MADHA
Expertise | Empathy | Excellence
ENGINEERING COLLEGE

DEPARTMENT OF BIOTECHNOLOGY

BT 3511-
MOLECULAR BIOLOGY & GENETIC ENGINEERING LABORATORY MANUAL

REGULATION 2021

III YEAR & V SEMESTER

MADHA ENGINEERING COLLEGE
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BT 3511 - MOLECULAR BIOLOGY LABORATORY SYLLABUS

Expt. No.: 1

Good Laboratory Practices – Safety Precautions in Laboratory

- Contact lenses are not to be worn in the laboratory.
- Not to take the observations on loose paper, always separate note book or laboratory manual are to be used.
- Blower in the hood / bio-safety cabinet are to be run during cleanup.
- Disinfectant are to be applied to any area and allowed minimum 20 minutes contact time before starting the work to ensure germicidal action of disinfectant.
- Equipments are to be bleached (1:10 bleach) followed by water and then 70% ethanol or isopropanol.
- Not to look at UV light directly as the damage may not be noticed until 30 minutes to 24 hours after exposure, view only through a filter or safety glasses that absorb harmful wavelengths.
- Always loosen the bottle caps before microwaving and autoclaving as it may explode.
- Always ampoules containing liquid or lyophilized culture material are to be opened in the safety cabinet or in a hood (use of gloves when opening ampoules or cryovials are advisable).
- Always cover-glasses and slides are handled with forceps.
- Slides, petriplates, beaker, conical flasks are to be marked with glass markers or labels mentioning the name of the organism, media, date of making and experiment name in short and the initials of the persons responsible etc…
- Petridishes are to be labeled on the cover and the test organism to be written on the lower surface and not to hinder observation.
- All cultures are to be discarded after inactivation according to the instructions given by the lab instructor.
- Contaminated plastic wares are to be disposed after treating with bleach (10% v/v final concentration) for at least 30 minutes before disposal and also consult the lab instructor before the disposal.

- Glassware containing liquefiable solid media was cleaned by heating and pouring out the material in liquid condition and then autoclaving.
- Solid media, liquefied by heat, never to be thrown in the sink, as it solidify and clog up the traps and drains.
- Flasks, test tubes, Petri dishes, etc. , containing cultures must be heated one hour in flowing steam before cleaning. Spore containing cultures should be autoclaved previous to cleaning.
- Agar and liquid wastes are to be displaced only in the closed box provided for the purpose or by the instructions given by the lab instructor.
- If cultures or media become dry, water was added before heating. A special care is taken in cleaning glassware containing mercuric chloride or any other disinfectant.
- A concentrated solution was used to destroy microorganisms by withdrawing water from their cells (plasmolysis), in the preservation of food by concentrated salt or sugar solutions.
- Microorganisms suffer plasmolysis (bursting of the cell) if placed in a chosen concentration medium,. Such that death is delayed or prevented.
- Desiccation was destructive to many microbes, especially those which do not form spores.
- Sterilization was adopted for sterilizing platinum, copper wires and iron, nickel spatulas, forceps, etc. in a naked flame or in an ether flame or in a muffle furnace or by hot air to heat to redness in a flame. A platinum needle is always be carefully dried before sterilization, by holding it near the flame. This avoids sputtering, which scatters microorganisms, especially if moist material Sterilization in an Ether Flame.
- In an emergency, small instrument, needles, etc., may be sterilized by dipping them in ether or absolute alcohol and after removal lighting the adherent fluid and allowing it to burn off the surface of the instruments repeatedly.
- Breakage or spill are to be covered immediately with paper towels and then saturated the area with disinfectant such as 1:10 dilution of household bleach and cleaned after 15-20 minutes.
- Access to exits, sinks, wash room and fire extinguishers are not to be blocked.

- All the used materials are to be placed in its proper place before leaving the lab and the instruments are to be checked for the shutdown except those which need to be remain working.
- For any biological disposal the instructions by the lab instructor are to be followed.
- Sterility of the biological cabinet is to be checked periodically.

Expt. No.: 2

Isolation of DNA from Bacterial cell

Aim:

To isolate the genomic DNA from the given *E.coli* culture.

Introduction:

Bacterial strain has it own double stranded chromosomal DNA in circular form. It has to be purified from the bacterial cell for the purpose of analysis and manipulation of DNA. Genomic DNA has molecular weight large than the plasmid DNA and finds uses in RFLP, AFLP, PCR and Analysis of Gene Targeting.

Principle:

DNA extraction involved 3 major steps: Cell lysis, separation of DNA from proteins and other cell debris and precipitation of DNA. The overnight cultured bacterial cells will be washed by adding solution-I which chelates the metal ions from the cells, thus making the cell wall susceptible to breakage by solution-II. Osmolality is change by solution-III. After adding lysozyme treatment, cell lysis by adding solution-IV. Proteins and debris are precipitated by solution-V. DNA in the aqueous solution will be concentrated /precipitated by solution-VI and VII (ethanol and isopropanol).

Materials required:

Conical flask, centrifuge tubes, *E.coli* culture, solution-I, solution-II, solution-III, solution-IV, solution-V, solution-VI, solution-VII, LB broth, RNase, Rotary shaker, Eppendorf tube. Agarose, TEB, Lysozyme, Gel loading dye, TE buffer and DNA marker.

Procedure:

1. The *E.coli* culture was inoculated in freshly prepared LB broth, after that the culture was incubated in the rotary shaker for overnight.
2. Once the bacterial growth has been observed, 1.5 ml of culture was aliquot into micro-

centrifuge tube.

3. The tube was centrifuged at 10, 000 rpm for 15 minutes and the supernatant was discarded.
4. 0.5 ml of solution-I was added and the pellet was suspended by vortexing or repeat pipetting and the tube was centrifuged at 10,000 rpm for 10 minutes and the supernatant was discarded.
5. The pellet was resuspended in 0.5 ml solution-II and centrifuged at 10,000 rpm for 10 minutes and the supernatant was discarded.
6. The pellet was resuspended in 0.5 ml solution-III. To this, 25ml of lysozyme was added and gently the tube was taped. Left the tube for 15 minutes at 37^{O}C.
7. 50 µl of Solution-IV was added and mixed well by gently taping (Do not vortex). The tube was heated at 55^{O}C for 10 minutes
8. Then 250 ml of solution-V was added and mixed well by gently taping. The tube was centrifuged at 10,000 rpm for 5 minutes.
9. Carefully the supernatant was transferred in to a fresh microfuge tube.
10. 0.6 ml of solution-VI was added in to the above supernatant and mixed by inverting the tube (3-4 times) and centrifuged at 10,000 rpm for 15 minutes.
11. The supernatant was decanted and the pellet was washed with 0.5 ml of solution VII by centrifuging at 10,000 rpm for 10 minutes.
12. The supernatant was decanted and the pellet was dried at 37^{O}C in the incubator till there is no trance of solution-VII.
13. The pellet was suspended in 20 µl of TE buffer.
14. To the pellet 5 ml of RNase was added and incubated at 37^{O}C for 1 hour.
15. The presence of Genomic DNA was confirmed by 0.8% Agarose gel electrophoresis.

Observation:

A single band was observed under UV Tran illuminator indicates the presence of genomic DNA.

Result:

The genomic DNA from *E.coli* was isolated.

Expt. No.: 3

Isolation of DNA from plant cell

Aim:

To extract DNA from the given plant leaf and confirm the presence of plant DNA.

Introduction:

The application of molecular biology techniques to the analysis of complex genomic depend on the ability to prepare pure high molecular weight DNA for further manipulations. Plants provide several special challenges for researchers, interested in recombinant DNA research. The high molecular weight plant nuclear genomic DNA (> 150 Kbp) is used to construct genomic DNA libraries and to probe the plant genome for the presence of DNA markers such as RAPD and RFLP.

Principle:

First lysing the cell and solubilizing the DNA, which is followed by enzymatic or chemical method to remove contaminating proteins RNA and other macromolecules. The rigid cell wall of the plant cell is weakened by EDTA in the solution-I. then the cells are subjected to osmotic shock at 65^{o}C to lyse the rigid cell wall of the plant cells. The cellular proteins are denatured by the SDS in Solution-II. Extraction with phenol denatures the protein, Chloroform removes SDS-Protein / polysaccharides complexes as white interface between the aqueous and organic phase after centrifugation and isoamyl alcohol prevent foaming. The high molecular weight genomic DNA in aqueous phase is precipitated with 100% ethanol (solution-III) and further the salts are removed by washing with solution-IV.

Material required:

Solution-I, Solution-II, Phenol, Chloroform, Isoamyl alcohol, Ethanol-I, Ethanol-II, TEB, Gel loading dye, Ethidium Bromide, TE Buffer Agarose, 1.5 Microfuge tubes, RNase and DNA marker.

Procedure:

1. 100 mg of young plant tissues were weighed and was sterilized with acetone and distilled water.
2. In a mortar 250 µl of solution-1 was added and the tissues were grinded using pestle to get a fine paste.
3. The homogenization process was repeated with additional 250 µl of extraction buffer for another 20 seconds.
4. Approximately 100 µl of homogenized leaf mixture was taken in a microfuge tube and 20 ìl of solution-II was added, vortexed for 30 seconds.
5. The tubes were warmed at 65^{O}C in a water bath for 1 hour.
6. The content was extracted with equal volume of Phenol: chloroform: Isoamyl alcohol (25:24:1). Mixed gently to avoid shearing of Genomic DNA
7. The sample was centrifuged at 10,000 rpm for 20 minutes at Room temperature.
8. The aqueous phase was collected carefully in a fresh microfuge tube with the pipette tip having wide opening to avoid shearing of DNA
9. Double the volume of 100% ethanol was added to the collected aqueous phase and mixed gently by inverting the tube to precipitate the DNA.
10. The tube was kept at -20^{O}C for 30 minutes to allow the DNA to precipitate.
11. The sample was centrifuged at 10000 rpm for 10 minutes at room temperature.
12. The supernatant was decanted carefully and the traced were removed by inverting the tube over the tissue paper.
13. The pellet was washed with 70% ethanol by centrifugation at 6000 rpm for 10 minutes at room temperature.
14. The supernatant was decanted carefully and the traced were removed by inverting the tube over the tissue paper.
15. The pellet was dried at 37^{O}C to remove ethanol completely and resuspended in 20 µl of TE buffer.
16. The sample was added with 5 µl of RNase and left for 30 minutes.
17. The sample was resolved at 20 µl of the extracted DNA in 1 % Agarose gel.

Observation:

A band pattern of different plant samples was observed under UV Tran illuminator indicates that the plant DNA was extracted.

Result:

The DNA was extracted from the given plant and confirmed by agarose gel electrophoresis.

Expt. No.: 4

Isolation of DNA from animal cell

Aim:

To isolate genomic DNA from the given animal cell tissue.

Principle:

DNA in the animal tissues is released by using lysis buffer. The cell membrane of the animal tissue is weakened by EDTA and SDS denatures the cellular proteins in the solution-I. Extraction with phenol denatures the protein, chloroform removes SDS-Protein/polysaccharide complexes as white interface between the aqueous and organic phase after centrifugation and isoamyl alcohol prevents foaming. The high molecular weight genomic DNA in aqueous phase is precipitated with 100% ethanol or isopropanol (solution-III) and further the salts are removed by washing with 70% alcohol.

Material required:

Solution-I, solution-II, solution-III, solution-IV, 70% alcohol, TEB, Gel loading dye, Ethidium Bromide, TE Buffer, Agarose, 1.5 Microfuge tubes, RNase, DNA marker and proteinase-K.

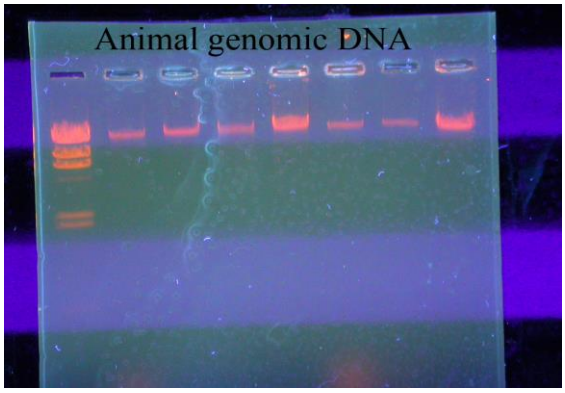
Procedure:

1. 0.1g of animal tissue was taken and to that 1ml of solution-I was added and homogenized well until tissue completely homogenize well in to slurry.
2. 20 µl of proteinase-K was added and gently the tubes were taped to mix well and left for 30 minutes at 65^{O}C.
3. 0.5 ml of solution-II was added, mixed well and centrifuged at 6000rpm for 5 minutes at 4^{O}C.
4. The aqueous phase was taken and 0.75 ml of solution-II was added and mixed well and centrifuged at 7000 rpm for 5 minutes at 4^{O}C.
5. The aqueous phase was taken and 0.75 ml of solution-III was added and mixed well and centrifuged at 7000 rpm for 5 minutes at 4^{O}C.

6. The aqueous phase was transferred to a fresh tube.
7. Double the volumes of ice-cold solution-IV was added and mixed gently to precipitate the DNA and incubated at -20^{O}C for 15 minutes.
8. The tube was centrifuged at 10000 rpm for 10 minutes at 4^{O}C and the supernatant was discarded.
9. The DNA pellet was washed once with 70% alcohol and short spin was given to remove the traces of alcohol.
10. The pellet was dried properly and was dissolved in 25 ìl of TE buffer and with 3ìl RNase and was incubated at 4^{O}C for 30 minutes.
11. 0.8% agarose solution was prepared with TEB buffer and the DNA samples were loaded on to the wells and electrophoresed.

Observation:

The band patterns were visualized under UV-Tran illuminator indicating the animal tissue DNA was isolated.



Lane-1 Molecular weight Marker

Lane 2-8 Animal genomic DNA

Result:

Thus the DNA was isolated from the given animal tissue sample.

Expt. No.: 5

Agarose Gel Electrophoresis

Aim:

To perform agarose gel electrophoresis by loading the DNA samples.

Introduction:

DNA can be electrophoresed through gel prepared by melting and re-gelling agarose. Agarose gel electrophoresis is useful in identification, separation of DNA molecule between the size of 500 – 2500 bp and subsequent purification of DNA molecule of interest in a heterogeneous mixture. Inclusion of a series of size standards on a gel means that the size of a DNA fragment can be determined from its distance of migration. Resolution of DNA species on Agarose gels made it a widely used reliable method in gene manipulation experiments and is a rapid and relatively inexpensive method. If stained with ethidium bromide, florescent substances, 1 – 10 ng of DNA could be visualized using a light source.

Principle:

Agarose is a copolymer of D-galactose and 3, 6, anhydro L- galactose. It forms a gel by hydrogen bonding and pore size depends on the agarose concentration. The movement of DNA fragments within the gel matrix is influenced by agarose concentration. Low agarose concentration improves the resolution of large fragments, but reduces the resolution of smaller fragment and vice versa. The DNA molecule are separated by electrophoresis on the basis of their molecular size between 500 – 2500 bp small pieces of DNA migrate through the gel matrix faster than larger pieces under the influence of electric current, shape or the confirmation of the molecule and magnitude of net charges on the molecule or the applied current (DNA molecules have a net negative charge and migrate towards the anode). Agarose gel electrophoresis is conducted in a horizontal configuration; there is less distortion (collapse) during electrophoresis and the bands of DNA are less distorted. The easiest gel system to operate seems to be one that has the gel completely

submerged by only one millimetre in the electrophoresis buffer. The ethidium bromide dye intercalated between the bases of DNA molecule and fluoresces bright orange when irradiated with UV light.

Caution:

- Wear gloves because of Ethidium Bromide.
- UV light can burn skin and eyes.
- Minimize exposure of UV light.

Material required:

Horizontal gel electrophoresis apparatus, Gel casting platform, gel combs (slot formers) , DC power supply unit, Microwave Oven, UV Transilluminator, Micropipettes, Para film, Adhesive tapes, Eppendorf tubes, tips, gel scoop, Glass wares, Tris Borate – EDTA (TBE), Agarose and DNA molecular weight markers.

Ethidium Bromide solution:

(Dissolve 0.2 g in 20 ml distilled water, mix well store at 4^{O}C)

Sample buffer / loading buffer 10X, 40% Sucrose, 0.25% Bromophenol blue(All w/v in 1 X TBE)

Procedure:

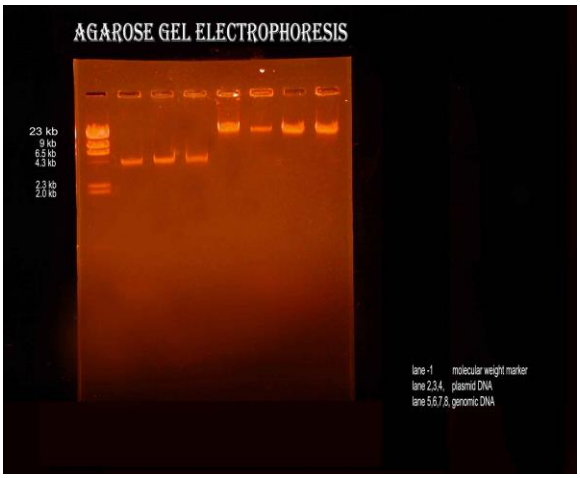
1. 1 gm of agarose was weighed and transferred to a conical flask. To the flask 100 ml of 1X TBE buffer was added.
2. The agarose was melted completely into a clear solution, using a microwave oven. The molten agarose was cooled to approximately 50^{O}C and ethidium bromide was added to a final concentration of 0.5 µl of 10 mg/ml solution of ethidium bromide to 100 ml gel mixture) and mixed well using a shaker.
3. The molten agarose was poured in a pre-set template with well forming comb (in advance, an agarose gel template was cleaned with 70% ethanol and sealed its end with adhesive tapes).
4. Placed horizontally on a levelling table and an appropriate well-forming comb was

placed. If the gel plate is not horizontal, wedge-shaped gel will form and DNA fragments will migrate peculiarly within it. The teeth of comb should not touch the bottom of the plate leaving at least 1 mm between comb and the gel plate while pouring.

5. The electrophoresis chamber was filled with 1X running buffer until the gel is covered by a couple of millimetres.
6. DNA sample concentration was adjusted (app. 5 to 8 µg genomic DNA; 0.5 µg plasmid DNA per lane).
7. The DNA samples was loaded (with 1/10 volume of tracking dye and heat shocked for 5 min at 65) carefully with a pipette P20 by slowly expelling the solution into a well, with the pipette tip slightly below the top of the well. Do not hold the pipette too far in the well: the sample will sometimes come out the bottom. Try not to plug the pipette tip against the side of the well either: the sample will usually squirt out when there gets to be enough pressure from the pipette.
8. The appropriate marker DNA flanking the sample lanes was loaded.
9. The lid on the electrophoresis kit was closed. The DNA was run toward the red (+) terminal. Electrophoresis the gel at 11 volts overnight or 100 volts for a couple of hours.
10. Stop electrophoresis by turning off the power supply, when the dye, Bromophenol blue has migrated a distance judged sufficient for separation of the DNA fragments (crosses more then 2/3 of the length of the gel and Bromophenol blue co migrates with 500 bp fragments). Visualize DNA band(s) on an UV Tran illuminator. (If required, this profile can be photographed through a red filter). View through an image analyzer and take a thermal print.

Observation:

The band patterns of plasmid DNA and the genomic DNA samples were visualized under UV-Transilluminator.



Note:

The above procedure is for making 100 ml of 1% agarose gel. Depending on the nature of DNA sample to be analyzed and the volume of the template, the experimenter should decide the percentage and the volume of the gel. For instance, plasmid DNA could be analyzed on 0.6 – 0.8 % gel. For analyzing chromosomal DNA, 0.4% gel is preferable. However, as the handling of 0.4% gel is difficult, chromosomal DNA can be analyzed on a higher percentage gel. For analyzing PCR products 1.5 – 2.0 % gel is required.

Agarose %	Range of separation
1.5	200 – 3000
1.2	400 – 7000
1.0	500 – 10000
0.7	800 – 12000
0.5	1000 – 30000

Result: Thus the agarose gel electrophoresis was performed by loading the DNA samples.

Expt. No.: 6

Restriction Enzyme Digestion

Aim:

To perform single and double digestion of DNA by using restriction enzymes.

Introduction:

Restriction digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called Restriction Endonucleases (sometime called as Restriction Enzymes or RE's). Restriction enzymes are bacterial enzymes which cut (hydrolyze) DNA into defined and reproducible fragments. In bacteria, they form part of restriction modification defence mechanism against foreign DNA. They are basic tool for cloning. These special enzymes recognize specific sequences called palindromic sequences in the DNA molecules (wherever that sequences occur in the DNA) and cleaves symmetrically in both strands. Restriction digests begin by mixing the DNA and RE, but it is unfortunately not quiet as simple as that. Those actual reaction conditions vary from one enzyme to the next and include temperature, NaCl and or $MgCl_2$ concentration, pH, etc. All of these variables except temperature are optimized by mixing the enzyme and DNA with a buffer specific for the enzyme of choice. Once all the ingredients are mixed in the reaction tube, the tube is incubated at the restriction enzyme's optimal temperature for 1 hour or longer. The finally when the digest has run for the appropriate amount of time, the reaction tube is put back on ice to prevent non specific degradation of DNA. Once the restriction digest is completed, agarose gel electrophoresis is preformed to separate the digest fragments by size and visualize the fragment and perhaps purify them for further experiments.

Principle:

Estimate the amount of DNA needed in your digest and scale up accordingly. To visualize a digest on an ethidium bromide- stained agarose gel, the size of the fragments and the total size of the clone DNA into account (e.g. 10-50 ng of intact lambda sized genomes (~

50 Kb) are easily seen on gels but if cut into small (~1 kb fragment), the relative proportion of the clone DNA in each fragment is ~ 1/50 and more DNA (500- 1000 ng) should be loaded in order to see them). If preparing a large number of digests at a time and the DNAs are the same concentration, prepare a cocktail of the reaction mix them divide it among the tubes of DNA.

Material required:

Water bath - set at 37^{O}C, microwave oven, Micropipettes, UV transilluminator, Vortex mixture, Incubator, Agarose gel electrophoresis unit, Eppendorf tubes and micropipettetips (sterile), Double distilled water, Restriction enzymes, 10x enzyme buffer, DNA samples, TBE buffer, Ethidium Bromide, Gel loading dye.

Procedure:

SINGLE DIGEST:

1. Digestion of DNA with a single enzyme is known as single digestion.
2. A new sterile 0.5 ml Microfuge tube was taken.
3. The following reagents were added in the order listed to the tube for 20 µl of reaction

Restriction buffer	- 2 µl
DNA source	- 3 µl
Enzyme (Eco RI / Hind III)	- 2 µl
Sterile Double Distilled water	- 13 µl
Total	**- 20µl**

DOUBLE DIGEST:

1. Digestion of DNA with two enzymes is known as double digestion.
2. A new sterile 0.5 ml Microfuge tube was taken.
3. The following reagents were added in the order listed to the tube for 20 µl of reaction

Restriction buffer	- 2 µl
DNA source	- 3 µl
Enzyme (Eco RI / Hind III)	- 2 µl
Sterile Double Distilled water	- 11 µl

Total - 20µl

1. Gently mixed by repeated pipetting or tapping, Briefly spin for few seconds and the reaction mix was incubated at 37^{O}C for 3 hours
2. 1% Agarose Gel electrophoresis was performed and the bands were observed under UV transilluminator.

Note:

The unit definition of restriction enzyme activity is based on the amount of enzyme required to cut bacteriophage lambda DNA to completion in one hour time. Often the laboratory conditions are not as ideal, and a slight excess of enzyme or a longer period of incubation is used to ensure complete digestion.

Observation:

- ✓ The given sample DNA has 7 and 5 restriction sites for Hind III and Eco RI respectively. When a digestion of DNA is set up with the one enzyme Hind III and Eco RI separately (single digest) 7 and 5 band were seen.
- ✓ In double digest of DNA with both the enzyme result in 12 bands.

Result: The single and double digestion was performed with the given restriction enzymes.

Expt. No.: 7

Competent cell Preparation

Aim:

To prepare competent cell from the given *E.coli* cells by calcium chloride method.

Principle:

Treatment of *E.coli* cells harvested at 0.6 OD with the ice cold solution of divalent cations ($CaCl_2$) induces a transient state of competence. The positively charged divalent cations interact with negatively charged DNA. The DNA uptake from the extracellular source is enhanced by sudden heat shock given to the chilled cells.

Materials required:

E.coli strain, LB broth, $CaCl_2$ solution, 15% glycerol, Micropipettes, micropipette tips, vortexer, refrigerator, centrifuge, incubator

Procedure:

1. A loopful of *E.coli* strain was inoculated onto 100 ml of LB broth and incubated at 37^{o}C with agitation to obtain 0.6 OD and the culture was chilled in ice for 30 minutes.
2. 1.5 ml of culture was aliquot into 2 sterile microfuge tubes using sterile micropipette tips.
3. The cells were pelleted by centrifugation at 4000 rpm for 10 minutes at 4^{o}C.
4. The supernatant was removed by aspiration with pipette and was resuspended in ¼ volumes (350 µl) of ice cold $CaCl_2$ solution. The cells were incubated in ice for 15 minutes.
5. The cells were pelleted by centrifugation at 4000 rpm for 10 minutes at 4^{o}C.
6. The supernatant was removed by aspiration and was resuspended in ½ volume (700 µl) of ice cold $CaCl_2$. The cells were incubated in ice for 30 minutes.

7. The cells were pelleted by centrifugation at 4000 rpm for 10 minutes at 4^{o}C.
8. The supernatant was removed by aspiration. For long term storage of competent cells was the pellet was resuspended in 100 µl of $CaCl_2$ + 15% glycerol mix, or resuspended in 100 µl of $CaCl_2$.

Observation:

Competent cells were prepared and the cells were resuspended in $CaCl_2$ and glycerol mix or further use.

Result:

Competent cells were prepared by using calcium chloride.

Expt. No.: 8

Transformation and screening for recombinant

Aim:

To perform the transformation process and to select the transformants with appropriate antibiotic, IPTG and X-gal.

Principle:

The given host *E.coli* is ampicillin sensitive and contains a gene expressing only ω portioin of β-galactosidase gene that can then complement with α peptide of the vector (plasmid DNA) to produce active functional enzyme.

Treatment of *E.coli* cells harvested at 0.6 OD with the ice cold solution of divalent cations ($CaCl_2$) induces a transient state of competence. The positively charged divalent cations interact with negatively charged DNA. The DNA uptake from the extra cellular source is enhanced by sudden heat shock given to the chilled cells. The expression of the gene for the enzyme is induced by IPTG (isopropyl β-D thio galacto pyranoside) which induce β-galactosidase enzyme synthesis. The enzyme then converts the chromogenic substrate in the medium X-gal (5-bromo 4- chloro 3-indolyl β-D galacto pyranoside) forming an intense blue product.

The transformants are selected by plating on the solid medium with appropriate antibiotic, IPTG and X-gal.

Materials required:

Competent cells, plasmid DNA, water bath (set at 42^{o}C), L-rod, Ampicillin (10mg / ml), petriplates, microfuge tubes, micropipettes and tips, LB medium.

Procedure:

Preparation of agar plates:

- LB agar plates (Plates 1and 2) were prepared with ampicillin at a concentration of 10mg/ml.
- By using a sterile L-rod, 40µl of X-Gal was spread on to plate-1 and plate-2 and allowed to diffuse for 20 minutes.
- 40 µl of IPTG was added on to both plates, spread evenly and allowed to stand for 20 minutes.

1. A loopful of *E.coli* strain was inoculated on to 100ml of LB broth and incubated at 37^{o}C with agitation until the culture reaches 0.6 OD.
2. The culture was chilled in ice for 30minutes.
3. 1.5 ml of culture was aliquot into sterile microfuge tubes by using sterile micropipette tips.
4. The tube was centrifuge at 4000rpm for 10 minutes at 4^{o}C.
5. The supernatant was removed by aspiration with pipette and the pellet was resuspended with 300µl of ice cold $CaCl_2$ solution and the cells were incubated in ice for 15 minutes.
6. The tube was centrifuged at 4000rpm for 10 minutes at 4^{o}C.
7. The supernatant was removed by aspiration and the pellets were resuspended with 700µl of ice cold CaCl2 solution. The cells are incubated in ice for 30 minutes.
8. The tubes are centrifuged at 4000rpm for 10 minutes at 4^{o}C.
9. The supernatant was removed by aspiration and resuspended in 100 µl of $CaCl_2$ and stored at - 20^{o}C.

Transformation:

10. Among the 2 tubes label one as test sample (Tube-1) and 5 µl of the given plasmid DNA was added to it and mixed well by taping the tube. To the other tube no plasmid or any DNA was added to maintain as negative control (Tube-2).
11. The tubes were incubated in ice for 30 minutes.

12. Heat shock was given by transferring the microfuge tubes to 42^{o}C water bath for not more than 90 seconds.
13. The microfuge tubes are placed in ice for 10 minutes and 400µl of sterile broth medium was added to them.
14. The microfuge tubes are incubated at 37^{o}C with agitation for 45 minutes.
15. Using L-rod, 100 µl of cells from the each tubes were evenly spread on LB agar plates 1 & 2 (Plate one for Tube – 1 and Plate two for Tube – 2).
16. The plates are incubated at 37^{o}C for 16 hours and the results are examined.

Observation:

Blue colour colonies were found on the plate-2 indicating the transformants whereas the plate -1 serves as control.

Result:

Thus the transformation was performed and the transformants were visualized as blue coloured colonies.

Expt. No.: 9

Plating of Bacteriophage

Introduction:

Isolation of bacterial viruses (Bacteriophage) can be obtained from Varity of Natural sources. Their isolation from these environments is not a easy task, because the phage particles are usually present in low concentration, therefore isolation requires a serious of steps:

- Collection of phage containing samples as its source
- Addition of an enriched susceptible host cell culture to the sample to increase the number of phage particles for subsequent isolation.
- Following incubation, centrifuge of the enriched sample fro the removal of gross particles.
- Filtration of the subsequent liquid through bacterial retaining membrane filter
- Inoculation of the bacteria- free filtrate onto a lawn of susceptible host cells grown on the soft agar plate medium
- Incubation and observation of the culture for the presence of phage particles, which is indicated by plaque formation.

Material required:

Tryptone, Calcium chloride, Sodium chloride, Potassium chloride, *E.coli* host, Phage lysate, Agar.

Procedure:

All the dilution tubes and media were labelled as follows

a) 9 ml of mine Tryptone tube 10^{-1} to 10^{-9}

3 ml of Tryptone soft agar plates tube 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}

Five Tryptone hard agar plates 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}

1. The five labelled soft agar tubes were placed into a water bath. The water should be of a depth just slightly above that of the agar in the tube. The water bath was brought to

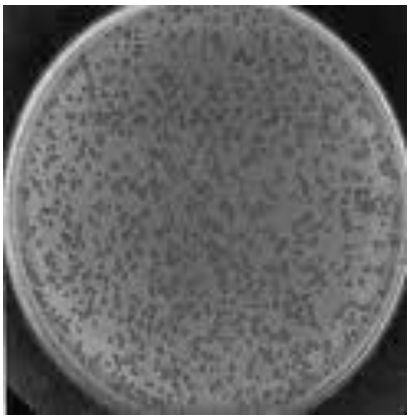
100 ^{o}C to melt the agar and cooled to maintain the melted agar at 45^{o}C.

2. By using 1 ml pipettes, aseptically a 10 fold serial dilution of the provided phage culture using the nine 9-ml tubes of tryptone was performed.
3. To the tryptone soft agar tube labelled 10^{-5} aseptically two drops of the *E.coli* culture was added with a Pasteur pipette and 0.1 ml of 10^{-4} tryptone broth phage dilution. Rapidly mixed by rotating the tube between the palms of the hands and the contents were poured over the hard tryptone agar plate labelled 10^{-5} thereby forming a double layered plate culture preparation. The plate culture was swirled gently and allowed to harden.
4. Using separate Pasteur pipette and 1 ml sterile pipettes, the step-3 was repeated for the tryptone broth phage dilution tubes labelled 10^{-5} through 10^{-8} to affect the 10^{-6} through 10^{-9} Tryptone soft agar.
5. Followed by solidification of the soft agar overlaid, all plate cultures were incubated in the inverted position for 24 hours at 37^{o}C.

Observation:

1. All plates were observed for the presence of plaque formation units that develop on the bacterial lawn.
2. The number of PFUs in the range 30 to 300 on each plate was counted.
3. The number of phage particles per ml of the stock phage culture based on the PFU count was calculated.
4. The counts were recorded in the chart.

Control plate



Plaque Forming Units (PFU's)

(10^{-6} dilution)

Example for calculation:

174 PFU's are counted in the 10^{-6} dilution tube.

(174) x (10^{6}) = 174 x 10^{6} PFU's per ml of stock phage culture (or) 1.74x10^{5} PFU's in 10 ml of the phage culture.

Result:

Phage Dilution	**Number of PFUs**	**Calculation: PFU x Dilution factor**	**PFU/ml of Stock Phage culture**
10^{-5}	280	280 x 10^{5} = 20x10^{5}	2.0x10^{5}
10^{-6}	174	174 x 10^{6}= 17.4x10^{5}	1.74x10^{5}
10^{-7}	125	125 x 10^{7}=12.5x10^{6}	1.25x10^{6}
10^{-8}	96	96 x 10^{8}= 9.6x10^{7}	0.96x10^{7}
10^{-9}	42	42 x 10^{9}= 4.2x10^{8}	0.42x10^{8}

Expt. No.: 10

λ phage titration

Introduction:

To understand the process of infection of bacteria by a temperate phage and its lytic pathway.

Principle:

Viruses that infect bacteria are referred to as bacteriophages, meaning bacteria eaters. These were first identified and described by Frederick Twort and Felix D' Herelle in 1917. Over the years, phages have become indispensable molecular tools in genetic engineering and related areas. Structurally three kinds of phages are recognized - Icosahedral head (tail less), Icosahedral head with tail and Filamentous phage. The phage genome can be either circular or linear, single or double stranded DNA or linear RNA with one or more proteins. The proteins form a capsid around the nucleic acid and protect them from host nucleases Bacteriophages remain in a state of dormancy in the environment and do not express any genes using this state and essentially persist until they come in contact with a susceptible host cell. On entering bacteria, the phage genome either integrates with the host genome (lysogeny) or independently produces prophages and lyse the host (lytic infection), resulting in death of the host cell and release of progeny phage particles. Each phage adsorbs to one cell and initiates an infection resulting in the release of about 100 phages/cell, these viruses infect the surrounding bacteria thereby releasing more virus particles. Due to successive rounds of infection, a spreading zone of lysis results in a clear area against a turbid background (due to growth of host bacteria). This clearing is referred to as plaque. The number of plaques formed is equivalent to the number of phage particles. Phage capable of only lytic growth is called virulent.

In case of lysogenic growth, the phage genome replicates as part of the host chromosome when the host cell divides. This prophage can be triggered to excise out of the host chromosome and begin lytic growth. Such phages are referred to as temperate phages.

E. coli lambda phage is a temperate phage. The DNA of λ phage is linear double stranded

having 48,502 base pairs. At the 5' terminus of each strand, it has a 12 53 nucleotide single stranded extension, complementary to each other, called the cohesive (cos) ends. The phage recognizes specific receptor on the surface of the host cell i.e., maltose binding protein for the purpose of adsorption. Hence, the host E.coli is grown in a medium containing maltose and magnesium which further facilitates the process of adsorption. On entering the host cell, the DNA circularizes due to base pairing between cohesive ends of the DNA. This serves as a template for transcription of phage genes during early stages of infection. The phage DNA then replicates by rolling circle mechanism synthesizes new capsid proteins, tail fiber protein and packages its DNA into capsids. Once assembled, prophage then brings about lysis of host cells and releases new infectious virus particles.

Phage titration (determination of the number of phage particles in a stock) is an important molecular biology technique. When genetic libraries in phage vectors are screened for positive clones, the plates that are being screened should have approximately 50-500 phage plaques per plate for optimal results. Less plaque will mean that too many plates will have to be screened, while more plaques will make it difficult to identify individual positive plaques. Plates in the ideal range are produced by preparing sequential dilutions of phage stocks. A streak plate for single colonies will also be prepared.

Plate cultures of bacteriophage are prepared by combining phage with susceptible host cells in top agar overlays (on top of regular nutrient agar plates). Top agar preparations contain lower concentrations of agar (7 g/L) than normal solutions used to prepare agar plates (15 g/L). The low agar concentration allows progeny phage from lysed cells to diffuse through the media and infect neighbouring bacterial cells. When these cells are lysed as well, a plaque (zone of lysed cells) is produced on the plate. Because phage can only reproduce in actively growing cells, the size of the plaques produced will depend on how soon the bacteria in the agar reach stationary phase and stop reproducing. Plaques will stop spreading at this point.

Top agar overlays are prepared by mixing phage dilutions with susceptible bacteria, and C. Overlays are gently mixed, and then°then adding ~ 3 ml of liquid top agar at 45-50 poured on top of pre-warmed nutrient agar plates. (Top agar must be maintained at 45 °C until immediately before use – higher temperatures will kill bacteria, and lower 50°C temperatures will allow agar to solidify prematurely.)

Materials Required:

Centrifuge, Incubator, Spectrophotometer, Shaker (37°C), Conical flasks, Petri plates, Measuring cylinder, Test tubes, Distilled water., Capped centrifuge tubes, Crushed ice, Tips, Micropipettes, $MgSO_4$ (1M), soft agar plates, SM buffer

Preparation of soft agar:

To prepare soft agar, add 0.8% of agar to LB broth. Boil to dissolve the agar. Aliquot 5 ml into test tubes and autoclave. Keep soft agar in molten state in a water bath or oven, adjusted to 42-45°C, just before use.

Preparation of 10 mM MgSO4:

Dilute $MgSO_4$ (1M), 100 times with sterile water, under aseptic conditions to get a working concentration of 10 mM $MgSO_4$.

Preparation of SM buffer (suspension medium):

Reagent	**Amount**	**Final Concentration**
NaCl	5.8g	100mM
$MgSO_4.7H_2O$	2g	8mM
Tris-HCl	50ml	50mM
H_2O	To 1 Litre	

SM phage diluent and storage buffer contains Mg_2 and is used for routine manipulation of phage suspensions. Lambda capsids require Mg^{2+} ions for stability. If dilutions are done in dH_2O (or especially TE, which contains EDTA, a chelating agent for divalent cations)

viable phage counts will be reduced.

Procedure:

Day 1: Revival of Host:

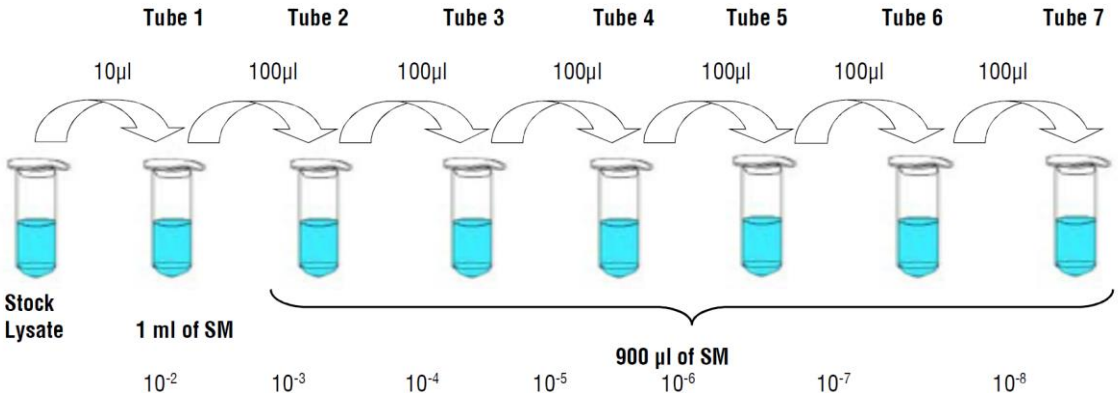
1. Break open the lyophilized vial and resuspend the sample by adding 0.1 ml of LB broth.
2. Streak a loopful (each) of this suspension on to two LB plates.
3. Incubate the plates overnight at 37ºC.

Day 2:

4. Inoculate single colony from revived plate in 5 ml of LB broth with 0.2% Maltose and 10mM $MgSO_4$.
5. Incubate at 37ºC in a shaker, overnight.

Day 3: Preparation of plating cells:

6. To 25 ml of LB broth, add 0.2 % Maltose and 10 mM $MgSO_4$. Inoculate the broth with 1% (0.25 ml) of overnight grown culture. Incubate at 37° C till the 0.D. reaches 0.6 at A600. (Approximately 2-3 hours.)
7. Chill on ice for 10 minutes.
8. Centrifuge the cells at 5000 rpm for 10 minutes in sterile centrifuge tubes. Discard supernatant.
9. Resuspend the cell pellet gently in 5-6 ml of 10 mM $MgSO_4$ and store at 4ºC.
10. Pipette 1ml of SM buffer each into six serially labeled 1.5 ml vials.
11. Transfer 10 µl of stock lysate into vial # 1 for diluting it to 10 –2. Mix and transfer 10 µl of 10–2 dilution to vial # 2.(i.e. 10-4 dilution). Repeat the same till the last dilution of 10-12 as shown below.
12. Label six LB agar plates and six sterile vials as 10- 2 , 10- 4 .... 10- 12.



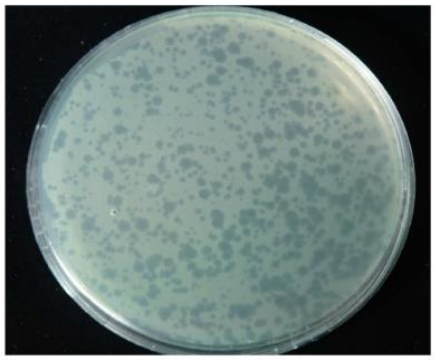
13. Pipette 100 µl of plating cells into each one of the vials. Add 10 µl of the respective phage dilution, mix gently and keep at 37°C for 15 minutes for adsorption of the phage onto host cell. (Note: Do not vortex.)
14. Pipette out the content of vial labeled as 10-2 into a test tube containing 5 ml of soft agar and swirl to mix. Care must be taken to ensure that the temperature of soft agar does not exceed 45°C (as the host will die) or fall below 40°C (as soft agar will solidify). (Note: Change the tip for each dilution.)
15. Pour the mix (phage-plating cells with soft agar) immediately on the respective LB agar plate and let the agar solidify.
16. Repeat steps 14 & 15 for each dilution.
17. Close the lids and incubate at 37°C for 16-18 hours.

Calculation of Phage Titre value:

Phage Titre value = Number of plaque forming units/ml of lysate.

Observation & Result:

The plates were observed for clear and distinct plaques. The number of plaques for each dilution was noted down and the results were interpreted in the table given below



Tube No.	Dilutions	Number of plaques	Phage titre value
1.			
2.			
3.			
4.			
5.			
6.			
7.			

Formula for calculating the phage titre value:

Phage titre value = Number of plaque forming units/ml of lysate

For example, if the number of plaques observed at dilution 10^{-5} is 50 then the phage titre value per ml of lysate will be:

Phage titre value = $50 \times 10^5 / 100$ µl
= 5×10^7 / ml

Expt. No.: 11

PREPARATION OF PLASMID DNA

AIM: To isolate plasmid DNA from the given bacterial cells (*E.coli* Strain DH5α)

PRINCIPLE:

Plasmid isolation is done by breaking the cell wall and denaturation of cellular proteins and the chromosomal DNA. The cellular debris is subsequently removed by centrifugation, and plasmid DNA and RNA are recovered. Plasmid DNA can be further isolated away from the contaminating RNA by treating the sample with RNAase. On average there are 15 copies of plasmid per E.coli cell, and upon amplification this number can be increased to 1000-3000 copies per cell. Addition of the antibiotic to the growing bacterial culture inhibits protein biosynthesis and, as a RESULT the replication of the chromosomal DNA. Plasmid replicates their DNA in the absence of *de novo* protein biosynthesis because they possess a origin of replication. Consequently, plasmid DNA accumulates in the cell and can attain levels as high as 40% of the total DNA. The cells are lysed by the addition of detergent, e.g., SDS; chromosomal DNA is denatured by treatment with alkali and removed in the form of a salt precipitate. Both chromosomal and plasmid DNA are denatured by alkali treatment but following neutralization with potassium acetate the plasmid, but not the chromosomal DNA, regains its super helical conformation. The reason is that the two strands of the plasmid DNA cannot be separated because of their super coiling.

CHEMICALS REQUIRED:

Solution I (50 mM TRIS pH 8.0, 10 mM EDTA, 0.1 g/L RNAse A)
Solution II (200 mM NaOH, 1% w/v SDS)
Solution III (ice cold; 3 M potassium acetate, pH 5.5)
100% isopropanol, 70% ethanol, Sterile ddH2O

1. Using a sterile tooth pick, innoculate a single bacterial colony from a plate with antibiotic selection into 2 mL LB broth with appropriate antibiotic selection (see table at end) in a sterile culture tube.
2. Incubate overnight at 37°C (if *E. coli*; 28°C if *Agrobacterium*) with shaking at 250 rpm.
3. Vortex each culture briefly and decant into labeled 2.2 mL microfuge tubes.
4. Centrifuge at fμll speed for two min to harvest cells. Aspirate off the supernatant.
5. Resuspend the cell pellet in 200 μL of Solution I by vortexing.

6. Add 200 μL of Solution II and mix gently by inversion. (This step lyses the cells, and the suspension should change from opaque to translucent. Gentle mixing is important to prevent shearing the high molecular weight DNA.)
7. Add 200 μL of Solution III and mix gently, but thoroughly, by inversion. (A white, flocculent, precipitant, consisting of cellμlar components and high molecular weight DNA, will form. Gentle but thorough mixing is important.)
8. Place the samples on ice for 5 min.
9. Centrifuge the tubes for 10 min. (In preparation for step 10, label 1.6 mL microfuge tubes and add 0.7 volumes of isopropanol [~600 μL x 0.7 = 450 μL, but round up to 450 μL and use a repeater pipet.])
10. Carefully transfer the supernatant from step 9 to labeled 1.6 mL microfμge tubes containing 450 μL isopropanol, avoiding any precipitated material, and mix thoroughly by inversion. Discard the old tubes.
11. Let the new tubes stand for five minutes, and centrifuge at full speed for 10 min to pellet the plasmid DNA.
12. Aspirate off the supernatant being careful not to disturb the precipitated DNA, which may form a visible pellet, or a smear on the side of the tube.
13. Gently wash DNA pellet with ~1 mL 70% ethanol (using a 10 mL glass pipet to fill the tube works well—do not mix as this may dislodge the pellet). Remove as much of the 70% ethanol as practical.
14. Dry the pellet in a vacuum centrifuge for five minutes, or air-dry on bench for >30 minutes. Pellets that are not thoroughly dry will not resuspend easily, and residual ethanol can affect subsequent manipulations.
15. Resuspend the DNA in 50 μL ddH_2O and store at -20^{o}C (TE buffer and TRIS pH 8.0 are also commonly used; the EDTA in TE can inhibit subsequent reactions)

INFERENCE:

RESULT:

Expt. No.: 12

WESTERN BLOTTING AND HYBRIDISATION WITH ANTISERA

AIM: To detect the presence of desired protein using western blotting and hybridization with antisera technique.

PRINCIPLE:

Certain synthetic membranes bind proteins tightly enough that they can be used as supports for solid-phase immunoassays. Bound proteins retain their antigenicity and are accessible to probes. Techniques that have been developed for probing proteins bound to synthetic membranes are collectively known as "blots". The most common blotting technique for protein is western blotting. In this technique, proteins are transferred from an electrophoresis gel to a support membrane and then probed with antibodies. Hence this technique is otherwise called as "immunoblotting". It combines the resolution of PAGE (1-D or 2-D) with the specificity of immunoassays allowing individual proteins in complex mixtures to be detected and analyzed.

The immunoblotting procedure is as follows. (i) Proteins are transferred from an electrophoresis gel to a membrane surface. The transferred proteins become immobilized on the surface of the membrane in a pattern that is an exact replica of the gel. (ii) Unoccupied protein-binding sites on the membrane are saturated to prevent non-specific binding of antibodies. This step is called either "blocking" or "quenching". (iii) The blot is probed for the protein of interest with a specific primary antibody. (iv) The blot is probed a second time. The second probe is an antibody that is specific for the primary antibody type and is conjugated to a detectable enzyme. The site of the protein of interest is thus tagged with an enzyme through the specificities of the primary and secondary antibodies. (v) Enzyme substrates that are converted into insoluble, detectable products are incubated with the blot. The products leave a colored trace at the site of the band or spot representing the protein of interest

Equipment and reagents

- Electrotransfer apparatus, tank or semidry,with filter papers, sponges and power supply
- Blotting membrane, nitrocellulose or PVDF
- Transfer buffer[a]
- TBS[b] and TTBS[c]
- Primary antibody
- Secondary antibody-enzyme conjugate[d]
- Substrate for the enzyme conjugated to the secondary antibody[e]

A. Transfer buffers.

For tanks (25 m*M* Tris, 192 m*M* glycine, 20% methanol, pH 8.3), dissolve 3.0 g of Tris and 14.4 g of glycine in 800 ml of water, then add 200 ml of methanol.

B. TBS is Tris-buffered saline (0.02 *M* Tris-Cl, 0.5 *M* NaCl, pH 7.5). To make TBS, dissolve 2.4 g of Tris and 29.2 g of NaCl in approximately 800 ml of water. Adjust the pH to 7.5 with HCl and bring the volume to 1 liter with water.

C. TTBS is TBS containing 0.05% Tween 20. Add 0.5 ml of Tween 20 to 1 liter of TBS.

D. The usual enzymes conjugated to antibodies are alkaline phosphatase and horseradish peroxidase.

E. Substrates. For alkaline phosphatase, the substrate is 0.15 mg of BCIP and 0.3 mg of NBT per ml of 0.1 ml Tris-Cl, 0.5 m*M* MgCl2. The buffer consists of 1.2 g of Tris and 10 μl of 4.9 *M* MgCl2 per 100 ml adjusted to pH 9.5 with HCl. Stock BCIP is 30 mg of BCIP (toluidine salt) per ml of dimethylformamide, and stock NBT is 60 mg of NBT per ml of 70% dimethylformamide. To make the substrate solution, add 50 μl of stock BCIP and 50 μl of stock NBT to each 10 ml of buffer. For horseradish peroxidase, the substrate solution contains 0.015% hydrogen peroxide and 0.05% 4-(chloro-1-naphthol) in TBS containing 16.7% methanol. To make this substrate, dissolve 60 mg of 4-(chloro-1-naphthol) in 20 ml of methanol; protect this solution from light. Add 600 μl of 3% hydrogen peroxide to 100 ml of TBS. Mix the two solutions together and use the resultant solution immediately. An alternative substrate s prepared with 50 mg of diaminobenzidine and 100 μl of 3% hydrogen peroxide in 100 ml of TBS.

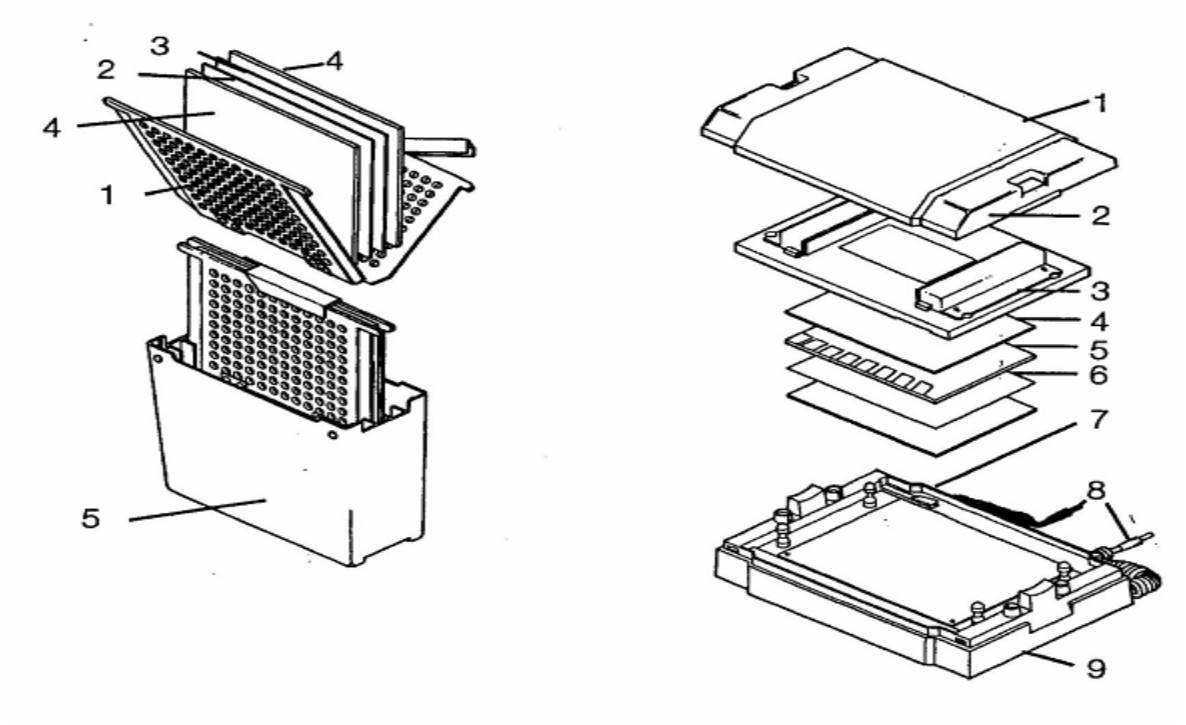
Electrotransfer apparatus

Two types of Electrotransfer apparatus

(A) A tank transfer cell is shown in an exploded view.
The cassette (1) holds the gel (2) and membrane (3) between buffer-saturated filter paper and buffer pads (4). The cassette is inserted vertically in the buffer-filled tank between the positive and negative electrodes (not shown). A lid with connectors and leads for applying electrical power is not shown.

(B) An exploded view of a semidry transfer unit is shown.

The gel (4) and membrane (5) are sandwiched between buffer-saturated stacks of filter paper (3 and 6) and placed between the cathode assembly (2) and anode plate (7). The safety lid (1) attaches to the base (9). Power is applied through cables (8).



(A) TANK TRANSFER

(B) SEMI DRY TRANSFER

PROCEDURE:

1. Prepare transfer buffer appropriate to the electrotransfer apparatus. Refer to the recommendations of the manufacturer of the apparatus or use those given here.
2. Make about 1 liter of buffer more than is required to fill the apparatus. Do not adjust the pH of transfer buffers; just confirm that they are close to the expected pH.
3. Remove the gel from the cassette and soak it in transfer buffer for about 10 min. It is helpful to cut off the stacking gel, if one was used, since the soft gel will stick tothe transfer membrane.
4. Follow the manufacturer's instructions for setting up the transfer apparatus. Cut filter paper to size if necessary. Soak filter paper and sponge pads (if used) in transfer buffer.

5. Cut the transfer membrane to size with a clean, sharp scalpel or razor blade. Do not touch the membrane with bare hands. Use gloves and (or) blunt, flat-blade forceps to manipulate the membrane.
6. Completely wet the transfer membrane with transfer buffer. PVDF must be wetted in methanol prior to being placed in aqueous solutions. Avoid air bubbles in the membrane by slowly sliding it into buffer (or methanol) at a slight angle or by floating it on buffer. Immerse the membrane in buffer and let it soak for 15 min. Do not allow the membrane to dry out before beginning the transfer.
7. Place about 1 liter of transfer buffer in a large tray and assemble the transfer array in it. Use the buffer in the tray to keep all elements of the transfer array well wetted during the assembly process.
8. To avoid trapping air bubbles between the gel and the membrane, lay the membrane on the on the gel from the center to the ends then gently roll a test tube or pipette on top of the membrane to push out pockets of air.
9. Put the transfer array into the transfer apparatus. Follow the manufacturer's instructions for electrotransfer.
10. Wash the membrane for 5-10 min in TBS.
11. Incubate the membrane for 30 min to 1 hr at room temperature in TBS containing 5% (w/v) nonfat dry milk to block excess protein binding sites on the membrane (5 g of nonfat dry milk per 100 ml of TBS).
12. Wash the membrane twice, for 5 min each time, with TTBS.
13. Incubate the membrane for 1 to 2 hr at room temperature with primary antibody or antiserum diluted in TTBS containing 5% nonfat dry milk. Dilutions of primary antibody vary with the source, but are generally of the order of 1:100 to 1: 3,000.
14. Wash the membrane twice with TTBS, for 5 min each time.
15. Incubate the membrane for 1 to 2 hr at room temperature with secondary antibody enzyme
16. conjugate appropriately diluted (*e.g.*, 1:3,000) in TTBS containing 5% nonfat dry milk.
17. Wash the membrane twice, for 5 min each time, with TTBS.
18. Wash the membrane with TBS to remove the Tween 20.
19. Incubate the membrane with substrate solution for about 1 hr or until the desired intensity is obtained.
20. Wash the completed blot with water. Washed immunoblots can be stored dry.

RESULT:

INFERENCE:

DEPARTMENT OF BIOTECHNOLOGY

BT 3561-
IMMUNOLOGY LABORATORY MANUAL

REGULATION 2021

III YEAR & V SEMESTER

MADHA ENGINEERING COLLEGE
MADHA NAGAR
CHENNAI- 600 069

BT3561 **IMMUNOLOGY LAB** **L T P C**

0 0 4 2

AIM

The develop skills of students in Immunology by performing simple experiments in the laboratory.

OBJECTIVES

At the end of the course the student would have gained knowledge to perform techniques like blood grouping, ELISA, & identification of T-cell, Immuno fluorescence etc. This will be of help in facilitating the students for project work.

1. Handling of animals, immunization and raising antisera
2. Identification of cells in a blood smear
3. Identification of blood group
4. Immuno diffusion & immuno electrophoresis
5. Testing for typhoid antigens by Widal test
6. Enzyme Linked Immuno Sorbent Assay (ELISA)
7. Isolation of peripheral blood mononuclear cells
8. Isolation of monocytes from blood
9. Immuno fluorescence
10. Identification of t cells by T-cell rossetting using sheep RBC.

TOTAL : 60 PERIODS

TABLE OF CONTENTS

Expt. No. 1

HANDLING OF ANIMALS

All animals will respond in some way to the presence of a human and most species will be nervous of strangers. Hence it is important to establish a friendly relationship with the animals to reduce nervousness on both sides.Good animal handling techniques will reduce the risk of injury from bites and scratches and reduce stress, increasing confidence of both animal and handler. A relaxed and confident animal will be more co-operative and enable easier workout of procedures.

1. MOUSE

The mouse, *Mus musculus* is the most commonly used laboratory animal. One of its popularity as research animal is that the genome of mouse is well known than any other animals.

Housing

Cages may be of stainless steel or plastic and are usually of shoebox type. Bedding should be provided, such as wood chips or ground corn cobs, which should be screened to ensure that it is uncontaminated before we placing cardboard rolls and objects to climb will enrich the environment and reduce stereotypic behaviour.

Feeding

Mice are fed with complete pelleted mouse diet, suspended above the floor to prevent faecal contamination. Generally, mice will consume 3-5g of pelleted diet and 6-7 ml of water per day. Water is required for lubrication of the food as well as hydration.

Handling

- Grasp the base of the tail gently but firmly and lift the mouse.
- Place the mouse down on a non-slip surface without releasing a tail.
- Slide the thumb and index finger of the other hand up the animal body and grasp the scruff of the neck to restrain the head.
- Extra restraint may be achieved by handling holding the tail with the fourth and fifth fingers.

2. RABBIT

Many strains of rabbit are available; commonest of them are Newzealand white and smaller Dutch rabbit. Suitable methods of marking include coloured coats, microchip implants, load dyes, or marker pen.

Housing

Single caging should be used only when group housing is inappropriate, as it may increase risks of trachures and nerve damage. They should always be housed where they can see other rabbits. Excessive cleaning or strong smelling disinfectants should be avoided, as it may reduce their secured feeling. Housing should be cleaned weekly once at least.

Feeding

Rabbits are coprophagic and require a diet with high fibre content. A diet of 12-22% fibre, 12% proteins for maintenance or 15-17% for growth is recommended. They need 6.8g of high energy food per 100g of body weight and 10ml/100g body weight.

Handling

- Rabbit should never be lifted by ears, without any support to the back, as it may injure the spine.
- Grasp the animal by the scruff and lift the front end.
- Place the free hand beneath the animals hind quarters and the animal scooped toward the handler.

After clear lifting, it should be held against chest or resting on the arm with its head tucked under armpit, with scruff securely held.

Expt. No. 2

BLOOD SMEAR PREPARATION AND IDENTIFICATION OF BLOOD CELLS

AIM:

To prepare blood smear and identify the blood cells.

INTRODUCTION:

The blood consists of a suspension of special cells in liquid called plasma. In an adult man, the blood is about 1/12th of the body weight and this corresponds to 5-6 litres. Blood consists of 55 % plasma, and 45 % by cells called formed elements. The blood performs a lot of important functions. By means of the hemoglobin contained in the erythrocytes, it carries oxygen to the tissues and collects the carbon dioxide (CO_2). It also conveys nutritive substances (e.g. amino acids, sugars, mineral salts) and gathers the excreted material, which will be eliminated through the renal filter. The blood also carries hormones, enzymes and vitamins. It performs the defense of the organism by mean of the phagocitic activity of the leukocytes, the bactericidal power of the serum and the immune response of which the lymphocytes are the protagonists.

The Plasma

Cells free serum or plasma, can be obtained by centrifugation. The plasma is a slightly alkaline fluid, with a typical yellowish color. It consists of 90 % water and 10% dry matter. Nine parts of it are made up by organic substances, whereas one part is made up by minerals. These organic substances are composed of glucides (glucose), lipids (cholesterol, triglycerides, phospholipids, lecithin, fats), proteins (globulins, albumins, fibrinogen), glycoproteins, hormones (gonadothropins, erythropoietin, thrombopoietin), amino acids and vitamins. The mineral substances are dissolved in ionic form, that is dissociated into positive and negative ions.

The Hematic cells

In the blood are present special cells, classified in: **erythrocytes** and **leukocytes**. There are also **platelets**, which are not considered real cells. In the following, we will deal the different categories of blood cells.

Erythrocytes (red cells)

The erythrocytes are the most numerous blood cells i.e. about 4-6 millions/mm^3. They are also called red cells. In man and in all mammals, erythrocytes are devoid of a nucleus and have the shape of a biconcave lens. In the other vertebrates (e.g. fishes,

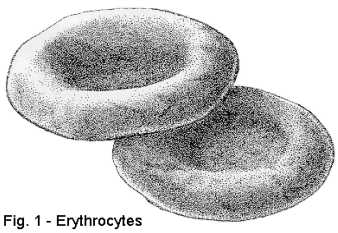


Fig. 1 - Erythrocytes

amphibians, reptilians and birds), they have a nucleus. The red cells are rich in hemoglobin, a protein able to bind in a faint manner to oxygen. Hence, these cells are responsible for providing oxygen to tissues and partly for recovering carbon dioxide produced as waste. However, most CO_2 is carried by plasma, in the form of soluble carbonates.

In the red cells of the mammalians, the lack of nucleus allows more room for hemoglobin and the biconcave shape of these cells raises the surface and cytoplasmic volume ratio. These characteristics make more efficient the diffusion of oxygen by these cells. In so-called "sickle-cell anaemia", erythrocytes become typically sickle-shaped. With the electron microscope, biologists saw that red cells can have different shapes: normal (discocyte), berry (crenated), burr (echinocyte), target (codocyte), oat, sickled, helmet, pinched, pointed, indented, poikilocyte, etc. The mean life of erythrocytes is about 120 days. When they come to the end of their life, they are retained by the spleen where they are phagocyted by macrophages.

Platelets

The main function of platelets, or thrombocytes, is to stop the loss of blood from wounds (hematostasis). To this purpose, they aggregate and release factors which promote the blood coagulation. Among them, there are the serotonin which reduces the diameter of lesioned vessels and slows down the hematic flux, the fibrin which trap cells and forms the clotting. Even if platelets appear roundish in shape, they are not real cells. In the smears stained by Giemsa, they have an intense purple color. Their diameter is 2-3 µm about, hence they are much smaller than erythrocytes. Their density in the blood is 200000-300000 /mm^3.

Leukocytes (white cells)

Leukocytes, or white cells, are responsible for the defense of the organism. In the blood, they are much less numerous than red cells. The density of the leukocytes in the blood is 5000-7000 /mm^3. Leukocytes divide in two categories: granulocytes and lymphoid cells or agranulocytes. The term granulocyte is due to the presence of granules in the cytoplasm of these cells. In the different types of granulocytes, the granules are different and help us to distinguish them. In fact, these granules have a different affinity towards neutral, acid or basic stains and give the cytoplasm different colors. So, granulocytes distinguish themselves in neutrophil, eosinophil (or acidophil) and basophil. The lymphoid cells, instead, distinguish themselves in lymphocytes and monocytes. As we will see later, even the shape of the nucleus helps us in the recognition of the leukocytes.

Each type of leukocyte is present in the blood in different proportions:

Neutrophil	50 - 70 %
Eosinophil	2 - 4 %
Basophil	0,5 - 1 %
Lymphocyte	20 - 40 %
Monocyte	3 - 8 %

Neutrophils are very active in phagocyting bacteria and are present in large amount in the pus of wounds. Unfortunately, these cells are not able to renew the lysosomes used in digesting microbes and dead after having phagocyted a few of them.

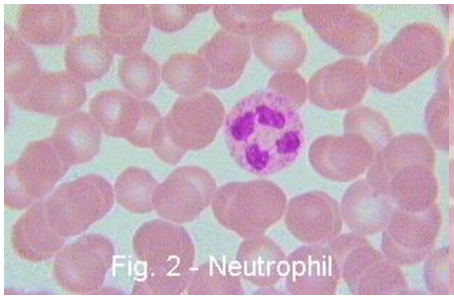


Fig. 2 - Neutrophil

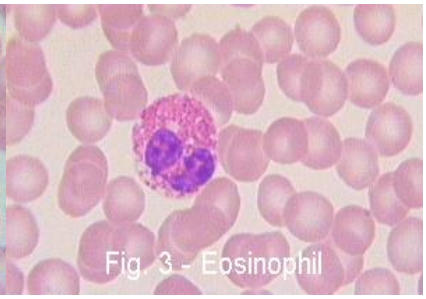


Fig. 3 - Eosinophil

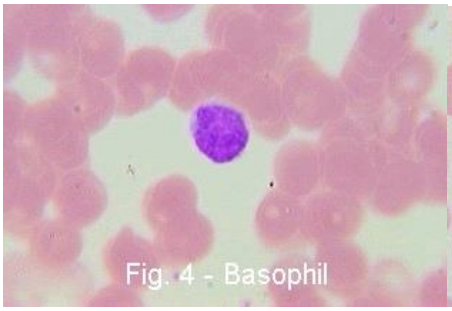


Fig. 4 - Basophil

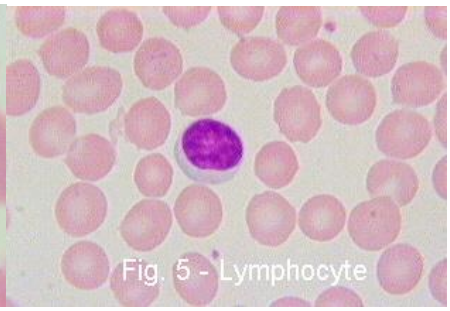


Fig. 5 - Lymphocyte

Eosinophils attack parasites and phagocyte antigen-antibody complexes.

Basophil secrete anti-coagulant and vasodilatory substances as histamines and serotonin. Even if they have a phagocytory capability, their main function is secreting substances which mediate the hypersensitivity reaction.

Lymphocytes are cells which, besides being present in the blood, populate the lymphoid tissues and organs too, as well as the lymph circulating in the lymphatic vessel. The lymphoid organs include thymus, bone marrow (in birds bursa), spleen, lymphoid nodules, palatine tonsils, Peyer's patches and lymphoid tissue of respiratory and gastrointestinal tracts.

Most lymphocytes circulating in the blood is in a resting state. They look like little cells with a compact round nucleus which occupies nearly all the cellular volume. As a consequence, the cytoplasm is very reduced. The lymphocytes of the lymphoid tissues and organs can be activated in a different amount following antigenic stimulation. In the blood, lymphocytes are 20-40 % of all leukocytes and are slight larger than red blood cells.

The lymphocytes are the main constituents of the immune system which is a defense against the attack of pathogenic micro-organisms such as viruses, bacteria, fungi and protista. Lymphocytes yield antibodies and arrange them on their membrane. An antibody is a molecule able to bind itself to molecules of a complementary shape called antigens, and recognize them. As for all proteins, even the antibodies are coded by genes. On the basis of a recombination mechanism of some of these genes, every lymphocyte produces antibodies of a specific shape.

Hence, lymphocytes perform an action which is called specific in that each of them recognize the complementary antigen only. Even if every lymphocyte is so selective to recognize only one molecule, the number of circulating lymphocytes is so large that they are able to recognize practically all substances which are in the organism, both its own and foreign. It is a question of recognizing hundreds of millions of different molecules.

The cells of the immune system, chiefly lymphocytes, cooperate amongst themselves to activate, boost or make more precise the immune response. To attain this scope, there exist different types of lymphocytes, with different functions: T and B lymphocytes. When the B cells are activated, they breed quickly (clonal selection) and they become plasmacells which secrete a great deal of antibodies in the blood stream (humoral response**)**. When free antibodies meet micro-organisms with complementary shape (epitopes), they bind to them and form complexes which immobilize the micro-organisms. Later, other cells which are not specific, but which are able to recognize antibodies,phagocyte these complexes.

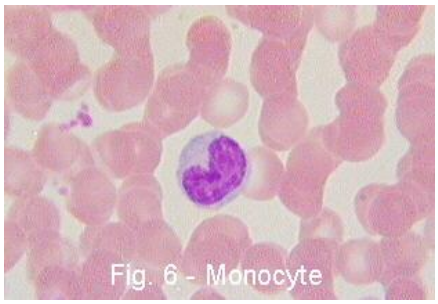
In their turn, the T cells are divided into three categories: Tc (cytotoxic), Th (helpers), Ts (suppressors). Even the Cytotoxic lymphocytes breed quickly when they are activated. They do not release antibodies in the bloodstream, but they keep the antibodies on their membrane and use them to recognize cells mainly of its own organism infected by virus or tumoral cells. The cytotoxic lymphocytes kill cells by means of the release of perforins, substances which produces lesions in the membrane of the target cell and cause its death by osmotic lysis **(**cell-mediated response**)**. The helper lymphocytes are needed to activate both B and Tc lymphocytes which, even though they recognize extraneous agents, seldom enter into direct action. Suppressor lymphocytes reduce the intensity of the immune response.

However, the immune system must not attack the cells of it's body as the autoimmune reaction can damage the organism and lead to death. How does the immune system distinguish between self and not self? We have seen that B and Tc lymphocytes which have recognized an antigen, do not enter in action, but they need to be activated by a helper lymphocyte. A few times after the organism's birth, some of the new lymphocytes pass through the thymus where they become T lymphocytes. Here, these cells are compared with all antigens of the organism (autoantigens). It seems that lymphocytes which recognize an antigen, as they are still immature, will die. In this way, as the autoreactive Th lymphocytes are been killed, only the B and Tc lymphocytes which have recognized extraneous antigens can be activated. The system of cellular cytotoxicity mediated by Th cells is evolved as a defense against their own infected, modified or aberrant cells. In fact, B and Tc lymphocytes can activate themselves against bacteria even without the agreement of the helpers.

The B and Tc activated lymphocytes, besides to producing antibodies and killing foreign cells, multiply quickly. During the cellular division, rearrangements often occur in the sequence of the genes, which code for the antibody. In this way, the antibody of the new cell takes a slightly different shape in comparison to that of its "mitotic parent". If the new shape matches the antigen better, this cell will be induced to divide more. The next generation of clones is therefore more efficient and, in its turn, can induce more selective varieties. This process and that of clonal selection make the immune response more effective. Finally, the immune system produces memory cells, i.e. deactivated lymphocytes ready to be reactivated on the occasion of further meeting with the same antigen.

Besides the Th and B cells, there is a third population of lymphocytes in the peripheral blood and lymphoid organs which do not have receptors for antigens. These lymphocytes have a non-specific defense function which is not activated by Th lymphocytes. These cells represent the more ancient

component of the immune system and they are characterized by their cytotoxic activity. For these reasons, they are named NK, Natural Killer. Apart from killing viruses, bacteria, infected and neoplastic cells, these lymphocytes also regulate the production of other hematic cells such as erythrocytes and granulocytes.



Monocytes are the precursors of macrophages. They are larger blood cells, which after attaining maturity in the bone marrow, enter the blood circulation where they stay for 24-36 hours. Then they migrate into the connective tissue, where they become macrophages and move within the tissues. In the presence of an inflammation site, monocytes quickly migrate from the blood vessel and start an intense phagocytory activity. The role of these cells is not solely in phagocytosis because they have also have an intense secretory activity. They produce substances which have defensive functions such as lysozyme, interferons and other substances which modulate the functionality of other cells. Macrophages cooperate in the immune defense. They expose molecules of digested bodies on the membrane and present them to more specialized cells, such as B and Th lymphocytes.

Materials Required

- Sterilized lancet or needle
- Clean microscope slides and coverslips
- Canada balsam or other medium for permanent preparations 95% ethyl or methyl alcohol
- Distilled water
- Giemsa stain
- Microscope which magnifies 200 times at least

PROCEDURE

Drawing the Blood

All the materials are kept ready to protect from dust, particularly the clean microscope slides. The

finger is cleaned with a sterile swab and the puncture is made on the finger tip.

Making the Smear

A small drop of blood is placed near the end of the slide, and the edge of another slide is brought in contact with the drop and the drop is allowed to spread evenly behind the spreader. The angle between the two slides has to be 30-40 degrees. Now, push to the left in a smooth, quick motion. The smear should cover about half the slide.

It is important that the quantity of blood is not excessive, otherwise the red cells could hide the leukocytes. With the microscope, you should observe the smear to check that some of them are properly made. The red cells must not overlap each other, nor be so scared to spread out.

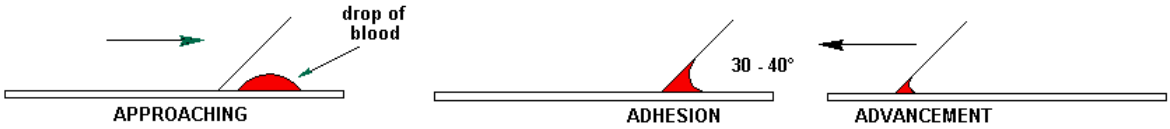


Fig. 7 - How to prepare a blood smear

Fixing

If the stain is applied to a smear without having fixed it beforehand, the cells will explode because of the so-called osmotic or hypotonic shock. This happens because the saline concentration inside the cells is much higher than that of staining fluid which is diluted in distilled water. In the attempt to equal the internal saline concentration to the values of the external one, the cells undergo swelling by osmosis. To attain the same saline concentration of the external liquid, the cells should swell more than their membrane allows, in fact they explode. The cell contents are released, and the preparation becomes unusable. To avoid this, before staining, smear has to be fixed. This operation hinders the inflation of the cells which keep sound when they are stained. A simple and effective fixing technique consists of dipping the smear in a vessel containing 95% ethyl or methyl alcohol for 3-5 minutes. In order to put alcohol on the smear, you can also use a dropper or a bottle dispenser.

Staining

The erythrocytes are slightly visible but the leucocytes are too pale, almost invisible and you will not see anything inside them. To be able to observe and recognize the different kinds of leukocyte, you must stain them. For this purpose, normally Giemsa stain is used. It is a mixture of stains, based on methylene blue and eosin. It is cheaply available commercially in volumes of 100 cc. It consists of a concentrated solution which you have to dilute in the proportion1/10, that is one part of Giemsa in nine of distilled water, or buffer solution (pH = 6.8-7.2). You can buy the stain in a store of chemicals

and laboratory equipment.

To stain a smear, take a slide with a fixed and dry smear. Put on the slide a drop of stain until it is fully covered. Stain for about 16 minutes, renewing the stain about four times. Then rinse the slide with distilled water at room temperature. Drain off the water and leave the slide to dry.

OBSERVATION

A magnification of 200 times is enough to allow you to observe and identify the different types of cells. If you use a higher power, you can also see the cells details better. You can examine either with dry objectives or with the oil immersion technique. In this last case, if you have put on a coverslip, you must wait a day to allow the balsam to set, otherwise, when you move the slide, oil will displace the coverslip.

Erythrocytes

The red cells are very numerous in the blood. Usually, they measure 6,6-7,5 µm in diameter. However, cells with a diameter higher than 9 µm (macrocytes) or lower than 6 µm (microcytes) have been observed. In the observation field of the microscope, you will see a lot of erythrocytes and, sometimes, some isolated leukocytes. Erythrocytes are without nucleus (among vertebrates, only the red cells of mammalians are lacking a nucleus). Their typical shape is that of a cake depressed in the center (fig. 1). Under the microscope, they look like pink discs clearer in the middle (fig. 2-6: pink cells around the leukocytes). Sometimes, they are piled up like coins. As we saw, the red cells can also have different shapes from those we described. Sometimes, this is normal, other times; this is due to diseases or to defective process of preparation and staining of the smear.

Platelets

Platelets are not true cells. They gemmate from big leukocytes called megakaryocytes. They are small sized diskettes about 3µm in diameter. They appear a purple color and are more intense than red cells (you can see some platelets in figures 5 and 6).

Leukocytes

Unlike red cells, leukocytes have a nucleus. It is easily visible under the microscope, but only after having stained the smear. The nucleus of these cells can show multiple lobes, or be indented or kidney-shaped (reniform). Usually, the shape of the nucleus of various kind of leukocytes is different. Together with the different colors of granules, the shape of nucleus helps us to recognize these cells. Leukocytes are divided into granulocytes and lymphoid cells. In the drawings which follow, besides nuclei and granules, you can see even mitochondria, Golgi apparatus, endoplasmic reticula and

ribosomes.

Granulocytes

They come from the bonemarrow.Their cytoplasm is rich in granules, which take typical colors which help their recognition. The nucleus is condensed in a little masses or **lobes**. In the blood, there are immature cells as well. They distinguish themselves by having a less segmented nucleus. As we have said, there are three types of granulocyte: neutrophil, eosinophil, basophil.

Neutrophil Granulocytes

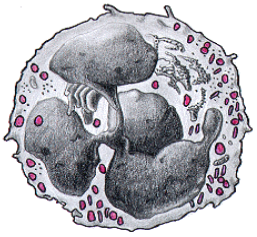


Fig. 8 - Neutrophil

The neutrophil are the more common leukocytes. They have a diameter of 12-15 µm. You can recognize them as their nucleus is divided into 2 - 5 lobes connected by a fine nuclear strand or filament (fig. 8). The cytoplasm is transparent because its granules are small and faintly pink colored. Immature neutrophils have a band-shaped or horseshoe-shaped nucleus and are known as **band cells**. In the nucleus of the neutrophil of cells from females, you may see an appendage like a little drumstick. It is the second X chromosome, inactivated.

Eosinophil Granulocytes

The eosinophils are quite rare in the blood. They have the same size as the neutrophils. Generally their nucleus is bi-lobed. But even nuclei with three or four lobes have been observed. The cytoplasm is full of granules which assume a characteristic pink-orange color (fig. 9). As for the neutrophil, the nucleus is still easily visible.

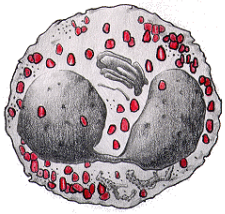


Fig. 9 - Eosinophil

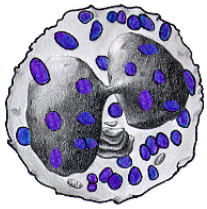


fig. 10 - Basophil

Basophil Granulocytes

Basophils are the rarest leukocytes: less than 1 %. They are quite small: 9-10 µm in diameter. Cytoplasm is very rich in granules which take a dark purple color. The nucleus is bi- or tri-lobed, but it is hard to see because of the number of granules which hide it (fig. 10).

Lymphoid Cells (or agranulocytes)

Because these cells appear lacking in granules, they are also named agranulocytes. They have a compact nucleus and a transparent cytoplasm. There are two types of lymphoid cells: lymphocytes and monocytes. Their look is similar, but their origin is different. In fact, whereas lymphocytes spring from lymphatic organs, monocytes have the same origin as the granulocytes.

Lymphocytes

Lymphocytes are quite common in the blood: 20-40%, 8-10 µm in diameter and generally they are smaller than the other leukocytes but they are still a few larger than red cells (fig. 11). The cytoplasm is transparent. The nucleus is round and large in comparison to the cell and it occupies most of it. In any case, some of the cytoplasm remains visible, generally in a lateral position. According to the quantity of cytoplasm, lymphocytes are divided into small, medium and large. With Giemsa stain, we cannot distinguish the different types of lymphocyte (B, T, NK), either in the blood because they are not activated, or because it would be necessary to perform special immunochemical staining.

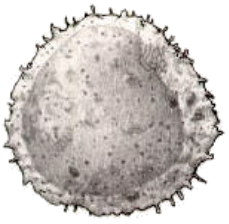


Fig. 11 - Lymphocyte

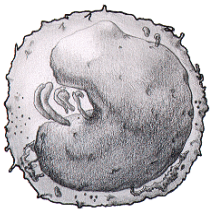


Fig. 12 - Monocyte

Monocytes

Monocytes are the biggest leukocytes: 16-20 µm. They have a great reniform or horseshoe-shaped nucleus, in some cases even bi-lobed. The cytoplasm is transparent, but with an appearance of "ground glass" (fig. 12).

CONCLUSION

Preparation of blood smear was done and different types of blood cells were observed under microscope.

Expt. No. 3

BLOOD GROUPING

AIM:

To determine the ABO blood group and Rh factor of the blood sample through agglutination reaction.

INTRODUCTION

The human blood varies among individuals due to genetic difference. There are nearly 300 blood group systems discovered so far. Some of the blood grouping systems is ABO groups, MN, SS, Lewis, kell and Duffy groups [minor blood groups] and Rh antigen. Of these ABO and Rh are the major, clinically significant and most important of all the blood group system.

Transfusion of incompatible blood into patient may result in the destruction of the transfused cells by hemolysis or by opsonization and increased phagocytosis. They can also result in fever and or alergie reaction. To avoid transfusion reactions the donor's and recipient's blood groups are cross-matched primarily for ABO and Rh compatibility.

All people (with few exceptions) of ABO system can be divided into 4 major groups. They are A, B, AB and O group. This depends on the agglutination reaction obtained by mixing their blood cells with two different reagents, known as anti-A and anti-B serum.

Rhesus factor-Rh: The Rhesus (Rh) group system is clilnically the second most important blood group system in humans, after the ABO system. Rh is a highly complex system in which more than 110 antigens have been identified. The five more common are-Rho, rh', rh", hr', hr". Rh factor is tested by using Anti-D monoclonal serum. If it agglutinates, it indicates a positive test and presence of Rho antigen. Absence of agglutination indicates negative test and absence of Rho antigen.

It is based on agglutination. RBCs of the blood group 'O' contain surface antigen called H-antigen. The serum of this group contains anti-A and anti-B immunoglobulins. RBCs of the blood group A contain surface antigen, A which is a modified H-antigen. The serum contains anti-B immunoglobulins and no anti-A immunoglobulins as it will lead to autoimmunity. RBCs of the blood group B contain a surface antigen B. The serum contains anti-A immunoglobulins and no anti-B immunoglobulins, as it will lead to autoimmunity. RBCs of blood group AB contain both antigen A and antigen B as surface markers and neither anti-A nor anti-B immunoglobulins in the serum.

These antigens can be identified by agglutination reaction using specific immunoglobulin against them. Blood of group A will agglutinate when mixed with anti serum-A. Similarly, blood of group B will agglutinate when mixed with antiserum-B. Blood of group O will agglutinate neither with

antiserum A nor antiserum B.

Apart from ABO antigens, the other antigen of profound influence in blood transfusion is Rh factor. Blood groups with Rh antigen on the RBCs are deseribed as Rh+ and those without it as Rh-. The Rh+ individnals do not harbour anti-Rh immunoglobutins in serum. Mixing the blood with anti Rh immunoglobulins can identify rh factor. Agglutination indicates Rh+ absence of agglutination indicates Rh-.

REAGENTS

- Reagents: Antiserum A (monoclonal), antiserum B (monoclonal), antiserum D (monoclonal).
- Sample: Blood obtained by finger prick.
- Glass slides, applicator sticks, cotton, 75% alcohol, dettol solution, lancent.

PROCEDURE

Sterile glass slide was taken and wiped with 70% alcohol.

The slide was divided into three halves. Circles of about 1.5 cm diameter was drawn and marked as A, B and D respectively.

A drop of the antiserum (commercially prepared antibody) solutions to the particular blood type within the circle was added.

To obtain blood, the tip of a finger was rubbed with alcohol and pricked with the help of a sterile lancet. Massage the finger to obtain several drop of blood, each drop was placed on circles containing Anti-serum A, Anti-serum B and Anti-serum D respectively.

Immediately Anti-serum and blood in each circle was stirred with clean tooth picks in a rotary motion.

The mixture against a high background for the appearance of red granules, indicating the agglutination or the clumping of red blood cells was observed and the results has been recorded.

RESULT

The blood sample failed to agglutinate with antiserum A and antiserum B. Hence the blood belongs to ‘O’ group. The sample agglutinated with anti-Rh immunoglobulin. This implies that the sample is Rh^{+}. Thus the blood group is O^{-}positive [O^{+}].

Expt. No. 4

SINGLE RADIAL IMMUNO DIFFUSION

AIM

To estimate the concentration of antigen in the given test samples by the technique of radial immunodiffusion.

PRINCIPLE

Single radial immunodiffusion (RID) is used extensively for the quantitative estimation of antigens. The antigen-antibody precipitation is made more sensitive by the incorporation of antiserum in the agarose. Antigen (Ag) is then allowed to diffuse from wells cut in the gel in which the antiserum is uniformly distributed. Initially, as the antigen diffuses out of the well, its concentration is relatively high and soluble antigen-antibody adducts are formed. However, as Ag diffuses farther from the well, the Ag-Ab complex reacts with more amount of antibody resulting in a lattice that precipitates to form a precipitin ring.

Thus by running a range of known antigen concentrations on the gel and by measuring the diameters of their precipitin rings, a calibration graph is plotted. Antigen concentrations of unknown samples, run on the same gel can be found by measuring the diameter of precipitin rings and extrapolating this value on the calibration graph.

MATERIALS REQUIRED

- Agarose, 10 X Assay buffer: standard Antigens, Test antigen and antiserum provided in the kit: gel punch with syringe: glass plate: template
- **Glass ware:** conical flask, measuring cylinder.
- **Reagents:** Alcohol, Distilled water
- **Other requirements:** micropipettes, tips, moist chamber.

PROCEDURE

A. PREPARATION OF AGAROSE GEL PLATES

1. To 10 ml of normal saline, 100mg of agarose was added to prepare 1% Agarose solution.
   The Agarose was boiled until the solution gets completely dissolved and no obvious particles of agarose remain in the the suspension. It is cooled to around 40°C - 50°C (palm bearable warmth)

2. To the cooled molten agarose 200µl of antibody was added and about 9 ml of molten agarose was poured onto the glass microscopic slide kept on a levelled surface.
3. The agarose was allowed to solidify. Before cutting the wells, the slide was kept at 2°C - 8°C for a short period, so as the cut edges of the wells do not breakdown when the agarose plugs are removed.

B.CUTTING WELLS IN AGAROSE

1. The wells on the agarose slide was cut by using a gel punch as per the template provided. The well is 3mm Diameter and 14 mm from the edge of the slide.
2. The agarose plugs was removed from the wells by using the metal cannula attached to a vacuum line. Alternatively 20-guage hypodermic needle was inserted into the plug to lift it free of the agarose gel.
3. Optimal results are obtained if reagents are added to the wells just after the plugs are perfectly removed.

C.FILLING WELLS

1. 10µl of antigen (undiluted) was added to the 1st well with a micropipette.
2. The antigen was diluted and loaded to other wells. The method of dilution: 50µl of normal saline was taken in four wells of a microtitre plate. 50 µl of antigen was added to the first well of microtitre plate and mixed thoroughly, then serially diluted it into another wells. Thus antigen dilutions of 1:2, 1:4 and 1:8 were obtained. into three wells of the 96-well microtitre plate. 50 µl of the diluted antigen from the last well of the microtitre was be discarded.
3. 10 µl of the three diluted antigen was added to the 2 nd, 3 rd, and 4 th wells respectively by using a micropipette.
4. 10 µl of test antigen was added to the 5th (last) well.
5. After the wells are loaded, the slide was kept in a flat-bottomed container such as a plastic breadbox with a tight fitting cover. The interior of the container should be kept moist by keeping a damp paper towel or wet cotton.
6. The slides were kept at room temperature. Lower temperature may slow the formation of precipitin line and prolong the test. The slide was kept undisturbed in the humid box at room temperature for 8-12 hours.

D. OBSERVATION OF SRID TEST

1. The slides were examined after 8-12 hours.
2. The precipitin discs were viewed clearly by keeping the slide on the dark background.
3. The diameter of the precipitin discs will vary with the concentration of antigen present in the sample.

OPTIONAL: If the precipitin discs are not clearly visible, the slide was dipped in 4% enhancer solution. About 30ml of the enhancer solution was added in to a 90mm petri dish and the slide was kept for 5-10 minutes to observe the rings. After enhancer treatment, the rings would be clearly seen. However, the measurement of the ring diameter needs to be made immediately since this enhancement is only a temporary phenomenon.

Sample (Well No.)	Std Ag conc (In mg/ml)	Ring Diameter (in mm)
1st	2.00	12
2nd	1.00	10
3rd	0.50	8
4th	0.25	6
5th-TEST SAMPLE	1.5	11

RESULT

From the standard curve the concentration of antigen in the given sample was found to be 1.5 mg/ml

Expt. No. 5

ROCKET IMMUNOELECTROPHORESIS

AIM:

To determine the concentration of unknown antigen using rocket immunoelectrophoresis.

INTRODUCTION

Rocket immunoelectrophoresis is also known as electro-immunodiffusion. In this technique, an antigen is electrophoresed in a gel containing antibody. This precipitate formed between antigen and antibody has the shape of a rocket, the height of which is proportional to the concentration of antigen in the well. This method is more sensitive and precise than radial immuno diffusion.

One limitation of rocket immuno electrophoresis is the need for the antigen to be negatively charged for electrophoretic movement within the agarose matrix. Rocket immunoelectrophoresis involves a specific antibody mixed with the agarose gel and is allowed to react with the antigen in a well by electrophoresis. During electrophoresis, antigen migrates towards the anode, reacts with the antibody in the gel to form a soluble antigen-antibody complex. The antigen-antibody complex reacts further, with more antibodies to form a lattice of the complex which precipitates in the gel. The height of the rocket is directly proportional to the antigen concentration in the well. If the antigen amount in the well is more, a longer rocket is formed. This is because ther antigen or antigen-antibody complex has to travel a longer distance to react with more amount of antibody in the gel for complete precipitation of the complex.

MATERIALS REQUIRED

Agarose, 1x buffer, antiserum, test antigens, standard antigens provided in kit, Micropipette, moist chamber, Immuno electrophoresis equipment, graph sheet.

PROCEDURE

A. PREPARATION OF AGAROSE GEL PLATES

1. To 5 ml of 1X running buffer, 50mg of agarose was added to prepare 1% Agarose solution. The Agarose was boiled until the solution gets completely dissolved and no obvious particles of agarose remain in the the suspension. It is cooled to around 40°C - 50°C (palm bearable warmth)
2. To the cooled molten agarose 150µl of antibody was added and about 4.5 ml of molten agarose was poured onto the glass microscopic slide kept on a leveled surface.
3. The agarose was allowed to solidify. Before cutting the wells, the slide was kept at 2°C -

8°C for a short period, so as the cut edges of the wells do not breakdown when the agarose plugs are removed.

B.CUTTING WELLS IN AGAROSE

1. The wells on the agarose slide was cut by using a gel punch as per the template provided. The well is 3mm Diameter and 14 mm from the edge of the slide.
2. The agarose plugs was removed from the wells by using the metal cannula attached to a vacuum line. Alternatively 20-guage hypodermic needle was inserted into the plug to lift it free of the agarose gel.
3. Optimal results are obtained if reagents are added to the wells just after the plugs are perfectly removed.

C.FILLING WELLS

1. 10µl of antigen (undiluted) was added to the 1st well with a micropipette.
2. The antigen was diluted and loaded to other wells. The method of dilution: 50µl of normal saline was taken in four wells of a microtitre plate. 50 µl of antigen was added to the first well of microtitre plate and mixed thoroughly, then serially diluted it into another wells. Thus antigen dilutions of 1:2, 1:4 and 1:8 were obtained. into three wells of the 96-well microtitre plate. 50 µl of the diluted antigen from the last well of the microtitre was be discarded.
3. 10 µl of the three diluted antigen was added to the 2 nd, 3 rd, and 4 th wells respectively by using a micropipette.
4. 10 µl of test antigen was added to the 5th (last) well.
5. After filling the wells the slide was kept on the bridge of the electrophoresis tank that has been filled with the running buffer (use 1X running buffer). The antigen containing wells was placed on the cathode (Negative side) of the electrophoresis tank (black terminal).
6. Connect the slide with filter paper strips (Wicks) with the respective chambers of the tank.
7. Electrophoresis at 200-250 V for about 4 hours was carried out and the power supply was disconnected. The slide was removed and kept at room temperature for 2-5 minutes.
8. A precipitin line in the shape of a rocket was formed at some height over the wells loaded with the antigen.
9. This rocket shaped precipitin arc was viewed clearly by keeping the slide on a dark background.
10. The height of the rocket will vary with the concentration of antigen present in the sample.

OPTIONAL: If the precipitin discs are not clearly visible, the slide was dipped in 4% enhancer solution. About 30ml of the enhancer solution was added in to a 90mm petri dish and the slide was kept for 5-10 minutes to observe the rings. After enhancer treatment, the rings would be clearly seen. However, the measurement of the ring diameter needs to be made immediately since this enhancement is only a temporary phenomenon.

Sample in wells (Well No.)	Std Ag conc (In mg/ml)	Length of the Rocket (in Cm)
1st	16	1.6
2nd	12	1.4
3rd	8	1.2
4th	4	1.0
5th-TEST SAMPLE	10	1.3

RESULT

From the standard curve the concentration of antigen in the given sample was found to be 10 mg/ml

Expt. No. 6

WIDAL TEST

AIM

To detect the specific antibodies present in the human serum for *Salmonella typhi* and *Salmonella parathyphi* by rapid slide test using Widal reagents for the diagnosis of Typhoid.

PRINCIPLE

Salmonella typhi & *Salmonella paratyphi* are the causative agents of Enteric Fever. In enteric fever once the patient is on medication it becomes difficult to isolate the organisms. In serological tests the antibodies produced as a result of infection are detected by using the killed bacterial antigens. The antibodies from the patient serum react with the corresponding antigens to cause clumping or agglutination.

The antigens of typhoid and paratyphoid consist of two distinct fractions- the stable somatic 'O 'Antigen and the labile flagellar 'H" antigen. The paratyphoid antigens are further classified into A&B Antigens. In typhoid and paratyphoid, the 'H" antigens type specific where as the 'O" antigen is group specific.

Antigens are the standardized smooth suspension of killed bacterial antigen for qualitative and semi quantitative detection of *S. Typhi* and *S. parathyphi* antibodies. The different colour to each antigen facilitates the differentiation. As undiluted serum is used in Slide test, it is a simple rapid and convenient screening test.

REAGENTS

- Sample: Blood obtained by finger prick.
- Positive and negative control samples provided in kit.
- Glass slides, applicator sticks, cotton, 75% alcohol, dettol solution, lancent.

PROCEDURE

RAPID SLIDE TEST (Screening Test)

The glass slide was wiped with 70% alcohol.

One drop of undiluted test serum in corresponding reaction circles and one drop of positive control (PC) and negative control (NC) serum in the marked PC and NC circles was added.

One drop of antigen O, H, A (H) and B (H) was added in respective circles. Any one of the H antigen (H, AH) or B (H) was added in Circles PC and NC.

The contents of each circle were mixed with separate wooden applicator stick.

The slide was kept undisturbed for 1 minute and agglutination was observed.

INTERPRETATION OF RESULTS

Agglutination with positive control and no agglutination with normal saline, validate test results. No agglutination upto one minute is a negative test for thypoid., and indicates the absence of corresponding antibodies .

Agglutination within one minute is a positive test for thypoid, and indicates presence of corresponding antibodies.

Expt. No. 7

ANTIGEN CAPTURE ELISA

OBJECTIVE

To determine the concentration of antigen by antigen capture ELISA or competitive ELISA method.

PRINCIPLE

ELISA or enzyme linked immunosorbent assay is a sensitive laboratory method used to detect the presence of antigens (Ag) or antibodies (Ab) of interest in a wide variety of biological sampleas. These asays require an immunosorbent ie. Antigen or antibody immobilized on solid surface such as wells of microtitre plates or membranes.

Antigen capture ELISA method is the most useful immunosorbent assay for detecting antigen, since it is 2-5 fold more sensitive than those assays in which antigen is directly bound on to the solid phase. In this assay, constant and limiting amount of antibody is immobilized on to a solid support. A fixed amount of labeled antigen [ie., antigen coupled with enzyme like Horse radish peroxidase (HRP) alkaline phosphatase (ALP) etc.] is added and allowed to compete with unlabeled antigen (standard or test sample) for the immobilized antibody. The amount of labeled antigen bouond is then estimated by a suitable assay for the label. The amount of labeled antigen that binds is inversely proportional to the amount of unlabeled antigen in the reaction mixture. Thus, the estimate of label in the well decreases with increase in the antigen concentration in the standard or test sample.

MATERIALS REQUIRED:

- Standard antigen, 100x antibody, test sample, 1000x HRP Labeled antigen, Blocking buffer, 10x TMB/H202 , Coating buffer, 10 x PBST ,5X Stop solution, microtiter wells.
- Glass Ware: measuring cylinder, test tube
- Reagent: distilled water
- Other Requirements: blotting paper, micropipette, tips

Preparation of sample diluent:

One ml of blocking buffer was taken and made upto a volume of 30 ml with 1 x PBS. This is used to dilute standard antigen and HRP labeled antigen.

Preparation of dilutions of standard antigen:

Stock standard antigen solution of concentration 1 mg/ml was prepared by dilution. This stock solution is diluted further to get a range of diluents concentrations as follows

Dilutions	Conc of std antigen
20ul of 1mg/ml (stock) + 480 µl of sample diluent	40 µg/ml (a)
200 µl of (a) + 800 µl diluent	8µg/ml (b)
500 µl of (b) + 500 µl diluent	4 µg/ml (c)
500 µl of (c) + 500 µl diluent	2µg/ml (d)
500 µl of (d) + 500 µl diluent	1µg/ml (e)
500 µl of (e) + 500 µl diluent	0.5µg/ml (f)
500 µl of (f) + 500 µl diluent	0.25µg/ml (g)
500 µl of (g) + 500 µl diluent	0.125µg/ml (h)

Preparation of Working Concentration of Test Samples

The test sample was diluted individually by mixing 10 µl of the sample with 2 ml of sample diluent.

Dilution Factor = sample : sample diluent

$= 10 * 10^{-6} : 2 * 10^{-3}$

$= 0.01 : 2$

$= 1:200$

Thus the dilution factor is 1:200

Preparation of Reagents

Reagents	Vol. to be taken	Vol. of distilled water to be added
10X TMB/H_2O_2	0.6 ml	5.4 ml
10X PBST	10 ml	90 ml
5X Stop solution	12 ml	48 ml

PROCEDURE

***DAY 1.*Coating of well with antibody**

Antibody of concentration 0 .1 mg /ml was diluted with coating buffer ie, 50 µl of stock was mixed with 4.95 ml of coating buffer, to get a working concentration 0.1 µg /ml.

200 µl of diluted, (1x) antibody was pipetted to each microtitre well (24 wells). The wells were

shaken or tapped to ensure that the antibody solution is evenly distributed over the bottom each well. Microtitre wells was incubated overnight at 4° C.

DAY 2

Blocking the residual binding sites on the well

The well contents were discarded. The wells were rinsed with distilled water 3 times, the water after each rinse drained out.

Each well was filled with 200 µl of blocking buffer and incubated at room temperature for 1 hour.

The well was rinsed 3 times (as in step 4) with distilled water. The water completely drained out by tapping the wells on a blotting paper.

Addition of Antigens to the Walls

100 µl of diluted std antigen (b to h) was added to different wells, and the diluted test sample was also added to an another well. A duplicate of each such addition was done in an another set of wells to verify the results. .

Addition of HRP Labelled Antigen

1x HRP labelled antigen using sample diluent was Prepared i.e. 3 µl of stock (1000x) was mixed with 3ml of sample diluent.

100 µl of 1x HRP labelled antigen was added to all the wells.

The microtitre plate was incubated at room temperature for 30 minutes.

The well contents were discarded. The wells was filled with 1x PBST. Allow to stand for 3 minutes, discard the contents and washing was repeated for 2 more times.

Addition of Substrate and Measurement of Absorbance

Required amount of TMB/H_2O_2 (substrate) solution was diluted to 1x using distilled water.

200 µl of 1x substrate was added to each well.

The plate was Incubated at room temp for 10 minutes.

100 µl of 1x stop solution was added to each well.

The contents of each well were transferred to individual tubes containing 2 ml of 1x stop solution.

Substrate blank was prepared by adding 200 µl of 1x substrate solution to 2.1 ml of 1x stop solution.

The absorbance was read at 450- nm after blanking the spectrophotometer with substrate blank and the readings was recorded as follows.

Sample	Dilution factor	A450		Average
b	1:100	0.078	0.066	0.072
c	1:200	0.063	0.061	0.062
d	1:400	0.052	0.05	0.051
e to h				
Test	1:200	.038	.042	0.04

Calculation of antigen concentration

A_{450} values of standard (b to h) was plotted on y axis (linear scale) vs the concentration of the antigen in µg/ml on x axis (log scale) on a semi-log graph sheet.

From the standard curve, the concentration of the antigen present in the diluted test sample was noted.

Concentration of antigen in the test sample = Concentration of the dilute test sample (in µg/ml from the graph) x dilution factor $/10^3$

For example, from the standard curve, the concentration of dilute test sample antigen was found to be 0.9 µg/ml

Therefore, concentration of antigen in test sample = 0.9 µg/ml x $200/10^3$

= 0.18 mg/ml

RESULT

The concentration of the antigen present in the test sample was found to be 0.18 mg/ml.

Expt.No. 8

ISOLATION OF LYMPHOCYTES FROM PERIPHERAL BLOOD BY FICOLL METHOD

AIM: To separate mononuclear cells from peripheral blood.

PRINCIPLE: Employing a mixture of Ficoll – Hypaque one can obtain gradients of specified density and osmolarity suited to the separation of cell types based on buoyant density. The specific density of the gradient that is needed for successful cell separation depends on the species. Human peripheral blood can be purified from diluted whole blood using Ficoll-Hypaque.

MATERIALS: Phosphate buffer saline (PBS), Blood sample, Ficoll-Hypaque, Heparin, centrifuge, Micropipettes, Sterile tips, Haemocytometer, Microscope.

PROCEDURE: Collect heparanized (10-50 U/ml) peripheral blood by venipuncture.

2. Dilute the blood sample in 1:2 ratios with PBS. Add 1ml of Ficoll-Hypaque solution such that it should form a layer on it.

3. Centrifuge at room temperature at 3000 rpm for 10 - 15 minutes.

4. Lymphocytes and monocytes are recovered at the Ficoll plasma interface where they form a white band. The RBC and PMN cells sediment through Ficoll-Hypaque and form a pellet. Aspirate the cells at the interface without removing Ficoll as it leads to granulocyte contamination.

5. Wash the cells in large volume of PBS at 2000 rpm for 10 minutes. Remove supernatant and mix the pellet. This procedure yields 90-95% pure mononuclear cells with more than 98% viability.

6. Count the cells in the four corners of the slide by loading the sample in hemocytometer.

NORMAL VALUE: 1800 TO 3600 Cells / cu.mm

RESULT: The number of lymphocytes present in the sample is _____.

Expt.No. 9 ISOLATION OF MONOCYTES

AIM:

To isolate monocytes from human peripheral blood sample.

PRINCIPLE: Heparinized or defibrinated whole blood is layered on top of a density gradient material (ficoll/hypaque) and subjected to a centrifugal force. Since erythrocytes (RBC) and PMN are denser than the gradient material and sediment to the bottom of the centrifuge tube. The lighter mononuclear cells (lymphocytes and monocytes) sediment to the plasma density gradient interface. At equilibrium, the mononuclear cells are carefully aspirated and saved.

MATERIALS REQUIRED:

Distilled water, Clinical centrifuge, Micropipette and tips, Monosep, HBSS solution and Acid wash glass beads.

PROCEDURE

1. The blood drawn from the peripheral vein was defibrinated by adding sterile glass beads and it was shaken well for 10-20 minutes until the bead sound stops.
2. The defibrinated blood was diluted in the ratio of 1:2 using PBS.
3. In a sterile fresh centrifuge tube 4ml of monosep was taken and 8ml of defibrinated blood sample was layered on monosep and centrifuged at 1600rpm for 20 minutes.
4. The volume volume was made upto 10 ml with HBSS and centrifuged at 1200 rpm for 5 minutes.
5. The pellet was washed thrice with HBSS and suspended in 3 ml of HBSS. The suspended pellet was stored for further use.
6. A drop of sample was smeared on the slide. Leishman's stain was added on glass slide for 2-3minutes and washed with water. Then it was observed under microscope.

RESULT

Monocytes were isolated and stored for future use. They can be further used for monolayer cell cultivation, cytotoxicity and chemotaxis assays.

Expt. No. 10

ISOLATION AND IDENTIFICATION OF HUMAN T-LYMPHOCYTES BY E-ROSETTE ASSAY

AIM

To isolate and identify the human T-lymphocytes by E-Rosette assay.

PRINCIPLE

Human T-lymphocytes can be identified and counted by their ability to form rosettes with SRBC. The cells have surface receptors CD2 that binds with sheep RBC and forms characteristic floral structure called rosettr were under the microscope (E-rosette). These receptors were earlier called TH-Human –T lymphocyte in the peripheral lymphoid organ bear receptor for SRBC on the surface. Each rosete consists of central to which are attached 3 or more SRBC.

MATERIALS REQUIRED

Lymphosep, PBS buffer, Acid wash glass beads, Conical centrifuge tube, pasteur's pipette, Microfuge tubes, Leishman's stain, Microscopic slides.

LYMPHOCYTE ISOLATION:

1. The blood drawn from the peripheral vein was defibrinated by adding sterile stone beads and it was shaken well for 10 to 20 minutes until the beads sound stops.
2. The defibrinated blood was diluted in the ratio of 1:2 using PBS.
3. In a sterile fresh centrifuge tube 4ml of lymphosep was taken and 8 ml of defibrinated blood sample was layered on lymphosep and centrifuged at 1600rpm for 20 minutes.
4. After centrifugation the interface (upper) peripheral blood lymphocyte was removed using a sterile Pasteur pipette and it was transferred to another centrifuge tube.
5. Then the volume was made upto 10 ml with PBS and centrifuged at 1200 rpm for 5 minutes.
6. The pellet was washed with PBS and the procedure was repeated thrice. The pure pellet was suspended in 5ml of PBS and stored.

E- ROSETTE ISOLATION

1. To 1 part of the sheep blood, 4 parts of PBS buffer was added and centrifuged.
2. The suspernatant was discarded and the pellet was washed with PBS thrice. Finally the SRBC pellet was suspended with 4 parts of PBS buffer.
3. To 0.5 ml of isolated lymphocytes 0.5 ml of SRBC was mixed and incubated at 37°C for 60 minutes.

4. After incubation, the mixture was centrifuged at 5000 rpm for 2 minutes.
5. The pellet was resuspended in PBS (equal volume) and kept in an ice bath or in the refrigerator at 4°C for 5 minutes or overnight.
6. The Rosette was gently transferred to a clean microscopic slide and 2 drops of leishman's stain was added and kept at room temperature for air dry. After 5 minutes, it was gently washed with tap water.
7. Finally the rosette under 40X objective lens was observed

RESULT:

Lyphocytes were isolated by E-rosette method and stored for future use.

DEPARTMENT OF BIOTECHNOLOGY

BT 3661-
BIOPROCESS LABORATORY MANUAL

REGULATION 2021

III YEAR & VI SEMESTER

MADHA ENGINEERING COLLEGE
MADHA NAGAR
CHENNAI- 600 069

LIST OF EXPERIMENTS

S.No	Name Of Experiment
1	Enzyme Kinetics – Determination Of Michaelis-Menten Parameters
2	Effect Of Temperature On Enzyme Activity And Deactivation Kinetics
3	Effect Of Ph On Enzyme Activity
4	Enzyme Inhibition Kinetics
5	Enzyme Immobilization Kinetics – Gel Entrapment
6	Enzyme Immobilization Kinetics – Cross Linking
7	Growth Of Bacteria-E.Coli
8	Growth Of Yeast

Ex.No:1 ENZYME KINETICS – DETERMINATION OF MICHAELIS-MENTEN PARAMETERS

Aim:

To study the Michaelis-Menten kinetics of α-amylase enzyme and hence to determine Vmax and Km.

Theory:

Kinetics of simple enzyme catalyzed reactions is referred to as Michaelis-Menten kinetics or saturation kinetics. These models are based on data from batch reaction weight constant volume in which the initial substrate (So) and enzyme (Eo) concentration are known. Saturation kinetics can be obtained from a simple reaction scheme that involves a reversible step for enzyme – substrate complex formation and a dissociation step of the ES complex.

Michaelis-Menten equation for steady state kinetics approximation is

$$V = \frac{Vmax\ S}{Km+S} \quad .....(1)$$

Where V_{max} – velocity of enzyme reaction of saturating substrate concentration

S - Substrate concentration

Km - Michaelis-Menten constant, the measure of affinity of enzyme for substrate

Km = [S] at V = V_{max} /2 from the graph V versus [S].

Lineweaver – Burk plot:

If is the reciprocal of Michaelis-Menten approximation, A plot of 1/V versus 1/[S] gives slope of Km/V_{max}; and Y-intercept of 1/V_{max} and X intercept of -1/Km.

Eddie – Hofsfee plot:

A plot of V versus s V/[S] results in a line of slope –Km and y-intercept of V_{max} and X intercept of V_{max} /Km.

Hans – Woolf plot:

A plot of [S]/V versus [S] results in a line of slope 1/ V_{max} ; Y intercept of Km/ V_{max} ;

X intercept of –Km.

In this experiment, the glucose formed can be estimated calorimetrically using GOD/POD kit.

Materials Required:

Starch Solution (1 %), DNS, Test tubes, pipette, spectrophotometer and α-amylase.

Procedure:

Calibration chart:

1. Standard glucose solution with varying concentration (0.1-1 mg/ml) was Prepared and makes its volume 2ml with distilled water.

2. 3 ml of DNS reagent was added in various test tubes.

3. Test tube content were kept in boiling water bath for 5 minutes

4. 1 ml of Rochelle's salt solution was added to all the tubes and then cooled

5 Intensity of dark red color was read at 540 nm using calorimeter.

6. A graph was plotted between the optical density and Concentration of the standards.

Study of substrate effect:

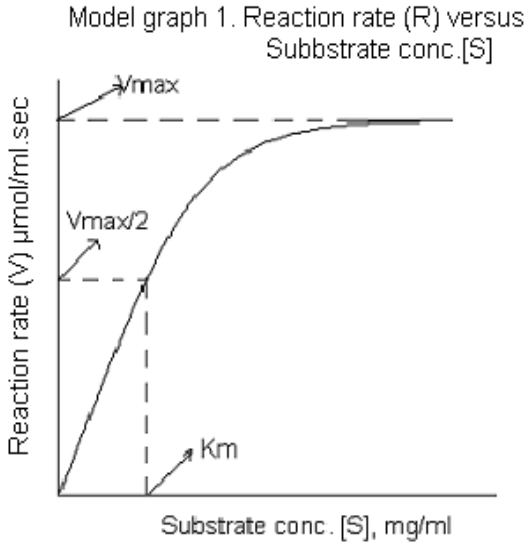
- Prepare 10 ml of 1% starch solution (10 mg/ml). Take 11 test tubes and mark them 0, 1 to10, "0" as blank.
- Add aliquots of 0.1 ml to 1 ml of 1% starch solution to test tubes labeled 1 to 10. Don't add starch solution to the tube'0'
- Add varying volumes of distilled water and raise the final volume of the reaction mixture to 2 ml and add 2 ml of distilled water to blank (0).
- Add 0.5 ml of α-amylase enzyme solution (10 mg/ml) in each tube including blank.
- Mix well and keep the test tubes at 35 – 37 ºC for 10 minutes.
- Add 0.5 ml 0.1 N HCl solution to stop the enzymatic digestion process.
- Find the amount of glucose formed by α-amylase using DNS method.
- Find reaction rate in μmol/ml.sec (V) which is equal to d[P]/dt.
- Plot a graph between reaction rate (V) and substrate concentration [S], and (Lineweaver Burk plot) reciprocal of substrate concentration (1/[S]) and reciprocal of reaction rate (1/V).
- Find the values of Vmax and Km from graphs.

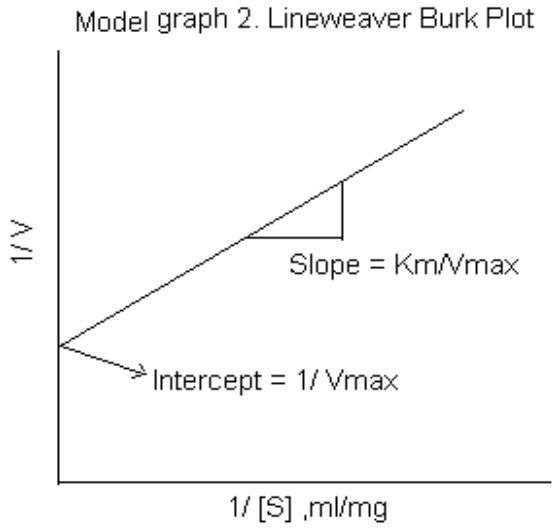
OBSERVATIONS:

TABLE 1.

Sl. No.	Vol. of 1 % starch sol. ml	Sub. Conc. (starch) [S] mg/ml	OD at 505 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] µmol/ml	V µmol/ml.sec	1/V	1/[S]

Model Graph





Model Calculation

Result:

The value of Vmax and Km are,

1. From optical density versus substrate concentration [S] plot

   Km =……mg/ml, and Vmax = …….. µmol/ml.sec

2. From Lineweaver Burk Plot,

   Km=……..mg/ml, and Vmax = …….. µmol/ml.sec.

Ex.No:2 EFFECT OF TEMPERATURE ON ENZYME ACTIVITY AND DEACTIVATION KINETICS

Date:

Aim:

To study the effect of temperature on activity of α-amylase enzyme and to find optimum temperature (T_{opt}), activation energy (Ea) and deactivation rate constant (K).

Theory:

The velocity of enzyme reaction increases when temperature of the medium is increased, reaches a maximum and then decreases due to denaturation. Temperature at which maximum amount of substrate is converted into product is called the optimum reaction time for that enzyme. As temperature is increased, more molecules get activation energy or the molecules at increased rate of motion, so the reaction velocity is enhanced. Denaturation of tertiary structure of protein occurs, when temperature is more than 50 ° C.so activity of enzyme is decreased.

According to Arrhenius equation, effect of temperature on enzyme activity (reaction rate) is given by

$Ka = Ae^{-Ea/RT}$……..(1)

Where, A is Arrhenius constant; Ea is the activation energy in joule/µmol; Ka is deactivation rate constant in min^{-}; R is gas constant ($8.314*10^{-6}$ joule/µmol K); T is temperature in K.

Materials required:

Equipments

- Beakers
- Graduated Cylinder
- Balance
- Pipette
- Calorimeter

Reagents

- Enzyme alpha amylase solution
- Starch (10gm/l)
- Rochelle's salt (40%)
- DNS Reagent: Dissolve by stirring 1g Dinitosalicylic acid, 200 mg of crystalline Phenol and 50 mg sodium sulphite in 100 ml of 1% NaoH, store at 4 °C.

Procedure:

Calibration chart:

1. Standard glucose solution with varying concentration (0.1-1 mg/ml) was Prepared and makes its volume 2ml with distilled water.

2. 3 ml of DNS reagent was added in various test tubes.

3. Test tube content were kept in boiling water bath for 5 minutes

4. 1 ml of Rochelle's salt solution was added to all the tubes and then cooled

5 Intensity of dark red color was read at 540 nm using calorimeter.

6. A graph was plotted between the optical density and Concentration of the standards.

Study of temperature effect:

7. Given sample were diluted to get a corresponding value in the calibration chart.

8. Temperatures from 25 °C to 95 °C were maintained in different 500ml beakers.

9. Starch solution (10g/L) of about 2ml was taken in each of the test tubes and was preincubated for 5 minutes, so that the temperature of the substrate solution is allowed to come to equilibrium with that of the water bath. Then 0.5ml of the enzyme was added in each of the tubes.

10. Reaction time of 10 minutes was allowed and then 3 ml of DNS solution was added to each of the test tubes. The mixture was kept in a water bath at 70 °C for 10 minutes. The reaction mixture was cooled and the color absorbance was measure at 540 nm in calorimeter after addition of Rochelle's salt.

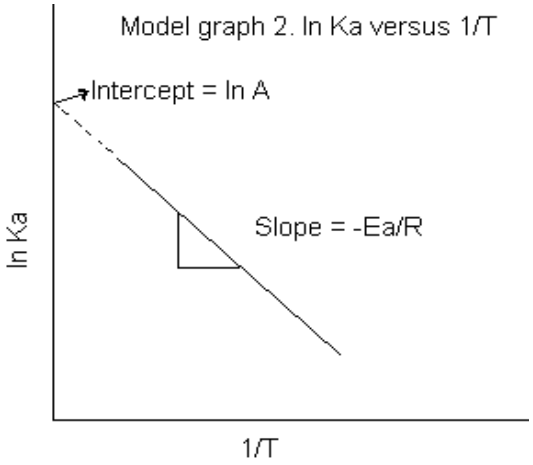
Result:

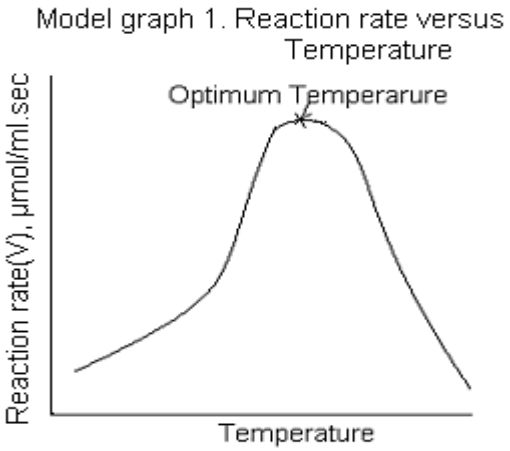
The effect of temperature on activity of α-amylase enzyme was studied and

Optimum temperature (T_{opt}) = ……..ºC,

Activation energy (Ea) = …….. joule/µmol.

Deactivation rate constant (K) = …….. Sec^{-}





Tabulation:

Standard curve:

S.No.	Concentration Of Standard(g/l)	O.D at 540 nm
1		
2		
3		
4		
5		

S.No	**Starch Conc.(50g/L)**	**Temperature (°C)**	**Absorbance @540 Nm**	**Conc. Of Glucose (G/L)**	**Rate Of Reaction (G/L Min)**
1					
2					
3					
4					

5					

ln V	**1/T * 10 ^{3}**

MODEL CALCULATION:

Rate of reaction = concentration / reaction time

= ------ / 10

= -------- g/ L min.

Ex.No: 3 **EFFECT OF pH ON ENZYME ACTIVITY**

Date:

Aim:

To study the effect of pH on α-amylase activity and to find optimum pH

Theory:

Enzymes are amphoteric molecules containing a large acid and basic groups, mainly situated on their surface. The charges on this group will vary according to their acid dislocation constants and with the pH of their environment. This will affect the total net charge of the enzyme and their distribution of charge on their surface, in addition to the reactivity of the catalytic active group. These effects are especially important in the neighborhood of the active site. Take together the changes in the charges with pH effect at the activity, structural stability and solubility of the enzyme.

There will be pH characteristics of each enzyme at which the net charge on the molecule is zero. This is called the isoelectric point [PI] at which enzyme generally has minimum (stability) solubility in aqueous solution. In a similar manner to effect on enzyme, the charge and charge distributions on substrates products and coenzymes will also be affected by pH changes. Increasing hydrogen ion concentration will increase the successful competition at hydrogen ion for any metal cationic binding sites on the enzyme reducing the bound metal cation concentration. DecreasingH_2 ion concentration, on the other hand, leads to increasing hydroxyl ion concentration, which compete against the enzyme ligands for divalent and invalent cations causing their conversion (to ligands) to hydroxides and at very high hydroxyl ion concentration, their complete removal from free enzyme.

Materials and methods:

Equipments

- Beakers
- Graduated cylinder
- Balance
- Pipe

- Syringe.

Reagents

- Enzyme: alpha amylase solution.
- Starch (10g/L) solution.
- Rochelle's salt (40%).
- DNS Reagent: Dissolve by stirring 1g Dinitrosalicylic acid, 200 mg of crystalline Phenol and 50 mg sodium sulphite in 100 ml of 1% NaoH, store at 4 °C.

Procedure:

Calibration chart:

1. Standard glucose solution with varying concentration (0.1-1 mg/ml) was Prepared and makes its volume 2ml with distilled water.

2. 3 ml of DNS reagent was added in various test tubes.

3. Test tube content were kept in boiling water bath for 5 minutes

4. 1 ml of Rochelle's salt solution was added to all the tubes and then cooled

5 Intensity of dark red color was read at 540 nm using calorimeter.

6. A graph was plotted between the optical density and Concentration of the standards.

Study of pH effect:

1. pH buffer solutions of 0.1 M ranging from pH 4 to 8 in increment of one pH unit was prepared with Starch solution (10 g/L) was prepared and Alpha amylase enzyme solution was added in various pH buffers.
2. Reaction time of 10 min was allowed and then 3 ml of the DNS solution was added to each of the test tubes. The mixture was kept in a water bath at 70 0C for 10 min. The reaction mixture was cooled and then the colors absorbance was measured at 540 nm in calorimeter after the addition of 1 ml of Rochelle's salt.

Observation:

S.no	pH of buffer (ml)	10 (g/l) Starch solution (ml)	0.5(g/l) alpha amylase solution (ml)	Absorbance @ 540 nm	Concentration of glucose (g/l)	Rate of reaction (g/lmin)
1. 2. 3. 4. 5	4 5 6 8 9					

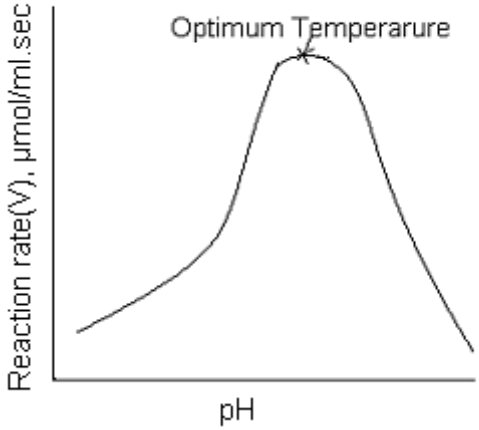
MODEL CALCULATION:

Rate of reaction = concentration / reaction time

= ------- / 10

= ------- g/ L min.

Model graph 1. Reaction rate versus pH



RESULT:

The optimum pH that favored α-amylase activity was found to be……

Ex.No:4 **ENZYME INHIBITION KINETICS**

Date:

Aim:

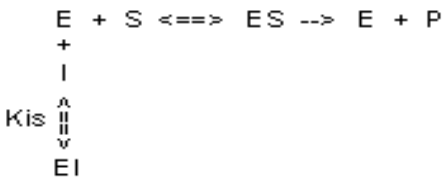
To study the effect of an enzyme inhibitor and determine the type of inhibition by copper sulphate on activity of α-amylase enzyme

Principle:

Certain compounds may bind to enzyme and reduce their activity. The compounds are called enzyme inhibitors. They may be reversible or irreversible. Reversible inhibitors may dissociate more easily from the enzyme after binding. The three major classes of reversible inhibitors are competitive, non-competitive and uncompetitive.

(i) Competitive inhibition:

Competitive inhibitors are molecules that bind to the same site as the substrate, preventing the substrate from binding as they do so but are not changed by the enzyme. They are usually substrate analogs and compete with substrate for active site of the enzyme. In the presence of a competitive inhibitor, it takes a higher substrate concentration to achieve the same velocities that were reached in its absence. So while $\mathbf{V_{max}}$ can still be reached if sufficient substrate is available, one-half $\mathbf{V_{max}}$ requires a higher **[S]** than before and thus $\mathbf{K_m}$ is larger.



$$v = \frac{VmS}{Km(1+I/Kis) + S} \quad \ldots\ldots(1)$$

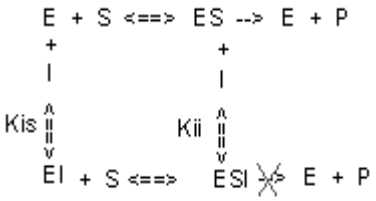
Rate of enzymatic conversion is given equation (1).

Where V_{max} – velocity of enzyme reaction of saturating substrate concentration, S - Substrate concentration, Km - Michelis Menton constant, measure of affinity of enzyme for substrate. Km = [S] at $V = V_{max}/2$ from the graph V versus [S].

(ii) Non-competitive inhibition:

They are not substrate analogs. Noncompetitive inhibitors are molecules that bind to some other site on the enzyme reducing its catalytic power. Physically usually found in allosteric

systems, not so common except in textbooks - usually find mixed. Corresponds to inhibitor binding to E and ES with identical inhibition constants. With noncompetitive inhibition, enzyme molecules that have been bound by the inhibitor are taken out of the game so enzyme rate (velocity) is reduced for all values of **[S]**, including $\mathbf{V_{max}}$ and one-half $\mathbf{V_{max}}$ but $\mathbf{K_m}$ remains unchanged because the active site of those enzyme molecules that have not been inhibited is unchanged. Effect is opposite of competitive, i. e. K_m unchanged, but V_{max} decreased by $1 + I/K_i$.



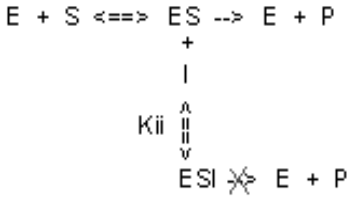
$$v = \frac{VmS}{Km(1+I/Kis) + S(1+I/Kii)} \qquad \text{.....(2)}$$

$$v = \frac{\left(\frac{Vm}{1+I/Ki}\right) S}{Km + S} \qquad \text{if } Kii = Kis$$

The rate of reaction for such a type of inhibition is given equation (2).

(iii) Uncompetitive inhibition:

This type the inhibitor does bind at the active site, but only after the S has bound to the active site so it does not compete with the S. Thus even when the [S] is saturating and all E is in ES, the inhibitor can still bind to the active site and produce an inactive ESI complex. The inhibitor only binds to ES so inhibitor stimulates formation of ES and increases binding of substrate to enzyme so Km is reduced. However the ESI complex is non-productive so Vmax lowered. The double reciprocal plot is series of parallel lines indicating lower Km and decreasing Vmax, and is characteristic of uncompetitive inhibition. The rate of such a reaction is given by equation (3).



$$v = \frac{VmS}{Km + S(1+I/Ki)} \qquad \text{.....(3)}$$

$$v = \frac{\left(\frac{Vm}{1+I/Kii}\right) S}{\left(\frac{Km}{1+I/Kii}\right) + S}$$

V/K_m = unchanged, i.e. both V_{max} and K_m are changed to the same extent.

Materials Required:

Starch Solution (1 %), DNS, Test tubes, pipette, α-amylase Spectrophotometer, alpha-amylase,

1% starch solution and 0.1M copper sulphate.

Procedure:

Calibration chart:

1. Standard glucose solution with varying concentration (0.1-1 mg/ml) was Prepared and makes its volume 2ml with distilled water.

2. 3 ml of DNS reagent was added in various test tubes.

3. Test tube content were kept in boiling water bath for 5 minutes

4. 1 ml of Rochelle's salt solution was added to all the tubes and then cooled

5 Intensity of dark red color was read at 540 nm using calorimeter.

6. A graph was plotted between the optical density and Concentration of the standards.

Study Of Enzyme Inhibition:

- Prepare 10 ml of 1% starch solution (10 mg/ml). Take 11 test tubes and mark them 0, 1 to10, "0" as blank.
- Add aliquots of 0.1 ml to 1 ml of 1% starch solution to test tubes labeled 1 to 10. Don't add starch solution to the tube'0'
- Add varying volumes of distilled water and raise the final volume of the reaction mixture to 2 ml and add 2 ml of distilled water to blank (0).
- Add 0.5 ml of α-amylase enzyme solution (10 mg/ml) in each tube including blank. Mix well and keep the test tubes at 35 – 37 ºC for 10 minutes. Add 0.5 ml 0.1 N HCl solution to stop the enzymatic digestion process.
- Find the amount of glucose formed by α-amylase using DNS method.
- Repeat the above steps with fixed amount of inhibitor solution (0.5 ml of 0.1 M copper sulphate solution) in each test tube.
- Find reaction rate μmol/ml.sec (V) which is equal to d[P]/dt. Plot the Lineweaver Burk plot (reciprocal of substrate concentration (1/[S]) and reciprocal of reaction rate (1/V)) for with and without inhibitor. Find the type of inhibition by given inhibitor, find the values of Vmax and Km for with and without inhibitor from graphs and calculate inhibition constant Ki.

Observations:

Sl. No.	Vol. of 1 % starch sol. ml	Sub. Conc. [S] mg/ml	OD at 540 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] µmol/ml	V µmol/ml.sec	1/V	1/[S]

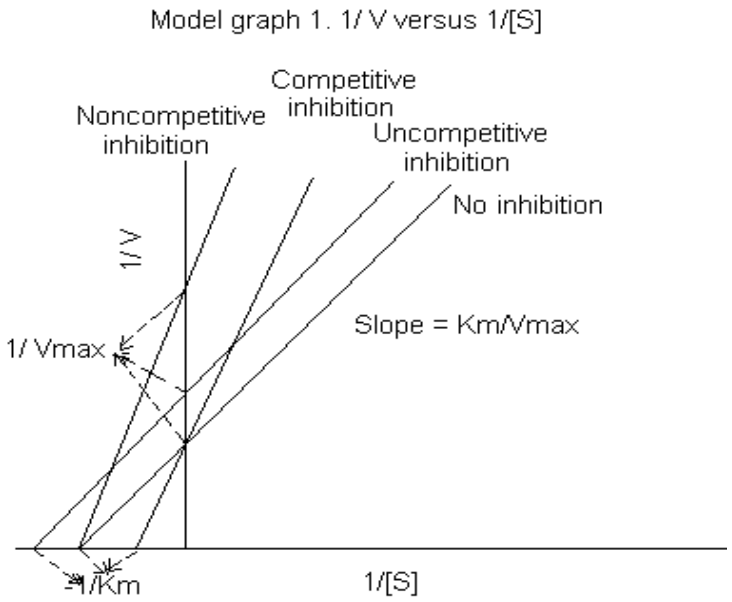
Table 1. Without Inhibitor

Sl. No.	Vol. of 1 % starch sol. ml	Sub. Conc. [S] mg/ml	OD at 540 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] µmol/ml	V µmol/ml.sec	1/V	1/[S]

Table 2. With Inhibitor

Model Calculation:

Model Graph:



Result:

The type of inhibition by copper sulphate was identified as ……..

The inhibition constant Ki for copper sulpahte was found to be …….. µmol/ml

Ex.No:5 **ENZYME IMMOBILIZATION KINETICS – GEL ENTRAPMENT**
Date:

Aim:

To study the effect of immobilization of α-amylase enzyme using calcium alginate, to find maximum reaction rate for free and immobilized enzyme and effectiveness factor (η).

Principle:

Entrapment is the physical enclosure of enzymes in a small space. Matrix entrapment and membrane entrapment, including micro-encapsulation are the two major methods of entrapment. Matrices used for enzyme entrapment are usually polymeric materials such as calcium alginate, agar, carangeenin, polyacrylamide and collagen. When immobilized in a polymer matrix, enzyme solution is mixed with polymer solution before polymerization takes place. Calcium alginate is just as widely used as polyacrylamide. This is the most commonly used method is entrapment in calcium alginate beads. This method does not alter the chemical nature of enzyme.

Sodium alginate + Enzyme + Calcium chloride

↓

Calcium alginate beads entrapped with enzyme.

Unlike polyacrylamide gels, gelation of calcium alginate does not depend on the formation of more permanent covalent bonds between polymer chains. Rather, polymer molecules are cross-linked by calcium ions. Because of this, calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yields of over 80% can be routinely achieved. However, just as easily as calcium ions can be exchanged for sodium ions, they can also be displaced by other ions. This property can both be advantageous and disadvantageous. If needed, enzymes or microbial cells can be easily recovered by dissolving the gel in a sodium solution. On the other hand, proper caution must be exercised to ensure that the substrate solution does not contain high concentrations of those ions that can disintegrate the gel.

Materials Required:

Erlenmeyer flasks, Beakers, Pipettes, Test tubes, Temperature bath, Spectrophotometer,

alpha-amylase, 0.2 M Calcium chloride, 3 % sodium alginate, DNS and 1% starch solution.

Procedure:

Calibration chart:

1. Standard glucose solution with varying concentration (0.1-1 mg/ml) was Prepared and makes its volume 2ml with distilled water.

2. 3 ml of DNS reagent was added in various test tubes.

3. Test tube content were kept in boiling water bath for 5 minutes

4. 1 ml of Rochelle's salt solution was added to all the tubes and then cooled

5 Intensity of dark red color was read at 540 nm using calorimeter.

6. A graph was plotted between the optical density and Concentration of the standards.

Study Of Immobilization Kinetics:

- Dissolve 0.3g of sodium alginate in 10 ml of distilled water to make 3% sodium alginate solution. After sodium alginate is completely dissolved by gentle warming, leave the solution undisturbed for 10 minutes to eliminate the air bubbles that can later be entrapped and cause the beads to float.
- Prepare 50ml of 0.2M calcium chloride solution in a beaker.
- Mix approximately 0.15 g of α-amylase enzyme with 10 ml of 3% sodium alginate solution.
- Add sodium alginate and α-amylase mixture drop wise to calcium chloride solution in the beaker using 1 ml micropipette or syringe at room temperature.
- Allow the beads in calcium chloride solution for 20 min to deplete the excess calcium.
- Remove excess calcium chloride on the surface of the beads by washing with distilled water.
- Transfer the beads to 50ml of 1% starch solution in a beaker, and equal quantity of α-amylase enzyme to 50ml of 1% starch solution in another beaker.
- Withdraw samples at 3 minutes internal from conical flasks with immobilized and free enzyme and find the amount of glucose [P] (µmol/ml) formed using DNS method.
- Calculate reaction rate (V) is equal to d[P]/dt.
- Find Vmax (maximum reaction rate) for both free and immobilized enzyme systems graphs.

❖ Find the effectiveness factor (η), which is the ratio of maximum reaction rate of immobilized enzyme to the free enzyme.

Observations:

Time sec	OD at 540 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] µmol/ml	V µmol/ml.sec

Table 1. Reaction rate of free enzyme

Table 2. Reaction rate of immobilized enzyme

Time sec	OD at 540 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] µmol/ml	V µmol/ml.sec

Model Calculation

Result:

Effect of immobilization of α-amylase enzyme using calcium alginate was studied and the

Maximum reaction rate (Vmax) for free enzyme **=** ……. µmol/ml.sec

Maximum reaction rate (Vmax) for Imm'd enzyme **=** ……. µmol/ml.sec

Effectiveness factor (η) = ……..

Ex.No:6 ENZYME IMMOBILIZATION KINETICS – CROSS LINKING

Date:

Aim:

To study the effect of immobilization of α-amylase enzyme by cross linking, to find maximum reaction rate for free and immobilized enzyme and effectiveness factor (η).

Principle:

Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix. Cross-linking an enzyme is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support, resulting in relatively low enzyme activity. Generally, cross-linking is best used in conjunction with one of the other methods. Preventing leakage from polyacrylamide gels has already been mentioned, but it is used much more widely as a means of stabilizing adsorbed enzymes. Since the enzyme is covalently linked to the support matrix, very little desorption is likely using this method. Marshall (1973), for example, reported that carbamyl phosphokinase cross-linked to alkylamine glass with glutaraldehyde lost only 16% of its activity after continuous use in a column at room temperature for fourteen days.

This technique is based on the polymerization of acrylamide with N,N'-methylene-bis-acrylamide (Bis) as the cross-linking agent. The degree of cross-linking, thus, can be partly controlled by adjusting the ratio of acrylamide to Bis used.

Materials Required:

Erlenmeyer flasks, Beakers, Pipettes, Test tubes, Temperature bath, Spectrophotometer, α-amylase, 1M Tris-HCl, 10% potassium persulphate, 10% SDS, TEMED, bis-acrylamide, acrylamide, DNS and graduated cylindrical Syringe.

Procedure:

Calibration chart:

1. Standard glucose solution with varying concentration (0.1-1 mg/ml) was Prepared and makes its volume 2ml with distilled water.

2. 3 ml of DNS reagent was added in various test tubes.

3. Test tube content were kept in boiling water bath for 5 minutes

4. 1 ml of Rochelle's salt solution was added to all the tubes and then cooled

5 Intensity of dark red color was read at 540 nm using calorimeter.

6. A graph was plotted between the optical density and Concentration of the standards.

Study Of Immobilization Kinetics:

- Monomer Solution: Add 0.8 g of Bis and 29.2 g of acrylamide to 100 ml of distilled water and dissolve completely.
- Dissolve 0.15 gm of α-amylase using 4.2 ml of distilled water in a petri-dish, add 760 µl of 1M Tris-HCl, 60 µl of 10% SDS and 20 µl of TEMED 10%, 1 ml of the buffered monomer solution, mix well and finally add 60 µl of 10% ammonim per-sulphate to initiate polymerization.
- Cover the petri-dish and its content using foil sheet for 20 min to facilitate the polymerization process.
- Cut the resulting gel into small cubes of approximately 3mm per side.
- Gently wash the free enzyme off the gel surface in 10 ml of the washing Solution.
- Transfer all the gel pieces to 50ml of 1% starch solution in a beaker, and equal quantity of α-amylase enzyme to 50ml of 1% starch solution in another beaker.
- Withdraw samples at 3 minutes internal from conical flasks with immobilized and free enzyme and find the amount of glucose [P] (µmol/ml) formed using DNS method.
- Find reaction rate (V) is equal to d[P]/dt.

- Plot a graph of reciprocal of reaction rate (1/V) versus reciprocal of substrate concentration (Lineweaver Burk plot) (1/[S]) for both free and immobilized enzyme systems.
- Find the values of Vmax and Km for both free and immobilized enzyme systems graphs.
- Find the effectiveness factor (η), which is the ratio of maximum reaction rate of immobilized enzyme to the free enzyme.

Observations:

Table 1. Reaction rate of free enzyme

Time sec	OD at 505 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] µmol/ml	V µmol/ml.sec

Table 2. Reaction rate of immobilized enzyme

Time sec	OD at 505 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] µmol/ml	V µmol/ml.sec

MODEL CALCULATION

Result

Effect of immobilization of α-amylase enzyme by cross-linking was studied and the

Maximum reaction rate (Vmax) for free enzyme **=** ……. µmol/ml.sec

Maximum reaction rate (Vmax) for Imm'd enzyme **=** ……. µmol/ml.sec

Effectiveness factor (η) = ……..

Ex.No:7 **GROWTH OF BACTERIA-E.COLI**

Date:

AIM:

To determine the specific growth rate and yield coefficient of the given microbial strain .

INTRODUCTION:

Batch culture system represents growth in a closed system. They use a flask or fermenter containing a suitable growth-supporting medium operated under optimum conditions of temperature, pH and redox potential. The medium is introduced with the cells grown until some essential component of the medium is exhausted or the environment changes because of the accumulation of a toxic product, pH change etc. In general microbial growth is determined by cell dry weight measurement.

The growth of curve can be divided into three phases:

1. **Lag phase**: During this period the cell adapts to the new environment by synthesizing necessary enzymes for the utilization of available substrates.
2. **Exponential phase**: The cell constituents in this phase increases at a constant rate so that the cell population doubles and continues to double at regular intervals.
3. **Stationary phase:** In this phase, cell death occurs because of depletion of essential nutrients, accumulation of toxic etc. The rate of cell death is balanced by the rate cell growth; hence there is no net growth or increase in cell number. This is followed by a death phase.

Specific Growth rate:

The growth of the microbial cells is autocatalytic

The mass balance equation used is:

Cell dry weight accumulation = growth – cell removal – cell death

Here we neglect cell death and no cells are removed in a batch reactor.

Hence

dX/dt = mX, where m is specific growth rate.

On integration we get,

$X = X_0 e^{mt}$,where X_0 is innoculum

$m = (\text{In } x_2 - \text{In } x_1)/(t_2-t_1) = (\text{In } OD_2 - \text{In } OD_1)/(t_2-t_1)$

Substrate utilization

Substrates are consumed to provide the necessary carbon, energy and structural components for the cell growth as well as for the maintenance of cell viability. The single substrate utilization can be determined from

Substrate accumulation = (substrate feed – substrate consumed for growth – substrate for product synthesis – substrate consumed for maintenance – substrate removal)

$ds/dt = FS/V - \mu X/Y_{x/s} - q_pX/Y_{p/s} - mX - F'S'/V$

Where,

F and F' are flow rates of medium into and out of behavior respectively (1/hr)

V is the volume of the culture (litre)

S and S' are the substrates concentration going in and out of the culture (g/l)

$Y_{x/s}$ is the biomass yield coefficient

$Y_{p/s}$ is the product yield coefficient

μ is the specific growth rate ($time^{-1}$)

Q is the product formation rate

In the batch reactor, with no feed or removal, the equation becomes:

$ds/dt = -\mu X/Y_{x/s} - q_p X/Y_{p/s} - mX$

In aerobic cultures if no products are formed and maintenance is negligible, the equation reduces to

$ds/dt = -\mu X/Y_{x/s}$

MATERIAL AND METHODS:

1. BIO REACTOR (Ex-situ type)

The studies that will be reported here were conducted on 5lit (working volume 3.0lit) reactor. This reactor is equipped with flat four-blade Rushton turbine. Height 265mm,diameter of tank 146mm, diameter of impeller 58mm, inter impeller distance 93mm, distance between last impeller and tank 212mm. The tank has two baffles with a width and height of 16 and 25mm.

Pre inoculums:

Type : 15-20% glycerol stock
Medium : LB medium
Culture OD : 0.5
Volume taken : 1 ml

Inoculums details:

Volume : 300 ml
PH : 5.9
Temp : 28ºC
Rpm : 300
Final OD :

Media composition:

Glucose : 0.5g
Yeast extract : 0.5g
Ammonium Nitrate : 0.1g

Fermentation condition:

Initial volume: 3.0 lit Medium + 300 ml of Inoculum
pH : 5.9
VVM : Anaerobic
Temp : 28 ºC
Rpm : 300

After inoculation, readings are taken every hour and tabulated. The values of OD, residual glucose and dry weight were thus recorded.

RESULT:

The specific growth rate and yield coefficient of the given microbial strain are determined through the Batch reactor.

INFERENCE:

For the batch reactor the following graphs are drawn.

i Variation of various parameters as a function of time.
ii ln OD Vs Time graph is plotted for the calculation of μ
iii Biomass Vs residual substrate concentration graph is plotted for $Y_{x/s}$ calculation
iv Using pure culture the quality parameters of the reactor could be verified.

Ex.No:8 **GROWTH OF YEAST**

Date:

AIM:

To determine the specific growth rate and yield coefficient of the given microbial yeast .

INTRODUCTION:

Batch culture system represents growth in a closed system. They use a flask or fermenter containing a suitable growth-supporting medium operated under optimum conditions of temperature, pH and redox potential. The medium is introduced with the cells grown until some essential component of the medium is exhausted or the environment changes because of the accumulation of a toxic product, pH change etc. In general microbial growth is determined by cell dry weight measurement.

Specific Growth rate: The growth of the microbial cells is autocatalytic.

The mass balance equation used is:

Cell dry weight accumulation = growth – cell removal – cell death

Here we neglect cell death and no cells are removed in a batch reactor.

Hence

dX/dt = mX, where m is specific growth rate.

On integration we get,

$X = X_0 e^{mt}$,where X_0 is innoculum

$m = (\ln x_2 - \ln x_1)/(t_2-t_1) = (\ln OD_2 - \ln OD_1)/(t_2-t_1)$

Substrate utilization

Substrates are consumed to provide the necessary carbon, energy and structural components for the cell growth as well as for the maintenance of cell viability. The single substrate utilization can be determined from

Substrate accumulation = (substrate feed – substrate consumed for growth – substrate for product synthesis – substrate consumed for maintenance – substrate removal)

$ds/dt = FS/V - \mu X/Y_{x/s} - q_p X/Y_{p/s} - mX - F'S'/V$

Where,

F and F' are flow rates of medium into and out of behavior respectively (1/hr)

V is the volume of the culture (litre)

S and S' are the substrates concentration going in and out of the culture (g/l)

$Y_{x/s}$ is the biomass yield coefficient

$Y_{p/s}$ is the product yield coefficient

μ is the specific growth rate ($time^{-1}$)

Q is the product formation rate

In the batch reactor, with no feed or removal, the equation becomes:

$ds/dt = -\mu X/Y_{x/s} - q_p X/Y_{p/s} - mX$

In aerobic cultures if no products are formed and maintenance is negligible, the equation reduces to

$ds/dt = - \mu X/Y_{x/s}$

MATERIAL AND METHODS:

1. BIOREACTOR (Ex-situ type)

The studies that will be reported here were conducted on 5lit (working volume 3.0lit) reactor. This reactor is equipped with flat four-blade Rushton turbine. Height 265mm,diameter of tank

146mm, diameter of impeller 58mm, inter impeller distance 93mm, distance between last impeller and tank 212mm. The tank has two baffles with a width and height of 16 and 25mm.

Pre inoculums:

Type : 15-20% glycerol stock
Medium : LB medium
Culture OD : 0.5
Volume taken : 1 ml

Inoculums details:

Volume : 300 ml
PH : 5.9
Temp : 28ºC
Rpm : 300
Final OD :

Media composition:

Glucose : 0.5g
Yeast extract : 0.5g
Ammonium Nitrate : 0.1g

Fermentation condition:

Initial volume: 3.0 lit Medium + 300 ml of Inoculum
pH : 5.9
VVM : Anaerobic
Temp : 28 ºC
Rpm : 300

After inoculation, readings are taken every hour and tabulated. The values of OD, residual glucose and dry weight were thus recorded.

RESULT:

The specific growth rate and yield coefficient of the given microbial strain are determined through the Batch reactor.

INFERENCE:

For the batch reactor the following graphs are drawn.

i Variation of various parameters as a function of time.
ii ln OD Vs Time graph is plotted for the calculation of μ
iii Biomass Vs residual substrate concentration graph is plotted for $Y_{x/s}$ calculation
iv Using pure culture the quality parameters of the reactor could be verified.

DEPARTMENT OF BIOTECHNOLOGY

BT 3611-
BIOINFORMATICS LABORATORY MANUAL

REGULATION 2021

III YEAR & VI SEMESTER

MADHA ENGINEERING COLLEGE
MADHA NAGAR
CHENNAI- 600 069

Exp No: Date:	BASIC UNIX COMMANDS	

AIM

To verify the basic UNIX Commands and Filters

1. Create a directory

```
Syntax: mkdir directory name
```

2. Open a directory

```
Syntax: cd directory name
```

3. To make a new file in the directory

```
Syntax: cat > filename
```

4. Display the contents of a file

```
Syntax: cat filename
```

5. To find the current working directory

```
Syntax: pwd
```

6. List the files

```
Syntax: ls
```

7. Concatenate two files into a new file

```
Syntax: cat file1 file2 > new file
```

8. Numbering a file

```
Syntax: cat-n filename
```

9. Who command

```
Syntax: who
```

10. Who am i command

```
Syntax: who am i
```

11. List the files in a long format

```
Syntax: ls-l
```

12. List all the files

```
Syntax: ls-a
```

13. Move a file to another file

```
Syntax: mv file1 file2
```

14. Remove a file

```
Syntax: rm filename
```

15. List files/directories by date/time

```
Syntax: ls-t
```

16. Date command

```
Syntax: date
```

17. Teletype command

```
Syntax: tty
```

18. Change mode

```
Syntax: chmod
```

19. Message command

```
Syntax: mesg
```

20. Move contents if one directory to another

```
Syntax: mv directory1 directory2
```

21. Change the current working directory

```
Syntax: cd directory name
```

22. Calendar command

```
Syntax: cal
```

23. Calendar for a year

```
Syntax: cal year
```

24. Calendar of a month for a year

```
Syntax: cal month year
```

25. Time command

```
Syntax: time
```

26. Display only the month

```
Syntax: date+%m
```

27. Display the month name

```
Syntax: date+%h
```

28. Password command

```
Syntax: passwd
```

29. Echo command

```
Syntax: echo text
```

30. Display date of the month

```
Syntax: date+%d
```

31. Display the current minute

```
Syntax: date+%M
```

32. Display the current second

```
Syntax: date+%S
```

33. Display the current hour of the day

```
Syntax: date+%H
```

34. Display the last two digits of the year

```
Syntax: date+%y
```

35. Count the words in a file

```
Syntax: wc filename
```

36. Counting only the number of lines in a file

```
Syntax: wc-l filename
```

37. Counting only the words in a file

```
Syntax: wc-w filename
```

38. Counting only the number of characters in a file

```
Syntax: wc-c filename
```

39. Check difference between two files

```
Syntax: diff file1 file2
```

40. Unix Calculator

```
Syntax: bc
```

41. Head command: displays first 10 lines of a file

```
Syntax: head filename
```

42. Tail command: displays 10 lines from of the bottom of the file

```
Syntax: tail filename
```

43. Finger command: displays the users in the connection

```
Syntax: finger loginname
```

44. More command: it displays the contents to fit on the screen

```
Syntax: more filename
```

45. Sort command: sort the contents of a file

```
Syntax: sort filename
```

46. Sending message to all users

```
Syntax:  wall
```

47. Write command: communicate with all users

```
Syntax: write user_name
```

48. List special characters in a file

```
Syntax: ls-F
```

49. List files in reverse order

```
Syntax: ls-r
```

50. Remove a file

```
Syntax:rm filename
```

51. Remove a directory

```
Syntax: rm directory name
```

RESULTS

Exp No: Date:	BASIC MATHEMATICAL OPERATIONS	

AIM

To perform Basic mathematical operations using PERL

PROGRAM

```
#!/usr/bin/perl -w
$x=10;
print”\tThe value of first variable,x is : $x\n”;
$y=5;
print”\tThe value of second variable,y is : $y\n”;
$sum=$x+$y;
print”\tThe sum of Two variables is : $sum\n”;
$diff=$x-$y;
print”\tThe difference of Two variables is : $diff\n”;
exit;
```

OUTPUT

RESULTS

Exp No: Date:	CONCATENATING DNA	

AIM

To Concatenating DNA sequences using PERL

PROGRAM

```
#!/usr/bin/perl -w
# Concatenating DNA
# Store two DNA fragments into two variables called $DNA1 and $DNA2
$DNA1 = 'ACGGGAGGACGGGAAAATTACTACGGCATTAGC';
$DNA2 = 'ATAGTGCCGTGAGAGTGATGTAGTA';
# Print the DNA onto the screen
print "Here are the original two DNA fragments:\n\n";
print $DNA1, "\n";
print $DNA2, "\n\n";
# Concatenate the DNA fragments into a third variable and print
# Using "string interpolation"
$DNA3 = "$DNA1$DNA2";
print "Here is the concatenation of the first two fragments (version
1):\n\n";
print "$DNA3\n\n";
# An alternative way using the "dot operator"
# Concatenate the DNA fragments into a third variable and print
$DNA3 = $DNA1 . $DNA2;
print "Here is the concatenation of the first two fragments (version
2):\n\n";
print "$DNA3\n\n";
print "Here is the concatenation of the first two fragments (version
3):\n\n";
print $DNA1, $DNA2, "\n";
exit;
```

OUTPUT

Exp No: Date:	**TRANSCRIBING DNA INTO RNA**	

AIM

To Transcribe DNA sequence into RNA sequence using PERL

PROGRAM

```
#!/usr/bin/perl -w
# Transcribing DNA into RNA
# The DNA
$DNA = 'ACGGGAGGACGGGAAAATTACTACGGCATTAGC';
# Print the DNA onto the screen
print "Here is the starting DNA:\n\n";
print "$DNA\n\n";
# Transcribe the DNA to RNA by substituting all T's with U's.
$RNA = $DNA;
$RNA =~ s/T/U/g;
# Print the RNA onto the screen
print "Here is the result of transcribing the DNA to
RNA:\n\n";
print "$RNA\n";
# Exit the program.
exit;
```

OUTPUT

Exp No: Date:	CONDITIONAL STATEMENT	

RESULTS

AIM

To use the conditional statement in PERL

PROGRAM

```
#!/usr/bin/perl -w
# if-elsif-else
$word = 'MNIDDKL';
# if-elsif-else conditionals
if($word eq 'QSTVSGE') {
print "QSTVSGE\n";
} elsif($word eq 'MRQQDMISHDEL') {
print "MRQQDMISHDEL\n";
} elsif ( $word eq 'MNIDDKL' ) {
print "MNIDDKL--the magic word!\n";
} else {
print "Is \"$word\" a peptide? This program is not
sure.\n";
}
exit;
```

OUTPUT

Exp No: Date:	ARRAY OPERATIONS	

AIM

To perform various array operations using PERL

PROGRAM 1: POP OPERATION USING ARRAYS

```
#!/usr/bin/perl –w
@bases = ('A', 'C', 'G', 'T');
$base1 = pop @bases;
print "Here's the element removed from the end: ";
print $base1, "\n\n";
print "Here's the remaining array of bases: ";
print "@bases";
```

PROGRAM 2: SHIFT OPERATION ON ARRAYS

```
#!/usr/bin/perl –w
@bases = ('A', 'C', 'G', 'T');
$base2 = shift @bases;
print "Here's an element removed from the beginning: ";
print $base2, "\n\n";
print "Here's the remaining array of bases: ";
print "@bases";
```

PROGRAM 3: UNSHIFT OPERATIONS ON ARRAYS

```
#!/usr/bin/perl –w
@bases = ('A', 'C', 'G', 'T');
$base1 = pop @bases;
unshift (@bases, $base1);
print "Here's the element from the end put on the beginning:";
print "@bases\n\n";
```

OUTPUT

Exp No: Date:	A SUBROUTINE TO APPEND ACGT TO DNA	

AIM

To append ACGT to DNA using subroutine

PROGRAM

```
#!/usr/bin/perl -w
# A program with a subroutine to append ACGT to DNA
# The original DNA
$dna = 'CGACGTCTTCTCAGGCGA';
# The call to the subroutine "addACGT".
# The argument being passed in is $dna; the result is saved in
$longer_dna
$longer_dna = addACGT($dna);
print "I added ACGT to $dna and got $longer_dna\n\n";
exit;
# Here is the definition for subroutine "addACGT"
sub addACGT {
my($dna) = @_;
$dna .= 'ACGT';
return $dna;
}
```

OUTPUT

Exp No: Date:	BIOLOGICAL DATABASES- GENBANK	

AIM

To retrieve the sequence of the Human keratin protein from GenBank database and to interpret the results.

DESCRIPTION

The **GenBank** sequence database is an open access, annotated collection of all publicly available nucleotide sequences and their protein translations. This database is produced at National Center for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration, or INSDC. GenBank and its collaborators receive sequences produced in laboratories throughout the world from more than 100,000 distinct organisms. GenBank continues to grow at an exponential rate, doubling every 18 month. Release 155, produced in August 2006, contained over 65 billion nucleotide bases in more than 61 million sequences.

GenBank is built by direct submissions from individual laboratories, as well as from bulk submissions from large-scale sequencing centers. Direct submissions are made to GenBank using BankIt, which is a Web-based form, or the stand-alone submission program, Sequin. Upon receipt of a sequence submission, the GenBank staff assigns an Accession number to the sequence and performs quality assurance checks. The submissions are then released to the public database, where the entries are retrievable by Entrez or downloadable by FTP. Bulk submissions of Expressed Sequence Tag (EST), Sequence-tagged site (STS), Genome Survey Sequence (GSS), and High-Throughput Genome Sequence (HTGS) data are most often submitted by large-scale sequencing centers. The GenBank direct submissions group also processes complete microbial genome sequences.

PROCEDURE

1. Open the GenBank website

2. Type the protein name in the text box titled enter keyword

3. Select the appropriate database as nucleotide /protein

4. On pressing search button the result page is displayed

5. Choose the first sequence by double clicking the accession number

6. Take the FASTA sequence.

7. Interpret the results.

OBSERVATIONS

RESULTS

Exp No: Date:	BIOLOGICAL DATABASE – PROTEIN DATA BANK	

AIM

To retrieve the structure of a protein and viewing it in RASMOL viewer.

DESCRIPTION

The **Protein Data Bank** (**PDB**) is a repository for the 3-D structural data of large biological molecules, such as proteins and nucleic acids. The data, typically obtained by X-ray crystallography or NMR spectroscopy and submitted by biologists and biochemists from around the world, can be accessed at no charge on the internet. The PDB is overseen by an organization called the Worldwide Protein Data Bank. The PDB is a key resource in areas of structural biology, such as structural genomics. Most major scientific journals, and some funding agencies, such as the NIH in the USA, now require scientists to submit their structure data to the PDB. If the contents of the PDB are thought of as primary data, then there are hundreds of derived (i.e., secondary) databases that categorize the data differently. For example, both SCOP and CATH categorize structures according to type of structure and assumed evolutionary relations; GO categorize structures based on genes.

PROCEDURE

1. Open the PDB website
2. Type the protein name in the text box titled enter keyword or type the PDB ID
3. On pressing search button the result page is displayed
4. Choose the appropriate structure by double clicking the PDB ID
5. A web page is displayed with details about the structure
6. Download the structure file from the right hand corner of the webpage
7. Save the file as PDB file.
8. Open the RASMOL viewer to view the downloaded structure.
9. Interpret the results.

OBSERVATIONS

Exp No: Date:	BIOLOGICAL DATABASES - UNIPROT	

AIM

To retrieve the sequence of the Human keratin protein from UniProt database and to interpret the results.

DESCRIPTION

The Universal Protein Resource (UniProt) is a comprehensive resource for protein sequence and annotation data. The UniProt databases are the UniProt Knowledgebase (UniProtKB), the UniProt Reference Clusters (UniRef), and the UniProt Archive (UniParc). The UniProt Metagenomic and Environmental Sequences (UniMES) database is a repository specifically developed for metagenomic and environmental data. UniProt is a collaboration between the European Bioinformatics Institute (EBI), the Swiss Institute of Bioinformatics (SIB) and the Protein Information Resource (PIR).

The UniProt Knowledgebase (UniProtKB) is the central hub for the collection of functional information on proteins, with accurate, consistent and rich annotation. In addition to capturing the core data mandatory for each UniProtKB entry (mainly, the amino acid sequence, protein name or description, taxonomic data and citation information), as much annotation information as possible is added. This includes widely accepted biological ontologies, classifications and cross-references, and clear indications of the quality of annotation in the form of evidence attribution of experimental and computational data.

The UniProt Knowledgebase consists of two sections: a section containing manually-annotated records with information extracted from literature and curator-evaluated computational analysis, and a section with computationally analyzed records that await full manual annotation. For the sake of continuity and name recognition, the two sections are referred to as "UniProtKB/Swiss-Prot" (reviewed, manually annotated) and "UniProtKB/TrEMBL" (unreviewed, automatically annotated), respectively.

PROCEDURE

1. Open the UNIPROT website

2. Type the protein name in the text box titled enter keyword

3. On pressing search button the result page is displayed

4. Choose the first sequence by double clicking the accession number

5. Take the FASTA sequence.

6. Interpret the results.

OBSERVATIONS

RESULTS

Exp No: Date:	SEQUENCE ANALYSIS TOOL - BLAST	

AIM

To find the similarity between sequences using BLAST

DESCRIPTION

Basic **L**ocal **A**lignment **S**earch **T**ool, or BLAST, is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold.

The BLAST programs have been designed for speed, with a minimal sacrifice of sensitivity to distant sequence relationships. The scores assigned in a BLAST search have a well-defined statistical interpretation, making real matches easier to distinguish from random background hits. BLAST uses a heuristic algorithm that seeks local as opposed to global alignments and is therefore able to detect relationships among sequences that share only isolated regions of similarity

There are many different types of BLAST available from the main BLAST web page. Choosing the right one depends on the type of sequence you are searching with (long, short; nucleotide protein), and the desired database. They are,

- blastn -Nucleotide-nucleotide BLAST
- blastp -Protein-protein BLAST
- PSI-BLAST -Position-Specific Iterative BLAST
- blastx -Nucleotide 6-frame translation-protein
- tblastx -Nucleotide 6-frame translation-nucleotide 6-frame translation
- tblastn -Protein-nucleotide 6-frame translation
- megablast -Large numbers of query sequences

PROCEDURE

1. Open the Basic BLAST search page.

2. From the "Program" Pull Down Menu select the appropriate program.

3. Open your FASTA formatted sequence in a text editor as plain text.

4. Copy the entire sequence and paste it in the field titled "Enter your input data here", by clicking it once.

5. Set the pull down menu to "Sequence in FASTA format".

6. Make sure you have selected the correct BLAST program and BLAST database.

7. If you have entered your FASTA sequence or an Accession or GI number, click the "Submit Query Button".

8. BLAST will now open a new window and tell you it is working on your search.

9. Once your results are computed they will be presented in the window.

OBSERVATIONS

RESULTS

Exp No: Date:	SEQUENCE ANALYSIS TOOL - FASTA	

AIM

To find the similarity between sequences using **FASTA**

DESCRIPTION

FASTA is a DNA and Protein sequence alignment software package first described by David J. Lipman and William R. Pearson in 1985 in the article Rapid and sensitive protein similarity searches. The original FASTP program was designed for protein sequence similarity searching. FASTA, described in 1988 added the ability to do DNA:DNA searches, translated protein:DNA searches, and also provided a more sophisticated shuffling program for evaluating statistical significance. There are several programs in this package that allow the alignment of protein sequences and DNA sequences. FASTA is pronounced "FAST-Aye", and stands for "FAST-All", because it works with any alphabet, an extension of "FAST-P" (protein) and "FAST-N" (nucleotide) alignment.

The current FASTA package contains programs for protein: protein, DNA: DNA, protein: translated DNA (with frameshifts), and ordered or unordered peptide searches. Recent versions of the FASTA package include special translated search algorithms that correctly handle frameshift errors (which six-frametranslated searches do not handle very well) when comparing nucleotide to protein sequence data.

In addition to rapid heuristic search methods, the FASTA package provides SSEARCH, an implementation of the optimal Smith-Waterman algorithm. A major focus of the package is the calculation of accurate similarity statistics, so that biologists can judge whether an alignment is likely to have occurred by chance, or whether it can be used to infer homology. The FASTA package is available from fasta.bioch.virginia.edu.The web-interface to submit sequences for running a search of the European Bioinformatics Institute (EBI)'s online databases is also available using the FASTA programs.

PROCEDURE

1. Open the Basic FASTA search page.

2. From the "Program" Pull Down Menu select the appropriate program.The program is based on the query sequence.

3.Select the database against which the search has to be carried out.

4 From the results drop down box select an option. Enter your email id in the text box titled “Your email”

3. Open your FASTA formatted sequence in a text editor as plain text.

4. Copy the entire sequence and paste it in the field titled "Enter or paste sequence data", by clicking it once.

5. Set the pull down menu to "Sequence in FASTA format".

6. Make sure you have selected the correct FASTA program and database.

7. If you have entered your FASTA sequence or an Accession or GI number, click the "Submit Query Button".

8. FASTA will now open a new window and tell you it is working on your search.

9. Once your results are computed they will be presented in the window.

OBSERVATIONS

RESULTS

Exp No: Date:	SEQUENCE ANALYSIS TOOL CLUSTALW	

AIM

To align more than two sequences and find out the similarity between those sequences

DESCRIPTION

Multiple alignments of protein sequences are important tools in studying sequences. The basic information they provide is identification of conserved sequence regions. This is very useful in designing experiments to test and modify the function of specific proteins, in predicting the function and structure of proteins, and in identifying new members of protein families. Sequences can be aligned across their entire length (global alignment) or only in certain regions (local alignment). This is true for pairwise and multiple alignments.

Global alignments need to use gaps (representing insertions/deletions) while local alignments can avoid them, aligning regions between gaps. ClustalW is a fully automatic program for global multiple alignment of DNA and protein sequences. The alignment is progressive and considers the sequence redundancy. Trees can also be calculated from multiple alignments. The program has some adjustable parameters with reasonable defaults.

PROCEDURE

1. Go to http://www.ebi.ac.uk/clustalw/

2. Put in your e-mail (if you want the results e-mailed to you not necessary) and an alignment title of your choice.

3. Paste sequences in the box below using the FASTA format:

    >Name of Sequence #1

    >Name of Sequence #2

4. Every sequence MUST have a different name given to it or the alignment will not work. Or Upload a file that includes all your sequences (such as a .doc file) in an acceptable format.

5. Press run button to start alignment reading.

6. When viewing your results, these are the consensus symbols used by ClustalW:

    a. "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment.

    b. ":" means that conserved substitutions have been observed.

    c. "." means that semi-conserved substitutions are observed.

7. If you would like to see your results in color, push the button that displays Show Colors. Click Hide Colors to get rid of color.

8. Click on the button named View Alignment File to see the alignment on a larger scale (bigger font).

OBSERVATIONS

RESULTS

Exp No: Date:	EMBOSS	

AIM

To perform Sequence analysis by using EMBOSS

DESCRIPTION

EMBOSS (European Molecular Biology Open Software Suite) is an open source package of sequence nanalysis tools. This software covers a wide range of functionality and can handle data in a variety of formats. Extensive libraries are provided with the package, allowing users to develop and release their own software. EMBOSS also integrates a range of currently available packages and tools for sequence analysis, such as BLAST and ClustalW. A Java API (Jemboss) is also available.

EMBOSS contains around 150 programs (applications). These are just some of the areas covered:

• Sequence alignment.

• Rapid database searching with sequence patterns.

• Protein motif identification, including domain analysis.

• Nucleotide sequence pattern analysis, for example to identify CpG islands or repeats.

• Codon usage analysis for small genome.

PROGRAMS USED FOR NUCLEIC ACID SEQUENCE ANALYSIS

1. Plot: Function : plot potential open reading frames in a nucleotide sequence
2. Restrict:Function : Report restriction enzyme cleavage sites in a nucleotide sequence
3. Transeq:Function : Translate nucleic acid sequences
4. Eprimer3:Function : Picks PCR primers and hybridization oligos
5. Backtranseq:Function : Back-translate a protein sequence to a nucleotide sequence
6. Dan: Function : Calculates nucleic acid melting temperature
7. Palindrome: Function : Finds inverted repeats in nucleotide sequence(s)
8. Revseq

   Function : Reverse and complement a nucleotide sequence

OBSERVATIONS

Exp No: Date:	**PHYLIP**	

AIM

To study the phylogenic relationships of nucleotide and protein sequence(s) by using PHYLIP Package.

DESCRIPTION

PHYLIP (for **phyl**ogeny **i**nference **p**ackage) is a package now consisting of about 30 programs that cover most aspects of phylogenetic analysis. PHYLIP is free and available for a wide variety of computer platforms (Mac, DOS, UNIX, VAX/VMS,and others).

PHYLIP is a command-line program and does not have a point-and-click interface.The documentation is well written and very comprehensive,and the interface is straightforward.A program within PHYLIP is invoked by typing its name, which automatically causes the data to be read from a file called ‘‘infile’’ or a file name you specify if no infile exists.This infile must be in PHYLIP format; this format is clearly described in the documentation, and most sequence analysis programs offer the ability to export sequences in this format.For example, if an alignment is produced using CLUSTAL W or edited using GeneDoc,the alignment may be saved in PHYLIP format and then used in PHYLIP programs directly. Once the user activates a given PHYLIP program and loads the infile, the user can then choose from an option menu or accept the default values.

The program will write its output to a file called ‘‘outfile’’ (and ‘‘treefile’’ where applicable). If the output is to be read by another program, ‘‘outfile’’ or ‘‘treefile’’ must be renamed before execution of the next program, as all files named outfile/tree file in the current directory are overwritten at the beginning of any program execution.The tree file generated is a widely used format that can be imported into a variety of tree-drawing programs, including DRAWGRAM and DRAWTREE that come with this package.However, these PHYLIP tree-drawing programs produce low-resolution graphics, so a program such as TreeView (described below) is instead recommended. Particulars of some of the PHYLIP tree-inference programs are discussed below

PROCEDURE

1. Retrieve the nucleotide sequences form the genbank entrez page.

2. Allign these sequences by using clustal W.

3. Save the multiple sequence alignment in phylip format.

4. The aligned sequences will act as a input to the ‘phylip’program

5. Select the phylip program from the phylip folder just by clicking the ‘dnadist’program.

6. The ‘DNA dist’ program have options for selecting the distance parameters, click on of them, it produces the distance table for the given sequences.

OBSERVATION

Exp No: Date:	MOLECULAR MODELING - SWISS MODEL	

AIM

To model a protein sequence using Swiss Model

DESCRIPTION

SWISS-MODEL (http://swissmodel.expasy.org) is a server for automated comparative modeling of three dimensional (3D) protein structures. It pioneered the field of automated modeling starting in 1993 and is the most widely-used free web-based automated modeling facility today. In 2002 the server computed 120 000 user requests for 3D protein models. SWISS-MODEL provides several levels of user interaction through its World Wide Web interface: in the 'first approach mode' only an amino acid sequence of a protein is submitted to build a 3D model. Template selection, alignment and model building are done completely automated by the server.

In the 'alignment mode', the modeling process is based on a userdefined target-template alignment. Complex modeling tasks can be handled with the 'project mode' using DeepView (Swiss-PdbViewer), an integrated sequence-to-structure workbench. All models are sent back via email with a detailed modeling report. WhatCheck analyses and ANOLEA evaluations are provided optionally. The reliability of SWISS-MODEL is continuously evaluated in the EVA-CM project. The SWISS-MODEL server is under constant development to improve the successful implementation of expert knowledge into an easy-to-use server.

PROCEDURE

1. Go to NCBI (http://www.ncbi.nlm.nih.gov/) click on PUBMED activate a “protein” search in the menu and search for a particular protein.

2. Follow links to the amino acids sequence and copy that sequence to the computer’s clipboard for use in the program BLAST. (you might also want to save it to a file as you will need it later).

3. Go back to the NCBI home page again and follow the “BLAST” link submit the sequence to BLAST selecting the PDB database alone. Click on the blue format button to see the results.

4. The resulting sequences are from sequences deposited with a known 3D structure and the four digit PDBcode is next to the words “pdb”.

5. Record the PDBcodes for the different known 3D structures which align with the sequence.

6. Go to the PDB. http://www.rcsb.org/pdb/ and either type in a four digit code for one (or more) of the structures OR – use the “searchlight” functionality and search for “hslV” for example to see many related files at once.

7. Check the different PDBcodes to find the one which structure was solved to the highest resolution.

8. Download one or more of these structures from the PDB. The files may have an “.ent” file designation. These are equivalent to “.pdb” files

9. Run PYMOL or RASMOL and view your protein.

10. Now that you know that a reasonable structure exists, submit the sequence to the Swiss-Model web site. http://www.expasy.ch/swissmod/SWISS-MODEL.html Submit the original sequence with your e-mail address. The SWISS-MODEL server may take ~0.5-3 hours to return the results of the modeling exercise. You don’t have to submit a PDB for SWISS-MODEL to use, it will use a mixture of all the top hits.

11. Receive several e-mails from SWISS-MODEL containing some introductory messages and the

results of the modeling and PHD exercise.

12. Save the models to a file and view with RASMOL.

OBSERVATIONS

RESULTS

Exp No: Date:	**HEX**	

AIM

To give practice in using docking software

DESCRIPTION

HEX is an interactive **protein docking** and **molecular superposition** program, written by Dave Ritchie. HEX understands protein and DNA structures in PDB format, and it can also read small-molecule SDF files. HEX will run on most Windows-XP, Linux and Mac OS X PCs. Most versions now include CUDA support for Nvidia GPUs. The latest version with CUDA is Ubuntu 10.10. Binaries for several earlier operating system versions are also available. On a modern PC, docking times range from a few minutes when the search is constrained to known binding sites, to about half an hour for a blind global search (or just a few seconds with CUDA).

Hex reads protein and DNA molecular structures from PDB-format files. Hex can also read SDF-format small-molecule structure files. PDB files can be downloaded from the main Protein Data Bank. Up to three input files can be loaded into Hex at any one time. These are treated as a receptor, a ligand and a reference complex.

It uses Spherical Polar Fourier (SPF) correlations to accelerate the calculations and its one of the few docking programs which has built in graphics to view the result.

Short-Cut Buttons

The column of buttons on the right-hand border of the main window implement handy or frequently used operations. For example, Hex stores a Home Position for the scene. Pressing the House button at the top right border resets the scene to the current home position. If you have oriented the molecule into a view that you like, you can make this orientation the Home Position position by pressing the Lock button, below the Home button. The Unlock button resets the Home Position to its the original setting (z-axis to the right, etc.). The next button (the “6.6” icon) is a text toggle, to control the display of summary text in the graphics window. The Axes button (two arrows at right angles) toggles the display of the coordinate axes (useful for screen shots). Below this is the Intermolecular Axis button (a double-headed arrow). This draws a white line between the centroids of the receptor and ligand molecules. If only one molecule has been loaded, a short white line is still drawn along the z-axis.

Function Buttons

The column of buttons on the left hand border of the main window implement various picking and editing functions. These operations differ from the short-cut buttons in that they generally involve changing the operating mode of the mouse. The inset icon in the top left border displays the current mouse mode. The default, Pointer Mode (a left arrow), represents the basic scene manipulation mode, described above. You can revert to this mode by pressing the Left Arrow button, which is the first button in the left-hand border.

Docking Molecules

In order to run a docking calculation in Hex, you will need to load a receptor and a ligand PDB structure using the File pull-down menu. If you want to test the docking algorithm by docking two separately determined sub-units of a complex for which the crystal structure is also available, you can also load the complex structure which will be used as a reference orientation to evaluate the accuracy of the docking prediction.

Generally, you will have to remove water molecules and any other hetero molecules prior to docking. You can do this globally using the Hetero Control menu panel. If more detailed control is required, you will probably have to edit each PDB file manually using a text editor. It may also be necessary to remove other chains in the PDB file or to shorten a chain to the domain of interest in docking.

When using a complex reference structure, you should find that on loading the molecules, the complex is drawn in grey and the complex molecule is superposed onto the receptor (it is the complex that physically moves here). If the ligand molecule originated from an edited complex file, then it will also be superposed over the complex because its coordinates are relative to the same coordinate frame as the receptor molecule. Otherwise, the ligand can be transformed into the receptor/complex frame using the Fit Ligand button in the Orientation Control panel.

Clustering Docking Results

The Controls ... Clustering control panel may be used to control the type of clustering and clustering parameters used, and how cluster membership affects the displayed solutions. By default, only the lowest energy member of each cluster is displayed or written to an output PDB file (Display Clusters = Best).

Saving Docking Results

There are two main ways in which docking results may be saved to disc. The most compact method is

to write a docking summary file using the File ... Save ... Transform menu item. This writes a file that lists the docking energies for all predicted orientations, along with the rotation and translation parameters which should be applied to the ligand coordinates in the original ligand PDB file in order to produce the corresponding docking orientation in the coordinate frame of the recptor PDB file. The actual file format is descibed in the first few "comment" lines of the output file. A similar but more detailed output file format is given by File ... Save ... Matrix. This lists the transformations in a more verbose format using keywords to label each piece of data. For rigid body docking, both formats are quite compact, but you will need to write your own code to process the docking summary file. Its not possible to read these files back into Hex; they are purely for output to external software.

The second, less compact output method is to write a separate PDB file for each docking orientation. The current docking orientation can be written to a single PDB file by selecting:

File ... Save ... Both

Alternatively, a range of docking solutions may be saved using:

File ... Save ... Range

The Mechanics of Docking

To perform a docking screen, the first requirement is a structure of the protein of interest. Usually the structure has been determined using a biophysical technique such as x-ray crystallography, or less often, NMR spectroscopy. This protein structure and a database of potential ligands serve as inputs to a docking program. The success of a docking program depends on two components: the search algorithm and the scoring function.

STEPS IN DOCKING

1. The target molecule can be visualized by

   Open --> Receptor --> Pathname of target protein
2. The drug molecule and their analysis can be visualized by

   Open --> Ligand-->Pathname of our drug
3. Modeling between receptor and target molecule can be performed by

   Control --> Matching
4. Docking can be performed by

   Control --> Docking

5. The docked molecule can be saved by

   File --> Save --> Both --> filename.pdb

OBSERVATIONS

RESULTS