EX.1: AIM:

To perform gradient plate replica plate technique to isolate mutants

Principle:

Mutation is a heritable change in the nucleotide sequence of DNA. Mutations may be characterized according to either the kind of genotypic change that has occurred or their phenotypic consequences. Mutations can alter the phenotype of a microorganism in several different ways. Morphological mutations change the microorganism's colonial or cellular morphology. Nutritional or biochemical variation may occur in a gene that encodes an enzyme involved in a metabolic pathway of amino acid synthesis. Changes in gene regulation occurs when mutation occur in a gene encoding a transcription factor. Lethal mutations prevent the reproducing capability of the organism, and when expressed, it results in the death of the microorganism.

Mutations often inactivate a biosynthetic pathway of the microorganism, and frequently make a microorganism unable to grow on a medium lacking an adequate supply of the pathway's end product. Based on this principle microorganism are classified as Prototrophic and Auxotrophic. Prototrophic organisms (wild type) have the same nutritional requirements as that of their ancestors. They need only inorganic salts, an organic energy source such as sugar, fat, protein and water to survive and grow. That is, the Prototroph's need only "Minimal medium" for their growth and survival. Auxotrophic mutants are unable to grow without one or more essential nutrients. Auxotrophs are mutant for particular nutrient synthesis pathway enzymes. Such an error is known as an inborn error of metabolism, whether it occurs in a bacterium or a eukaryote. An auxotroph can be grown only on an enriched medium that provides the particular nutrient that the mutant cannot metabolize on its own.

There are two classes of mutations: Spontaneous mutations and induced mutations.

Spontaneous Mutations:

A mutation without a known cause is called Spontaneous mutations. This occurs at low frequency leading to the chemical instability of purine and pyrimidine

bases and also due to low level of metabolic errors, or mistakes during the DNA replication.

Induced Mutations:

Mutations that results from exposure of organisms to mutagenic agents such as ionizing irradiation, ultraviolet light or various chemicals that react with nucleic acids. In experimental organisms, researchers often treat them with these mutagens in order to increase the frequency of mutation in them.

Generally, chemical mutagens induce point mutations, whereas ionizing radiations gives rise to large chromosomal abnormalities. Point mutations are simple changes in single base-pairs, the substitution of one base-pair for another, or duplication or deletion of single base-pairs. Point mutations occur at a single point on a chromosome. Missense mutation is a type of point mutation, in which a single nucleotide is changed that leads to substitution of a different amino acid and a nonsense mutation, is a point mutation, that changes a normal codon into a stop codon that does not code for an amino acid and arrest peptide synthesis without amino acid insertion resulting in a non functional protein product.

Frame shift mutation, is a kind of mutation caused by the addition or deletion of nucleotides which is not a multiple of three so that the codon is read incorrectly during translation. A silent mutation causes base substitution without amino acid substitution and thus has no effect. Such substitutions will not cause any change in their product and cannot be detected without genome sequencing. In any case, the mutation events are often reversible. The subsequent mutations in the nucleotide pair restore the original wild type phenotype. That is, a gene that has undergone mutation reverses to its original base composition. This is referred to as back-mutation, reverse mutation or reversion.

Genetic and biochemical investigations in bacteriology are often initiated by isolation of mutant strains. The spontaneous mutations due to resistance in antibiotics such as Streptomycin are easily detected because they grow in the presence of antibiotic concentrations that inhibit the growth of normal bacteria.

Gradient Plate Technique:

An excellent way to determine the ability of organisms to produce mutants that are resistant to antibiotic is to grow them on a gradient plate of a particular antibiotic. The gradient plate consists of two wedges like layers of media: a bottom layer of plain nutrient agar and top layer of antibiotic with nutrient agar. The antibiotic in the top layer, diffuse into the bottom layer producing a gradient of antibiotic concentration from low to high. A gradient plate is made by using Streptomycin in the medium. E. coli, which is normally sensitive to Streptomycin, will be spread over the surface of the plate and incubated for 24 to 72 hours. After incubation colonies will appear on both the gradients. The colonies develop in the high concentration are resistant to the action of Streptomycin, and are considered as Streptomycin resistant mutants. For isolation of antibiotic resistant of gram negative enteric bacteria, the antibiotics commonly used are Rifampicin, Streptomycin, and Erythromycin etc.

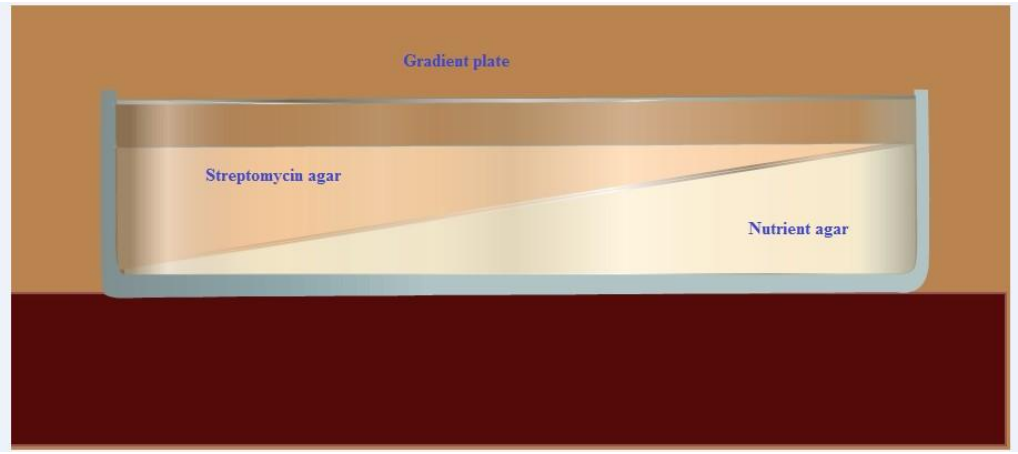


Fig 1: Gradient plate preparation steps

Replica Plating Method:

If an organism has the ability to produce mutant strains resistant to antibiotics, the nature of mutation, whether it is spontaneous or induced have to be tested. It

would be a difficult task to identify a few mutant colonies from a vast population of 100-500 colonies. This can be accomplished by a replica plating technique. The technique was developed by Joshua and Esther Lederberg in 1952 for providing the direct evidence for the existence of pre-existing mutations. This technique isolates both nutritional mutants and antibiotic resistant mutants. Their actual experiment concerned with replicating master plates of sensitive cells to two or more plates containing either streptomycin or T1 phage. Replica plating allows the observation of microbes under a series of growth conditions. The bacteria are grown in an environment that is not selective for given mutation. This technique is used to transfer the members of each colony to a selective environment. A simple velveteen covered colony transfer device is used to transfer the colonies in nutrient agar medium supplemented with or without a particular antibiotic or nutrient. The fibers of velvet act as fine inoculating needles, picking up the bacterial cells from the surface of this master plate. The velvet with its attached microbes is then touched to the surface of a sterile agar plate, inoculating it. In this manner, microbes can be repeatedly stamped onto media of differing composition. By comparing the presence of colonies following incubation we can indirectly determine the mutant colonies by their absence in the selective environment. A colony that develops on a complete medium fail to develop on a minimal medium that lacks a specific growth factor, the occurrence of a nutritional mutant is indicated. The microbes that do not grow on the minimal medium represent auxotrophic strains. A simple velveteen covered colony transfer device is used to transfer the colonies in nutrient agar medium supplemented with or without a particular antibiotic or nutrient. A colony that develops on a complete medium fail to develop on a minimal medium that lacks a specific growth factor, the occurrence of a nutritional mutant is indicated. The microbes that do not grow on the minimal medium represent auxotrophic strains. This method has been applied in numerous experiments to identify the occurrence of mutations. Many of the biochemical pathways in microbes were elucidated in this way by using nutritional mutants (Fig 2).

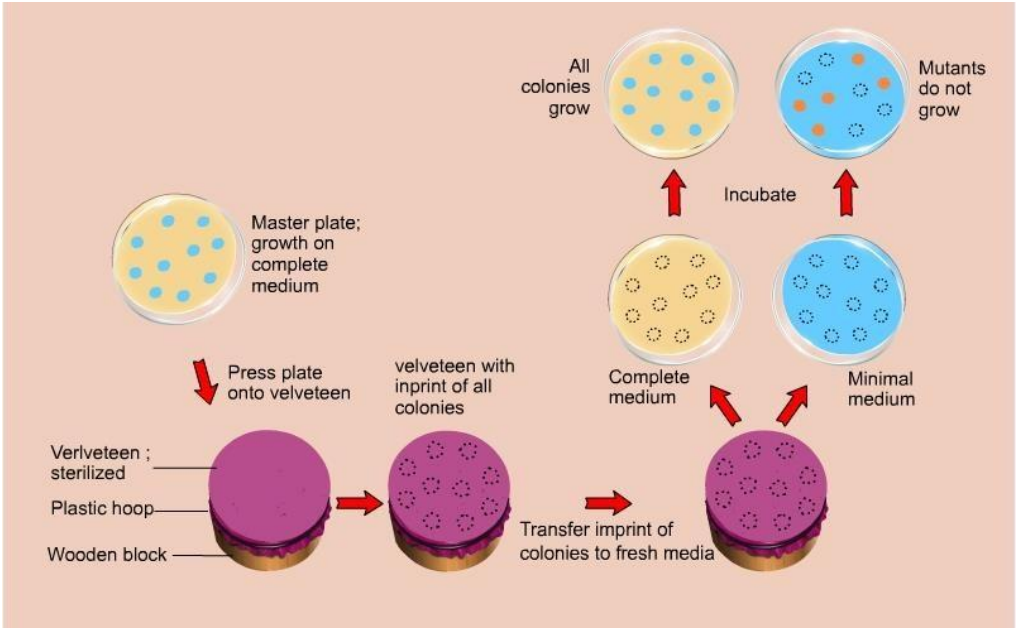


Fig 2: Replica plating technique

RESULTS

EX.2 TRANSFORMATION IN BACTERIA

AIM:

To perform transformation and transfer of foreign DNA into bacteria

THEORY

DNA can be exchanged among bacteria by three methods: transformation, transduction and conjugation. Transformation is one of the most popular techniques of molecular genetics because it is often the best way to reintroduce experimentally altered DNA into cells. This technique was first discovered in bacteria, but other ways have been designed to transform many types of animal and plant cell as well. Transformation was the first mechanism of bacterial gene exchange to be discovered. The initial experiment on transformation was performed by Frederick Griffith in England in 1928 when he was working with two strains of *Streptococcus pneumoniae*. Griffith's experiments were based on the fact that S.pneumoniae makes two types of different appearing colonies, one type made by pathogenic bacteria and the other type made by bacteria that are incapable of causing infections. The colonies made by the pathogenic strains appear smooth on agar plates, because the bacteria excrete a polysaccharide capsule. Bacterial transformation has been widely investigated in Pneumococcus, Haemophilus influenzae, Bacillus subtilis, and certain other bacteria. During the process of transformation, genes are transferred from one bacterium to another as 'naked' DNA solution. In nature, some bacteria, perhaps after death and cell lysis, release their DNA into the environment. Other bacteria can then encounter the DNA and, depending on the particular species and growth conditions, take up fragments of DNA and integrate them into their own chromosomes by recombination. Transformation works best when the donor and recipient cells are very closely related.

Two elements are required in a transformation system. The first element is a suitable host bacterium. For this, commonly we use E.coli as host organism. The strain of E.coli has been cultured in the laboratory and it has been selected for characteristics that make it especially useful in the molecular biology laboratory. Plasmid is the other important element in the transformation system. Plasmid encodes some enzymes and antibiotic resistant markers which are expressed in the bacterium after transformation. When transformation occurs, the DNA transferred is often a plasmid: small, circular DNA found naturally in many bacteria. Plasmid is

found as extra chromosomal DNA and it contains some genes that the bacterium would not normally possess. These extra genes can provide a growth advantage to bacteria by providing the gene for an enzyme such as amylase, beta- lactamase.

There are two forms of transformation: natural and artificial, each process depends on the ability of the organism to transform the DNA into the host cells. In natural transformation, bacteria are capable of DNA naturally which means they can take up DNA from their environment directly. That kind of bacteria is called as naturally transformable. In artificial transformation, bacteria are not naturally transformable which they do not take up DNA from the environment. Bacterial cells have been exposed to particular chemical or electrical treatments to make them more permeable and then only the cells can take up DNA efficiently. Bacteria can take up DNA artificially by using different techniques such as electroporation, heat shock, Ca^{2+} treatment of cells and protoplast uptake of DNA. In these techniques, cells made permeable to DNA by calcium ion treatment will take up both single stranded and double stranded DNA. Therefore, both linear and double stranded circular plasmids can be efficiently introduced into chemically treated cells. This fact has made calcium ion induced competence very useful for cloning and other applications that require the introduction of plasmid and phage DNA into cell.

Bacteria should possess requisite molecular characteristics to be susceptible for transformation- called competence. Transformation of naturally competent bacteria with plasmid or phage DNA usually occurs only with DNAs that are dimerized or multimerized into long concatemers. A dimerized or multimerized DNA is one in which two or more copies of the molecule are linked to head and tail. If a dimerized plasmid or phage DNA is cut only once, it still has complementary sequences at its ends that can recombine to recyclize the plasmid. The process of bacterial transformation and autonomous replication of the engineered plasmid DNA allows the production of large amounts of the DNA of interest within the bacterial host. This allows for further manipulations of the cloned DNA or for the expression of the gene of interest in the bacteria itself. The produced protein may result in a new bacterial phenotype, or the protein itself may be the desired end product.

Principle

Transformation process allows a bacterium to take up genes from its surrounding environment; that is transformation involves the direct uptakes of

fragments of DNA by a recipient cell and the acquisition of new genetic characteristics. There are two major parameters involved in efficiently transforming a bacterial organism. The first is the method used to induce competence for transformation. The second major parameter is the genetic constitution of the host strain of the organism being transformed. Competent cells are capable of uptaking DNA from their environment and expressing DNA as functional proteins. If a bacterium is said to be competent, it has to maintain a physiological state in which it can take up the donor DNA. Calcium chloride treatment is one of the best methods for the preparation of competent cells. Competence results from alterations in the cell wall that makes it permeable to large DNA molecules. This is a naturally occurring process and through this bacteria can transfer advantageous characteristics, such as antibiotic resistance. Bacteria can take DNA from the environment in the form of plasmid. Most of them are double stranded circular DNA molecules and many can exist at very high copy numbers within a single bacterial cell. Many naturally occurring plasmids carry an antibiotic resistant gene referred to as a marker.

In the process of transformation, the competent cells are incubated with DNA in ice. Then it is placed in a water bath at 42ºC and further plunging them in ice. This process will take up the DNA into the bacterial cell. Then it is plated in an agar plate containing appropriate antibiotic. The presence of an antibiotic marker on the plasmid allows for rapid screening of successful transformants. Blue –white selection (Alpha complementation) can be used to determine which plasmids carry an inserted fragment of DNA and which do not. These plasmids contain an additional gene (lac Z) that encodes for a portion of the enzyme β – galactosidase. When it transformed into an appropriate host, one containing the gene for the remaining portion of β –galactosidase, the intact enzyme can be produced and these bacteria form blue colonies in the presence of X – gal (5-bromo-4-chloro-3-indoyl-b-D-galactoside) and a gratuitous inducer called IPTG (Isopropyl β-D-Thiogalactopyronoside). These plasmids contains a number of cloning sites within the lac Z gene, and any insertion of foreign DNA into this region results in the loss of the ability to form active β –galactosidase. Therefore colonies that carry the plasmid with the insert, ie, Transformants will remain white and the colonies without the foreign DNA (Non-Transformants) will remain Blue. We can also calculate the efficiency of transformation by using the concentration of DNA and number of transformed colonies.

RESULTS

3. BACTERIAL CONJUGATION

OBJECTIVES

To demonstrate the technical procedure to monitor the conjugational transfer of genetic material from one cell to another.

PRINCIPLE

Conjugation is a reliable, robust method to transfer plasmids between bacteria. This is a general purpose protocol for conjugation using a DAP (diaminopimelic acid, 0.3 mM)- donor strain with a standard strain. Many steps can be optimized, but this is a good starting point for trying a new strain. For additional resources, concerns, and options, see the end of this document.

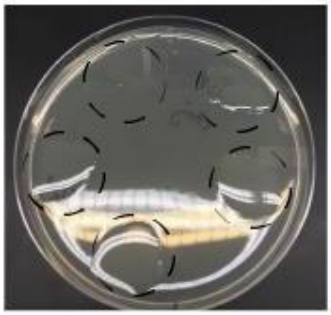
Required:

- **Donor strain** that contains your plasmid of interest (this should be a strain like Mu Free Donor (MFDpir) or B2155, which contain chromosomal integrated rp4 based transfer machinery). MFDpir can be requested from its creators. Your plasmid should contain an oriT sequence (known as the origin of transfer, this allows it to be transferred)
- **Recipient strain**
- **Growth media** for each individual strain.
- **Non-selective plates** containing **DAP** (0.3 mM) for conjugation. These plates should be made from media that **both** strains can grow on, and contain no antibiotic. You can set up ~4 conjugation mixtures per plate.
- **Selective plates**. These plates should contain the same antibiotic as the marker on the plasmid you want to transfer, but no DAP. To plate dilutions, you need 3-4 plates per conjugation mixture. You may also include additional counter selectable markers (ie, antibiotics your recipient is resistant to but the E. coli donor is not) to minimize E. coli background.
- **Non-selective** plates (optional). If desired, these can be used to determine the conjugation efficiency by comparing growth to the selective plates.
- Standard pipettes, microcentrifuge tubes, and incubator that supports growth of both strains.

Protocol:

1. Grow overnight cultures of your donor and recipient strains. Donor strain must be grown in the presence of DAP (0.3 mM) and appropriate antibiotic.
2. Gently spin down 1ml culture (~3000rpm for 5 minutes) and wash donor and recipient cells in PBS. Repeat. Resuspend in 500µL PBS. This removes residual antibiotic from the donor cell culture. Recipient cells can also be scraped off of plates and washed in PBS to prepare for conjugation.
3. Measure optical density and combine 1:1 ratio of donor and recipient cells in micro centrifuge tube (50µL : 50µL). Mix by pipetting twice. For particularly slow growing strains that will be outcompeted rapidly by E. coli, a larger recipient:donor ratio will yield more transconjugants (see notes).
4. Plate 100uL of mixture onto non-selective plate containing DAP. Do not spread. You can plate up to 4-5 conjugations on the same plate by carefully plating into quadrants on the plate (See image 1 for example of 5 conjugations on the same plate).
5. Incubate conjugation plate overnight (See image 2 for example of overnight growth).
6. Scrape up each conjugation mixture into a micro centrifuge tube with 1mL PBS. Vortex and gently spin down. Repeat.
7. Resuspend conjugation mixture in 1mL of PBS. Plate 100µL of this mixture and 100µL of a 10-fold dilution onto selective plates.
8. Pick single colonies and restreak or grown in selective media. Confirm transfer of the plasmid via PCR amplification of the

5 Conjugation mixtures (100µL) spotted on one plate. Lid is marked (along with line across edge and base) to distinguished mixtures



insert.

RESULTS

5. TITRATION OF PHAGES

Objectives:

1. To demonstrate the ability of bacteriophage to replicate inside a susceptible host cell.
2. To determine the concentration of phage particles in a suspension.

Principle:

Bacteriophages (phage) are obligate intracellular parasites. They multiply inside a bacterium by making use of some or all of the host (ie., bacteria) biosynthetic machinery (viruses that infect bacteria are known as bacteriophage). They enter the bacterial cell by 'landing' on the cell wall and injecting their DNA into the bacterial cytoplasm. After entry, the phage DNA acts as a template for production of phage proteins. These proteins replicate the phage and subjugate the cell, eventually causing lysis and death of the host cell. A bacteriophage particle is even harder to see than a bacterium. Viruses are beyond the limits of resolution of the light microscope and can be seen only with electron microscopes. Fortunately, we can use a technique very similar to the colony-counting technique used to measure the number of bacteria to count phage particles, known as the plaque assay. Lytic phages are enumerated by this method.

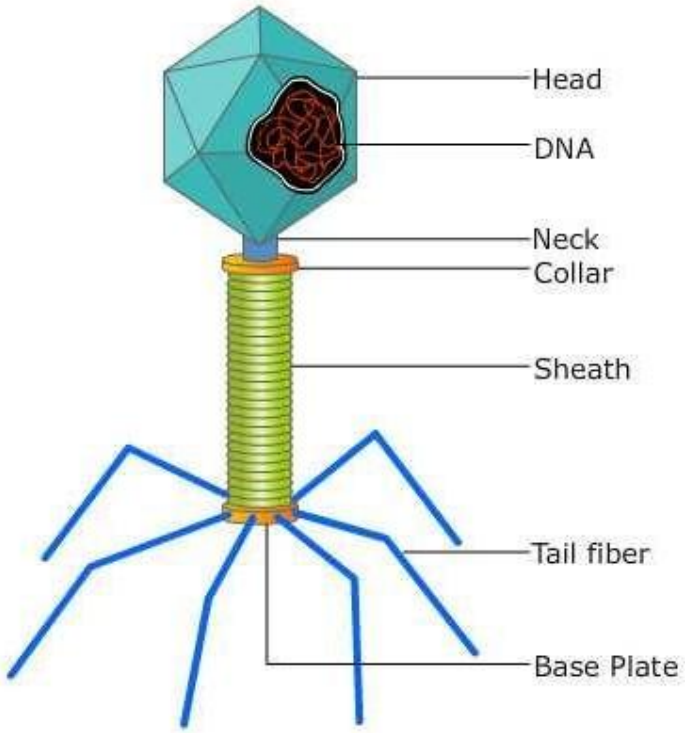


Fig:- A typical phage

The plaque assay is originally a virological assay employed to count and measure the infectivity level of the bacteriophages. But later, it was applied to measure and count the mammalian viruses as well. This assay is the most widely used technique for the isolation of virus and its purification, and to optimize the viral titers. The basis of plaque assay is to measure the ability of a single infectious virus to form a "plaque" on a concurrent monolayer culture cells. A plaque is developed as a part of infection of one cell by a single virus particle that is followed by the replication of that virus, and finally, the death of the cell. The newly replicated virus particles will later infect and then kill surrounding cells.

For this technique, we will be provided with a virulent phage stock and a susceptible host cell culture. 10-fold dilutions of the phage stock are prepared. The procedure requires the use of a Double-Layer Agar (DLA) technique also known as double agar overlay method, in which the hard agar serves as a base layer (to form gel), and a mixture of few phage particles (diluted stock) and a very large number of host cells in a soft agar forms the upper overlay. When the plates are incubated,

susceptible Escheria coli cells multiply rapidly and produce a lawn of confluent growth on the medium. When one phage particle adsorbs to a susceptible cell, penetrates the cell, replicates and release new phage particles which infect other bacteria in the vicinity of the initial host cell. The growth or spread of the new viruses is then restricted or limited to the neighbouring cells by the gel. This cycle is repeated until large numbers of bacteria have been destroyed. The destroyed cells produce single circular, non turbid areas called plaques in the bacterial lawn, where there is no growth of bacteria because the phage progeny originating from single virus particles have multiplied sufficiently to kill bacteria over an easily visible area. Eventually the plaque becomes too large to be visible to our naked eye. Each plaque represents the lysis of a phage-infected bacterial culture and can be designated as a plaque-forming unit (PFU) and is used to quantitate the number of infective phage particles in the culture. Dyes that stain the living cells are frequently used to enhance the contrast between the plaques and the living cells. Therefore the dead cells in the plaque will appear as unstained against the colored background. Only viruses that have the ability to cause visible damage of cells can be assayed using this way.

The number of phage particles contained in the original stock phage culture is determined by counting the number of plaques formed on the seeded agar plate and multiplying this by the dilution factor. For a valid phage count, the number of plaques per plate should not exceed 300 nor be less than 30. Plates showing greater than 300 PFUs are too numerous to count (TNTC); plates showing fewer than 30 PFUs are too few to count (TFTC).

The main difference between Plaque Assay and Colony-Counting is that, to count bacteria, we spread about 100 - 400 bacteria over the surface of the agar and incubate the plate. To count phage, we spread 100 - 400 phage particles mixed with a very large number of host bacterial cells over the surface of the agar and incubate the plate. In colony-counting, we directly observe the accumulation of large numbers of bacteria, each pile being a colony. In plaque formation, we directly observe the absence of bacteria against a background of dense bacterial growth, each zone of destruction being a phage plaque (hole) in the lawn of bacteria. Each hole represents one phage in the sample applied to the plate.

RESULTS

PROTEIN ESTIMATION

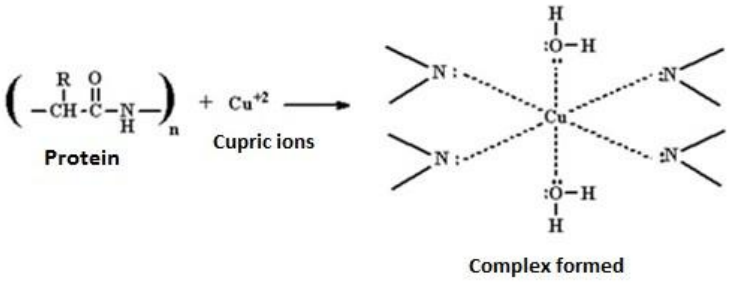
AIM:

To estimate the protein concentration using BSA as standard

Theory :

Lowry's assay for total protein is one of the most commonly performed colorimetric assays. This procedure is sensitive because it employs two colour forming reactions. It uses the Biuret reactions in which Cu2+ in presence of a base reacts with a peptide bond of protein under alkaline conditions resulting in reduction of cupric ions (Cu2+) to cuprous ions (Cu+), and Lowry's reaction in which the Folin Ciocaltaeu reagent which contains phosphomolybdic complex which is a mixture of sodium tungstate, sodium molybdate and phosphate, along with copper sulphate solution and the protein, a blue purple colour is produced which can be assessed by measuring the absorbance at 650-700nm.

The blue purple color is formed due to the reduction of phosphomolybdotungstate to hetero-polymolybdenum blue by the copper catalysed oxidation of aromatic amino acids tryptophan and tyrosine. Thus the color intensity depends on the amount of these aromatic amino acids present and will thus vary for different proteins.



The measurement of protein with Folin's reagent has certain advantages. Firstly, it is a sensitive assay which requires no digestion. Secondly, It is 10 or 20 times more sensitive than measurement of the ultraviolet absorption at 280 nm and is much more specific and much less liable to disturbance by turbidities, thirdly, it is several fold more sensitive than the ninhydrin reaction and is simpler, as well as much easier to adapt for small scale analyses. Also it is 100 times more sensitive than the biuret reaction.

There are disadvantages also for the Folin's reaction, like the amount of color varies with different proteins, it is lessconstant than the biuret reaction, but more constant than the absorption at 280 nm. The color is not always proportional to concentration.Inspite of all these concerns, folin's method can be used in measurement of protein during enzyme fractionations, mixed tissue proteins, measurement of very small absolute amounts of protein, or highly diluted protein and analyses of large numbers of similar protein samples.

Most protein estimation techniques use Bovine Serum Albumin (BSA) as a standard protein, because of its easy availability and low cost with improved purity. Constructing a protein standard curve is of prime importance in studying the activity of an enzyme as this analysis relies on accurate quantitation of protein concentration.

RESULTS

LAC OPERON

AIM:

To induce lac operon

PRINCIPLE

There are three proteins: LacZ-β-galactosidase, LacY-the lactose permease, andLacA-lactose transacetylase are encoded by the lactose operon. The LacI repressor gene thatclose to lac operon represses the operon and the presence of an inducer will inactivate therepressor. Although lactose is not an inducer, but it can indirectly induces the lac operon aftera small amount has been isomerized to allolactose. This is an isomer of lactose, generated in a side reaction by the low basal levels of β -galactosidase which are found before induction.IPTG (isopropyl-thio-β-D-galactosidase) is often used as inducer. IPTG is not metabolizedand is of no use to cell as it is a gratuitous inducer.The use of less favoured substrates such as lactose is prevented when favoured carbonsource such as glucose presents. Catabolite repression depends largely on the intracellularlevel of cyclic AMP. Catabolite Activator Protein (CAP) binds with cyclic AMP and thecomplex is known as cyclic AMP receptor protein (CRP). The level of CRP is constant.Transcription of catabolite sensitive operons such as the *lac*operon requires binding of CRP-cAMP complex to the promoter region. Thus, RNA polymerase is able to bind to andtranscribe the operon. The regulation of cyclic AMP levels is due mostly to changes inactivity of adenylate cyclase which catalyses the conversion of ATP to cyclic AMP plusinorganic pyrophosphate. The presence of glucose will cause a drop in the activity ofadenylate cyclase and hence there will be a drop in cyclic AMP levels. Glucose must betransported for this to happen, but it does not need to be broken down and metabolized. Non-metabolizable analogs of glucose, such as 2-deoxyglucose, cannot be degraded but can betransported and also cause catabolite repression.

Materials:

The materials involved in this experiment are

E.coli

(Lac

+) culture, 0.002M lactose, 0.002Mglucose, 0.002M IPTG, 0.002M PBG, 1.0mg/ml sodium deoxycholate, Toluene, 0.01MONPG, 0.01M sodium phosphate buffer(pH7), and 2M sodium carbonate. The apparatus usedare 37 C water bath shaker, sterile capped test tubes, ice bath, spectrophotometer, cuvettesand micropipettes.

Procedures:

A.Cell Growth Induction of Enzyme 4ml of starved *E.coli* cells (1x107 cell/ml) was added into four large sizes (18mm) labeled testtubes respectively. Then, 0.2ml of 0.002M inducer, which are LAC, GLU, IPTG and dH20were added in four labelled tubes respectively. Then each tube was closed with a cap, andthey were placed in a 37C water bath and shaken for 30 minutes.C.

Assay for Enzyme1.

Disruption of selective permeabilityEach 4.2ml of an induced *E.coli* culture was added with 50µL of sodiumdeoxycholate(1.0mg/ml) and 50µL of toluene. Then they were capped and placed in a 37 Cwater bath and shaken for 10 minutes. They were kept in ice bucket until needed for assays.2.

Enzyme assays

2.0 ml of 0.1M sodium phosphate buffer (pH7), 2.0ml lyse *E.coli*

preparation and 0.2ml of0.01M ONPG were added into each small labelled culture tube. Then these tubes wereincubated for 15 minutes at 37 C without shaking. The reaction was stopped by adding 0.5mlof 2M sodium carbonate.

RESULTS

PAPER CHROMATOGRAPHY

AIM

Experiment to separate amino acids in a mixture by paper strip chromatography

Principle:

It is based on the fact that paper chromatography separates compounds on the basis of their different rates of migration of filter paper (cellulose). The rates of migration depend upon the solvent which is flowing up or down the paper and on the relative adsorption which holds the molecules more or less tightly to the paper.

If the solvent flows towards upper side on the paper it is called ascending chromatogram, and if it flows towards lower side then it is known as descending chromatogram.

Requirements:

Chromatography paper, test tubes (2), capillary tubes (3), coupling jar (1), distilled water, amino acids (glycine and aspartic acid), phenol and ninhydrin.

Method:

Take three strips of chromatography paper of equal size (12 cm in length and 1.5 cm in width).

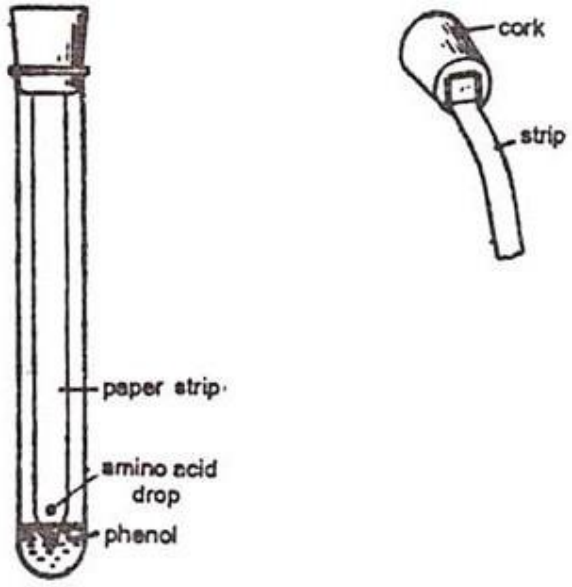
2. Draw a fine line with a lead pencil, parallel to and 1.5 cm from one edge of the paper. This line will indicate the bottom of your chromatogram.

3. On this line draw a circle on each strip, about 1.5 cm from one edge. These circles will indicate the position of your samples.

4. Put a drop of glycine in the circle of one strip, aspartic acid on the second and that of a mixture of both amino acids on the 3rd strip with the help of separate capillary tubes. (Note : Avoid excess handling of the chromatography paper, since your hands may contaminate it with amino acids. Touch it only at the edges).

5. Take 5 ml of 80% phenol in three large test tubes.

6. Fix each strip in the cork as shown in Fig.



7. Insert one strip in each test tube and see carefully that lower edge of the strip touches the phenol. (Note carefully that the amino acid spot should not he touched by the phenol and the paper should not touch the wall of the test tubes).

8. Keep these test tubes in the stand and wait for 20 to 30 minutes or more until the solvent has risen within 0.5 cm from the top of the paper.

9.Remove the strips from the tube and let it dry in an oven at about 100°C for 3-5 minutes.

10.Dry strips should be sprayed by 0.1 % ninhydrin- acetone reagent and set it aside to dry.

11. Keep it again in the oven for 2 to 3 minutes. Do not overheat the paper.

12. Remove the paper and immediately outline with pencil the spots that you see. Amino acids will appear as purple spots on the filter paper.

Rf value can be measured by the following formula:

$$Rf = \frac{\text{Distance travelled by a given spot}}{\text{Distance travelled by solvent front}}$$

Distance travelled by a given spot is measured from the center of the spot.

RESULTS

Thin Layer Chromatography

AIM

To identidy components using Thin Layer Chromatography

PRINCIPLE

TLC is a type of planar chromatography.

- It is routinely used by researchers in the field of phyto-chemicals, biochemistry, and so forth, to identify the components in a compound mixture, like alkaloids, phospholipids, and amino acids.
- It is a semi quantitative method consisting of analysis.
- High performance thin layer chromatography (HPTLC) is the more sophisticated or more precise quantitative version.

Principle

Similar to other chromatographic methods, thin layer chromatography is also based on the principle of separation.

1. The separation depends on the relative affinity of compounds towards stationary and the mobile phase.
2. The compounds under the influence of the mobile phase (driven by capillary action) travel over the surface of the stationary phase. During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, separation of components in the mixture is achieved.
3. Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or character are identified by means of suitable detection techniques.

System Components

TLC system components consists of

1. **TLC plates,** preferably ready made with a stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.

2. **TLC chamber.** This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.
3. **Mobile phase.** This comprises of a solvent or solvent mixture The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.
4. **A filter paper.** This is moistened in the mobile phase, to be placed inside the chamber. This helps develop a uniform rise in a mobile phase over the length of the stationary phase.

Procedure

The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are preferred.

1. With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
2. Then, samples solutions are applied on the spots marked on the line in equal distances.
3. The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect this way).
4. Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
5. The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent — as shown in the picture) for development.
6. Allow sufficient time for the development of spots. Then remove the plates and allow them to dry. The sample spots can now be seen in a suitable UV light chamber, or any other methods as recommended for the said sample.

RESULTS

Semester – I

19MBP112 **BASIC PRACTICAL– II** **4H –2C**

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

End Semester Exam: 9 Hours

COURSE OBJECTIVES

The contents of this course would enable the student

- To acquire practical knowledge on the different molecular mechanism of gene transfer, mutations and separation of nucleicacids.
- To understand the molecular mechanism of compound separation and isolation using chromatography techniques.

COURSE OUTCOME

1. A student undertaking this course will be learning the principles behind the molecular techniques which would enable him to work in competent molecular biology based laboratories.

EXPERIMENTS

1. Spontaneous Mutation – gradient platetechnique
2. Induced Mutagenesis-chemical and physical -UV
3. Replica plating technique.
4. Transformation in Bacteria
5. Bacterial Conjugation
6. Induction of Lac operon
7. Measurement of growth-one step growth curve using a T evenphage
8. Titration of phages(T4)
9. Nuclear staining for nucleic acid identification.
10. Protein Purification using microfiltration.
11. Analysis of amino acid by Paper chromatography
12. Analysis of amino acid by Thin layer chromatography
13. Purification of proteins by column chromatography
14. Analysis of amino acid by HPLC –Demonstration

Suggested Reading:

1. Benson, H.J. (1998). *Microbiological Application (Laboratory Manual in General Microbiology)*, (7thed.). WCB.
2. Palanivelu, P. (2004). *Analytical Biochemistry and Separation Techniques*, (3rded.). Twenty First Century Publication, Madurai.
3. Chakraborthy, P., and Pal, N.K., (2008). *Manual of Practical Microbiology and Parasitology*, New Central Book Agency (P) Ltd,India.
4. Gaud, R.S., and Gupta, G.D., (1999). *Practical Microbiology*, 1st Ed.). Nirali Prakashan, Pune.