Semester – II

I9MBP211 **ADVANCED PRACTICAL – III** **4H – 2C**

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**
End Semester Exam: 9 Hours

SCOPE

This practical is to provide the student with a basic knowledge in microbial biotechnology and acquire knowledge of the advanced techniques at molecular levels

OBJECTIVES

To obtain a good outstanding practical skill in various techniques in microbial biotechnology and agricultural microbiology

EXPERIMENTS

1. Isolation of plasmid DNA from Bacteria
2. Isolation of chromosomal DNA from Bacteria
3. Determination of molecular weight by SDS Polyacrylamide gel electrophoresis
4. Isolation of microbes from soil
5. Isolation of free-living N_2 fixation from soil - Azotobacter
6. Isolation of symbiotic nitrogen fixers from root nodule - Rhizobium
7. Isolation of phosphate solublisers, ammonifiers and denitrifers
8. Study of Mycorrhizae, Cynobacteria and Azolla
9. Determination of Dissolved oxygen of water
10. Determination of BOD (Biochemical Oxygen Demand) of water
11. Determination of COD (Chemical Oxygen Demand) of water
12. Estimation of Protein by Lowry's Method

REFERENCES

1. Aneja K.R. (2001). Experiments in Microbiology, Plant Pathology, Tissue Culture and Mushroom Production Technology, (3rd ed.). New Age International (P) Limited Publishers, New Delhi
2. Cappucino, J.G., and Sherman, N., (2001). Microbiology A Laboratory Manual, (6th ed.). Benjamin Cummings, New York.
3. Chirikjan, J.G., Kisailus, E.C., King, B., Krasner, R., and Mortensen, H., (1995). Biotechnology. Theory and Techniques, Vol II, Jones and Bartlett Publishers, London.
4. Palanivelu, P. (2004). Analytical Biochemistry and Separation Techniques, (3rd ed.). Twenty First Century Publication, Madurai.
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ADVANCED PRACTICAL III

EX.NO: 1

Preparation of Plasmid DNA by Alkaline Lysis with Sodium Dodecyl Sulfate: Minipreps

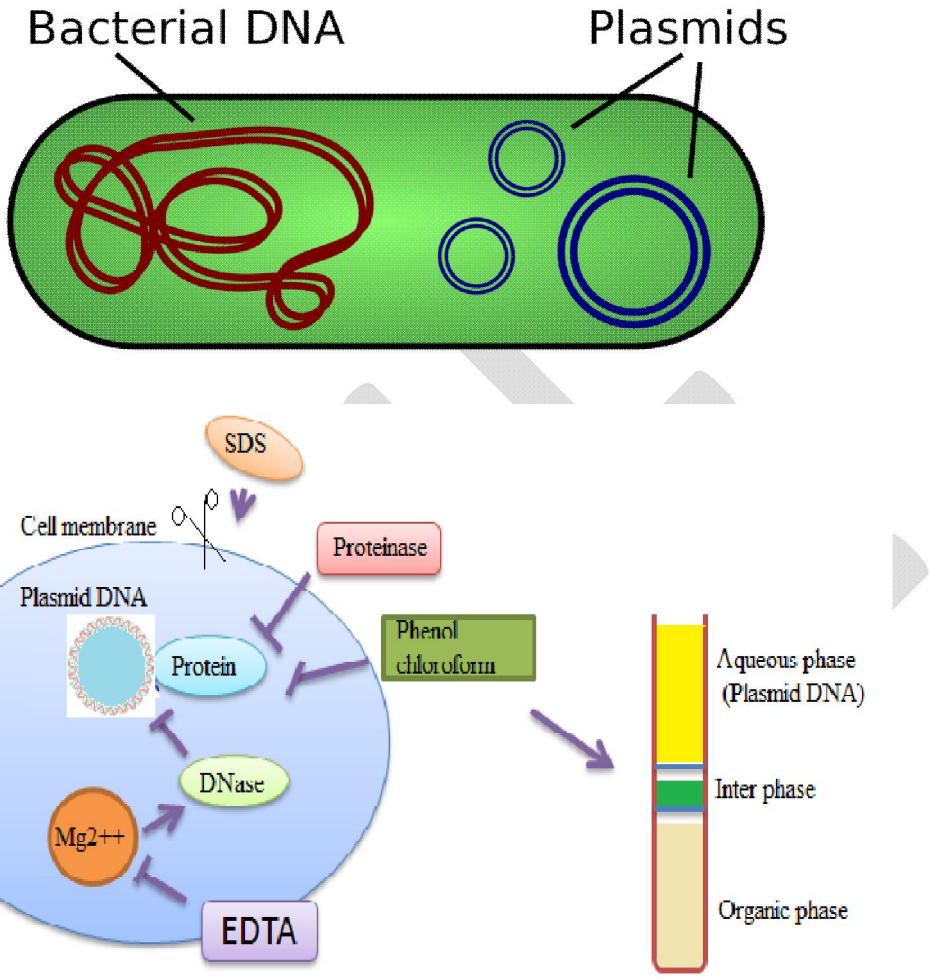
Aim: To isolate plasmid DNA from bacterial cells.

Principle:

Many microorganisms contain small pieces of circular DNA called plasmids that exist separately from the host-cell genome. In studies that use recombinant DNA techniques, plasmid DNA is often preferred over chromosomal DNA because it is smaller and easier to manipulate. Isolating pure DNA is a necessary step in studies that incorporate cloning, gene sequencing, gene mapping, or any other recombinant DNA technique. 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.

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 cell membrane, Tris is a buffering agent used to maintain a constant pH8. 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 neutralise 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 plasmid. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. Once the plasmid DNA is released, it must be precipitated in alcohol. The plasmid DNA in the aqueous phase is precipitated with ice cold (0^{o}C) ethanol or isopropanol. The precipitate is usually redissolved in buffer and treated with phenol or organic solvent to remove the last traces of protein, followed by reprecipitation with cold ethanol.

Schematic diagram of principle of Plasmid DNA Isolation



Materials Required:

Luria Broth, Bacterial cells containing plasmid, Incubator shaker, preset to 37°C, TE buffer (pH 8.0), Solution I, Solution II, Solution III, Phenol-chloroform mixture, Isopropanol, 70% ethanol, Autoclaved Distilled Water, Eppendorf tubes 1.5 ml Micropipette, Micro tips, Microfuge.

Alkaline Lysis Solution I

50 mM glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Prepare Solution I from standard stocks in batches of approx. 100 mL, autoclave for 15 min at 15 psi (1.05 kg/cm2) on liquid cycle, and store at 4°C.

Alkaline Lysis Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)

1% (w/v) SDS

Prepare Solution II fresh and use at room temperature.

Alkaline Lysis Solution III

5 M potassium acetate, 60.0 mL

Glacial acetic acid, 11.5 mL

H_2O, 28.5 mL

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.

Procedure

Preparation of Cells

1. Inoculate 2 mL of rich medium (LB Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking. Ensure that the culture is adequately aerated.

i. The volume of the culture tube should be at least four times greater than the volume of the bacterial culture.
ii. The tube should be loosely capped.
iii. The culture should be incubated with vigorous agitation.

2. Pour 1.5 mL of the culture into a micro centrifuge tube. Centrifuge at maximum speed for 30 sec at 4°C in a micro centrifuge. Store the unused portion of the original culture at 4°C. Discard the supernatant and repeat same tube filling again with more bacterial culture. The purpose of this step is to increase the starting volume of cells, so that more plasmid DNA can be isolated per preparation. Spin tube in micro centrifuge for 1 min. Pour off supernatant and drain tube on paper towel.

3. When centrifugation is complete, remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

4. Resuspend the bacterial pellet in 100 µL of ice-cold Alkaline lysis solution I by vigorous vortexing.

5. Add 200 µL of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. Do not vortex, store the tube on ice.

6. Add 150 µL of ice-cold Alkaline lysis solution III. Close the tube, and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube for 3–5 min on ice.

7. Centrifuge the bacterial lysate at maximum speed for 5 min at 4°C in a microcentrifuge. Transfer the supernatant to a fresh tube.

8. Add an equal volume of phenol:chloroform. Mix the organic and aqueous phases by vortexing, and then centrifuge at maximum speed for 2 min at 4°C in a micro centrifuge. Transfer the aqueous upper layer to a fresh tube. (Optional)

Recovery of Plasmid DNA

9. Precipitate the nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing, and then allow the mixture to stand for 2 min at room temperature.

10. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 min at 4°C in a micro centrifuge.

11. Remove the supernatant by gentle aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.

12. Add 1 mL of 70% ethanol to the pellet, and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 min at 4°C in a micro centrifuge.

13. Again remove all of the supernatant by gentle aspiration. Remove any beads of ethanol that form on the sides of the tube. Dry the tube open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5–10 min).

15. Re-suspend the DNA pellet in 50 µl of TE buffer or nuclease free water (NFW).

16. Measure optical density of DNA in a Nano drop Spectrophotometer at 260/280nM and 260/230 nm wavelength absorbance.

260/280 Ratio

- ✓ The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

260/230 Ratio

- ✓ This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants (EDTA, carbohydrates and phenol) which absorb at 230 nm.

17. After quantification run a 1% agarose gel to check the quality of the DNA. Store the plasmid DNA solution at −20°C.

Observation

The Plasmid DNA was visible as precipitate during ice cold absolute ethanol step. The absorbance of the plasmid DNA was ~1.83 at 260/280 nm. When an uncut plasmid DNA was loaded on agarose gel showed three bands (i.e.), super coiled, linear and open circular. The super coiled, which typically has a faster mobility during agarose gel electrophoresis than linear plasmid and open circular, which typically migrates higher than the linear form.

Result

The plasmid DNA was isolated from bacterial cells by alkaline lysis method with sodium dodecyl sulfate.

EX.NO:2

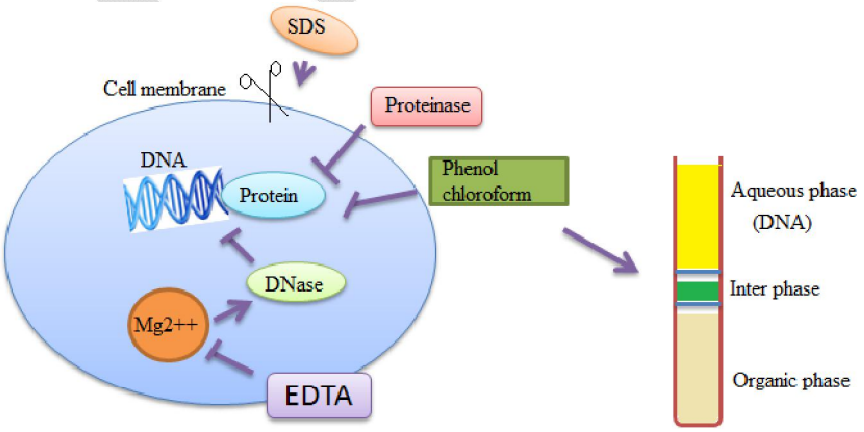
Isolation of Genomic DNA from E. coli

Aim: To isolate the genomic DNA from E .coli DH5α cells.

Principle:

The isolation and purification of DNA from cells is one of the most common procedures in modern molecular biology and embodies a transition from cell biology to the molecular biology (from *in vivo* to *in vitro*). The isolation of DNA from bacteria is a relatively simple process. The organism to be used should be grown in a favourable medium at an optimal temperature, and should be harvested in late log to early stationary phase for maximum yield. The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl sulphate) is used to disrupt the cell membrane. Once cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. DNA can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for action of most of the nucleases. Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K. Proteinase enzyme is used to degrade the proteins in the disrupted cell soup. Phenol and chloroform are used to denature and separate proteins from DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol.

Schematic diagram showing the principle of isolation of genomic DNA from *E. Coli*



Materials Required:

LB Broth, *E. coli* DH5α cells, TE buffer (pH 8.0),10% SDS, Proteinase K, Phenol-chloroform mixture, Isopropanol, 70% ethanol , Autoclaved distilled water, 1.5 ml Eppendorf tubes, Micropipette, Micro tips, Microfuge.

Reagent Preparation

Tris-Cl

To prepare a 1 M solution, dissolve 121.1 g of Tris base in 800 mL of H_2O. Adjust the pH to 8 by adding concentrated HCl. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 L with H_2O. Dispense into aliquots and sterilize by autoclaving.

EDTA (ethylene diamene tetra acetic acid)

To prepare EDTA at 0.5 M (pH 8.0): Add 186.1 g of disodium EDTA•$2H_2O$ to 800 mL of H2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.

Tris-EDTA buffer

To prepare Tris-EDTA buffer

Reagent	Amount to add	Final concentration
Tris-Cl (1 M, pH 8.0)	1 mL	10 mM
EDTA (0.5 M, pH 8.0)	200 µL	1 mM
Distilled H_2O	98.8 mL	

Procedure

1. Transfer 1.5 ml of the overnight *E. coli* culture grown in LB medium to a 1.5 ml eppendorf tube and centrifuge at max speed for 1min to pellet the cells. Discard the supernatant without disturbing the cell pellet.
2. Resuspend the cell pellet in 600 µl of lysis buffer and vortex to completely dissolve cell pellet, add 30 µl of Proteinase K, (20 mg/ml) and mix well by inverting the tube and incubate at 37 °C for 1 h.
3. After 1 h of incubation at 37 °C, add equal volume of phenol/chloroform and mix well by inverting the tube several times until the phases are completely mixed. *Do not vertex the tube it may shear the DNA.*

- ✓ Phenol is a very strong acid that causes severe burns. Chloroform is a carcinogen. Wear gloves, goggles and lab coat, and keep tubes capped tightly. To be safe, work in the hood if possible.

4. Centrifuge at 12,000 rpm for 5 min at room temperature. The upper aqueous phase alone was carefully transferred to a new 1.5 ml eppendorf tube without disturbing the other layers. The white layer in between the aqueous and organic interface is protein. Repeat the phenol/chloroform step until no *protein* is visible at the *interface*.
5. To the aqueous phase, add 2.5 ml volumes of ice cold absolute ethanol and mix well by inverting the tube several times. The DNA precipitation will be visible like a cotton thread.
6. Centrifuge at 12,000 rpm for 10 min at 4 °C and discard the supernatant without disturbing the DNA pellet.
7. To the DNA pellet 1 ml of 70% ethanol (stored at room temperature) was added and gently rinsed by inverting the tube several times and centrifuged at 12,000 rpm for 10 min at room temperature.
8. Carefully discard the supernatant and the DNA pellet was air-dried in a sterile environment for 10-15 min to remove traces of residual ethanol.
9. Re-suspend the DNA pellet in 50 µl of TE buffer or nuclease free water (NFW).
10. Measure optical density of DNA in a Nano drop Spectrophotometer at 260/280nM and 260/230 nm wavelength absorbance.

260/280 Ratio

- ✓ The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

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11. After quantification run a 1% agarose gel to check the quality of the DNA.

Observation

The DNA was visible like a white cotton thread during ice cold absolute ethanol precipitate step. The absorbance of the DNA was ~1.83 at 260/280 nm.

Result

The genomic DNA from *E .coli* was isolated, loaded and visualization on agarose gel electrophoresis.

Determination of molecular weight of protein by Sodium Dodocyle Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Aim: To determine the molecular weight of protein by Sodium Dodocyle Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Principle:

SDS-PAGE stands for Sodium Dodocyle Sulfate-Polyacrylamide Gel Electrophoresis. It is an electrophoresis technique used to separate proteins based on their mass. SDS is a detergent and used to give a negative charge to the denatured protein. One molecule of SDS binds every 2 amino acids of the protein. The proteins are denatured, and thus the gel used is called denaturing gel. There are two different gels used in SDS-PAGE. One is called separating gel or resolving gel, and other is called stacking gel. As the name suggests, the resolving gel is dedicated to the separation of the proteins, and the stacking gel is meant to stack the proteins in one band.

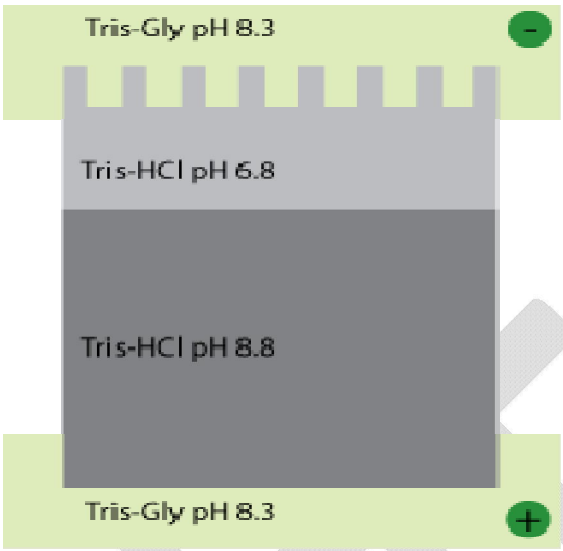
The gel is prepared from Acrylamide and Bis-acrylamide. Acrylamide is the monomeric subunit for the Polyacrylamide and the Bis-acrylamide is used for cross-linking between the polymer. Thus, Bis-acrylamide is used for producing the pores. The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. Greater the concentration of the Bis-acrylamide, smaller would be the pore size. Other components required for preparing the gel are TEMED (Tetra methyl ethylene diamine), APS (Ammonium per Sulfate) and Buffer. APS is used for generating the acrylamide free radical so that free radical polymerization can be initiated. Riboflavin can also be used as the free radical source. TEMED is a free radical stabilizer and added to promote polymerization.

Stacking Gel:

The buffer used is 1M Tris-Hcl (pH-6.8), the pH of the gel is 6.8. This lower ionic strength implies higher electrical resistance and consequently a higher electric field, provoking the faster movement of the proteins and of every other charged particle in the gel. Such a high electric field coupled with the glycine in the running buffer (that will not go into the resolving gel due to the pH), helps to clean the sample from the Cl- ions from the Tris-HCl buffer. The HCl provide Cl- ions and Glycine at this pH have net neutral charge. The glycine moves slowly as compared to the protein whereas the Cl- ions move faster than proteins. Thus, in the stacking gel, the proteins are sandwiched between the Cl- ions and Glycine.

Separating gel or Resolving Gel:

The buffer used is 1.5 M Tris-Hcl (pH-8.8), the pH of the gel is 8.8. When the stacking gel stacks the proteins and reaches the resolving gel, the pH changes. At this pH, the glycine would have net negative charge and moves faster and leaves the Cl- ion, and protein behind. Now, the protein would move in the gel based on their mass.



Protein sample preparation

The protein samples need to be denatured.

To denature the protein sample,

1. DTT (Dithiothreitol)
2. Beta-mercaptoethanol

These reagents would reduce the disulfide linkage between the cysteine in the proteins. Apart from this, EDTA and buffer are also added. EDTA will chelate the Ca2+ and Mg2+ ions which are co-factor for activation of proteases activity. The sample is also heated for denaturing it.

The SDS-PAGE electrophoretic unit is vertical. The two gels are prepared between two glass plates which are separated by a 1 mm spacer. First, resolving gel is poured and allowed to polymerize, and then the stacking gel is poured. The wells are created by putting a comb between the plates before pouring the stacking gel.

The sample concentration should be 2 mg/ml. In one well, 10 µl of the sample can be loaded. To ensure that the sample sinks to the bottom of the gel, sample buffer is supplemented with additives that increase the density of the sample. For such purpose, sucrose and glycerol solution is used.

Once the sample has been loaded, the electric field is applied. The applied voltage is between 100–150 volts. After the run (time depends on the voltage), the protein bands are fixed by addition of methanol and

acetic acid. Coomassie dye is used to stain the protein. After that, destaining is also carried out by increasing the methanol and acetic acid. Other staining method includes Zinc staining and Silver staining.

Materials

Equipment

1. SDS-PAGE gel apparatus.
2. Power pack.

Reagents

Acrylamide/Bis Acrylamide

Acrylamide - 29 g in 40 ml

N N Bis-Methylene-Acrylamide - 1 g in 20 ml

Make up to 100 ml with distilled water

Filter and store at 4º C in the dark

10 % (W/V) SDS (100 ml)

SDS 10.00 g in 50 ml

Make up to 100 ml with distilled water

1M Tris-Hcl Solution (pH-6.8)

Tris 6.057 g in water 30 ml of DH_2O

Adjust the pH to 6.8 and make up 50 ml with distilled water

1.5 M Tris-Hcl Solution (pH-8.8)

Tris 9.085 g in water 30 ml of DH_2O

Adjust the pH to 8.8 and make up 50 ml with distilled water

10 % (W/V) APS (Freshly prepared)

Ammonium persulfate – 100 mg/ml

Laemmli 2X buffer/Sample Loading Buffer (2X, 8 ml) (pH-6.8)

62.5mM Tris-Hcl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol blue

1 mM Tris-Hcl, pH 6.8 - 0.5 ml

25% Glycerol - 2.0 ml

1 % Bromophenol blue - 0.08 ml

10% SDS - 1.6 ml

DH_2O - 3.42 ml

Store as 1-2 ml aliquots at -70 C and β-Mercaptoethanol (0.4 ml) or 3% DTT (Dithiothreitol) immediately before use.

10X running buffer/(Tris-Glycine/SDS) (pH-8.3)

Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H_2O. The pH of the buffer should be 8.3 and no pH adjustment is required. Store the running buffer at room temperature and dilute to 1X before use.

Procedure

Pouring SDS-polyacrylamide Gels

1. Assemble the glass plates according to the kit manual.

2. Determine the volume of the gel mold provided by the manufacturer. In a 15 ml falcon tube, prepare appropriate volume and desired concentration of acrylamide for the resolving gel or separating gel, using the concentration provided below in the table, after adding the TEMED and APS to the separation gel solution, the gel will polymerize quickly, so add these two reagents when ready to pour. Pour the acrylamide solution into the gap between the glass plates. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm)

TABLE A8-9 Solutions for Preparing Resolving Gels for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

⇓ Components / Gel Volume ⇒	Volume (ml) of Components Required to Cast Gels of Indicated Volumes and Concentrations							
	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
10% gel								
H_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% acrylamide mix <!>	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate <!>	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED <!>	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

Modified from Harlow and Lane (1988).

3. Layer the top of the separating gel with isopropanol, the overlay prevents oxygen from diffusing into the gel and inhibiting polymerization, remove bubbles and will also keep the polymerized gel from drying out.
4. After polymerization is complete (30 minutes), pour off the isopropanol and wash the top of the gel several times with deionized H_2O to remove any unpolymerized acrylamide and then remove remaining H_2O with the edge of a paper towel.
5. Prepare the stacking gel in a disposable 15 ml falcon tube. Mix the components in the order shown in the table below. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly.

TABLE A8-10 Solutions for Preparing 5% Stacking Gels for Tris-glycine SDS-polyacrylamide Gel Electrophoresis

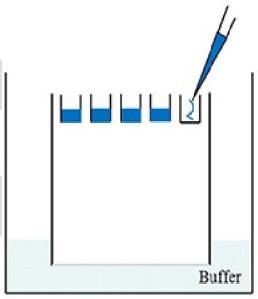
	Volume (ml) of Components Required to Cast Gels of Indicated Volumes							
Components ⇓ / Gel Volume ⇒	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
H_2O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acrylamide mix <!>	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% ammonium persulfate <!>	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED <!>	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Modified from Harlow and Lane (1988).

6. Pour the stacking gel solution directly onto the surface of the polymerized resolving gel. Teflon combs should be cleaned with H_2O and dried with ethanol just before use. Immediately insert a clean Teflon comb into the stacking gel solution, being careful to avoid trapping air bubbles.

Preparation of samples and running the gel

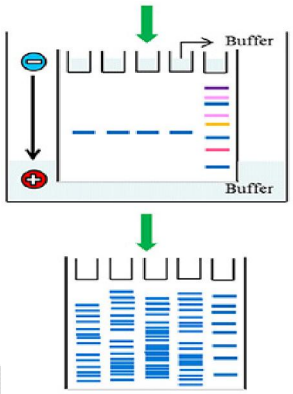
7. While the stacking gel is polymerizing, prepare the samples in appropriate volume of 1X sample solubilisation buffer and heat the samples at 100º c for 10–15 min to denature the proteins. Solubilise sample at 1 mg/mL and run 1–2 µL/lane (1–2 µg/lane).
8. After polymerization is complete (30 minutes), remove the Teflon comb carefully. Use a squirt bottle to wash the wells immediately with deionized H_2O to remove unpolymerized acrylamide. Mount the gel in the electrophoresis apparatus. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs. Remove bubbles that become trapped at the bottom of the gel between the glass plates.
9. Load up to 15 µl of each samples and 6 µl protein marker into the bottom of the wells. This is done with a micro pipette. Load an equal volume of 1X SDS gel loading buffer into wells that are unused.



Protein samples and marker loaded in vertical SDS-PAGE system

10. Attach the electrophoresis apparatus to an electric power supply (the positive electrode should be connected to the bottom buffer reservoir). Apply a voltage of 50 V to the gel. After the dye front has moved into the resolving gel, increase the voltage to 100 V and run the gel until the bromophenol blue reaches the bottom of the resolving gel approximately 4 hours.

11. When the dye front reaches the bottom of the gel, turn off the power, disassemble the gel apparatus, and place the gel in 200 - 300 ml of fixer/destainer. Gently shake for 16 h pour off spent fixer/destainer and add CBB. Gently shake for 30 min. Destain the gel in several changes of fixer/destainer untill the background is almost clear. Then place the gel in dH_2O, and gently mix untill the background is completely clear. The peptide bands will become a deep purple-blue. The gel can now be photographed. To store the gel wet, soak the gel in 7% glacial acetic acid for 1 h, and seal in a plastic bag.



Direction of migration of samples in vertical SDS-PAGE system

SDS-PAGE gel after Coomassie blue staining

Observation

The after staining with coomassie blue proteins appeared as discrete bands in the gel. The relative molecular weights of the protein with respect to their bands were observed in Kilo Daltons.

Result

The protein samples were separated by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis.

EX.NO: 4

ISOLATION OF MICROBES FROM SOIL

AIM

To isolate different microbial population from soil.

BACKGROUND

The soil is home to a wide range of plant and animal life. Roots are the largest form of plant life and certain rodents, insects, and earthworm and other largest animals through higher plants are the primary producers of chemical energy that sustains the terrestrial ecosystem. Plants are dependent on the invisible (other than certain fungal structures) community of soil microorganisms for continued supply of many essential nutrients. Nutrient elements bound in organic combination would be inaccessible to plants without microbial decomposition of matter and mineralization of these elements, not only would nutrient cycling step but also biological N-fixation.

Soil microorganisms are indispensable to life on earth. Soil microorganisms live in their film of water that surrounds soil particles. These organisms include microflora bacteria, fungi, and actinomycetes along with microfauna, protozoa and nematodes. In terms of numbers and biological activity, the microfloraare dominant. Bacteria are small (about 10µm) and occur in three general shapes. Bred "bacillus" spherical (coccus) and spiral bacilli and cocci are more common in soil. The branched hyphae exhibit cell division and fungal mycelia (hyphae mass) are often macroscopic. Actinomycetes are also filamentous and branched by smaller.

AGAR PLATE METHOD FOR MICROBIAL COUNT

In this method, soil is dispensed in an agar medium so that individual microbial cells, spores, or mycelial fragments develop into macroscopic colonies. The procedure involves successive dilution of soil. Depending upon the extent of dilution, plates may be filled with a huge enumeration of colony- forming units initially present in the soil is from plates, in between these extremes. This method requires sterile technique to avoid introduction of extraneous microbes, anyone of several different growth.

Medium can be used but no single growth medium is optimal for all microorganisms that inhibit soil. Thus, growth of certain organisms is favoured in the chosen medium and growth of organisms is studied. Antagonistic or antibiotic is added to the agar in the agar plate method.

MATERIALS REQUIRED

- Nutrient agar, CzapekDox agar, Starch Casein- nitrate agar.
- Flask containing 100ml of sterile saline.
- Test tubes with 9ml of sterile saline for serial dilution.

PROTOCOL

- Agar media plates such as nutrient agar, czapekdox agar, and starch casein nitrate agar were prepared for bacteria, fungi and actinomycetes respectively.
- 1g of the collected soil was suspended in 100ml of sterile saline and shaken vigorously.
- This suspension was serially diluted and until 10^{-7}, using spread plate technique. 1ml of this suspension was plated for each kind of microorganism as given below.
- For bacteria, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions were used to inoculate nutrient agar plates and incubated at 37°C for 24 hrs.
- For fungi 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions were used to inoculate czapekdox agar plates and incubated at room temperature for 3-5 days.
- For actinomycetes 10^{-3}, 10^{-4}, and 10^{-5} dilution were used to inoculate starch casein nitrate agar plates and incubated at room temperature for 3-7 days.
- Single colonies of bacteria, fungi, actinomycetes isolates were transferred to respective agar plates and maintained for further investigation.

RESULT

Nutrient agar

Colony morphology: most of the colonies were flat, wrinkled, irregularly margined with pear white colors that appeared after 84 hrs of incubation.

Czapekdox agar

Colony morphology: colonies with long branching filaments and fluffy/ cottony appearance. Different spore colors were seen at the back side of the plate after 5 days of incubation.

Starch casein nitrate agar:

Colony morphology: the colonies were flat with irregular margins. Fungi like filaments pick some colonies produce pigments that were seen at the back side of the plate after 5 days of incubation

EX.NO:5

ISOLATION OF FREE LIVING N2 FIXERS FROM SOIL AZOTOBACTER

AIM:

To isolate the free living nitrogen fixing bacteria, Azotobacter spp from rhizosphere soil.

BACKGROUND:

Azotobacter is free living – heterotrophic nitrogen fixing bacteria that occur in the rhizosphere of variety of plants. The genus Azotobacter has six species viz, A.chroococcum, A.vinelandi, A.beiferinkil, A.nigricans, A.arnelandi, A.armeniacus, A.paspali. Except the last species, which is a rhizoplane bacterium , the other members are largerly rhizosphere borne. The potential of A.chroococcum and A.paspali are used as biofertilizer for various non- leguminous crops.

Azotobacter is an aerobic gram negative rod shaped bacteria occur singly, in chains, or in clumps. It doesn't form endospores but do form thick walled cysts, these cysts are resistant to dessication and to some detrorious chemical and physical agent. They however, cannot withstand extreme temperatures while in the cyst stage of their life, they do not fix nitrogen and are especially refractile. It may be motile by peritrichous flagella or non- motile. It can produce a water soluble pigment- yellow- green, fluorescent or red/violet/brownish black. It grows well at or optimum temperature between 20^0C to 30^0C and at P^H 7.0- 7.5, they are able to grow on various carbohydrates, alcohols, and organic acids

Azotobacter was first discovered using culture medium that is devoid of combined nitrogen source. Azotobacter is found on natural alkaline soils, in aquatic environments in the plant rhizosphereband phycosphere. A.chroococcum is the most common species of azotobacter present in the soil. Azotobacter spp are known to influence plant growth through their ability to fix molecular nitrogen, production of growth promoting substances like IAA, gibberlins or gibberalline like compounds and vitamins, excretion of ammonia in the rhizoshpere in the presence of root exudates production of anti- fungal metabolites and phoshphate solubulization.

Jensen's nitrogen free medium and ashby's medium is routinely used for preparation and mass production of Azotobacter. Seed inoculation of A.chroococcum increase the yield of crops about 10% and cereals about 5-10%. The response to inoculation was increased by manuring or by fertilizer application , coinoculation of Azotobacter with other bioinoculant like Rhizobium,

Azospirillum , Phosphate solubulizers, Vesicular Arbuscular Mycorrizhae(VAM) have been reported to enhance the growth and yield of legumes, cereals, and vegetable crops.

Beneficial effects of Azotobacter chroococcum inoculation has been reported on various cereal, vegetables, oil seeds, legume and cash crops, Inoculation experiment with Azotobacter gave better yield performance only ate lower levels of nitrogen (0 to 30kg N ha^{-1}). These diazotropic bacteria require large amounts of available carbon for their survival in soil addition of farmyard manure(FYM) compost and other organic amendments to agricultural soils improves the efficiency of Azotobacter and hence the plant growth and yield.

MATERIALS REQUIRED:

- Jensens's medium, Ashby medium.
- Rhizosphere soil from sugarcane/paddy/cotton.
- Test tubes

PROTOCOL:

- Agar plates with Jensen's and ashby's medium were prepared.
- 1g of the collected rhizosphere soil was suspended in 100ml of sterile distilled water and gently mixed to form uniform suspension.
- This suspension was serially diluted until 10^{-5} and 0.1ml of sample was spreaded onto the plates by spread plate technique.
- The plates were incubated at room at temperature for 3-5 days.

EX.NO: 6

ISOLATION OF SYMBIOTIC NITROGEN FIXERS FROM ROOT NODULE – RHIZOBIUM

AIM:

To isolate Rhizobium spp. from root nodules of leguminous plants.

Backgrounds:

Nitrogen is an essential nutrient for plant growth and development planers usually depend upon combined or fixed forms of nitrogen such as ammonia and nitrate because it is unavailable in its most prevalent form as atmospheric nitrogen much of this nitrogen is provided to cropping systems, in the form of industrially produced nitrogen fertilizers, use of these fertilizers has to world wide ecological problems as well as affects the human health, Biological nitrogen fixation (BNF) is the cheapest and environment friendly procedure in which nitrogen fixing microorganisms.

interacting with leguminous plants, fix aerobic nitrogen into soil.

Soil contains many types of microorganisms such as bacteria, actinomycetes, fungi and algae which are important because they affect the physical, chemical and biological properties of soil amongst the soil bacteria a unique group called rhizobia has a beneficial effect on the growth of plants. It can live either in the soil or within the root nodules, where it converts atmospheric nitrogen to ammonia and provides organic nitrogenous compounds to the plants.

Legumes have been used in agriculture since concerns time and legume seeds or purses were among the first source of human feed and their domestication. In leguminous plant, the bacteria live in small outgrowths on the roots called nodules, within this nodules the bacteria do nitrogen fixation and the plants absorbs the ammonia, and the legume plants absorbs the ammonia, a unique ability to establish symbiosis with nitrogen fixing bacteria of the family Rhizobiacoas such group of Rhizobium, BradyrhizobiumAllorhizobium, Sinorhizobium and Mesorhizobium, these bacteria can fix atmospheric nitrogen only in root nodules of legumes and that too when it is in the bacteroid stage of its life cycle. It possess the entire complement of genes for nitrogen fixation,which are normally latent and become active only under species conditions.

Rhizobium are Gram negative motile rods, the size and morphology of the nodules formed by the Rhizobia vary with plant species, they can fix 150-200 kilograms of nitrogen contain the enzyme nitrogenase which catalyzes the following reaction,

$$N2 + 3H2 \longrightarrow 2N + H3$$

Yeast mannitol Agar with congo red is used for the cultivation of rhizobium species and for studying root nodulation. Yeast extract serves as a good source of readily available amino acids. Vitamin B complex and accessory growth factors for rhizobia, it also posses the oxidation reaction potential of medium in the range favorable for rhizobia and serves as hydrogen donor in respiratory process, mannitol is the fermentable sugar alcohol source. Magnesium provides cations, essential for the growth of rhizobia.

Congo red inhibits penicillin – susceptible strain colonies of rhizobia stand out as white, translucent and elevated with entire margins.

MATERIALS REQUIRED:

- ✓ Fresh pinkish root nodules,
- ✓ Yeast extract mannitol agar (YEMA) with congo red,
- ✓ 0.1% mercuric chloride (OR) 3% sodium hypochloride,
- ✓ 70% ethanol,
- ✓ Sterile – forceps, glass rods, blade and distilled water.

PROCEDURE:

1. A plant was carefully and root system was washed in running tap water to remove the adhering soil particles.
2. Healthy, unbroken, pink nodules were selected and washed in distilled water.
3. The nodules were then immersed in 70% ethanol for 5-10 seconds and followed by rinse in sterile distilled water.
4. The nodules were then surface sterilized by soaking in 0.1% acidified mercuric chloride for 1-2 minutes and following by rinse in sterile distilled water.
5. The process was repeated for three times and finally washed thoroughly in sterile distilled water and plated in sterile petridish.

METHOD-I:

1. By using a sterile blade, the nodule were cut into two harves and squeezed on the surface of the medium using sterile forceps.
2. Using this as a mother inoculum a quadrant streaking was performed with a inoculation loop.

METHOD-II:

1. The surfaced sterilized nodules were collected in a test tube and crushed using a sterile glass rod in the presence of few drops of sterile distilled water.
2. With an inoculation loop a loopful of the suspension was streaked on the medium.
3. Alternatively, a serial dilution of the suspension can be performed and plated either by spread or pour plate method.
4. All plates, used in either method were incubated at room temperature for 3-5 days.

OBSERVATION:

After incubation at room temperature for 4 days the YEMA plates showed white gristering colonies which may be Rhizobium spp.

EX.N0:7

ISOLATION OF PHOSPHATE SOLUBULIZERS, AMMONIFIERS, AND DENITRIFIERS

AIM:

To isolate phosphate solubulizing microorganism from soil.

BACKGROUND:

Phosphorous is one among the three important nutrients required for plants, animals, and microbial growth. It can occur in two forms.viz, Soluble phosphorous and Insoluble phosphorous. Soluble form can be easily assimilated by plant and microbes, It is not so in the case of organic and inorganic phosphorous that cannot be utilized by plants ,they are called as Phosphate solubulizing Microorganism(PSB).

Mechanism of mineral phosphate solubulization of PSB strains is associated with the release of low molecular weight organic/inorganic acids through with their hydroxyl and carboxyl groups chelate the cation bound to phosphate, there by converting it into soluble forms. Bacteria such as B.Subtilis, Polymyxa, Pseudomonas, Mycobacterium, Micrococcus flave bacteria and the fungi Aspergillus and Penicillium are some of the best Phosphate solubulizers.

PSB have been introduced to the agricultural community as biofertilizers.PSB have attracted the attention of Agriculturulist as soil inoculums to improve the plant growth and yield, wgen PSB used with rock phosphate, it can save about 50% of the crop requirement of Phosphate fertilizer , the use of PSB as inoculants increases uptake by plants by simple inoculation of seed with PSB gives crop yield responses equivalent to 30kg $P_2 0_5$/ha or 50%of the need for phosphate solubulizers.

Pikovaskaya's agar is used for detection of phosphate solubulizing bacteria from soil.Yeast extract in the medium provides nitrogen and other nutrients necessary to support bacterial growth. Dextrose acts as energy source, different salts and yeast extract supports the growth of organisms, phosphate Solubulizing bacteria will grow on the medium and form a clear zone around the colony, formed due to phosphate solubulization in the vianity of the colony.

MATERIALS REQUIRED:

- Soil Sample.

- Sterile Blanks for Dilution.
- Pikovskaya's Agar
- Sterile L- Rods

PROTOCOL:

- One gram of the collected soil sample was mixed in 100ml of sterile distilled water and it was further serially diluted upto 10^{-6} dilutions.
- From each dilution 0.1 ml of the sample was evenly spread over the agar medium by spread plate technique.
- The plates were incubated at room temperature for 3-5 days

EX.NO: 8

DETERMINATION OF DISSOLVED OXYGEN OF WATER

AIM:

To determine the dissolved oxygen content of water sample by winkler`s method.

BACKGROUND:

The test was originally developed by Ludwing winkler, while, working as a Doctoral student at Budapest university. In 1888 the DO determination measure the amount of dissolved (or free) oxygen present in water or waste water, to maintain healthy water, the amount of oxygen must be as high as possible when the maximum amount of oxygen possible is dissolved in water, it is said to be saturated, the saturation concentration decreases with increasing temperature and is smaller for salt water than for sea water. E.g.for 25°c the saturation concentration of oxygen in fresh water it is 6. 7mg L-1 the concentration dissolve oxygen it also temperature dependent at 10°c the saturated concentration of oxygen in fresh water is 11.3 mg L-1 while at 20°c it is 9.2 mg L-1, aerobic bacteria and aquatic life such as fish must have do to survive, aerobic waste water treatment processes are aerobic and facultative bacteria to break down the organic compounds found in waste water into more stable, products the will not harm the receiving waters, waste water treatment facilities such as lagoons or povels trickling filters and activated sludge plants depend on these aerobic bacteria to treat sewage. If sufficient oxygen is not naturally supplied through wind and turbulence to replace the depleted oxygen, the body of water will develop a low DO and became anaerobic.

Two methods are commonly used to determine DO. Concentration used to determine DO concentration is the method which is a titration-based method and depends on oxidizing property of DO and to the membrane electrode procedure which works based on the rate of diffusion of molecular oxygen across a membrane.

In the iodonetric method, divalent manganese solution is added to the solution following by adding of strong alkali in a glass-stopper bottle DO rapidly oxidise an equivalent amount of the dispersed divalent manganese hydroxide precipitates to hydroxides precipitates to hydroxides of higher valence states. In the presence of iodine ions in an acidic solution, the oxidized manganese reverts to the divalent state, with the liberation of iodine ions in an acidic solution, the oxidized manganese reverts to the divalent state, with the liberation of iodine equivalent of the original DO content, the iodine is then titrated with a standard solution

of throsulfate the titration with a standard solution of thiosulfates. The titration and point can be detected visually with a starch indicator some oxidizing and reducing agents present in solution can interfere with the iodometric method agents liberate iodine from agents positive interference and some reducing agents reduce iodine to iodide (Negative interfered).

Also organic matter present in solution can be oxidized purifically in the presence of oxidized manganese precipitate, thus causing negative errors, thus some modification of procedure is required.

Steps in the Winkler method of oxygen-determination:

- Manganese (II) ions liberated from the manganese sulfate are loosely bound with excess hydroxide
  mn2++2oH---->Mn(oH2)
- Manganese (H) is oxidized to manganese (II) in the presence of a strong base and binds the dissolved oxygen.
  2Mn(OH2) +1/2 o2+H2O---->2Mn(OH3)
- Free iodine is produced upon oxidification of the sample at a rate of one l2 molecule for each atom of oxygen.
  2Mn(OH3) +2i+6+i---->2Mn2+12+6H2O
- Free iodine complexes with excess iodine ions.
  i2+i---->i3
- The iodine/iodide complex is reduced to iodide with thiosulfate.
  i3+2S2O2-3---->3+S4O62-

MATERIALS REQUIRED:

APPARATUS: -

300 ml BOD bottle, 500 ml beaker, BOD incubator, burette, pipetes.

REAGENTS: -

1. Nutrient solution:

A) Phosphate buffer:

Dissolve 8.5g kH2po4, 2i, 75g, k2Hpo4, 33, 4g Na2Hpo4, 7H2o and 1.7g NH4cl in approx 500ml reagent water. Dilute F2 II the pH should be 7.2 store in 4°c refrigerated check before each use for contamination (if there is any indication of microbial growth, discard remaining reagent and prepare fresh).

B) Magnesium sulfate solution:

Dissolve 22.5g mgso4, 7H2o in reagent water, dilute to 1L.

C) Calcium chloride solution:

Dissolve 27.5g cacl2 in reagent water, dilute to 1L.

D) Ferric chloride solution:

Dissolve 0.25g Fecl3, 6H2o in reagent water, dilute to 1L.

2. Manganese sulfate solution:

Dissolve 480g mnso4, H2o in reagent water, filter, dilute to 1L.

3. Alkali-iodide-axide reagent :

Dissolve 500g NaoH and 135g NaI in reagent water, dilute to 1L. Add 10g NaN3 dissolved in 40ml reagent water, this reagent should not give a color with starch solution when diluted and acidified.

4. Concentrated sulfuric acid:

5. Standard sodium thiosulfate 0.0250N/0.18M:

6. Starch solution:

Prepare an emulsion of 5g soluble starch in a martar or beaker with a small amount of distilled water, pour thus emulsion into 1L of boiling water, allow to boil for few minutes, and let settle overnight use the clear supernatant.

7. Dilution water:

If may be prepared immediately before use by adding 1ml of each nutrient solution per litre of dilution water, when volumes of sample used exceed 150ml, additional nutrients should be added to the sample bottke. Add an additional 0.1ml of nutrients for each 50ml of sample used in excess of 150ml.

8. Preparation of glucose-glutamic acid standard (GGA):

Dry reagent grade glucose and glutamic acid at 103°c for 1hrs and cool for one hrs in the desicattor. Dissolve 150mg (0.15g) of glucose and 150mg (0.15g) of glutamic acid in disttilrd water and bring up to 1/L

note. Sear the bottles and sterilize them these sterilized portions can than be cooled and stored at 4°c. When a known standard is ren, 6ml of GGA standard from one of the sealed, sterilized container is added to each BODs bottles are filled 3/4 full with solution water (198+30.5mg oxygen/L is based on 9.27 dilution of GGA 6ml/300).

WINKLER TITRATION TECHNIQUE: -

PROCEDURE:

- ✓ Slowly drain off three portions of aerated dilution water into three separate BOD bottles, avoid adding atmospheric o2 to dilution water.
- ✓ To two of the three BOD bottles, add 1ml mnso4 solution, followed by 1ml alkali-iodine azide reagent-submerge pipette tips in sample when adding reagent rinse tips well between uses.
- ✓ Stepper carefully to exclude air bubbles. Mix by investing bottle several times.
- ✓ When precipitate has settle to about half the bottle volume, carefully remove the stopper and mix by gentle invasion until the iodine is uniformly distributed throughout the bottle.
- ✓ Transfer 203ml of sample into a white 500ml beaker and titration with 0.0250N sodium thiosulfate to a pare straw color. Add 1-2ml of starch solution and continue to titration of first disappearance of the blue colour (200ml of original dilution water is equal to 203ml of dilution water plus reagent).
- ✓ Titration two of the three sample results should be within 0.1ml.
- ✓ Completely fill two bottles with dilution water of be incubated as blanks.
- ✓ Label each bottle carefully as to sample and volume used.

RESULTS:

EX.NO: 9

DETERMINATION OF BOD (BIOCHEMICAL OXYGEN DEMAND OF WATER)

AIM

To determine the biochemical oxygen demand o various water samples including sewage.

BACKGROUND

The biochemical oxygen demand test measures the ability of naturally occurring microorganisms to digest organic matter usually in a 5 day incubation. BOD at 20°C by analysing the depletion of oxygen which measures biodegradable organic matter and are normally expressed as mg/L. The BOD gives an indication of the amount of oxygen needed to stabilize or biologically oxidise the waste. The advantage of BOD test is that it measures only the organic matter oxidised by the bacteria. The disadvantage is the 5 days time lag and the difficulty is in obtaining consistent repetitive values.

BOD samples are normally incubated in 300 ml BOD bottles and are diluted as necessary to allow atleast 1-0mg/L of DO(dissolved oxygen) to remain at the end of the 5 day period to be a varied test. Atleast 2mg/L of oxygen must be depleted during the incubation. If necessary samples must be pretreated to assure proper PH, temperature, and absence of toxic materials(chlorine) thus consuming a suitable environment for survival of the BOD consuming bacteria.

There is a limited amount of oxygen which will dissolve in the sample(about 9mg/L).These for dilution are prepared with a buffered mineral nutrient containing water.

The test does not determine the total amount of organic materials present, since many compounds are not decomposed by biological and biochemical reactions in the set conditions. In conducting the test several different dilutions are to be used to ensure that at least one will deplete the oxygen content by about 50%.Two differentnt dilutions of the same sample should generate valid results. The BOD values of the two dilutions are normally averaged. The BOD values for glucose/glutamic acid standared should be 60%-70% of the COD values for the same sample.

The method consists of filling with diluted and seeded sample in two overflowing airtight bottles of specified size and incubation.

MATERIALS REQUIRED

- Ferric chloride solution

Dissolve 0.025g Fecl3,6H2O in reagent water.Dilute to 1L.

- Manganese surface solution:

Dissolve 480g MnSO4 in reagent water, dilute to 1L.

- Alkali-iodide-Azide reagent:

Dissolve 500mg NaOH and 135g Nacl in reagent water. Dilute to 1L.Add 10g NaN3 dissolved in 240ml reagent water, this reagent should not give a colour with starch solution when diluted and acidified.

- Concentrated sulphuric acid
- Standared sodium thiosulfate
- Starch solution

Prepare an emulsion of 5g soluble starch in a mortar or beaker with a small amount of distilled water, pour this emulsion into1L1 of boiling water. allow to boil for few minutes,and let settle overnight. Use the supernatant.

- Dilution water

It may be prepared immediately before use by adding 1ml of each nutrient solution per litre of distilled water. When volume of samples used exceed 150ml additional nutrients should be added to the sample bottle. Add on additional 0.1ml of nutrient for each 50ml of sample used in excess of 150ml.

- Preparation of Glucose-Glutamic acid standared(GGA)

Dry reagent grade glucose and glutamic acid at 103 degree celcius or 1 hour in the desiccator .Dissolve 150mg(0.15g) of glucose and 150mg(0.15g)glutamic acid in distilled water and bring up to 1L

NOTE

Seal andSterilize the bottles. then they are cooled and stored at 4 degree Celsius. When the known standared is run 6ml,o GGA standared from one of the sealed/sterilized containers is added to each BOD bottles and filtered.

Winkler filtraton technique:

PROCEDURE

- Slowly siphon three portions of aerated dilution water into 3 separate BOD bottles. avoidadding atmospheric oxygen to dilution water.
- To two of the three BOD bottles add 1ml,MnSO4 solution followed by 1ml-alkali-iodide-Azide reagent submerge pipette tips in sample when adding reagent. Rinse tips well.
- Keep stopper carefully to exclude air bubbles, mix by inverting bottles several times.
- When precipitate has settled to above half the bottle volume carefully remove the stopper and 1ml concentrated sulphuric acid. restopper and mix by gentle inversion until the iodine is uniformly distributed throughout the bottle.
- Transfer 200ml of sample into a 500 ml beaker and titrate with 0.025N sodium thiosulphate to develop a pale straw colour.Add 1-2ml of starch solution and continue to titrate first disappearance of blue colour
- Titrate two of the three samples.
- Completely fill two bottles with dilution water to be incubated as blanks.
- Label each bottle carefully as to sample and volume used.

INOCULATION OF BOD IN SAMPLE

BOD5 is calculated by the following formulae

$$BOD5 = (DO-D5)/P$$

Where

DO is the dissolved oxygen of the diluted solution after preparation.

D_5 is the DO of the diluted solution after 5 day incubation.

P is the decimal dilution factor.

OBSERVATION

The biological oxygen demand of the given water sample after 5 days are found to be 4.8mg/L

Dissolved oxygen of water sample before incubation:

D_1 = 8x1000x0.25xg/d5= 8mg/l

Oxygen of water sample after incubation :

D_2 = 8x1000x0.025x0.8/D_5 =3.2mg/L

BOD of the water sample is D_2-D_1= 8-3.2=4.8Mg/l

EX.NO: 10

DETERMINATION OF COD CHEMICAL OXYGEN DEMAND OF WATER

AIM:

To detemine the chemical oxygen demand of various water sample.

BACKGROUND:

The chemical oxygen demand test most commonly used indirectly measure the amount of organic compounds in water, most applications of cod determine the amount of organic pollutants found in surface water (lakes and river)making COD a useful measure of water quantity. Its expressed in milligrams per liter(mg/l) which indicates the mass of oxyen consumed per liter of sollution. It is the measurement of the amount of oxygen in water consumed for chmical oxidation of pollutants,if detemines the quantity of oxygen required to oxidize the oragnic matter in water of waste water sample,under specific conditions of oxidizing agent, temperature and time,the applicable range is 3-900mg/l. The ratio of BOD to COD is useful toarror the amenability of waste for biological treatment.

Ratio of BOD to COD greater than or equal to 0-8 indicates that needs biological treatment BOD value is always industries waste water, COD value is about 2.5times than that of BOD values.

The organic matter present in the sample gets oxidized completely by pottassium dichromate k2(r207) in the presence of surpluric acid(H2S04) to produce C02 and H20,the sample is refused with a known amount of pottasium dichromate in the sulphuric acid medium and the excess pottasium dichromate determined by titration against ferrous ammonium sulphate,using ferrioin as an indicater,the dichromate consumed by the sample, is equivalent to the amount of oxygen required to oxidize the organic matter, the limitation of the test lies in its inability to differentiate between the biologically oxidizable and biologically inert material and to final out the system rate constant of aerobic biological stabilization.

MATERIALS REQUIRED:

Standard pottasium dichromate digestion solution (0.01667m)

- ✓ Sulphuric acid reagent
- ✓ Ferrion indicator solution
- ✓ Standard ferrous ammonium 0.10m

PROTOCOL:

- ✓ In a container (tubes or ampules) with 2.5ml of a sample ,1.5ml of pottasium dichromate solution were added.
- ✓ 3.5ml of sulphuric acid was added inside the vessels where an acid layer is formed under the sample digestion solution layer.
- ✓ After tightly capping the tubes,it was inverted several times to mix completely.
- ✓ The tubes were placed in blank digestor procheatoal to 150C and refluxed for 2 hours ,behind a a protective shield.
- ✓ It was then cooled to room temperature and placed in test tube rack
- ✓ One or two drops of ferrioin indicator was added to the tubes and stirred rapidly on magnetic stirrer,while titrating against standardized 0.10MEAS.
- ✓ The end point is a color change from blue –green to reddish brown although the blue-green may reappears within minutes.
- ✓ The procedure was repeatedely titrated with the blank containing.
- ✓ The reagent and a volume of distilled water equals to that of the sample.
- ✓ COD is given by,

$$COD(mg\ 0.1/l) = (AB)*m*8000)/\ V\ sample$$

Where;

A= Volume of FAS used for blank (ml)

B= Volume of FAS used for sample (ml)

C= Molarity of FAS

8000-mili equivalent weight of oxygen (8)*1000ml/l

EX.NO: 11

Study of mycorrhizae, Cyanobacteria and Azolla

Mycorrhizae

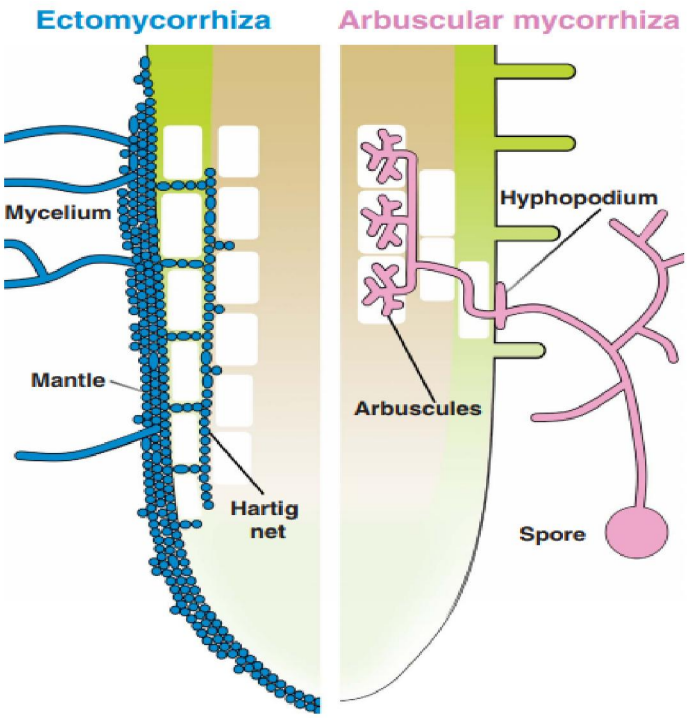
Owing to their filamentous organization, fungi exploit very diverse substrates on the basis of their nutritional strategy. Saprobes thrive in soil, water and on decaying animal and plant tissues. A smaller group of fungi, the parasitic and mutualistic symbionts, feed on living organisms. Such a classification cannot easily be applied to mycorrhizal fungi, a heterogeneous group of species spread over diverse fungal taxa. Although they can spend part of their life cycle as free-living organisms, mycorrhizal fungi always associate with the roots of higher plants, indeed over 90% of plant species, including forest trees, wild grasses and many crops. Both partners benefit from the relationship: mycorrhizal fungi improve the nutrient status of their host plants, influencing mineral nutrition, water absorption, growth and disease resistance, whereas in exchange, the host plant is necessary for fungal growth and reproduction.

Mycorrhizal fungi colonize environments such as alpine and boreal zones, tropical forests, grasslands and croplands. They have a major role in nutrient cycling through the specific activity of their mycelium in absorbing soil nutrients and supplying them to the plant, although their role in carbon flux is less well defined. The term mycorrhiza is derived from the Greek words for ‘fungus’ and ‘root’. Mycorrhizal fungi develop an extensive hyphal network in the soil, the aptly named wood-wide web, which can connect whole plant communities offering efficient horizontal transfer of nutrients. Mycorrhizas develop specialized areas, called symbiotic interfaces, to interact with the host plant.

There are two broad categories of mycorrhizal associations with plant roots, ectomycorrhiza and endomycorrhiza, which are differentiated by how they physically interface with the plant. The ectomycorrhizae (EM) occur mainly in the roots of woody plants (i.e. forest trees) and form a dense hyphal covering (fungal sheath or mantel) over the root tip from which hyphae grow into the intercellular spaces forming a net (Hartig net) of hyphae around the root cortex cells, but do not penetrate the cell walls. In contrast, the endomycorrhizae fungal hyphae grow into the root cortex and enter the cells forming fan-like, highly branched structure known as an arbuscule that remain separated from the cytoplasm by the plant plasma membrane. The endomycorrhiza can be further divided into the more widespread arbuscular mycorrhiza (AM) and the specialized orchid and ericoid mycorrhizas which, as the name implies, are colonizers of orchids and ericoid (e.g., cranberry) plant species. The AM fungal associations are the most abundant of all mycorrhizal associations. In both cases, the Hartig net and the arbuscules increase the contact area between the fungus and the plant through which the transfer of nutrients to the plant and carbon to the fungus occurs. Unlike the ectomycorrhiza, the endomycorrhiza are wholly dependent on the plant for

their carbon and when associations occur, both endomycorrhiza and ectomycorrhiza can demand up to 20-40% of the total photosynthetically fixed carbon the plant produces.

Schematic picture showing the difference between ectomycorrhizae and endomycorrhizae colonization of plant roots.



Cyanobacteria

Blue-green algae, also called cyanobacteria, any of a large, heterogeneous group of prokaryotic, principally photosynthetic organisms. Cyanobacteria resemble the eukaryotic algae in many ways, including morphological characteristics and ecological niches, and were at one time treated as algae, hence the common name of blue-green algae. Algae have since been reclassified as protists, and the prokaryotic nature of the blue-green algae has caused them to be classified with bacteria in the prokaryotic kingdom Monera.

Like all other prokaryotes, cyanobacteria lack a membrane-bound nucleus, mitochondria, Golgi apparatus, chloroplasts, and endoplasmic reticulum. All of the functions carried out in eukaryotes by these membrane-bound organelles are carried out in prokaryotes by the bacterial cell membrane. Some cyanobacteria, especially planktonic forms, have gas vesicles that contribute to their buoyancy. Chemical, genetic, and physiological characteristics are used to further classify the group within the kingdom. Cyanobacteria may be unicellular or filamentous. Many have sheaths to bind other cells or filaments into colonies.

Cyanobacteria contain only one form of chlorophyll, chlorophyll a, a green pigment. In addition, they contain various yellowish carotenoids, the blue pigment phycobilin, and, in some species, the red

pigment phycoerythrin. The combination of phycobilin and chlorophyll produces the characteristic blue-green colour from which these organisms derive their popular name. Because of the other pigments, however, many species are actually green, brown, yellow, black, or red.

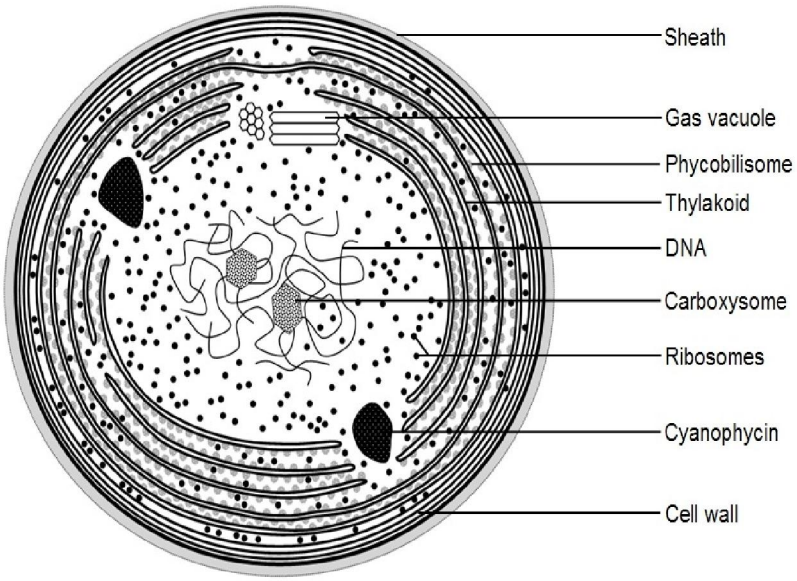
Most cyanobacteria do not grow in the absence of light (i.e., they are obligate phototrophs); however, some can grow in the dark if there is a sufficient supply of glucose to act as a carbon and energy source. In addition to being photosynthetic, many species of cyanobacteria can also "fix" atmospheric nitrogen—that is, they can transform the gaseous nitrogen of the air into compounds that can be used by living cells. Particularly efficient nitrogen fixers are found among the filamentous species that have specialized cells called heterocysts. The heterocysts are thick-walled cell inclusions that are impermeable to oxygen; they provide the anaerobic (oxygen-free) environment necessary for the operation of the nitrogen-fixing enzymes. In Southeast Asia, nitrogen-fixing cyanobacteria often are grown in rice paddies, thereby eliminating the need to apply nitrogen fertilizers.

Cyanobacteria range in size from 0.5 to 60 micrometres, which represents the largest prokaryotic organism. They are widely distributed and are extremely common in fresh water, where they occur as members of both the plankton and the benthos. They are also abundantly represented in such habitats as tide pools, coral reefs, and tidal spray zones; a few species also occur in the ocean plankton. On land, cyanobacteria are common in soil down to a depth of 1 m (39 inches) or more; they also grow on moist surfaces of rocks and trees, where they appear in the form of cushions or layers.

Cyanobacteria flourish in some of the most inhospitable environments known. They can be found in hot springs, in cold lakes underneath 5 m of ice pack, and on the lower surfaces of many rocks in deserts. Cyanobacteria are frequently among the first colonizers of bare rock and soil. Various types of associations take place between cyanobacteria and other organisms. Certain species, for example, grow in a mutualistic relationship with fungi, forming composite organisms known as lichens.

Cyanobacteria reproduce asexually, either by means of binary or multiple fission in unicellular and colonial forms or by fragmentation and spore formation in filamentous species. Under favourable conditions, cyanobacteria can reproduce at explosive rates, forming dense concentrations called blooms. Cyanobacteria blooms can colour a body of water. For example, many ponds take on an opaque shade of green as a result of overgrowths of cyanobacteria, and blooms of phycoerythrin-rich species cause the occasional red colour of the Red Sea. Cyanobacteria blooms are especially common in waters that have been polluted by nitrogen wastes; in such cases, the overgrowths of cyanobacteria can consume so much of the water's dissolved oxygen that fish and other aquatic organisms perish.

Cross section of Cyanobacterial cell



Azolla

Azolla commonly known as mosquito fern, duckweed fern, fairy moss, and water fern, is a small free floating aquatic fern native to Asia, Africa, and the America. It grows in swamps, ditches, and even in lakes and rivers where the water is not turbulent. The name Azolla is derived from the two Greek words, Azo (to dry) and Ollyo (to kill) thus reflecting that the fern is killed by drought. Due to their symbiosis with atmospheric nitrogen (N_2) fixing microorganisms (diazotrophs), the primary production of the plants is hardly ever N-limited under natural conditions. The diazotrophs live inside Azolla's leaf cavities and include the cyanobacteria Nostoc/Anabaena azollae that forms unbranched, multi-cellular chains that contain both photosynthetic, vegetative cells and N_2 fixing heterocysts. The endosymbiont, which is nitrogen-fixing, provides sufficient nitrogen for both itself and its host. The fern, on the other hand, provides a protected environment for the algae and also supplies it with a fixed carbon source. It has capability to fix atmospheric nitrogen as well as to produce biomass at a very high rate. Using nitrogenase enzymes, the diazotrophs reduce atmospheric N_2 to ammonium (NH_4^+), which is then excreted into the Azolla leaf cavity and taken up by the fern. In response to N-limitation, when there is no exogenous N available to Azolla, the heterocyst fraction increases in diazotroph chains. Azolla is commonly used as biofertilizer as well as green manure in the paddy field. Now a days Azolla (either fresh or in dried) is also used as a feed ingredient for ruminants and nonruminants type of livestock. Besides its utilization as biofertilizer and livestock feed, azolla, the 'green gold mine' of the nature is also used as medicine, water purifier, human food and for production of biogas.