Semester – II

I7MBP211 **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

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.
5. CIMMYT. 2015. Laboratory Protocols: CIMMYT Applied Molecular Genetics Laboratory. Third Edition. Mexico, D.F.: CIMMYT.

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ISOLATION OF PLASMID DNA FROM BACTERIA

AIM

To isolate and analyse the purity of the plasmid DNA from bacteria by alkaline lysis method from bacteria.

BACKGROUND

The term plasmid was coined by *Joshua Lederberg*. Plasmid is circular, extra chromosomal DNA present in bacteria. Naturally they occurs to carry genes responsible for antibiotic resistance to degrade certain substance such as hydrocarbon. It is also used in molecular biology/recombinant techniques for cloning genes from various other sources and amplified them for further steps. The size of plasmid range from 1-1000 kilo base pairs. Plasmid DNA miniprob is a fundamental techniques in molecular biology and efficient plasmid DNA isolation and purification from bacterial cultures is a key to many molecular based experiments. They are important tool in genetic and biotechnology laboratories aa well as industries where they are used to make large amount of protein.

Alkaline lysis method of plasmid isolation was originally developed by *Birnboim* and *Doly* in 1979. In this procedure bacteria containing the desired plasmid are harvested from culture medium by centrifugation and suspended in isotonic solution which is subsequently subjected to lysis by an alkaline solution containing a detergent (SDS and NaOH). While detergent serves to lyse cells and denature protein subsequent step is neutralized results in denaturation of plasmid and genomic DNA. Since plasmid DNA covalently closed it reappears properly and remain in solution insoluble from white genomic DNA reappears randomly

resulting in the formation of precipitate. This precipitate is separated by high speed centrifugation. Plasmid from the supernatant can be recovered by precipitation using isopropanol or ethanol.

HARVESTING

Generally bacterial cells containing the plasmid growth in a liquid media. Therefore it is essential to separate the bacterial cells from the culture medium. Almost all protocols use centrifugation to separate the bacterial cells. Centrifugation speed is optimized in such a way that results in accumulation of all the bacterial cells in the form of pellet should be loose enough to be resuspended early in resuspension buffer leads to incomplete lysis resulting in low yield of plasmid separation of bacterial cells from the rest of culture medium is essential as bacterial culture medium may contain inhibitors which can inhibit the enzyme activity (Eg: Restriction enzyme)

RESUSPENSION OF PELLET

Bacterial pellet isolated from first step is resuspended in resuspension buffer. Resuspension buffer contains Glucose, EDTA, and Tris buffer. Glucose is required to make the solution isotonic. EDTA chelate the divalent cations (Calcium and Magnesium) which are released upon bacterial lysis. EDTA results in inactivation of many enzymes which may harm plasmid DNA. Tris buffer acts as a buffering agent. Addition of. RNase to remove RNA from the plasmid preparing.

LYSIS OF BACTERIA

In this step bacterial suspension is treated with lysis solution which contains sodium dodecyl sulfate (SDS and NaOH). SDS is a detergent which the solution is phospholipid and denatures protein. Component of the cell membrane

leading to lysis and release of the cell contents. High alkaline condition due to NaOH denature the plasmid and genomic DNA.

NEUTRALIZATION OF LYSATE

Addition of neutralization solution acidic acetate brings the pH of the lysate back to neutral resulting in precipitation of protein and genomic DNA. Both plasmid and genomic DNA denature in correct confirmation due to it's circular and covalent nature. Therefore remains in the solution, genomic DNA precipitate due to random association of both the strands. SDS reacts with potassium acetate and form insoluble potassium dodecyl sulfate (KDS).

LEANING OF LYSATE

Precipitate formed upon addition of neutralization solution is separated in aqueous solution by high speed centrifugation.

RECOVERY OF PLASMID FROM CLEARED LYSATE

Supernatant from the previous step contains plasmid DNA. Most protocols use precipitation to recover plasmid from solution. Plasmid DNA can be precipitated by either addition of two volumes of absolute alcohol or 0.7 volume of isopropanol to the cleared lysate. Centrifugation at high speed results in collection of plasmid in the form of pellet.

WASHING OF PLASMID PELLET

Precipitation of plasmid DNA contains salts which need to be removed from the plasmid. For this purpose 70% ethanol wash is given to the pellet.

MATERIALS REQUIRED

1. **SOLUTION I (RESUSPENSION BUFFER)**
   - 50mm Glucose

- 10mm EDTA
- 25mm Tris (pH-8.0)
- Autoclaved and stored at 4° C.

2. **SOLUTION II (LYSIS BUFFER)**
   - Freshly prepared 0.2N NaOH
   - 1% SDS, stored at room temperature (RT)
3. **SOLUTION III (NEUTRALIZATION BUFFER)**
   - 3m potassium acetate pH 6.0
   - 3m potassium acetate were prepared by using 29.45g in 60ml of distilled water, to this 11.5ml glacial acetic acid was added and maked upto 100ml by adding 28ml of distilled water.
4. Isopropanol : Stored at 20°C
5. 70% of Ethanol
6. **TE Buffer (pH 8.0)**
   - 10mm Tris
   - 20mm Acetic acid
   - 1mm EDTA

PROCEDURE

- 5ml LB medium containing proper antibiotic was inoculated with a single bacterial colony and incubated at 37°C overnight with vigorous shaking (at least 200rpm).
- Bacterial cells were pelleted by centrifugation at 4000 rpm for 5 minutes at room temperature.
- The supernatant was discarded and bacterial pellet was resuspended in 150 μl of ice cold solution I. The mixture was pipetted up and down or vortexes as necessary to fully resuspended the bacterial cells. The content was stored for 5 minutes at room temperature.
- 300μl of solution II was added to the suspension and mixed thoroughly by repeated gently inversion. Do not vortex the mixture was incubated for 5 minutes on ice.

- 225μl of ice cold solution III was added to the lysate and mixed thoroughly by repeated gently inversion. Do not vortex the tube was stored on ice for 5 minutes.
- The content was centrifuged at 13000 rpm for 15 minutes at 4° C and the supernatant was transferred to fresh tubes.
- 0.7 volume of propanol was added to the supernatant to precipitate the plasmid DNA. The content was mix thoroughly by repeated gently inversion (do not vortex) and incubated for 5 minutes at room temperature.
- The tube was then centrifuged for 10 minutes at 13000 rpm at 4° C and the supernatant was discarded.
- The pellet was then rinsed in 70% ethanol and air dried for about 10 minutes to allow the ethanol to evaporate.
- 50ml of distilled water or TE buffer was added to dissolve the pellet. After addition of RNase (10mg/ml) the mixture was incubated for 20 minutes at room temperature to remove RNA.
- **AGAROSE GEL ELECTROPHORESIS –** Agarose gel were prepared at a final concentration of 0.8% in TAE buffer and the sample was loaded along with standard molecular weight DNA marker electrophoresis was carried out for 1 hour at 110v using TAE as the running buffer. The gel was then stained with (1ml/1ml) ethidium bromide and visualized under UV light in a trans illuminator.

RESULTS

ISOLATION OF CHROMOSOMAL DNA FROM BACTERIA

AIM

To isolate and analyse chromosomal DNA from bacteria.

BACKGROUND

Nucleic acid are vital macro molecular in all living cells. The DNA contains the basic genetic information as such cellular DNA is located at the site of primary genetic activity within the cell in prokaryotic cells. Genetic activity occurs throughout the cell while in eukaryotic cell. It is in discreet particles within the cell many method have been described for isolating DNA from required for the analysis to be performed. The efficiency and recovery of extraction depends on the sample nutrient ionic conditions of the extraction medium type of lysing agent used.

Some DNA analysis (eg: those using restriction enzymes) require DNA of high relatively large amounts. Thus DNA can be obtained using protocols that include steps to purify DNA one released from cells. In contact analysis based on polymerase chain reaction PCR only require very small amount of DNA whose quality can be crude, simple, rapid method allowing DNA to be released from bacterial cells are sufficient for most PCR application. Bacterial DNA can be prepared using extraction kits marketed by several manufacturers. Most of the kits are resins or membranes without organic extraction and/or the alcohol precipitation steps to purify.

In general techniques with extraction kits are easily and rapidly performed but are more expensive than their in-house developed counterpart. Most protocols for the purification of bacterial genomic DNA consists of lysis, following the

incubation with a non specific protease and a series of extraction prior to precipitation of nucleic acid sub produce effectively remove contaminating proteins. If large amounts of clean DNA are required. The procedure can be scaled up and the DNA purified on a CsCl gradient.

The mechanical barriers in bacteria base to be disrupted to release cellular contents including DNA. Tris buffer containing EDTA is found to be useful for this purpose. Since EDTA can chelate bivalent action present in lipid bilayers thus weakening the membrane. Glucose is added to prevent cells from bursting suddenly. Tris is followed by lysozyme treatment to attack N-acetyl glucosamine residues of bacterial cell wall making the already cell wall porous forming and exposing peri plasmic spacers, subsequently Tris is subjected to SDS treatment to disrupt cell membrane followed by treatment with phenol chloroform to denature protein and also to separate aqueous and organic phases. The use of isoamyl alcohol as organic solvent reduces foam during DNA extraction. Chilled isopropanol treatment is given that arises the precipitation of DNA from the aqueous phase solution. Finally precipitate with 70% ethanol is done of eliminate divalent actions. The DNA pellet thus obtained is relatively pure and is suspended in Tris, EDTA buffer. As Tris buffer of pH 8.0 helps to store DNA is a stable form. EDTA protects the DNA from DNase degradation by chelating the divalent cations Mg2+/ Ca2+which are added for enzyme activity.

MATERIALS REQUIRED

- LB broth
- Bacterial culture
- Eppendorf tube
- Distilled water
- Micropipette with tips

- Centrifuge tube

REAGENTS

- TE buffer – 1m Tris HCL (pH 8.0)
- 0.5m EDTA (pH 8.0)
- 10% SDS
- 5m NaCl
- 0.1% ammonium bromide
- TBE buffer

TE BUFFER

- 1m Tris HCl – 3.152g
- 0.5m KCl – 3.722g
- Distilled water – 20ml
- 10% SDS – Dissolve 10g SDS in 100ml of distilled water
- 5ml NaCl – Dissolve 18g of NaCl in 100ml of distilled water
- 0.1% CTAB – Dissolve 01g of CTAB in 100ml of distilled water

LYSIS BUFFER

- 10mm Tris HCl – 15.8g
- 50mm KCl – 37.2g
- 0.1% Tween 20 – 100μl
- Distilled water – 100ml

10X TBE BUFFER (g/l)

- Tris base – 108
- Boric acid – 55
- 0.5m EDTA – 7.44

To prepare 1X TBE buffer, dissolve 1ml of 10X TBE buffer in 9ml of distilled water.

PROCEDURE

- 1.5ml of overnight bacterial culture is to be taken and harvest the cell are by centrifugation at 8000 rpm for 5 minutes.
- 600μl of TE buffer is to be added to cell pellet and resuspend the cell.
- Add 30μl of 10% SDS to the suspended cells and incubate at 37°C for 1 hour then mix well.
- 100μl of 5m NaCl and 0.1% CTAB need to be added and incubate the tubes at 65°C for 10 minutes.
- Add 400μl of chloroform, isoamyl alcohol (24:1) and centrifuge the mixture at 12,000 rpm for 5 minutes.
- Collect the upper phase and mix with 300μl of ice cold isopropanol and centrifuge at 13,000 rpm for 5 minutes.
- To collect the pellet wash it with 500μl ethanol and again centrifuge at 5000 rpm for 1 minute.
- Air dry the mixture and to the dry pellet add 20μl of TE buffer and store at 4°C.
- Load the sample in 0.8% Agarose gel added with ethidium bromide and run the DNA using 1X TBE buffer at 50v for 1 hour and visualize using a gel documentation system. (1X TBE buffer is used for the preparation of 0.8% Agarose gel)

RESULTS

DETERMINATION OF PROTEIN MOLECULAR WEIGHT BY SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

AIM

To separate proteins of different molecular weight using sodium dodecyl sulfate – poly acrylamide gel electrophoresis.

BACKGROUND

The separation of macro molecules in an electric field is called electrophoresis. Discontinuous poly acrylamide gel electrophoresis (PAGE) is probably the most common analytication technique used to separate and characterize proteins. The most commonly used system is called the Laemmle method after U. K Laemmle who was the first to publish a paper employing SDS-PAGE in a scientific study.

A solution of acrylamide and bisacrylamide is polymerized. Acrylamide alone forms linear polymers. The bisacrylamide introduces crosslink between poly acrylamide chains. The "pore size" is determined by the ratio of acrylamide. A high ratio of bisacrylamide to acrylamide and a high acrylamide concentration cause low electrophoretic mobility. Polymerization of acrylamide and bisacrylamide monomers is induced by ammonium per sulphate (APS) which spontaneously decomposes to form free radicals. TEMED a free radicle stabilizer is generally included to promote polymerization.

Sodium dodecyl sulfate (SDS) are also called as sodium lauryl sulfate (SLS) is an amphipathic detergent. It has an anionic head group and a lipophilic tail. It binds non covalently to proteins with a stoichiometry of around the SDS molecule per two amino acids. SDS cause proteins to denture and dissociate from each other (exceeding covalent cross linking). It also confers negative charge. In the

presence of SDS, the intrinsic change of a protein is marked. During SDS PAGE all protein is migrate towards the anode (positively charged electrode). SDS treated protein have very similar charge to mass ratio and similar shapes. During PAGE, the rate of migration of SDS treated protein is effectively determined by molecular weight.

Polyacrylamide gels restain larger molecular from migration as fast as smaller molecules because the charge to mass ratio is nearly the same among SDS denatured polypeptides. The final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. In a gel of uniform density the relative migration of a protein (RF) the F as subscript is negatively proportional to the leg of it's mass. If protein of known mass are run simultaneously with the unknowns. The relationship between RF and mass can be plotted and the masses of unknown protein can be estimated.

Regardless of the system, preparation requires casting two different layers of acrylamide between glass plates, the lower layer (separating O2 reserving gel) is responsible for actually separating polypeptides by size. The upper layer (stacking gel) includes the sample wells. If is designed to sweep up protein in a sample between two moving boundaries. So that they are compressed into micrometer, thin layers when they reach the separating gel.

Protein separation by SDS PAGE can be used to estimate relative molecular mass to determine the relative abundance of major protein in a sample and to determine the distribution of protein among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed. Different staining method can be used to detect protein such as coomasive brilliant blue staining or silver staining.

ACRYLAMIDE CONCENTRATION (%w/v)	RANGE OF SEPERATION
5	60 - 350
10	20 - 300
15	10 - 100

RANGE OF SEPERATION OF PROTEIN IN POLYACRYLAMIDE GELS

The protein sample is mixed with the sample buffer and boiled for 3 – 5 minutes then cooled to room temperature before it is loaded into the sample well of gel. Protein samples prepared for SDS PAGE analysis are denatured by heating in the presence of a sample buffer containing Tris buffer to maintain pH of SDS to denature protein reducing agent such as 20mm DTT or mercapto ethanol to break disulphide bond. Glycerol to make the sample denser/heavier then water and sink neatly to the bottom of the well. A tracking dye (bromophenol blue) is used to monitor the progress of the sample in the gel. Bromophenol blue is a small molecule which travels just behind the ion front moving down towards the bottom of the gel. Few protein molecular travel a head of this tracking dye, when the dye front reaches the bottom of the running gel. The current is turned off to make sure that proteins do not electrophoresis but of the gel into the buffer tank.

To access the relative molecular weight of protein in a gel, a sample containing several proteins in a molecular mass run along side the test sample in

one or more lanes of the gel. Such sets of known proteins are called protein molecular weight markers or protein ladder. Several kinds of ready to use protein molecular weight markers are available that are labelled or prestained for different model of detection. These are pre reduced and therefore preliminarily suited for SDS PAGE rather than native PAGE (mw) markers are detectable via their specialized labels (eg. Fluorescent tags) and by ordinary protein staining method.

Gels are removed from the glass plates and stained with a dye coomasive brilliant blue dye. It binds strongly to all protein and unbound dye is removed by extensive washing of the gel. Blue protein bond can there offer be located and quantified since the amount of bound dye is proportional to proteins content stained gas can be dried and preserved, photographed or scanned with a recording densitometer to measure the intensity of the colour in each protein band.

In general, casting stands are used to prepare the mini slab gels. Two clean plat with two Teflon spares make a single cassette. The cassette are stacked upright in the stand with the bottom of the cassette filled tightly to the bottom of the stand.

Using freshly prepared 10% ammonium per sulfate (APS) is always recommended.

Once the catalyst are added polymerization may occur quickly. Thus if it necessary to have the casting stand and to have the overlay solution ready.

Acrylamide polymerizes spontaneously in the absence of oxygen. So the polymerization process involves complete removal of oxygen from the solution. Polymerization is more uniform if the mix is degassed to remove much of the

dissolved oxygen by placing it under a vaccum for 5 minutes or so before polymerization.

Immediately after pouring the gel mix it must be overlaid with water saturated butanol to an additional height of 0.5cm or the purpose of butanol is to produce a smooth completely level surface on top of the separating gel. So that bounds are straight and uniform.

Combs are inserted taking care not to catch bubbles under the teeth and adjust to make them evenly, if necessary.

Acrylamide is a toxic substance and hence proper handling is required with wearing gloves is mandatory while handling the solution.

Any left over should be discarded only after the polymerization in to the gel.

MATERIALS REQUIRED

30% ACRYLAMIDE STOCK SOLUTION

- Acrylamide – 30g
- Bisacrylamide – 0.8g

SEPARATING BUFFER

- 1.5m Tris (pH 8.8) – 100ml

STACKING GEL BUFFER

- 0.5m Tris (pH 6.8)

RUNNING BUFFER

- 50mm Tris
- 192mm Glycine

- 0.1% SDS
- pH 8.3

5X LOADING BUFFER

- 250mm Tris (pH 6.8)
- 10% SDS
- 4% Sucrose/30% Glycerol
- 5% β mercapto ethanol
- 10% Bromophenol blue (0.5% w/v solution in water)

GEL STAINING SOLUTION

- Coomasive brilliant blue – 0.1g
- Methanol – 40ml
- Glacial acetic acid – 10ml
- Distilled water – 50ml

DESTAINING SOLUTION

- Methanol – 40ml
- Glacial acetic acid – 10ml
- Distilled water – 50ml

10% SDS SOLUTION

PROCEDURE

- The glass plates were thoroughly cleaned with soap, rinsed in distilled water and finally wiped with alcohol and air dried.
- It was then assembled properly using the spaces rubbed with petroleum jelly/Vaseline, clipped and clamped in an upright position.

- 2% agar was then applied around the edges of the spacers to hold them in place and seal the chamber. So as to be leak proof (water maybe added to the sealed glass plates to check for any leakage).
- The following ingredients were mixed together for the preparation of 10% separating gel mixture was added in the given order.

- 30% Acrylamide stock solution – 6.65ml
- Tris HCl – 4.0ml
- Deionized/Distilled water – 9.0ml
- 10% SDS – 200μl
- Ammonium per sulfate – 100μl
- TEMED – 10μl

- The contents were mixed gently and poured in the space between the plate to gel the desired length/height of separating gel. Butanol/70% ethanol was layered on top of the mixture and left for about 30 – 60 minutes for polymerization.
- After making sure that the solution has polymerized into gel, the layered butanol/70% ethanol was removed and washed with streaking gel buffer.
- 4% Stacking gel was prepared by mixing the following solution.

- 30% Acrylamide stock solution – 1.35ml
- Tris HCl – 1.0ml (pH 6.8)
- Deionized/Distilled water – 7.5ml
- 10% SDS – 100μl
- Ammonium per sulfate – 50μl
- TEMED – 10μl

- The stacking gel mixture was poured, comb was placed properly and left for 30 – 60 minutes for polymerization.

- After the gel has polymerized the comb was removed without desorting the shape of the wells. This gel can be used immediately or can be stored in a leak proof container containing the running buffer. It was advisable to use the gel after overnight storage.
- After removing the clips and agar the gel plate was carefully installed in the electrophoresis apparatus. It was then filled with the running buffer and any trapped air bubbles at the bottom of the gel were removed.
- After determining the protein concentration ideal range (10 – 50μl) the sample were mixed with the sample buffer and heated in a boiling water for 5 minutes.
- After the sample have cooled, it was carefully loaded into the wells using a micropipette. Standard marker protein were loaded in the first/middle/last well, according to the requirement.
- The current (25mA) was turned on unit the sample reached the end of the stacking gel and then increases to 75mA till the samples reached the end of the separating gel.
- After the run was completed, the gel was carefully removed from the plates and soaked in staining solution placed over a recking platform for about 2 – 3 hours.
- After staining the gel is immersed in destaining solution and shaked continuously over a reaching platform overnight or until the background of the gel is colourless. Initially the destaining solution was changed frequently least for 3 – 5 times.
- The proteins bands may appear in blue colour and can be documented by gel documentation system. Alternatively, it can also be scanned using a scanner by placing the gel in a polythene bag.

RESULTS

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 from plant life and certain rodents, insects and earthworms. The 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 structure) community of soil micro organism for continued supply of many essential nutrients. Nutrient elements bound in organic combination would be inaccessible to plants without microbial decomposition of organic matter and mineralization of three elements. Not only would nutrient cycling step but also biological N – fixation.

Soil micro organism are indispensable to life on earth. Soil micro organism live in their film of water that surround soil particles. These organism include microflora, bacteria, fungi and actinomycetes along with microfauna, protozoa and nematodes. In terms of numbers and biological activity. The microflora are 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 (Mass hyphae) are often macroscopic. *Actinomycetes* are also filamentous and branched by smaller.

AGAR PLATE METHOD FOR MICROBIAL COUNT

In this method soil is dispersed 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 extent of dilution. Plates may be filled with huge enumeration of colony forming units initially present in the soil is from plates in-between these extremes. This method required sterile technique to avoid introduction of extraneous microbes. Any one of several different growth medium can be used but no single growth medium is optimal for all micro organism that inhibit soil. Thus growth of certain organism is favour in the chosen medium and growth of other is systemised. Antagonistic or antibiotic in the agar plate method.

MATERIALS REQUIRED

- Nutrient agar, C zaper – Dox agar, Starch casein nitrate agar.
- Flask containing 100ml of sterile saline.
- Test tubes with 9ml of sterile saline for serial dilution.

PROCEDURE

- Agar medium plates such as nutrient agar, C zaper – Dox 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 add 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 are used to inoculate nutrient agar plates and incubated at 37°C for 24 hours.
- For fungi 10(-2), 10(-3), 10(-4), and 10(-5) dilution were used to inoculate zaper – dox agar plate 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 and *actinomycetes*. This isolated were transferred to respective agar plates and maintained further investigation.

RESULTS

ISOLATION OF FREE-LIVING N^2 FIXING FROM SOIL – *AZOTOBACTER*

AIM

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

BACKGROUND

Azotobacter is a free living heterotrophic nitrogen fixing bacteria that occur in the rhizosphere of variety of plates. The genus *Azotobacter* has six species viz, *A. chroococcum, A. vinelandii, A. beijerinckii, A. nigricans, A. armeniacus, A. paspali* except the last species which is a *rhizoplane* bacteria. The other membrane largely soil borne and *rhizospherits.* The potential of *A. Chroococcum,* and *A. paspali* as a bio fertilizer for various non legume crops is well documented.

Azotobacter is an aerobic, gram negative rod shaped bacteria occurs singly in chains or in clumps. It doesn't form endospores but it form thick walled cysts. These cysts are resistant to desiccation and to some deleterious chemical and physic agent. They however cannot withstand extreme temperature while in the cyst stage of their life cycle. They don't fix nitrogen and are optically refractive. It maybe 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 optimum temperature range between 20°C to 30°C and the range of pH 7.0 – 7.5. They are able to grow on various carbohydrates, alcohol and organic acids.

Azotobacter was first discovered using culture that was devoid of combined nitrogen source. *Azotobacter* is found on natural to alkaline soils. In

aquatic environment in the plant *rhizosphere* and *phycosphere*. *A. chroococcum* is their most common species of *Azotobacter* present in the soil. *Azotobacter spp* are know to influence plant growth through their ability to fix molecular nitrogen, production of growth promoting substances like IAA, gibberellin like compound and vitamins, excretion of ammonia in the rhizosphere in the presence of root exudates production of antifungal metabolites and phosphate solubilisation.

Jensen's N – free medium, Ashby's medium is routinely used for propagation and mass production of *Azotobacter* seed inoculation of *A. chroococcum* increase the yield of field crops by about 10% and of cereals by about 5 – 20%. The response to inoculation was increased by manurrring or by fertilizer application, co – inoculation of *Azotobacter* with other bio inoculum like *Rhizobium, Azospirillum, Solubilizers, Vesicular Asbuscular Mycorrhizae (VAM)* have been reported to enhance the growth and yield of legumes, cereals and vegetative crops.

Beneficial effects of *Azotobacter chroococcum* inoculation has been reported on various cereals, vegetables, oil, seed, legume and cash crops. Inoculation experiments with *Azotobacter* gave better yield performance only at lower levels of nitrogen (0 to 30kg N ha-1). These diazotrophic bacteria require large amount of available carbon for their survival in soil. Addition of farmyard manure (FYM) compost and other organic fundaments to agricultural soil improves the efficiency of *Azotobacter* and hence the plant growth and yield.

MATERIALS REQUIRED

- Jensen's agar medium, Ashby agar medium.
- *Rhizosphere* soil from sugarcane / paddy / cotton.
- Flask containing 100ml of sterile saline test.
- Tubes with 9ml of sterile saline for serial dilution.

PROCEDURE

- Agar plates with Jensen's medium and Ashby's medium were prepared.
- 1g of the collected *rhizosphere* soil was suspended in 100ml of sterile saline and gently mixed to form uniform suspension.
- This suspension was serially diluted until 10(-5), using spread plate techniques. 1ml of these serially diluted suspension 10(-3), 10(-4), and 10(-5) was plated on to the agar medium.
- The plates were incubated at room temperature for 3 – 5 days.

RESULTS

ISOLATION OF SYMBIOTIC NITROGEN FIXERS FROM ROOT NODULE – *RHIZOBIUM*

AIM

To isolate *rhizobium spp.* from root nodule of leguminous plants.

BACKGROUND

Nitrogen is an essential nutrients for plant growth and development planners usually depend upon combined or fixed forms of nitrogen such as ammonia and nitrate because it is unavailable in it's most prevalent form as atmospheric nitrogen much of this nitrogen is provided to cropping system in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has to world wide ecological problems as well as it 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 microorganism 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 effects 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 compound to the plants.

Legumes have been used in agriculture since concept time and legume seeds or pulses were among the first source of human feed and their domestication. In leguminous plant the bacteria live-in small outgrowth on the roots called nodules. When these nodules the bacteria do nitrogen fixation and

the plants absorbs the ammonia. Legume plant absorbs the ammonia, a unique ability to establish symbiosis with nitrogen fixing bacteria of the family *Rhizobia* as such group of *Rhizobium, Brady rhizobium, Allorhizobium, Sino rhizobium,* and *Mesorhizobium.* These bacteria can fix atmospheric nitrogen only in root nodules of legumes and that to when it is in the bacteriod stage of it's life cycle. It possesses 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 – 200kg of nitrogen contain the enzyme nitrogenase which catalyses the following reaction yeast mannitol agar with Congo red is used for the cultivation of *rhizobium* species and for studying the root nodulation.

$$N2 + 3H2 \text{ ----------} \rightarrow 2N + H3$$

Yeast extract serves as a good source of readily available amino acid. Vitamin B complex and accessory growth factor for *rhizobia*. It also poises the oxidation reaction. Potential of medium in the range favourable 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 strains, colonies of *rhizobia* stand out as white, translucent gristening 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 hypo chloride.
- 70% Ethanol.

- Sterile – forceps, glass rod, blade and distilled water.

PROCEDURE

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

METHODS I

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

METHODS II

- 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.
- With an inoculation loop a loop full of the suspension was streaked on to the medium.

- Alternatively, a serial dilution of the suspension can be performed and plated either by spread or pour plate method.
- All plates used in either method were incubated at room temperature for 3 – 5 days.

ISOLATION OF PHOSPHATE SOLUBILIZERS

AIM

To isolate phosphate solubilizing microorganism from soil.

BACKGROUND

Phosphorus is one among the three important nutrients required for plants, animals and microbial growth. It can occur in two forms:

1. Soluble phosphorus and
2. Insoluble phosphorus.

Soluble form can be easily assimilated by plant and microbes. It is not so in the case of organic and inorganic phosphorus that cannot be utilized by plants. Certain microorganism act upon the insoluble form and make the bound phosphate available to plants. They are called as phosphate solubilizing microorganism (PSB) mechanism of mineral phosphate solubilisation of PSB stains is associated with the release of low molecular weight. Organic / inorganic acids through with their hydroxyl and carboxyl group chelate the cations bound to phosphate. There by covering it into soluble forms. Bacteria such as *Bacillus subtilis, B. polymyxa, Pseudomonas, Mycobacterium, Micrococcus, Flave* bacteria and fungi like *Aspergillus,* and *Penicillium* and some of the best phosphate Solubilizers.

PSB have been introduced to the agricultural community as bio fertilizers. PSB have the attention of agriculturist as soil inoculum to improve the plant growth and yield. When 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. Simple inoculation of seed with PSB gives crop yield

responses equivalent to 30kg P2O5/ha or 50% of the need for phosphate fertilizers.

The use of PSB are pikovskaya's agar is used for detection of phosphate solubilizing bacteria from soil. Yeast extract in the medium provides nitrogen and other nutrients necessary to support bacteria growth. Dextrose acts as an energy source. Different salts and yeast extract supports the growth of organism. Phosphate solubilizing bacteria will grow on their medium and form a clear zone around the colony formed due to phosphate solubilisation in the viability of the colony.

MATERIALS REQUIRED

- Soil sample.
- Sterile water blanks for dilution.
- Pikovskaya's agar plates.
- Sterile L- rod.

PROCEDURE

- 1g of the collected soil sample was mixed in 100ml of sterile distilled water and it was further serially diluted upto 10(-6) dilution.
- From each dilution 0.1ml of the sample was evenly spread over the agar medium by spread plate technique.
- The plate were incubated at room temperature for 3 – 5 days.

RESULTS