and 10 mL sample:

$$\text{Lactic Acid (\% w/v)} = \frac{0.1 \times V_{NaOH} \times 90.08}{10 \times 10} = \frac{9.0 \times V_{NaOH}}{100}$$

(Where 90.08 is the equivalent weight of lactic acid).

4. Observation and Result:

EXPERIMENT 4

ESTIMATION OF MICROBIAL POLYUNSATURATED FATTY ACIDS (PUFA) BY VOLUMETRIC ANALYSIS

The estimation of microbial Polyunsaturated Fatty Acids (PUFA) by volumetric analysis is generally achieved through the determination of the

Iodine Value (IV) or **Acid Value (FFA)** via titration. While gas chromatography (GC) is the standard for detailed identification, volumetric methods are used to determine total unsaturation, where higher iodine values indicate higher amounts of PUFA (more double bonds).

Below is an experimental procedure for estimating microbial oil unsaturation using the Wijs method (a type of volumetric analysis) and general FFA determination.

1. Materials and Reagents:

- **Microbial Biomass:** Dried and ground microbial cells (e.g., fungi, microalgae, marine bacteria).
- **Solvents:** Chloroform, Methanol, Hexane.
- **Wijs Solution:** Iodine monochloride in acetic acid (for iodine value).
- **Titrants:** 0.1 N Sodium thiosulfate ($Na_2S_2O_3$), 0.1 N Potassium hydroxide (KOH).
- **Indicators:** 1% Phenolphthalein in 95% ethanol, 1% Starch solution.
- **Other:** Potassium iodide (KI) solution (10%), Cyclohexane.

2. Experimental Procedure:

A. Biomass Harvesting and Lipid Extraction

1) **Harvesting:** Cultivate the microorganism and harvest the biomass by centrifugation (e.g., 8000 rpm for 10 min).
2) **Drying:** Dry the biomass at 50°C overnight or use lyophilization.
3) **Extraction:** Extract lipids using the modified Bligh and Dyer method: Mix 1 g of dried biomass with chloroform/methanol/water (11:08:10 v/v). Vortex and separate the phases.
4) **Concentration:** Dry the extracted lipid under nitrogen or in a rotary evaporator at 35°C.

B. Volumetric Analysis: Iodine Value (Total Unsaturation):

1) **Sample Prep:** Weigh 0.1–0.5 g of the extracted microbial oil into a 250 mL conical flask.

2) **Dissolution:** Dissolve the oil in 10 mL of chloroform or cyclohexane.
3) **Reaction:** Add 25 mL of Wijs solution. Stopper the flask, shake, and keep in the dark for 30–60 minutes.
4) **Addition of KI:** Add 10 mL of 10% KI solution and 100 mL of distilled water.
5) **Titration:** Titrate with 0.1 N Sodium thiosulfate until the yellow color disappears.
6) **End Point:** Add 1 mL of starch indicator and continue titrating until the blue color disappears.
7) **Blank:** Run a blank titration (without oil) following the same steps.

C. Volumetric Analysis: Free Fatty Acid (FFA) Value

1) **Preparation:** Dissolve 1–10 g of oil in 25 mL of hot neutral alcohol (neutralized with KOH).
2) **Indicator:** Add 2-3 drops of phenolphthalein.
3) **Titration:** Titrate against 0.1 N KOH until a faint pink color persists for 15 seconds.

3. Calculations:

- **Iodine Value (IV):**

$$\mathbf{IV=(B-S)\times N\times 12.69WIV}$$

Where

B=mL of Na2S2O3 for blank

S=mL for sample

N=Normality

W=Weight of sample

FFA % (as Oleic Acid):

$$\mathbf{FFA\ \%=V\times N\times 28.2mFFA}$$

Where

V=volume of KOH

N=Normality

m=mass of sample in gm

MOLECULAR BIOLOGY:

EXPERIMENT 1

ISOLATION OF GENOMIC DNA FROM E. COLI (DH5A)

Aim:

To isolate the genomic DNA from E.coli (DH5α) cells.

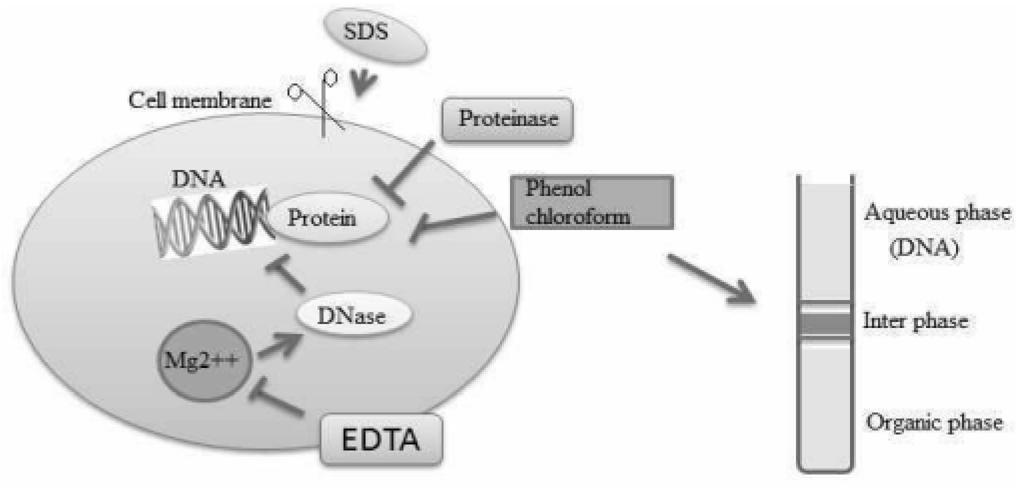
Background:

DNA was first isolated in 1869 by Friedrich Miescher at the University of Tübingen. He obtained DNA from human leukocytes washed from pus-laden bandages amply supplied by surgical clinics in the time before antibiotics usage, and he referred DNA as nuclein. He continued to study DNA and switched from leukocytes to salmon sperm as his starting material. Meisher's choice of starting material was based on the knowledge that leukocytes and sperm have large nuclei relative to cell size. DNA isolated from salmon sperm and from (bovine) lymphocytes is still available commercially.

Principle:

In prokaryotes, DNA is double-stranded and circular and is found throughout the cytoplasm. The cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl or lauryl sulfate) is used to disrupt the cell membrane. DNA can be protected from endogenous nucleases by chelating Mg2++ ions using EDTA. Mg2++ ion is considered as a necessary cofactor for most nucleases. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup. Phenol/ chloroform is used to denature and separate protein from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and a pure phenol layer. Proteins can also be removed by salting out proteins by sodium acetate. The denatured protein forms a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol. This method of DNA isolation gives good results for most of the Gram- negative bacteria. A few E. coli strains contain high molecular weight polysaccharides that may interfere with DNA. It needs to consider the genotype of E. coli strain to know whether we need to modify any steps to get a good outcome.

Schematic diagram of the principle of isolation of genomic DNA from E. coli



Materials Required:

1. LB Broth 2. E. coli DH5α cells 3. Reagents 4. TE buffer (pH 8.0) (10 mM Tris, 1mM EDTA) 5. 10% SDS 6. Proteinase K 7. Phenol-chloroform mixture 8. 5M Sodium Acetate (pH 5.2) 9. Isopropanol 10. 70% ethanol 11. Autoclaved Distilled Water 12. Eppendorf tubes 2 ml 13. Micropipettes 14. Microtips or pipette tips 15. Microfuge

Preparation of Reagents 1.TE BUFFER (pH 8.0): 10 mm Tris HCl (pH 8.0) 1 mm EDTA (pH 8.0) 2. 10% SDS: Dissolve 10 g of SDS in 100 ml autoclaved distilled water. 3.PROTEINASE K: Dissolve 10 mg of Proteinase K in 1 ml Autoclaved distilled water. 4.PHENOL/ CHLOROFORM MIXTURE: Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer and store it on ice. 5. 5M SODIUM ACETATE: Dissolve 41 g of sodium acetate in 100 ml distilled water and adjust pH to 5.2 with diluted acetic acid.

6. ISOPROPANOL

7. 70% ETHANOL

Procedure:

2 ml overnight culture was taken and cells were harvested by centrifugation for 5 minutes (2500g). 875 µl of TE buffer was added to the cell pellet and cells were resuspended in the buffer by gentle mixing. 100 µl of 10% SDS and 5µl of Proteinase K were added to the cells. The above mixture was mixed well and incubated at 37º C for 20 min in an incubator. 1ml of the phenol-chloroform mixture was added to the contents, mixed well by inverting and the samples were incubated at room temperature for 5 minutes. The contents were centrifuged at 10000 rpm for 10 minutes at 4º C. The highly viscous jelly-like supernatant was collected using cut tips and was transferred to a fresh tube. The process was repeated once again with t h e phenol-chloroform mixture and the supernatant was collected

in a fresh tube. To this 100 µl of 5M sodium acetate was added and mixed gently. 1.5 ml of isopropanol was added and mixed gently by inversion till white strands of DNA precipitated. The contents were centrifuged at 5000 rpm for 10 minutes. The supernatant was removed and 1ml 70% ethanol was added. The above contents were centrifuged at 5000 rpm for 10 minutes. After air-drying for 5 minutes, 50 µl of TE buffer or distilled water was added. The DNA samples were stored for further experiments.

Precautions:

Cut tips should be used so that the DNA is not subject to mechanical disruption. Depending on the source of DNA the incubation period of Proteinase K should be optimized. The phenol/chloroform extraction should be repeated depending on the source of DNA to obtain pure DNA. DNase free plastic wares and reagents should be used.

- **Results and Discussion:**

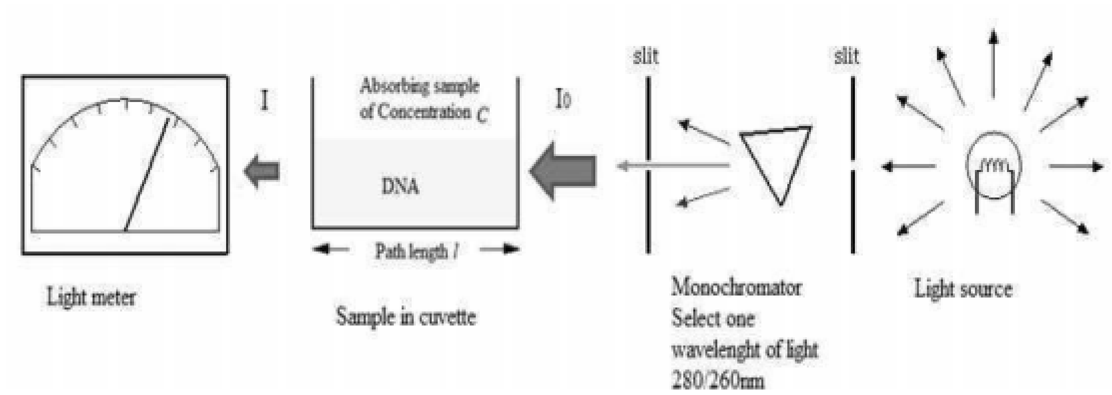
EXPERIMENT 2

QUANTITATIVE ANALYSIS OF GENOMIC DNA

Aim: To determine the amount, concentration and purity of the given DNA sample.

Principle:

This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by the following expression: The device UV spectrophotometer works on this principle and used to find the concentration of the sample. The concentration and quality of a sample of DNA are measured with a UV spectrophotometer. A standard graph can be drawn using different concentrations of DNA and OD (optical density) values.



The diagram above shows that a beam of monochromatic radiation (Io) is directed to a sample solution. Absorption takes place by the sample and the beam of radiation is leaving out (I).

Materials Required:

DNA Sample

- TE buffer
- UV spectrophotometer

Procedure:

Take the DNA sample (10 ul) in TE buffer. Now dilute the above sample by the factor of 100 i. e, by taking 10µl of the sample in 990µl of TE buffer. After doing this take the optical density value at A260 & A280 and calculate the amount of DNA recovered.

Use the following formula to determine the concentration of DNA:

Total DNA (µg) = 50 µg/ml x Absorbance at A260 x dilution factor x 0.05 ml

Where 0.05 ml is the total volume of the DNA.

Quality:

DNA quality measurement is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure DNA. If there is a contaminant, there is some additional OD, which decreases the OD ratio between 260 and 280 nm.

Clean DNA has an OD260/OD280 between 1.8 and 2.0

Results and Discussion:

EXPERIMENT 3

PLASMID DNA ISOLATION

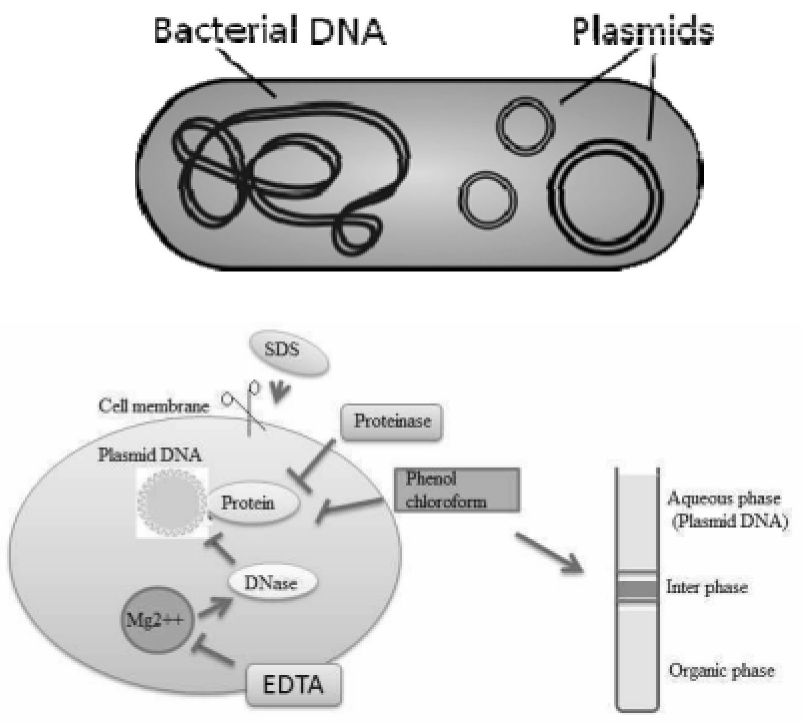
Aim: To isolate plasmid DNA from bacterial cells.

Principle:

When bacteria are lysed under alkaline conditions both DNA and proteins are precipitated. After the addition of acetate-containing neutralization buffer, the large and less supercoiled chromosomal DNA and proteins precipitate, but the small bacterial DNA plasmids can renature and stay in solution.

In prokaryotes, the plasmid is double-stranded, circular, and is found in the cytoplasm. The cell membranes must be disrupted in order to release the plasmid in the extraction buffer. Solution 1 contains glucose, Tris, and EDTA. Glucose provides osmotic shock leading to the disruption of the cell membrane, Tris is a buffering agent used to maintain a constant pH 8. The plasmid can be protected from endogenous nucleases by chelating Mg2++ ions using EDTA. Mg2++ ion is considered as a necessary cofactor for most nucleases. Solution II contains NaOH and SDS and this alkaline solution is used to disrupt the cell membrane and NaOH also denatures the DNA into single strands. Solution III contains acetic acid to neutralize the pH and potassium acetate to precipitate the chromosomal DNA, proteins, along with the cellular debris. Phenol /chloroform is used to denature and separate proteins from the plasmid. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and a pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. Once the plasmid DNA is released, it must be precipitated in alcohol. The plasmid DNA in the aqueous phase is precipitated with cold (0oC) ethanol or isopropanol. The precipitate is usually redissolved in the buffer and treated with phenol or an organic solvent to remove the last traces of protein, followed by reprecipitation with cold ethanol.

Schematic diagram of the principle of Plasmid DNA Isolation



Materials Required:

- Luria Broth
- Bacterial cells containing plasmid
- Reagents
- TE buffer (pH 8.0)
- Solution I, Solution II and Solution III
- Phenol-chloroform mixture
- Isopropanol
- 70% ethanol
- Autoclaved Distilled Water
- Eppendorf tubes 2 ml
- Micropipette
- Microtips
- Microfuge

Preparation of Reagents:

1) TE BUFFER (pH 8.0): 10 mm Tris HCl (pH 8.0) 1 mm EDTA (pH 8.0)
2) Resuspension solution (Solution-I):50 mM glucose, 10 mM EDTA, 25 mM Tris (pH 8.0), Store at 40 °C.
3) Lysis solution (Solution-II) - 0.2 N NaOH, 1% SDS, Store at room temperature.
4) Neutralizing solution (Solution-III) - 3 M KOAc (pH 6.0) - For 100 ml solution, 60 ml 5 M potassium acetate (49.07 g potassium acetate in 100 ml H2O). 11.5 ml glacial acetate and 28.5 ml H2O, store at room temperature.
5) Phenol – Chloroform Mixture: Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer and store it in dark.
6) Isopropanol

Procedure:

Take 2 ml of overnight culture and harvest cells by centrifugation for 5 minutes. Discard the supernatant carefully. Add 100 µl of solution I to the cell pellet and resuspend the cells by gentle mixing. Incubate the above mixture at room temperature for 5 minutes. Add 200 µl of solution II to the mixture and mix by inverting the tubes for 5 minutes. Incubate for 5-10 minutes at room temperature. Add 500µl of ice-cold solution III to the mixture and mix by inverting the tube. Incubate on ice for 10 minutes. Centrifuge at 10,000 rpm for 5 minutes. Transfer the supernatant into the fresh tube. Add 400 µl of the phenol-chloroform mixture to the contents, mix well by inverting and incubate them at room temperature for 5 minutes. Centrifuge at 10000 rpm for 5 minutes. Collect the supernatant (viscous) using micro tips and transfer to a fresh tube. Add 0.8 ml of isopropanol and mix gently by inversion. Incubate for 30 min at room temperature. Centrifuge the contents at 10,000 rpm for 10 minutes. Discard the supernatant after centrifugation. The contaminated salt in the DNA pellet can be removed with 70% ethanol washing. After washing air-drying for 5 minutes, add 100 µl of TE buffer or autoclaved distilled water to the pellet to resuspend the plasmid DNA. Take 10 µl of plasmid sample and dilute to 1 ml with distilled water for spectrometric analysis. The concentration of plasmid is determined using a spectrophotometer at 260/280 nm. An aliquot of plasmid DNA is used for agarose electrophoresis for quantitative and qualitative analyses.

Precautions:

Cut tips should be used so that the DNA is not subject to mechanical disruption. Depending on the source of DNA the incubation period of Proteinase K should be optimized. The phenol/chloroform extraction should be repeated depending on the source of DNA to obtain pure DNA. DNase free plastic wares and reagents should be used. Results and Discussion:

EXPERIMENT 4

QUALITATIVE ANALYSIS OF RNA

Aim:

To separate and visualize RNA bands by Agarose gel electrophoresis.

Introduction:

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

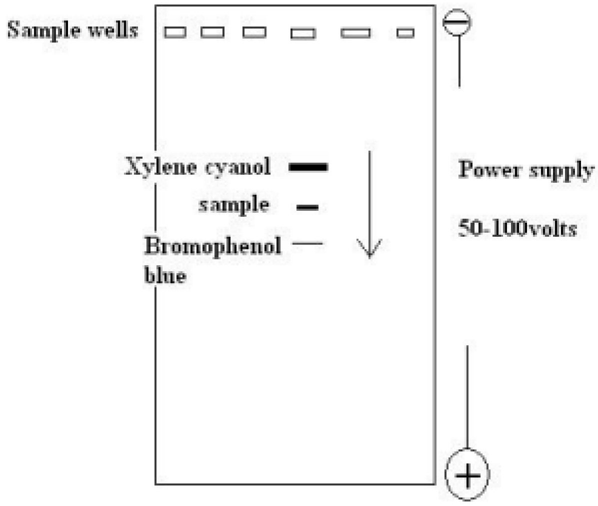
Principle:

The agarose gel contains molecule-sized pores, acting like molecular sieves. The pores in the gel control the speed that molecules can move. DNA molecules possess a negative charge in their backbone structure due to the presence of - PO4 groups thus this principle is exploited for its separation. Smaller molecules move through the pores more easily than larger ones. Conditions of charge, size, and shape interact with one another depending on the structure and composition of the molecules, buffer conditions, gel thickness, and voltage. Agarose gels are made with between 0.7% (provides good resolution of large 5–10 kb DNA fragments) and 2% (good resolution for small 0.2–1 kb fragments).

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

Purpose of Gel Loading Buffer:

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



Xylene cyanol gives a greenish-blue color while bromophenol blue provides a bluish colored zone. The successful DNA run is determined by the presence of both the colored dye in the gel. Materials Required:

Electrophoresis buffer: 1x TAE buffer

Agarose ultra-pure (DNA graded)

Electrophoresis tank, gel tray, sample comb, and power supply

Plastic or insulation tape

Ethidium bromide: 10 mg /ml stock solution

5x Gel loading dye

DNA marker solution, RNA sample, and gloves.

Procedure:

1) Making a 1% Agarose Gel Weigh 0.5 g agarose and dissolve it in 50 mL of 1x TAE Buffer. (Note: Use 250 ml conical flask for preparing 50 ml solution to avoid overflow of gel solution while heating and to avoid its loss.) Heat the solution over a hot plate to boiling constituency marked with a clear solution Leave the solution to cool and add 2µl of EtBr solution mix it well by gentle swirling. Pour it in the gel tray-comb set up. Also, be sure the gel plates have been taped securely and contain the well combs prior to pouring Allow the solution to cool and harden to form a gel.

2) Loading of Samples Carefully transfer the gel to the electrophoresis tank filled with a 1x TAE buffer. Prepare your samples [8 ul of RNA sample (0. 1 ug to 1 ug) and 2 ul of 5x gel loading dye] Remove the comb and load the samples into the well. Connect appropriate electrodes to the power pack and run it at 50- 100volts for 20min. Monitor the progress of the gel with reference to tracking dye (Bromophenol blue). Stop the run when the marker has run 3/4th of the gel. 3. Examining the gel Place the gel on the UV-transilluminator and check for orange- colored bands in the gel.

Precautions:

Wear gloves during the addition of EtBr and while handling the casted gel (EtBr is a potent carcinogen). Handling the gel should be careful as the gel may break due to improper handling. While performing the UV-trans illumination for visualizing the bands, avoid direct contact and exposure to eyes.

Results and Discussion:

EXPERIMENT 5

QUANTITATIVE ANALYSIS OF RNA

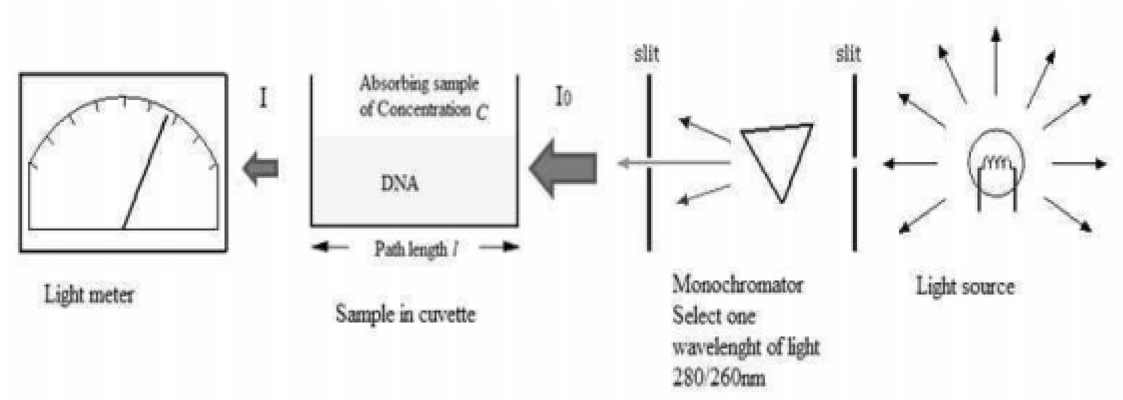
Aim:

To determine the amount, concentration and purity of the given RNA sample.

Principle:

This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by the following expression:

The device UV spectrophotometer works on this principle and used to find the concentration of the sample. The concentration and quality of a sample of RNA are measured with a UV spectrophotometer. A standard graph can be drawn using different concentrations of RNA and OD (optical density) values.



The diagram above shows that a beam of monochromatic radiation (Io) is directed to a sample solution. Absorption takes place by the sample and the beam of radiation is leaving out (I).

Materials Required: RNA sample, TE buffer, UV spectrophotometer

Procedure:

1. Take the RNA sample (10 ul) in TE buffer. 2. Now dilute the above sample by the factor of 100 i. e, by taking 10µl of the sample in 990µl of TE buffer. 3. After doing this take

the optical density value at A260 & A280 and calculate the amount of RNA recovered. 4. Use the following formula to determine the concentration of RNA: Total RNA (µg) = 40 µg/ml x Absorbance at A260 x dilution factor x 0.03 ml Where 0.03 ml is the total volume of the RNA. Quality: RNA quality measurement is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure RNA. If there is a contaminant, there is some additional OD, which decreases the OD ratio between 260 and 280 nm.

Results and Discussion: