KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: I B.Sc MB COURSE NAME: Microbial Genetics Practical

COURSE CODE: 18MBU213 SYLLABUS BATCH: 2018-2021

Semester – II

18MBU213 **MICROBIAL GENETICS- PRACTICAL** **(4H –2C)**

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

End Semester Exam: 6Hours

COURSE OBJECTIVES

- To focus on the basic principles of Genetics incorporating the concepts of classical, molecular and population genetics.
- Compilation is required for recent advances in genetic principles for strong foundation in Biotechnology.

COURSE OUTCOME (CO'S)

- Students undertaking this practical shall be able to describe the steps involved in the basic Microbial Genetics
- Effectively understand the implication of mutation and its characteristics.
- Further, the experiments would allow students to recall and relate the information gained from Microbial Genetics theory paper.

EXPERIMENTS

1. Preparation of Master and Replica Plates.
2. Study the effect of chemical (HNO2) and physical (UV) mutagens on bacterial cells.
3. Study survival curve of bacteria after exposure to ultraviolet (UV) light.
4. Isolation of Plasmid DNA from *E.coli.*
5. Estimation of DNA
6. Study different conformations of plasmid DNA through Agaraose gel electrophoresis.
7. Demonstration of Bacterial Conjugation.
8. Demonstration of bacterial transformation and transduction.
9. Demonstration of AMES test.

SUGGESTED READINGS

1. Klug, W.S., Cummings, M.R., Spencer, C., Palladino, M. (2011). Concepts of Genetics, 10th edition, BenjaminCummings
2. Krebs, J., Goldstein, E., Kilpatrick, S. (2013). Lewin's Essential Genes, 3rd edition, Jones and Bartlett Learning.
3. Pierce, B.A. (2011) Genetics: A Conceptual Approach, 4th edition, Macmillan Higher Education Learning.
4. Watson, J.D., Baker, T.A., Bell, S.P., et al. (2008) Molecular Biology of the Gene, 6th edition, BenjaminCummings.
5. Gardner, E.J., Simmons, M.J., Snustad, D.P. (2008). Principles of Genetics. 8th edition,Wiley-India.
6. Sambrook, J., and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual. 4thedition, Cold Spring Harbour Laboratorypress.
7. Maloy, S.R., Cronan, J.E.,andFriefelder, D. (2004) Microbial Genetics 2nd edition, Jones and Barlett Publishers.
8. Peter J. Russell, i Genetics – A molecular approach, 7th edition, 2010. Pearson Benjamin Cummings Publishers, Boston,USA.
9. David Freifelder, Microbial Genetics. Narosa Publishing House, 10th edition, 2004. New Delhi, India.

Experiment No: 1

PREPARATION OF MASTER AND REPLICA PLATES

Aim: To isolate antibiotic resistant bacteria using replica plating technique.

Principle: This technique permits comparison of cell growth on a primary plate to secondary plates, generating a means to screen cells for a selectable phenotype. First a primary, or master, plate is inoculated with cells either by spread-plating a dilution that produces single colonies or by transferring them to a plate in a spatial pattern specified by grid markings. Secondary plates containing media with growth inhibitors or media that lacks a particular nutrient are inoculated with cells from colonies on the primary plate. The spatial pattern of colonies is reproduced first by pressing a piece of velvet to the primary plate. Bacterial cells adhere to the velvet because they have greater affinity for the velvet than for the agar. The imprint of cells on the velvet then is transferred to multiple secondary plates with cell growth reflecting the same colony pattern as that of the primary plate. In other words, it is like having a rubber stamp, replicating the growth pattern from one plate to another. This technique is advantageous because it allows a relatively large number of colonies to be screened simultaneously for many phenotypes in a single experiment

Materials Required: Petriplates, conical flask, test tubes, test tube stand, Bunsen burner, inoculation loop, Nutrient agar media, velveteen cloth, wooden block.

Procedure

Prepare primary plate: Label around the edge of the bottom (not the lid) of an agar plate with at least your name, the date, and the type of growth medium.

1. Mark off a grid on the bottom of the plate with at least two equally spaced vertical lines and at least two equally spaced horizontal lines. Number the resulting squares.
2. Use a pre-sterilized toothpick to inoculate each square with a cell sample. For each sample, dab the center of the square. Do not cover the entire square with cells or else the sample will overgrow when incubated and contaminate surrounding squares.

   Each square will be inoculated with a different sample, derived either from broth cultures or colonies on another plate.
3. Invert and incubate the primary plate, which will be used to inoculate various secondary media.

Inoculate secondary plates: Stack the primary plate and all the secondary plates. With a marker, make an orientation mark on the side of the plates. Make sure the mark is on the bottom side of each plate, not the lid.

4. Obtain a sterile velveteen cloth and place it on the cylindrical block. Lock the velveteen cloth in place with the holder. Note the orientation mark on the block.

- The block should be the same size as the bottom of a Petri dish (10.2 cm diameter). It should come with a locking ring that clamps the velveteen cloth to the block while replica plating.
- The velveteen cloth (15.2 x 15.2 cm square) must be pre-sterilized. Stack 10 or 12 clean squares then wrap them in aluminum foil then place them in the autoclave at 121 °C on the dry cycle (gravity setting) for 30 minutes. Before using them in a replica plating experiment, make sure they are completely dry by placing them in a warm oven for several hours. Note that velveteen squares may need to be de- linted with masking tape prior to sterilization.

  Velveteen squares may be re-used. After used velveteen squares have been decontaminated in the autoclave, they should be rinsed in plain water and re- sterilized as described above.

The cylindrical block and clamp should be disinfected between uses with a brief rinse in 70% (v/v) ethanol or 10% chlorine bleach.

5. Remove the lid and invert the primary plate. Line up the orientation mark on the plate with the mark on the block. Lower the plate so that the surface of the agar is in contact with the velveteen cloth on the cylindrical block. Lightly but evenly press down with your finger tips on the back of the primary plate and then carefully lift the primary plate away from the block. Replace the lid on the plate.

   The velvet impression of cells from the primary plate can be used to inoculate approximately 7-8 secondary plates before an impression with a new velvet needs to be made.

6. Repeat step #7 with each of the secondary plates.

   As a positive control, the last plate in the series should be an agar medium in which all strains tested should grow. This way you can confirm that cells were transferred to all secondary plates in the series. Otherwise, lack of growth on a particular test medium may be ascribed to insufficient cell transfer rather than a phenotype of the strain.

   To avoid false positives, the secondary plates should be ordered from least to most favorable substrate. Otherwise, nutrients could be transferred between plates allowing cells to grow on an unfavorable medium.

7. Invert the plates and incubate.

   Note: When inspecting the secondary plates for growth, be sure to distinguish between growth and an imprint. The latter is a negative result.

Result:

Experiment No: 2A

STUDY THE EFFECT OF CHEMICAL (HNO_2) MUTAGENS ON BACTERIAL CELLS

Aim: To study the effect of chemical mutagens on the given bacterial culture.

Principle: Chemical mutagens are defined as the compounds that cause induced mutation. They vary in their potency since this term reflects their ability to enter the cell. The reactivity with DNA type of chemical change they introduce into the ND will be corrected by a repair system.

Nitrous acid (HNO_2) a potent chemical mutagen groups are converted to ketogroups and thus cystine residues for example will be converted into uracil. Uractil will be capable of pairing with adeine. Thus causing a change from a C-G pair to T-A similarly deamination creates the base hypoxanthaines which will base pair with cystosine.

Materials Required: Mid log culture of E. coli, 0.1 M sodium acetate buffer, nutrient agar plates, nitrous acid, things necessary for replica plating techniques.

Procedure:

- 10 ml of mid log phase culture of E. coli was taken and washed with 0.1 M sodium acetate buffer.
- The washes wells were resuspended in 1 ml of 0.05M fresh nitrous acid solution and incubated at room temperature at different time intervals (between 10 to 20 minutes).
- After treating with nitrous acid, the suspension was centrifuged and cells were collected. The the cells were collected and washed with sodium acetate buffer. Following this the cells were serially diluted.
- The diluted cell suspension was then plated on nutrient agar by spread plate technique and incubated overnight at 37°C.
- The colonies formed on the nutrient agar plates after incubations was transferred to animal agar medium by replica plating technique and incubated overnight at 37 °C to monitor the mutagenesis.

Result:

Experimental: 2B

STUDY THE EFFECT OF PHYSICAL (UV) MUTAGENS ON BACTERIAL CELLS

Aim: To isolate the auxotrophic mutant by UV induced mutagenesis that require one or more growth factors required by wild type or prototroph can synthesize.

Principle: Auxotrophs are produced in the laboratory by treating prototrophs with mutagenic agents such as UV radiation or nitosoguanidine or nitomycine C. In 1952, Joshua Leaderberg and E. M . Linderberg devised a technique called replica plating for detaching and isolating mutant strain of microorganism.This technique is used to detect auxotrophic mutants. The principle application of this technique is that it permits the simultaneous transfer of large number of colonies from one plating medium to another in a single operation by use of cotton, velveteen cloth.

The mutants are produced by treating a culture with a mutagen. This culture containing both with type ad auxotroph is plated on complete medium. After the colonies have developed the bacteria are transferred by replica plating technique. The sterile velveteen cloth carrier is passed on the surface of the plate to pickup bacteria from each colony and is passed on the surface of the plate to as well as minimal medium. The plates are incubated for the growth of colonies that lack compound which is present in the complete medium that lacks. The plates are incubated for the growth of colonies and both the replica plates compared to identify auxotrophic mutant colonies.

Material Required: L.B. broth, 24 h culture of *E. coli*, Nutrient agar plates, minimal agar plates, cotton velveteen cloth, 95 % alcohol, L rod, sterilized 1 ml pipette, UV lamp (15 watts), phosphate buffered saline, 0.1 $MgSO_4$, Mac Farland‟s standard tube.

Procedure:

- A mid log phase broth culture of *E. coli* was prepared and it was centrifuged at 5000 rpm for 5-10 min and the supernatant was discarded.
- The pellet was dissolved in equal volume of 0.1M Mangnesium sulfate. The turbidity was adjusted to half 0.5 MacFarlan's tubes.
- 1 ml from this sample was taken in sterilized pertriplates and exposed to UV at different time intervals (5, 10, 15, 20, 25 and 30 min). During UV exposure the petriplates containing the culture was placed at a distance of 30 cm from UV lamp.
- 0.5 ml of UV exposed culture was inoculated in 4.5 ml of L.B broth and incubated over night at 37 °C dilution under dark conditions.
- After incubation 0.5 ml of culture was transferred to 4.5 ml of sterile saline to give 10^{-1} dilution serial dilution was carried out up to 10^{-5} dilution.
- 0.1 ml from each dilution was seeded on nutrient agar by spread plate techniques and incubated at 37 °C for 24 hours.
- A plate with even distribution of colonies (approximately 5-100 colonies) was considered as master plates.
- Using this, replica plating technique was carried out into minimal agar plate and incubated at 37°C for 24 hours. The plates were checked for auxotrophs mutants by comparing replica plates with master – plates.

Result:

Experiment No: 3

STUDYING THE SURVIVAL CURVE OF BACTERIA AFTER EXPOSURE TO ULTRA VIOLET (UV) LIGHT

Aim: To study the survival curve of bacteria after exposure to ultra violet (UV) light by direct plate irradiation medium.

Principle: In their natural environment bacteria most often live in nutrient limiting conditions and are exposed to various stresses such as UV radiations. As a result they have enveloped a number of protective mechanism, such as the resonse, to prevent DNA damage. Bacteria in exponential, stationary or death phase may be prone to UV damage to different degrees depending on the fidelity and the accuracy, the DNA repair mechanism. The survival of E. coli in response to UV irradiation during log and stationary phase have been assesses by different researchers. In stationary phase, E. coli exhibits increased resistance to UV exposure compared to cells in exponential phase. This indirectly suggests either an up regulation of DNA repair mechanisms during stationary phase or a decreased rate of DNA replication which allows more time for DNA repair.

Materials Required: E. coli standard culture, LB broth and agar plates, UV chamber, black box, digital timer to note down the time interval and other basic laboratory items.

Procedure:

- E. coli were grown by inoculating in LB broth (20 ml final volume) overnight and allowed to reach an O.D of 0.7 to 0.8 at 660 nm with a cell density of $1x10^8$ cells/ml. Once the sub-culture reached the desired optical density, a series of dilution of the sub- culture was prepared (10^{-1} to 10^{-6}).
- These dilutions were then used to make final plates for the ultraviolet irradiation by spread plating 0.1 ml of the diluted sub-cultures onto LB agar plate.
- After plating, the plates were kept in a black box (plastic cooler box) before and after irradiation. The majority of the experiments were done in the dark to avoid photo reactivation.
- UV irradiation was done in a custom built UV chamber with a glass front. The UV lamp could be adjusted vertically to a desired height up to 1.2 meter.
- One plate from each treatment sets (10^{-2} to 10^{-4} dilution) were randomly placed in the centre of the chamber (lids of the treatment plates were removed before placing the plates) and irradiated for the set times (5,10, 15 sec) while 10^{-5} and 10^{-6} dilutions were the control.
- After irradiation, the lids were replaced and the plates were immediately placed into the black box. all the plates were incubated in the 37°C incubator for 24 to 36 hours.

Result:

Experiment No: 4

ISOLATION OF PLASMID DNA FROM *E. COLI*

Aim: To isolate the plasmid DNA from *E. coli*.

Principle: When bacteria are lysed under alkaline conditions both DNA and proteins are precipitated. After the addition of acetate-containing neutralization buffer the large and less supercoiled chromosomal DNA and proteins precipitate, but the small bacterial DNA plasmids can renature and stay in solution. In prokaryotes, plasmid is double stranded, circular, and is found in the cytoplasm. The cell membranes must be disrupted in order to release the plasmid in the extraction buffer. Solution 1 contains glucose, Tris, and EDTA. Glucose provides osmotic shock leading to the disruption of cell membrane, Tris is a buffering agent used to maintain a constant pH8. Plasmid can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for most nucleases. Solution II contains NaOH and SDS and this alkaline solution is used to disrupt the cell membrane and NaOH also denatures the DNA into single strands. Solution III contains acetic acid to neutralize the pH and potassium acetate to precipitate the chromosomal DNA, proteins, along with the cellular debris. Phenol /chloroform is used to denature and separate proteins from plasmid. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. Once the plasmid DNA is released, it must be precipitated in alcohol. The plasmid DNA in the aqueous phase is precipitated with cold (0^oC) ethanol or isopropanol. The precipitate is usually redissolved in buffer and treated with phenol or organic solvent to remove the last traces of protein, followed by re-precipitation with cold ethanol.

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

Preparation of solutions

1. TE Buffer (pH 8.0): 10 mm Tris HCl (pH 8.0) 1 mm EDTA (pH 8.0)
2. Solution I: Lysis solution (Prepare for 5 ml)
   50mM Tris HCl (pipette out 0.25 ml of 1M Tris HCl in a beakerP. 20mM EDTA
   Pipetter out 0.20 ml of 0.5M EDTA into the beaker.
3. Solution II: Denaturing solution (Prepare for 5 ml) 15% Glucose
4. Solution III: Neutralizing solution (Prepare for 5ml)
   0.2 N NaOH 1% SDS
   Dissolve the above solution in 3 ml of distilled water
   Phenol – Chloroform Mixture: Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer and store it in dark.
5. Isopropanol

Procedure:

- Take 2 ml overnight culture and harvest cells by centrifugation for 5 min. Discard the supernatant carefully.
- Add 100 µl of solution I to the cell pellet and resuspend the cells by gentle mixing. ¾ Incubate the above mixture at room temperature for 5 min.
- Add 200 µl of solution II to the mixture and mix by inverting the tubes for 5 min. ¾ Incubate for 5-10 min at room temperature.
- Add 500µl of ice cold solution III to the mixture and mix by inverting the tube. Incubate on ice for 10 min.
- Centrifuge at 10,000 rpm for 5 min. Transfer the supernatant into fresh tube.
- Add 400 µl of phenol-chloroform mixture to the contents, mix well by inverting and incubate them at room temperature for 5 min.
- Centrifuge at 10000 rpm for 5 min.
- Collect the supernatant (viscous) using cut tips and transfer to a fresh tube.
- Add 0.8 ml of isopropanol and mix gently by inversion. Incubate for 30 min at room temperature.
- Centrifuge the contents at 10,000 rpm for 10 min.

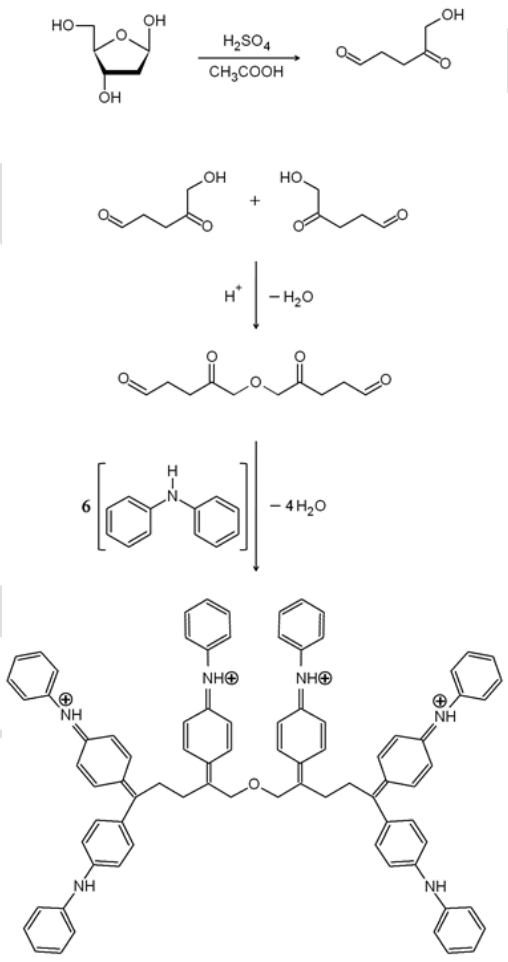
Result:

Experiment No: 5

ESTIMATION OF DNA

Aim: To determine the concentration of a given DNA sample using diphenylamine method

Principle: The principle underlying estimation of DNA using diphenylamine is the reaction of diphenylamine with deoxyribose sugar producing blue-coloured complex. The DNA sample is boiled under extremely acidic conditions; this causes depurination of the DNA followed by dehydration of deoxyribose sugar into a highly reactive ω-hydroxylevulinylaldehyde. The reaction is not specific for DNA and is given by 2-deoxypentoses, in general. The *ω*-hydroxylevulinylaldehyde, under acidic conditions, reacts with diphenylamine to produce a blue-coloured complex that absorbs at 595 nm. The mechanism of reaction of deoxyribose sugar with diphenylamine is shown in figure 6.1. As the sugar linked to only purine residues participates in the reaction, the readout is only from 50% of the total number of nucleotides. As this holds true for both the known standard and the given unknown sample, the concentration of the unknown sample can be directly calculated from the standard graph.



Materials:

Equipments:

1. A UV/Visible spectrophotometer
2. Vortex mixer
3. Weighing balance
4. Water bath

Reagents:

1. Diphenylamine reagent
2. Calf thymus DNA
3. Glacial acetic acid
4. Concentrated sulfuric acid

Glassware and plasticware:

1. Pipettes
2. Pipette tips
3. A 5 ml glass pipette
4. Pipette aid
5. A 100 ml measuring cylinder
6. A 250 ml amber coloured glass bottle
7. Test tubes
8. Caps for glass tubes
9. Distilled water
10. Quartz or glass cuvettes

Preparation of reagents:

Diphenylamine (DPA) reagent :

1. Weigh 1g of diphenylamine and transfer it into a 250 ml amber coloured glass bottle.
2. Add 100 ml glacial acetic acid and shake well to achieve complete dissolution.
3. Add 2.5 ml of concentrated sulfuric acid.
4. Store the reagent in dark at 2 – 8°C.

Calf thymus DNA (100 µg/ml)

Prepare 100 µg/ml of calf thymus DNA solution in distilled water.

Procedure:

1. As the concentration of the unknown DNA sample can be anything, the assay will be performed with a range of dilutions (1, 1:10, 1:100, and 1:1000). Prepare 1 ml of each of the dilutions.
2. Take 15 test tubes and label them from 1 to 15.
3. Pipette out 100 µl , 200 µl , 300 µl , …….., 1000 µl calf thymus DNA standard in the glass tubes labeled 1 – 10; leave blank the tube no. 11.
4. Add distilled water to make the final volume 1 ml in each of the tubes (including blank).
5. Take 1 ml of each of the unknown DNA dilutions in the tubes labeled 12 – 15.
6. Add 3 ml of DPA reagent in each of the 15 tubes and mix well by vortexing.
7. Cover each of the tubes with the caps and place them in boiling water bath for 10 minutes.
8. Take out all the tubes from water bath and allow them to return to room temperature.

9. Measure the absorbance of tubes 1 – 10 and 12 – 15 at 595 nm in the quartz/glass cuvette against the reagent blank (tube 11).
10. Record the readings in the suggested observation table below:

Observation Table:

Tube No.	Volume (μl)	Mass (μg)	Distilled water (μl)	Diphenylamine reagent (ml)	A_{595}
Standard DNA					
1	100	10	900	3	
2	200	20	800	3	
3	300	30	700	3	
4	400	40	600	3	
5	500	50	500	3	
6	600	60	400	3	
7	700	70	300	3	
8	800	80	200	3	
9	900	90	100	3	
10	1000	100	0	3	
11	Blank (0)	0	1000	3	
Unknown sample					
12	1000 (1:1000 dil.)	Unknown	0	3	
13	1000 (1:100 dil.)	Unknown	0	3	
14	1000 (1:10 dil.)	Unknown	0	3	
15	1000 (Undiluted)	Unknown	0	3	

Calculations:

Plot the absorbance values obtained for tubes 1 – 10 against the amount of standard DNA added to these tubes.

1. Fit the data points using linear regression [with intercept (0,0)].
2. Determine the slope $\left(\frac{\Delta Absorbance}{\Delta DNA\ amount}\right)$ of the regression line.
3. The concentration of the unknown DNA is given by:

$$DNA\ concentration\ (\mu g/ml) = \frac{Absorbance \times Dilution\ factor}{slope\left(\frac{1}{\mu g}\right) \times Volume\ of\ diluted\ DNA\ sample\ used\ for\ the\ assay\ (ml)}$$

Notes:

1. It is recommended to use freshly prepared diphenylamine reagent. The solution, however, can be prepared in advance and stored in dark at 2 – 8 °C.
2. Prepare all the samples in triplicate and the tubes should be labeled properly. To follow the numbering used in the procedure and table given above, the tubes can be labeled as *a*, *b*,and *c* ; for example the three samples for tube 1 can be labeled as 1*a*, 1*b*, and 1*c*. The calculations can then be performed taking the average absorbance of the three tubes.

3. Concentrated sulfuric acid should be carefully pipetted out using a 5 *ml* glass pipette with the help of a pipette aid in a chemical fume hood.
4. The dilution(s) of unknown sample that show absorbance between 0.05 – 1.0 should be used for calculations.
5. UV/Visible spectrophotometer should be switched on at least 30 *min* before use.

Result:

Experiment No: 6

STUDYING THE CONFIRMATION OF PLASMID DNA THROUGH AGAROSE GEL ELECTROPHORESIS

Aim: To separate the plasmid DNA fragment based on their Molecular Weight.

Principle: DNA with different conformation that has not been out with a restriction enzyme will migrate with different speeds. Nicked or open circular DNA will move slowly than linear and supercoiled DNA will move slowly than linear and super coiled DNA (slowest to fastest : nicked to open circular, linear or super coiled plasmid).

Agarose gel electrophoresis is the easiest and most popular way of separating and analyzing DNA. Here DNA molecules ar separated on the basis of charge by applying an electric field to the electrophoretic apparatus. Shorter moleculaes migrate more easily and move faster than longer process is called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of ethidium bromide.

Agarose is a polysaccharide obtained from the red algae Porphyra umbilicalis. Agarose makes an inert matrix. Most agarose gels are made between 0.7% and 2 % of agarose.

Agarose concentration versus optical range of DNA size

Agarose (%) Concentration	0.3	0.6	0.7	0.9	1.2	1.5	2.0
DNA (kb)	5~60	1~20	0.8~10	0.5~7	0.9~6.0	0.2~3.0	0.1~2

Materials required

- DNA sample and DNA ladders (DNA‟s of known size)/ An electrophoresis chamber and power supply.
- Gel casting trays - which are available in a variety of sizes and composed of UV- transparent plastic.
- Sample combs – around which molten agarose is poured to form sample wells in the gel. Electrophoresis buffer – Usually tris acetate or Tris borate buffer is used.
- Loading buffer – which contains dense (eg.glycerol) to allow the sample to ease into the sample wells and two tracking dyes (eg. Bromophophenol blue and Xyelne cyanol) which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
- Ethidium Bromide – A fluorescent dye used for staining nucleic acid.
- Trans illumination – can ultraviolet light box which is used to visualize ethidium bromide stained DNA in gels.

Buffer Composition: Running buffer – 0.5X TBE, 5.4g trisbase, 2.75 g of boric acid, 2 ml of 0.5 M EDTA in 1 litre water solution (pH8.0). The agarose solutions should be the same with the running buffer.

Procedure

- Prepare the suitable concentration of agarose solution and microwave it until boiling. Add 0.5 µg/ml of ethidium

bromide when it's cooled down to 55°C. Choose the suitable gel tank and pour the fluid agarose gel and then insert the comb.

- After a complete solidificatinon put the agarose gel in the electrophoresis tank. Pour enough running buffer into the electrophoresis tank. (The surface should be higher than the top of the gel and not overflow).
- Mix the sample with leading buffer sufficiently and load them into the sample lane together with the marker (Usually marker in the first well).
- Set an appropriate voltage and run the electrophoresis.
- After approximately 35 min (80 V) 25 min (100 V) put the agarose gel in an UV transilluminator and record the results.

Result

Experiment No: 7

DEMONSTRATION OF BACTERIAL CONJUGATION

Aim: To study the process of bacterial conjugation through transfer of genes coding for antibiotic resistance.

Introduction: Bacteria possess several methods for gene transfer for transmission of genes between individual cells. These mechanisms not only generate new gene assortments, they also help to move genes throughout populations and from species to species. The methods include transformation, transduction and conjugation. These methods occur by lateral gene transfer which is a potent evolutionary force that can create diversity within bacterial species. Conjugation is a recombination process where two live bacteria come together, and the donor cell transfers genetic material to the recipient cell. This process was first observed in 1946 by Joshua Lederberg and Edward Tatum in a series of experiments with *E. coli*.

Principle: Conjugation is the mode of gene transfer in many species of bacteria. In 1950 William Hayes, Francis Jacob and Elie L. Wollman established that conjugating bacteria are of two mating types. Certain "male" types (designated as F+) donate their DNA and other "female types" (designated as F-) receive the DNA as shown in Figure 1. Fcells become F + when they acquire a small amount of DNA. Hence the F factor is called as the Fertility factor. In contemporary microbiology, the donor's F factors are known to be plasmids which are the extra chromosomal elements. The factors (plasmids) contain about 20-30 genes, most of which are associated with conjugation. These genes encode enzymes that replicate DNA during conjugation and structural proteins needed to synthesize special pili at the cell surface. Known as F pili or sex pili, these hair like fibres contact the recipient bacteria, and then retract so that the surfaces of donor and recipient are very close or touching one another. At the area of contact, a channel or conjugation bridge is formed. Once contact via sex pili has been made, the F factor (plasmid) begins replicating by the rolling circle mechanism. A single strand of the factor then passes over or through the channel to the recipient. When it arrives, enzymes synthesize a complementary strand, and a double helix is formed. The double helix bends to a loop and reforms an F factor (plasmid), thereby completing the conversion of recipient from F- cell to F+ cell. Meanwhile, back in the donor cell a new strand of DNA forms, to complement the leftover strand of the F plasmid. The transfer of F factors involves no activity of the bacterial chromosome; therefore the recipient does not acquire new genes other than those on the F factor.

Materials Required: Donor strain A, Recipient strain B, Streptomycin sulphate, tetracycline hydrochloride, Luria Bertain Broth, Agar powder, Bacteriological.

Storage: Bacterial Conjugation Teaching Kit is stable for 6 months from the date of receipt without showing any reduction in performance. On receipt, store Donor Strain A, Recepient Strain B, Tetracycline hydrochloride and Streptomycin sulphate at 2-8 °C. Other kit contents can be stored at room temperature (15-25 ˚C).

Day 1

Using sterile flexi loop, streak a loopful of E.coli Donor Strain from the stab onto two LB plates with Tetracycline (30 µg/ml) and E. coli Recipient Strain onto two LB plates with Streptomycin (100 µg/ml). Incubate at 37oC for 18-24 hours

Day 2

Pick up a single colony from Donor and Recipient Strain grown overnight on LB plates and inoculate in 6 ml of LB broth having respective antibiotics. Incubate the test tubes overnight at 37°C.

Day 3

- Take 25 ml of LB broth and add 25 µl of tetracycline into it and inoculate 1 ml of overnight grown culture into it. Incubate at 37oC in a shaker.
- Take 25 ml of LB broth with streptomycin at a concentration of 100 µg/ml and inoculate 3 ml of overnight grown culture in it. Incubate at 37 °C in a shaker.
- Grow the cultures till O.D of the donor culture reaches 0.8-0.9 at A 600.
- Add 0.2 ml of each donor and recipient cultures in a sterile test tube labeled as conjugated sample. Mix by gentle pipetting and incubate at 37 °C for 1-1.5 hours.
- Take 2 sterile test tubes and label them as donor and recipient. Add 0.2 ml of respective cultures to the test tubes and incubate at 37 °C for 1-1.5 hours. NOTE: Do not place the tubes in shaker for conjugation and further incubation period.
- Add 2 ml of LB broth into each tube after incubation. Incubate the tubes at 37 °C for 1.5 hours.
- Plate 0.1 ml of each culture on the antibiotic plates.
- Incubate the plates overnight at 37 °C overnight.

Result:

Experiment No: 8a

DEMONSTRATION OF BACTERIAL TRANSFORMATION

Aim: To learn the process of transduction, a genetic transfer of a particular gene from one E. coli host to another through a bacteriophage.

Introduction: Transduction is a method of genetic recombination in bacteria, in which DNA is transferred between bacteria via bacteriophages. In this process, DNA of a bacterial cell is transferred into another bacterial cell with the help of a bacteriophage. Transduction is a common tool used by molecular biologists to stably introduce a foreign gene into a host cell's genome.

Principle Bacteria can exchange or transfer DNA between other bacteria in three different ways:

- Transformation: This process involves acquisition of DNA from the environment and susceptible to DNase.
- Conjugation: During this process DNA is directly acquired from another bacterium and cell-to-cell contact is required.
- Transduction: In this process DNA is incorporated to a bacterial cell via a bacteriophage intermediate. It does not require cell-to-cell contact and it is DNase resistant. In all these cases the source cells of the DNA are called donors and the cells that receive the DNA are called the „recipients'. In each case the donor DNA is incorporated into the recipients cell's DNA by recombination exchange.

If the exchange involves an allele of the recipient's gene, the recipient's genome and phenotype will change.

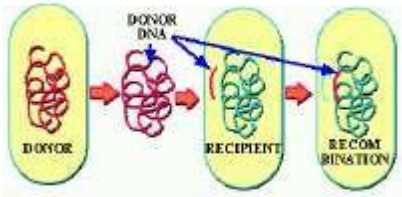
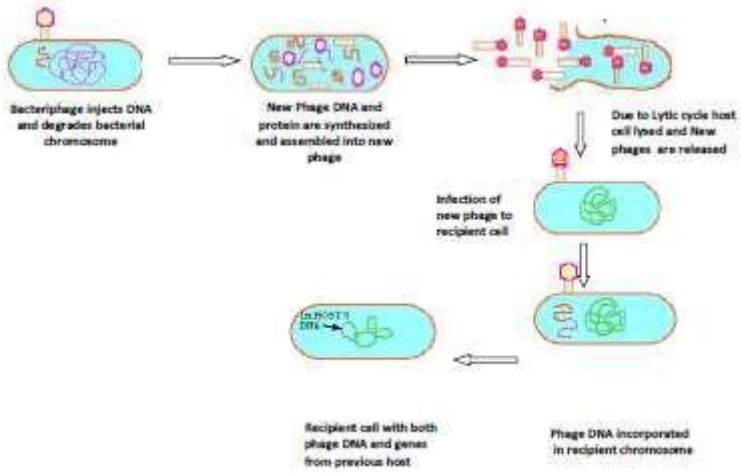


Fig 1: Exchange of genetic material between two bacterial cells. The ability of a virus (bacteriophage) to carry bacterial DNA between bacteria was discovered in 1952 and named as transduction.

The scientists found that, when a donor cell is lysed by P1 (bacteriophage), the bacterial chromosome is broken up into small pieces and sometimes the forming phage particles mistakenly incorporate a piece of the bacterial DNA into a phage head in place of phage DNA. The bacteriophage goes through either the lytic cycle or the lysogenic cycle. During the lysogenic cycle the phage chromosome is integrated into the bacterial chromosome and can remain dormant for several generations. If the lysogen is induced the phage genome is excised from the bacterial chromosome and initiates the lytic cycle, which culminates in lysis of the cell and the release of phage particles. The lytic cycle leads to the production of new phage particles which are released by lysis of the host.

The entire process of transduction involves the following steps:

- First, the phage infects a susceptible bacterium (donor) and injects its DNA into the host.
- The phage DNA utilizes the host's machinery and synthesizes phage components including phage DNA. During these process parts of bacterial chromosome is integrated into the phage DNA.
- As a final step in the phage life-cycle, all the phage components in the cytoplasm are assembled into complete phage and the cell is lysed to release the newly made phage particles.
- When this newly made virus particle infects the „recipient' bacteria, the phage DNA (containing parts of „donor"s DNA) is injected into it and the transduced bacterial genes can be incorporated by recombination.
- The transduction process is different from the usual gene recombination process. The most striking difference is the transfer of genetic material from cell to cell by viruses. The second feature is the fact that only a small part of the total genetic material of one bacterial cell is carried by the bacteriophage.



During transduction the donor's genetic material is transferred to the recipient cell through a bacteriophages

Materials Required: Donor strain, Receipient strain, Susceptible host, Phage lysate, ampicillin, chloramphenicol, LB broth, agar powder (bacteriological grade), collection tubes (polypropylene 2 ml), 10 % dextrose, 1M calcium chloride, 1M magnesium chloride.

Procedure:

Day 1:

- Streak a loopful of culture from stabs of Donor on LB C20 plate, Recipient on LB A100 plate and susceptible host on LB plate.
- Along with streaking, inoculate loopful of culture from given stabs in 5ml LB broth with respective antibiotics.
- Incubate the plates overnight at 37°C and Culture tubes at 37oC shaker for overnight with a speed of 300 rpm.

Day 2:

- Store the 5 ml culture tubes at 40C for inoculation on Day 3.
- Inoculate 10-15 colonies from revived donor plate into 5 ml LB C20 and label as Donor Tube.
- Incubate at 30°C in shaker for 2 hours.
- Keep a 5ml aliquot of sterile LB broth in water bath, set at 60-65°C.
- Add 100 µl of given phage lysate to above labeled donor tube, continue incubation for 30 mins at 30°C.
- Add 2 ml of preheated sterile LB broth to donor tube mix well and incubate this tube at 42°C for another 20 mins.
- Transfer this tube to 37°C and incubate for 3 hours.
- After incubation, spin this culture at 5000 rpm for 10 mins. Take the supernatant, filter it through 0.45 µm filter, label as Phage lysate 2.Store at 4°C for further steps.
- Inoculate single colony from recipient plate in 5 ml of LB broth with ampicillin (100 µg/ml). This is recipient tube; incubate this tube overnight at 37°C shaker.

Day 3:

- Inoculate 100 µl of overnight grown recipient culture in 5 ml of fresh LB broth with ampicillin (100 µg/ml), incubate on shaker at 37°C for 2 hours.
- After incubation take 50 µl of this culture in 2 ml collection tube, add 50 µl 0.1M CaCl2 along with 250 µl of Phage lysate 2 obtained and stored at 4°C on Day 2.
- Mix well and incubate further at 30°C for 2 hours. (Do Not keep on Shaker.)
- After 2 hours, take each 50 µl of this culture and plate on LB C20, LB A100 and LB C20 A100 plates.
- Along with it , take 50µl of overnight grown cultures of recipient strain and donor strain which are revived on Day 1, plate on LB C20, LB A100 and LB C20 A100 plates.
- Incubate all plates at 37°C for overnight.
- On next day store these plates at 40C for observation and results.

Day 4:

- Inoculate 20-25 colonies from revived plate of susceptible host (Day 1) in 15 ml of fresh LB broth.
- Incubate this culture on shaker at 37°C for 2 hours.
- After incubation spin down 1.5 ml of this culture at 8000rpm for 10 mins. In 7 different collection tubes (2ml) at RT. Then resuspend each pellet in 100 µl of fresh sterile LB broth. Use this as plating cells for titration.
- Before starting Titration protocol, keep 5 ml of LB broth at 60°C.
- To confirm presence of phages in lysogenized colonies of infected recipient culture, inoculate 10-15 Colonies from LB C20 A100 plate (of recipient infected with phage) in 5 ml LB C20 A100 broth. Label this as Lysogenized culture.
- Incubate this tube at 30°C for 3 hours. After incubation add 2 ml of hot LB broth (kept at 60°C) to this

Result:

Experiment No: 8b

DEMONSTRATION OF BACTERIAL TRANSDUCTION

Aim: To prepare competent cells and transform plasmid DNA**.**

Introduction: Bacterial transformation is a process which involves genetic alteration of bacteria by incorporation and stable expression of a foreign genetic material from the environment or surrounding medium. Since DNA is a very hydrophobic molecule, it will not normally pass through a bacterial cell membrane. In order to uptake the foreign DNA, the bacterial cells must first be made competent. Competence is the ability of a cell to take up extracellular DNA from its environment. There are different methods of carrying out transformation, e.g. chemical transformation, electroporation, gene gun, liposome mediated transfer and microinjection. Chemical transformation includes the usage of Calcium chloride ($CaCl_2$). This mode of transformation is easy to perform and requires minimum number of equipments.

Principle: For the incorporation of plasmid into a cell, bacteria must first be made "competent". This process includes the treatment of cells with bivalent calcium ions in ice-cold condition. As a result small pores are formed on the cell membrane, which makes it permeable. The plasmid DNA may adhere to the surface of the cell and uptake is mediated by a pulsed heat shock at 42 °C. A rapid chilling step on ice ensures the closure of the pores. These cells are allowed to propagate and selection of transformants can be done by growing the cells on a selective media which will allow only the plasmid containing cells to grow.

Plasmids are extra chromosomal DNA element capable of independent replication inside a suitable host. Plasmids encode a wide variety of genes, including those required for antimicrobial resistance. These genes act as selective markers when a transformation experiment is carried out. The E. coli plasmid pUC19 encodes a gene that can be used as a selectable marker during a transformation Plasmid is amplified within the cell Pl.

Plasmids are extrachromosomal DNA element capable of independent replication inside a suitable host. Plasmids encode a wide variety of genes, including those required for antimicrobial resistance. These genes act as selective markers when a transformation experiment is carried out. The *E. coli* plasmid pUC19 encodes a gene that can be used as a selectable marker during a transformation experiment. pUC19 has ampicillin resistance marker that enables only transformed cells to grow on LB– Ampicillin plates. Transformants, thus having the ability to grow on ampicillin plates can be selected. This process of direct selection of recombinants is called insertional-inactivation. pUC19 also carries the *N*terminal coding sequence for β-galactosidase of the *lac* operon. The *E. coli* host strain has a deletion at the amino terminal end of the LacZ gene, which codes for β-galactosidase. When pUC19 is transformed into the competent host cells, the truncated products from both complement each other and as a result enzymatically active β- galactosidase is produced. This is called α-complementation. The tranformants turn blue on X-gal and IPTG containing plates due to the production of β-galactosidase. X-gal is the chromogenic substrate of β- galactosidase and IPTG acts as the inducer for the expression of this enzyme.

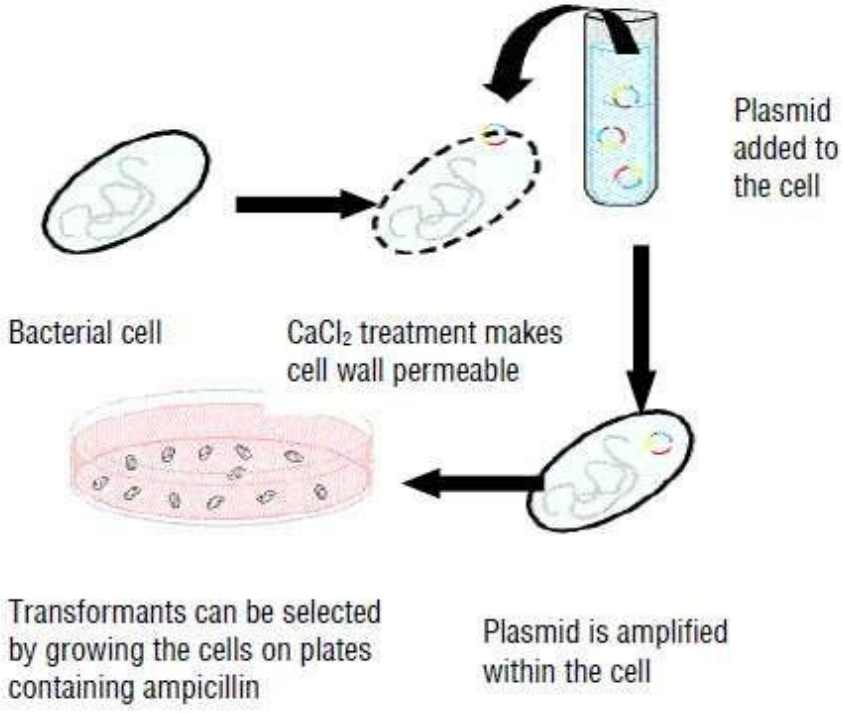
Materials Required

Ampicillin, LB broth, Agar powder, E. coli host, plasmid DNA, 1M Calcium chloride, X-Gal, IPTG, Collection tubes

Glass wares: Conical flask, Measuring cylinder, Beaker

Other requirements: Micropipettes, Tips, 50 ml Centrifuge Tubes, Water bath (42oC), 37oC Incubator, 37°C

Shaker, Centrifuge, UV Transilluminator, Crushed ice, Sterile double distilled water, Sterile loop and spreader.



The process of bacterial transformation includes treatment of cells with CaCl2, which makes cells permeable, and plasmid DNA can enter the cell.

Procedure Day 1

1. Open the bottle containing culture and resuspend the pellet with 0.25 ml of LB broth.
2. Pick up a loopful of culture and streak onto LB agar plate.
3. Incubate overnight at 37°C.

Day 2

1. Inoculate a single colony from the revived plate in 1 ml LB broth.
2. Incubate at 37°C overnight.

Day 3:

1. Take 50 ml of LB broth in a sterile flask. Transfer 1 ml of overnight grown culture into this flask.
2. Incubate at 37°C shaker at 300 rpm for 3-4 hours till the O.D600 reaches ~ 0.6.

A) Preparation of Competent Cells:

Note: Prepare competent cells within 3 days of reviving the strain.

1. Transfer the above culture into a pre chilled 50 ml polypropylene tube (not provided).
2. Allow the culture to cool down to 4°C by storing on ice for 10 minutes.
3. Centrifuge at 5000 rpm for 10 minutes at 4°C.
4. Decant the medium completely. No traces of medium should be left.
5. Resuspend the cell pellet in 30 ml prechilled sterile 0.1 M Calcium chloride solution.
6. Incubate on ice for 30 minutes.
7. Centrifuge at 5000 rpm for 10 minutes at 4°C.
8. Decant the calcium chloride solution completely. No traces of solution should be left.
9. Resuspend the pellet in 2 ml pre chilled sterile 0.1M Calcium chloride solution.
10. This cell suspension contains competent cells and can be used for transformation.

B) Transformation of cells:

1. Take 200 µl of the above cell suspension in two 2.0 ml collection tubes and label them as „control" and „transformed". Add 2 µl of plasmid DNA to the tube labeled as transformed and mix well.
2. Incubate both the tubes on ice for 30 minutes.
3. Transfer them to a preheated water bath set at a temperature of 42°C for 2 minutes (heat shock).
4. Rapidly transfer the tubes on ice-bath. Allow the cells to chill for 5 minutes.
5. Add 800 µl of LB Broth to both the tubes. Incubate the tubes for 1 hour at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
6. Take four LB agar plates containing ampicillin, X-Gal, IPTG and label them as control, A, B and C. Plate 200 µl of culture from the „control" tube and plate it on the corresponding plate with a sterile spreader. Plate 50 µl, 100 µl and 200 µl of cell cultures from the „transformed" tube on the plates labeled as A, B and C, respectively.
7. Store at room temperature till the plates are dry.
8. Incubate the plates overnight at 37µC.
9. Blue white colony selection plate

Result:

Experiment: 9

DEMONSTRATION OF AMES TEST

Aim: To demonstrate Ames test to assess the mutagenecity of the given sample.

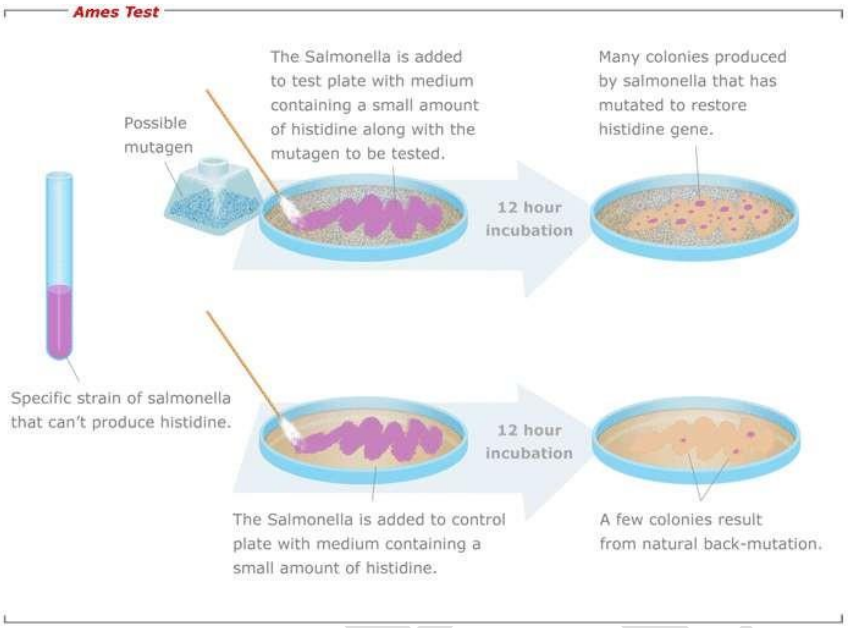
Principle: Ames test is developed by Bruce N. Ames in 1970s to test for determining if the chemical is mutagens. This test is based on the principle of reverse mutation or back mutation. So, the test is also known as bacterial reverse mutation assay.

Procedure:

- Test organism: Ames test uses several strains of bacteria (*Salmonella, E.coli*) that carry mutation. Eg A particular strain of *Salmonella* Typhimurium carries mutation in gene that encodes histidine. So it is an auxotrophic mutant which loss the ability to synthesize histidine (an amino acid) utilizing the ingredients of culture media. Those strains are known as His- and require histidine in growth media.
- Culturing His- salmonella is in a media containing certain chemicals, causes mutation in histidine encoding gene, such that they regain the ability to synthesize histidine (His+) This is the reverse mutation. Such chemicals responsible to revert the mutation are actually a mutagen. So, this Ames test is used to test mutagenic ability of varieties of chemicals.

1. Isolate an auxotrophic strain of *Salmonella* Typhimurium for histidine. (ie. His-ve)
2. Prepare a test suspension of his-ve *Salmonella* Typhimurium in a plain buffer with test chemical (let's say 2-aminofluorene). Also add small amount of histidine.
3. Ps: small amount of histidine is required for initial growth of bacteria. Once histidine is depleted only those bacteria mutated to gain the ability to synthesize histidine form colony.
4. Also prepare a control suspension of His-ve *Salmonella typhimurium* but without test chemicals.
5. Incubate the suspensions at 37 °C for 20 min
6. Prepare the two agar plate and spread the suspension on agar plate
7. Incubate the plates at 37 °C for 48 h
8. After 48 h count the number of colonies in each plate. The mutagenicity of chemicals is proportional to number of colonies observed. If large number of colonies on test plate is observed in comparison to control, then such chemical are said to be mutagens.

   *Very few number of colonies can be seen on control plate also. This may be due to spontaneous point mutation on hisidine encoding gene.


Ames Test
The Salmonella is added to test plate with medium containing a small amount of histidine along with the mutagen to be tested.
Many colonies produced by salmonella that has mutated to restore histidine gene.
Possible mutagen
12 hour incubation
Specific strain of salmonella that can't produce histidine.
12 hour incubation
The Salmonella is added to control plate with medium containing a small amount of histidine.
A few colonies result from natural back-mutation.

Result: