Semester – VI

15MBU611 APPLICATION ORIENTED PRACTICAL – VI 5H – 3C

Instruction Hours / week: L: 0 T: 0 P: 5 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 9 Hours

SCOPE

Acquire knowledge of the emerging and reemerging trend in molecular levels. Apply methods of infection control and hygienic standards. To know the precautions in industrial sectors to be followed in control and prevention of contamination.

OBJECTIVES

Integrated approach to various techniques of commercial importance and interpretation of new approach for diagnosis and needs to be emphasized in industrial application.

1. Isolation of genomic DNA from bacteria
2. Isolation of plasmid DNA from bacteria
3. Determination of molecular weight by Agarose gel electrophoresis
4. Determination of molecular weight by SDS PAGE electrophoresis (Demo)
5. Isolation of antibiotic producers from soil microbes
6. Production of Penicillin by batch fermentation
7. Cellulase production test
8. Identification of clinically important fungi – *Candida albicans*
9. Identification of *Aspergillus sp.* by Lacto phenol cotton blue mounting
10. Identification of *Mucor sp.* by Lacto phenol cotton blue mounting
11. Identification of *Rhizopus sp.* by Lacto phenol cotton blue mounting
12. Identification of *Fusarium sp.* by Lacto phenol cotton blue mounting
13. Identification of *Penicillium sp.* by Lacto phenol cotton blue mounting
14. Observation of parasites – *Entamoeba sp. Plasmodium sps. Ascaris sp. Taenia sp.*
15. Observation of parasites – Blood smear examination

REFERENCES

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9. Satyadev, G., 2004. Handbook of Medical Laboratory Technology. 1st Edition, Paras Medical Publisher, Hyderabad, India.

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15MBU611 APPLICATION ORIENTED PRACTICAL – VI 5H – 3C

List of Practicals

1. Isolation of genomic DNA from bacteria
2. Isolation of plasmid DNA from bacteria
3. Determination of molecular weight by Agarose gel electrophoresis
4. Determination of molecular weight by SDS PAGE electrophoresis (Demo)
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6. Production of Penicillin by batch fermentation
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8. Identification of clinically important fungi – *Candida albicans*
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15. Observation of parasites – Blood smear examination

2018

List of practicals – lecture plan

Experiment name	Hours
1. Isolation of genomic DNA from bacteria	1 weeks/ 5 hours
2. Isolation of plasmid DNA from bacteria	1 weeks/ 5 hours
3. Determination of molecular weight by Agarose gel electrophoresis	1 weeks/ 5 hours
4. Determination of molecular weight by SDS PAGE electrophoresis (Demo)	1 weeks/ 5 hours
5. Isolation of antibiotic producers from soil microbes	1 weeks/ 5 hours
6. Production of Penicillin by batch fermentation	1 weeks/ 5 hours
7. Cellulase production test	1 weeks/ 5 hours
8. Identification of clinically important fungi – *Candida albicans*	1 weeks/ 5 hours
9. Identification of *Aspergillus sp.* by Lacto phenol cotton blue mounting	1 weeks/ 5 hours
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11. Identification of *Rhizopus sp.* by Lacto phenol cotton blue mounting	1 weeks/ 5 hours
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13. Identification of *Penicillium sp.* by Lacto phenol cotton blue mounting	1 weeks/ 5 hours
14. Observation of parasites – *Entamoeba sp. Plasmodium sps. Ascaris sp. Taenia sp.*	1 weeks/ 5 hours
15. Observation of parasites – Blood smear examination	1 weeks/ 5 hours

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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 inresuspension 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. Resuspensionbuffer 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 actsas 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 NaOHdenature 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 makedupto 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 supernatantwas discarded and bacterial pellet was resuspended in 150μl of ice cold solution I. The mixture was pipetted up and downor vortexes as necessary to fully resuspended the bacterial cells. The content was stored for 5 minutes at room temperature.
- 300μl of solution IIwas 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 IIIwas 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° Cand 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 samplenutrient 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 membraneswithout 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

theaqueous 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.
- 600$\mu l$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 for1 hour then mix well.
- 100$\mu l$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

ACRYLAMIDE CONCENTRATION (%w/v)	RANGE OF SEPERATION

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 theyreach 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 andto 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.

RANGE OF SEPERATION OF PROTEIN IN POLYACRYLAMIDE GELS

5	**60 - 350**
10	**20 - 300**
15	**10 - 100**

The protein sample is mixed with the sample buffer and boiled for3 – 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 denatureprotein 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 differentmodel 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% βmercapto 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 numbersand 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