PRACTICAL MANUALS OF BSc BIOTECHNOLOGY SEMESTER III& IV

DAV COLLEGE JALANDHAR

BIOCHEMISTRY PRACTICAL MANUAL

Semester-3

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2.	The absorbance curve of two dyes.
3.	Determination of reducing sugar using 3,5 dinitrosalicylic acid.
4.	Chromatographic methods for separation of macromolecules • Thin layer chromatography • Gel permeation chromatography

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EXPERIMENT-1

AIM:- Demonstrate Beer- Lambert's Law

Requirements:-

Spectrophotometer, Test tube, Test Tube stand, Breakers, micropipettes, Bromophenol blue and Distilled water.

Principle:-

When a ray of monochromatic light with initial intensity 'Io' passes through a solution in a transparent vessel, some of the light is absorbed so that the intensity of the transmitted light 'I' is small. There is some loss of intensity from scattering by particles in the solution & reflection at the interphase but mainly from absorption by the solution. The relationship between 'I' & 'Io' depends on the path-length of absorbing medium 'l' & the concentration of absorbing medium 'c'.

LAMBERT'S LAW:- When a ray of monochromatic light passes through an absorbing medium, its intensity decreases exponentially as the length of absorbing medium increases.

BEER'S LAW:- When a ray of monochromatic light passes through a medium its intensity decreases exponentially as the concentration of absorbing medium increases.

These 2 laws are combined together in Lambert-Beer Law.

$I=I_0e^{-kcl}$

The ratio of intensity is known as transmittance & is usually expressed as '%'

$T=I/Io = e-^{kcl}$

Taking log,

$$\log_e Io/I = KCl$$

$$\log_{10} Io/I=2.303\ KCl$$

$$\log_{10} Io/I=KCl$$

The expression "$\log_{10} Io/I$" is known as Extinction (E) or Absorbance (A)

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Therefore, E= KCl

If the Beer Lambert Law is obeyed & the ‘l’ is kept constant, then plot a graph against ‘E’ & ‘conc.’ will give the straight line passing through the origin whereas a plot of (%) transmittance against conc. gives negative exponential curve.

MOLAR EXTINCTION COEFFICIENT:-

If ‘l’ is 1 cm & concentration is 1 mole /L then its absorbance is equal to ‘K’.

Therefore, E=K

The molar extinction coefficient which is characteristic of compound has dimensions of 1 mole/cm.

SPECIFIC EXTINCTION COEFFICIENT:-

The molar weight of some compounds such as proteins or nucleic acid in a mixture are not readily available & in this case the specific extinction coefficient is used. This is the extinction of 10g/l of the compound in the light part of 1cm.

PROCEDURE:-

i. Took 5 test tubes in a stand to make different concentration of dye & mark them as T_1, T_2, T_3, T_4, T_5.
ii. Took Bromophenol Blue solution as a stock.
iii. Then made different concentration from the stock solution by adding distilled water.
iv. In T_1, added 10ml of stock & No water & in T_2, 8ml of stock & 2 ml Distilled water to make 10ml, then add 6ml of stock & 4ml Distilled Water in T_3 & So On.
v. In T_6, added Distilled water & no stock, Mark it as blank.
vi. Now took absorbance of all the concentrations at particular wave length of 590nm.
vii. Noted down the absorbance of different cone & plotted graph b/w absorbance & concentration.

<u>Calculations:-</u>

<u>Stock solution</u>:-

Bromophenol blue = 100 mg/ 100ml

Concentration = Quantity/ volume = 100/100 = 1 mg/ml

= 1000µg/ml

Working solution:-

Volume of stock = 1ml

Distilled water = 99ml

$C_1V_1 = C_2V_2$

(Stock) (Working solution)

$1000 \times 1 = C_2 \times 100$

$C_2 = 10 \mu g/ml$

OBSERVATION:-

S.No.	CONCENTRATION	STOCK ADDED (in ml)	DISTILLED WATER	ABSORBANCE
Blank	1 µg/ml	0	5ml	0
T1	2 µg/ml	1ml	4ml	0.138
T2	4 µg/ml	2ml	3ml	0.213
T3	6 µg/ml	3ml	2ml	0.374
T4	8 µg/ml	4ml	1ml	0.440
T5	10 µg/ml	5ml	0	0.543

Result:-

The straight line is observed passing through origin according to beer lamberts law.

PRECAUTIONS:-

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i. Switch on the spectrophotometer half an hour before use.
ii. There must be no Ionization, association, dissociation, and solvation of the solute with concentration of time.
iii. The solution is the concentration giving intense colour, law holds upto a threshold maximum concentration for a given substance.
iv. Wavelength of light should be at the observation maxima of solution. This also gives the great sensitivity.

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EXPERIMENT-2

AIM: To Demonstrate the absorption maxima of 2 dyes (Bromophenol Blue & Methyl orange)

REQUIREMENTS:-

Spectrophotometer, micropipette, measuring cylinder, Test tubes, Test tube stand, Cuvettes, Beaker, Chemicals (Baomophenol Blue & Netluf orange) and distilled water.

CALCULATIONS:-

Stock solution 1 & working solution-

$$C1V1 = C2V2$$

$$1000bg * V1 = 10bg * 10ml$$

$$V1 = 100/1000 \text{ ml} = 0.1\text{ml}$$

THEORY:-

Colored compounds have their own characteristic absorption spectra & careful selection of the wavelength where maximum observation is found enables a mixture of 2 colored substances to be analyzed.

A spectrophotometer is the sophisticated type of colorimeter where prism. The bandwidth of the light passed by filter is quite board so that it may be difficult to distinguish b/w 2 compounds of closely related absorption with a colorimeter. A spectrophotometer is then needed when the 2 peaks can be selected on the monochromatic.

SOLUTIONS:-

Stock Solution 1:-

10mg dye in 10 ml of distilled water conc = 1 mg/ml

Stock Solution 2:-

Took 0.1 ml of solution 1 and added into 9.9ml of distilled water conc = 0.01 mg/ml

Working Solution:-

Took out 0.1 ml of stock solution 2 and added into 9.9 ml distilled water conc = 10mg/ml

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OBSERVATIONS:-

Wavelength (nm)	Absorbance of bromophenol blue	Absorbance of methyl orange
400		
420	0.1010	0.692
440	0.242	0.792
460	0.033	0.865
480	0.054	0.776
500	0.092	0.512
520	0.136	0.250
540	0.221	0.073
560	0.348	0.010
580	0.516	-0.05
590	0.554	-
600	0.382	0.00
620	0.135	-0.012
640	0.030	-0.009
660	0.01	-0.012
680	0.015	-0.018
700		

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PROCEDURE:-

i. Prepare stock solution one by adding 10mg dye (bromophenol blue) in 10 ml distilled water i.e. conc 1mg/ ml.
ii. Pipetted out 0.1ml stock '1' into another beaker & added 9.9ml distilled water. This was stock solution '2' i.e. 1mg/ml.
iii. Now again diluted the solution with distilled water to make its required concentration.
iv. Switch on the spectrophotometer & set the transmittance 200%.
v. Set the mode value
vi. Prepare 2 cuvettes filled with bromophenol blue solution & with distilled water each.
vii. Selected the wavelength & set the absorption at o, by blank solution.
viii. Set the wavelength to 400nm by inserting blank.
ix. Noted the reading of dye at 400nm.
x. Recorded the absorbance reading.
xi. Reported the procedure of measuring the bromophenol blue absorbance at 20nm intervals from 400-700nm.

Repeated the above procedures to determine the absorption maxima of meth orange & recorded its readings.

PRECAUTIONS:-

i. Carefully handle the micropipettes.
ii. Drop the pipette at its highest margins so as to relax it, for its longer life span.
iii. Wipe the cuvettes before inserting it into the spectrophotometer.
iv. Change the filter while taking reading above 600 nm.
v. Clean & dry cuvette should be used.

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EXPERIMENT-3

AIM:- Determination of reducing sugar using 3-5 dinitrosalicylic acid.

REQUIREMENTS:-

1. Standard Maltose solution:
   Maltose, Distilled water.
2. DNS Reagent
   Phenol
   Sodium sulphite
   NaOH
   Potassium sodium tartrate tetrahydrate (Rochelle salt)

CALCULATIONS:-

Standard Maltose solution (1mg / ml):-

Weight 100ml of maltose and transfer it into a volumetric flask, dissolve and make up the volume to 100 ml, with distilled water.

APPARATUS:-

Flask,, Beakers, spectrometer, pipette, vortex, test tubes, water bath.

PRINCIPLE:-

Reducing sugar convert di-nitro salicylate under alkaline condition to amino nitro salicylate and orange yellowish compound that has an absorption maxima at 540 nm.

REAGENTS:-

DNS Reagent:- Dissolve 1g of DNS & 200mg of phenol and 50mg of sodium sulphite in 100ml of 1% (w/v) NaDH. Store this reagent in refrigerator.

Potassium Sodium

Tartrate tetra hydrate 40% (W/V) [Rochelle Salt]

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OBSERVATION:-

S.No.	Standard Maltose (ml)	Distilled Water (ml)	Conc. Of Maltose (mg/ml)	DNS Reagent (ml)	Pot. Sod. Tartrate (ml)	Distalled water (ml)	Absorbance at 54nm
1.	Blank	1	0	2	1	7	0
2.	0.2	0.8	0.4	2	1	7	0.17
3.	0.4	0.6	0.8	2	1	7	0.27
4.	0.6	0.4	1.2	2	1	7	0.32
5.	0.8	0.2	106	2	1	7	0.34
6.	1.0	0	2.0	2	1	7	0.42
7.	Unknown	Unknown	?	2	1	7	0.37

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RESULT:-

From the graph:-

Cone. Of unknown = 0.9 mg/ml

Mathematically:

Conc. Of unknown = 0.D of unknown/0.D of known * conc. Of known*DP

= 0.37/0.32*1.2*1

1038mg/ml

PROCEDURE:-

i. To 1ml of the sugar solution (contain 0.2-1mg of maltose) or appropriately diverted unknown sugar sample solution, added 2ml of DNS reagent and vortex.
ii. Heated the tubes in a boiling water bath for 5 min, while the tubes are still warm, added 1ml of 40% potassium-sodium tartrate solution. Cooled the tubes to room temperature & added 7ml of DOW & vortex.

iii. Measured the absorbance against the reagent blank at 54nm. Construct a calibration curve on a graph paper, by plotting the sugar concentrate (0.2-1mg) on x-axis and absorbance at 540 nm on the y-axis.
iv. Conclude the concentration of the sugar in the sample for the calibration curve. While calculating the sugar concentration in the unknown sample, the dilution factor has to be taken into accounts.

PRECAUTIONS:-

i. Handle the spectrophotometer carefully.
ii. Glass ware used must be clean.
iii. Weighting should be done accurately.
iv. Micropipettes should be relaxed after used.

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EXPERIMENT-4

AIM:- To identify lipids in given sample by thin layered chromatography.

MATERIAL REQUIRED:-

1. Glass plates (20*20cm) (3mm thickness)
2. Chromatography glass jar with vaccum greased gel.
3. Capillary tube.
4. Silica gel-G
5. Sample (lipid, oil, fat, ghee)
6. Solvent system (Chloroform: methanol: H_2O) (65:25:4)

PRINCIPLE:- To the separation of compounds is TLC is based upon different absorption as well as partition of analysis b/w liquid stationary phase & mobile solvent phase. This technique is rapid as compare to paper chromatograph. Molecular can separated b/w the hydrophilic analytes have more affinity to polar stationary matrix while less hydrophilic molecules tend to be have more affivity towards mobile phase resulting in its faster movement & separation. The separated analytes are identified by comparing their Rf values to that of reference standard. The Rf values of an analytes depends upon:

1. Solvent system
2. Degree of saturation of mobile phase in chromotography chamber.
3. Particle size of the absorbent.
4. Type of absorbent
5. Temperature & humidity

Thus, Rf values for an analytes is constant for given set of experimental condition.

Rf = Distance travelled by the analyte/Distance travelled by mobile phase (solvent)

Commonly used stationary matrix for TLC include silica gel-G, silica gel-H, aluminum & then cellulose.

RESULTS:-

Rf of spot 'A' = distance travelled by spot 'A'/Distance travelled by solvent front = 4.5/18=0.25

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Rf of spot 'B' = distance travelled by spot 'B'/Distance travelled by solvent front = 8.5/18=0.47

Normally glass, aluminum or polyester codes are used for clothing stationary matrixes.

PROCEDURE:

1. Placed thoroughly clean & dried glass plates on a flat surface.
2. Wright 5g of silica gel-G and added 10-15 ml of distilled water & thoroughly shake the contents, immediately code the slurry into the glass plates, silica gel uniformly layered of thickness (25-35mm)
3. Air dry the plates at room temperature.
4. Active the coat TRC plate in hot air own at 110ºC for one hour.
5. Remove the plates from the own & allow to cool at room temp in desiccate chamber.
6. Spotted the activated TRC plate with a lipid sample with the help of capillary tube standard as well as unknown.
7. Now develop the plates in a solvent system consisting of chloroform, methanol, H2o. The chromatography developed by placing the TLC plates vertically in a TLC chamber saturated with mobile phase in such manner the spotted edge tips in solvent system.
8. Run the chromatography until the solvent from reaches the top edge of the plate.
9. Move the plate & mark the solvent front as soon as remove the plates from the chamber.
10. Located the lipids spots by spraying the iodine vapors.
11. Calculate the Rf value of lipid components in the sample & identify them by comparing the Rf value with lipid standard.

PRECAUTIONS:-

1. Thoroughly clean glass plates.
2. Do not touch or change coated areas & edges of the plates.
3. Thickness of silica gel should be uniform throughout the plate.
4. Surry should be free of stumps.

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EXPERIMENT-5

AIM:-Separation of bimolecular by gel prevention chromatography.

REQUIREMENTS:-

Chromatographic column, micropipette.

THEORY:-

Molecules can be separated on the basis of differences in their size by passing them down a column containing swollen particulars of gel smaller molecule can enter the gel but larger molecular are absorbed from the crossing network. This means that volume of a solvent is very much less for molecules totally excluded from the gel than from the smaller molecular which are free from separation. The separation of the molecules of the column gel preparations passed down the column is that the small molecule diffuse into the column & follow a longer path than larger molecules which are completed excluded from the gel particular complete separation occur with larger molecules clustering first as small molecule in last.

If molecular is completely excluded in gel then kd=0 & if the molecule has complete accessibility to the gel, then kd=1.

Molecule have normal kd values b/w 0 to 1, if kd >1 then absorption of the compound on the gel has occurred column porous bead & sauce is applied on the top of the column of chore beads of the cross linked gel, they can get separated as follows:

1. Large molecules cannot cuter the poses & Clute as 1st peak in the chromatogram. They elute fast & this from total elusion.
2. Intermediate molecules enter the poses & have an average residence time in the particles depending upon their size & shape. This portion of chromatogram is called selective permeation reagent.
3. Surface molecular enter the poses & have longest residence time: elute out as last peak in chromatogram.
4. This last peak is total permeation limit.

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PROCEDURE:-

1. Fixed the washed & cleaned column vertically to the stand.
2. Equilibrate the column with gel filtration buffer. Drawn out the buffer completely.
3. Suspended 4g of sepphadax (Aluminum) in sodium chloride soln & leave it to swell for 3 hrs. during this time stir the soln & removed any fumes by decantation.
4. Prepare the column of the gel by pouring the gel expansion into a column & allowed to settle under graving while maintain a slow flow rate to column.
5. Equilibrate the column with gel filtration buffer. Drain out the buffer completely.
6. Loaded the known amount of the mixture of methylene blue & cobalt chloride to the top of column & elute it with isotonic solute soln collected the colour fraction in diff tubes. Then fix the bottom cap & top cap to stop the flow of buffer & stored at 4ºC for next use.

PRECAUTIONS:-

1. Do not let the column to gel dry any time.
2. Always open the top cap of the column first & then bottom cap to start the flow of buffer.
3. There should not be any air bubble in the column.
4. Loading should be done carefully.

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CELL BIOLOGY-A
PRACTICAL MANUAL
Semester –III

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Experiment-1

Aim: - To study the compound microscope.

Theory: - The compound microscope is used to see object not visible to naked eyes.The first microscope was given by Johnson and Johnson in n1950. In compound microscope two lenses are used to magnify the object. Magnification of the object is multiplied by the magnification of the eye piece. Magnification is different from resolving power. Higher the numerical aperture greater will be resolving power.

Limit of resolution= 0.61 / NA=n ×sin α

n= Refractive index

Sin α= sin of angle of aperture

Limit of resolution: - Limit of resolution is inversely proportional to resolving power i.e. higher the resolving power, smaller is the limit of resolution.

Numerical aperture: - Sin value of aperture angle multiplied by relative index of medium filling the space between the front lens and cover slip given the numerical aperture.

NA= n× sin Ø.

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PARTS OF COMPOUND MICROSCOPE

- OPTICAL PARTS: -

1. Plane concave mirror: - It is the mirror having a plane side and a concave side. The mirror is an adjustable mirror for focussing light.

2. Objective lens: - It is the lens situated at the lower end of the body tube. It may be 10x, 20x, 40x and 100x.
3. Eye piece or Ocular lens: - It contains lenses of increase magnification. Eye piece of following magnification are generally used 5x, 6x, 8x, 10x and 12x.

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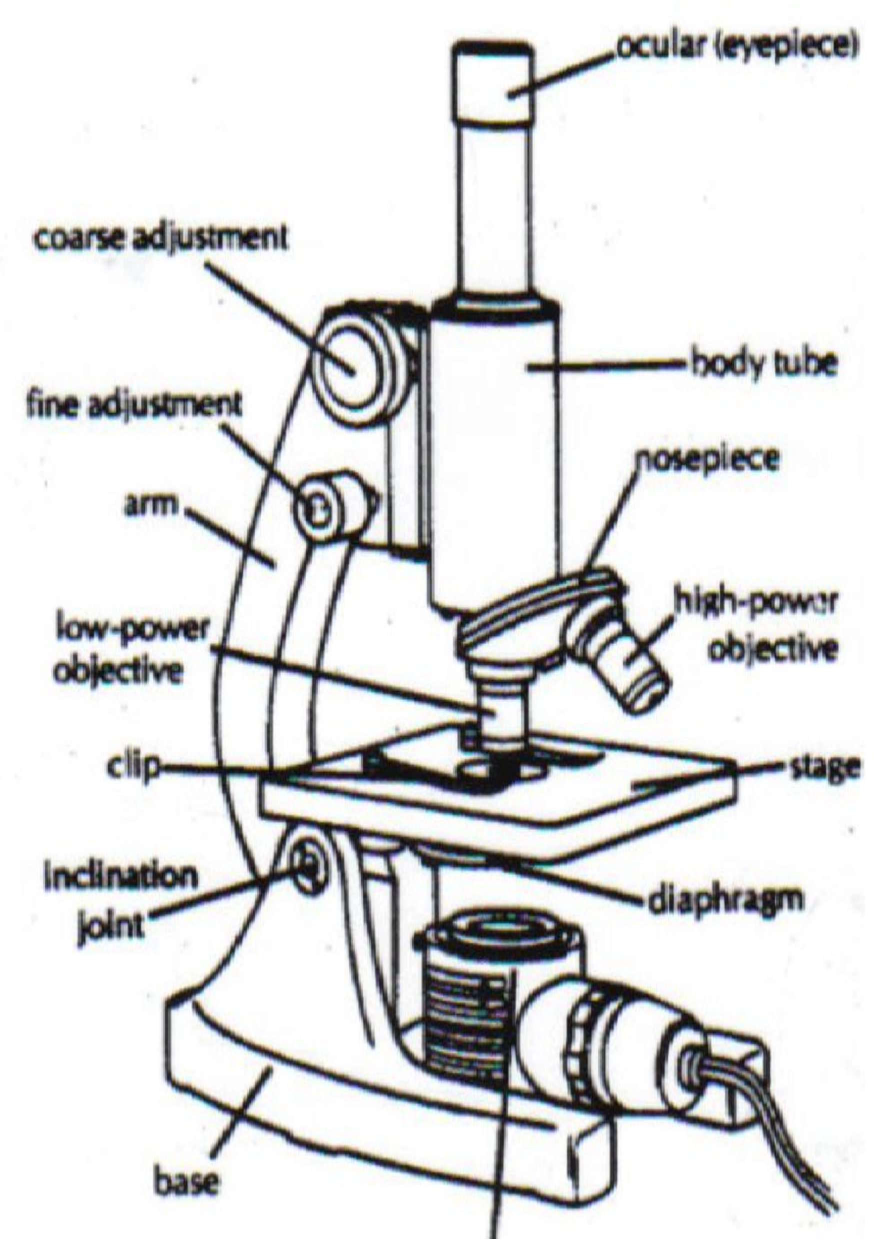

- MECHANICAL PARTS: -

1. U shaped horizontal base/foot: - It is the supporting basal part of the microscope. It is like horse-shoe shaped. It bears whole weight of microscope.
2. Pillar: - It is formed of two vertical plates which are attached to the foot. It gives support to handle the stage.
3. Stage: - It provides space for the object mounted on given slide in the fluid medium and often covered within a thin glass cover slip. It has a counter circular hole in a middle of which object mounted on a slide in set. The slide is covered by two simple clips.
4. Arms: - It is curved structure meant by holding the whole structure. It can be inclined with the pillar at an inclination point. It bears two screws for the coarse and give adjustment arm to support the body tube.
5. Body tube: -A wide tube is supported by arm by focussing the object with the help of these screws. It can be moved up and down.
6. Nose piece: -It is the rounded resolving part attached to the lower end of the body tube containing 2.3 objective of different magnification.
7. Adjustment Screw: - These are present on slides of upper part of the arm. They move the body tube up and down to focus the object. They are of two types- coarse and fine.
8. Iris Diaphragm: -It regulates amount of light passing through the specimen or material.

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USES:

- Compound microscope uses the visible light to illuminate the object and mainly used for studying the structure of an organism and cells, it’s to get large view in oil immersion.

Precautions: -

- When not in use microscope should be placed in a box.
- The objective lens should be protected from sticky material
- Before observing the mounted object a clear cover slip should be placed over it.
- All the lens objective and ocular should be kept clear and prevented from dust.

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Experiment-2

Aim: - To study electron microscope.

Theory: - Electrons are the illumination source of electron microscope. These take the advantages of the very short wavelength attainable in electron beams. This permits a considerable improvement in resolution over the light microscope.

All the space transverse by electron inside the electron microscope must be kept under a high vacuum. Otherwise the electrons of the beam which have poor power to pass through matter would be completely scattered and absorbed by gas molecule in the microscope.

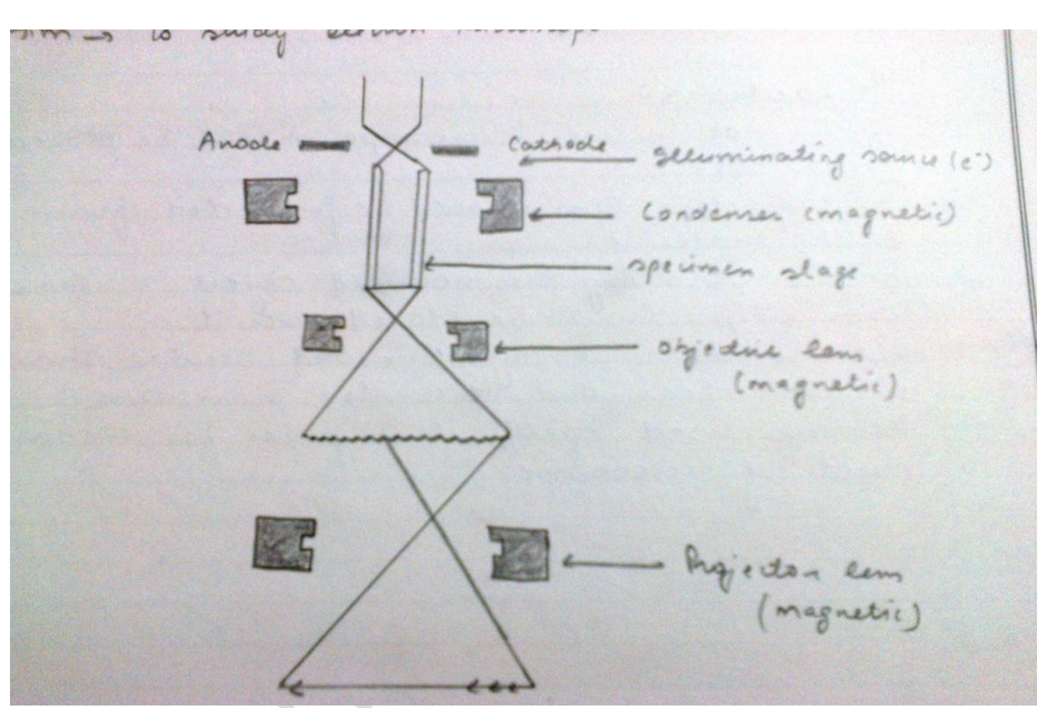
The electron microscopes are of two types:

1. Transmission electron microscope.
2. Scanning electron microscope.

Transmission electron microscope: -

It is so called because beams forming the image pass through the specimen. A TEM resembles an inverted microscope.

- Electric Gun: - It is present at the top of central column and the illumination source. It consists of a filament a thin tungsten wire and an anode. An electric current heats the filament a thin tungsten wire to a high temperature which causes electron to be driven from its surface.



The anode is grounded and is thus positive with respect to the filament. As s result electrons leaving the filament are strongly attached in the anode.

Scanning Electron microscope: -

It is widely used to examine the surface of the cells or isolated cellular structure. It differs extensively in its construction and the operation from the TEM.

a. Only the illumination source and condenser lens are similar.
b. An electron beam in the SEM is produced by an electron gun.
c. It is focussed into an intense spot on the specimen surface by the magnetic field system analogues to the condenser lens of TEM.
d. Rather than being stationary however the focussed spot moves rapidly plates between the condenser lens and the specimen.

e. Molecules on the surface of specimen being examined are excited by intense spot of electron to high energy level.

The excited molecules release this energy in several forms including high energy is called secondary electron. Other lens is present in SEM the secondary electron having a particular spot in the specimen surface are picked up by a detector at one side of specimen.

The detector constitutes of following elements: -

Condenser lens: - Just below the gun a series of two condenser lens focus a very small intense spot of electrons on the specimen. The specimen can be moved in different regions. It can also be held in the state position with movement number greater than a angstrom so to allow photographs to be made clear.

The projector: - It focuses the magnified image into a fluorescent screen at the bottom of the column. This screen is coated with crystals that respond to an electron by emitting visible light. By this process the electron image is converted to visual image. The image is permanently recorded by exposure of a photographic plate to the electron beam at the level of the screen. There is provision of airlocks of the screen. So that specimen and photographic plate can be exchanged without disturbing the microscope vacuum and large molecules such as protein can be resolved by TEM. The scattering by specimen atom is inversely proportional to the velocity of beam of electron.

Photomultiplier: - The emitted light is amplified by photomultiplier .It is projected into photoelectric cell.

Photoelectric cell: - It works on the same principle as the high meter on the camera, emits a current proportional to amount of light striking it.

An electron tube: - The tube produces a narrow beam of electrons that is focussed into a spot at the front of tube. Detector inside the tube, connected to the same electronic circuit scan the beam in the microscope move the spot back and forth the spot across the screen at the same rate in the same direction .the detector adjust the scanning spot on the television screen at the point and instant to high level of high level of brightness when the scanning spot on the microscope sticks a point on the specimen surface that emits large number of secondary electron.

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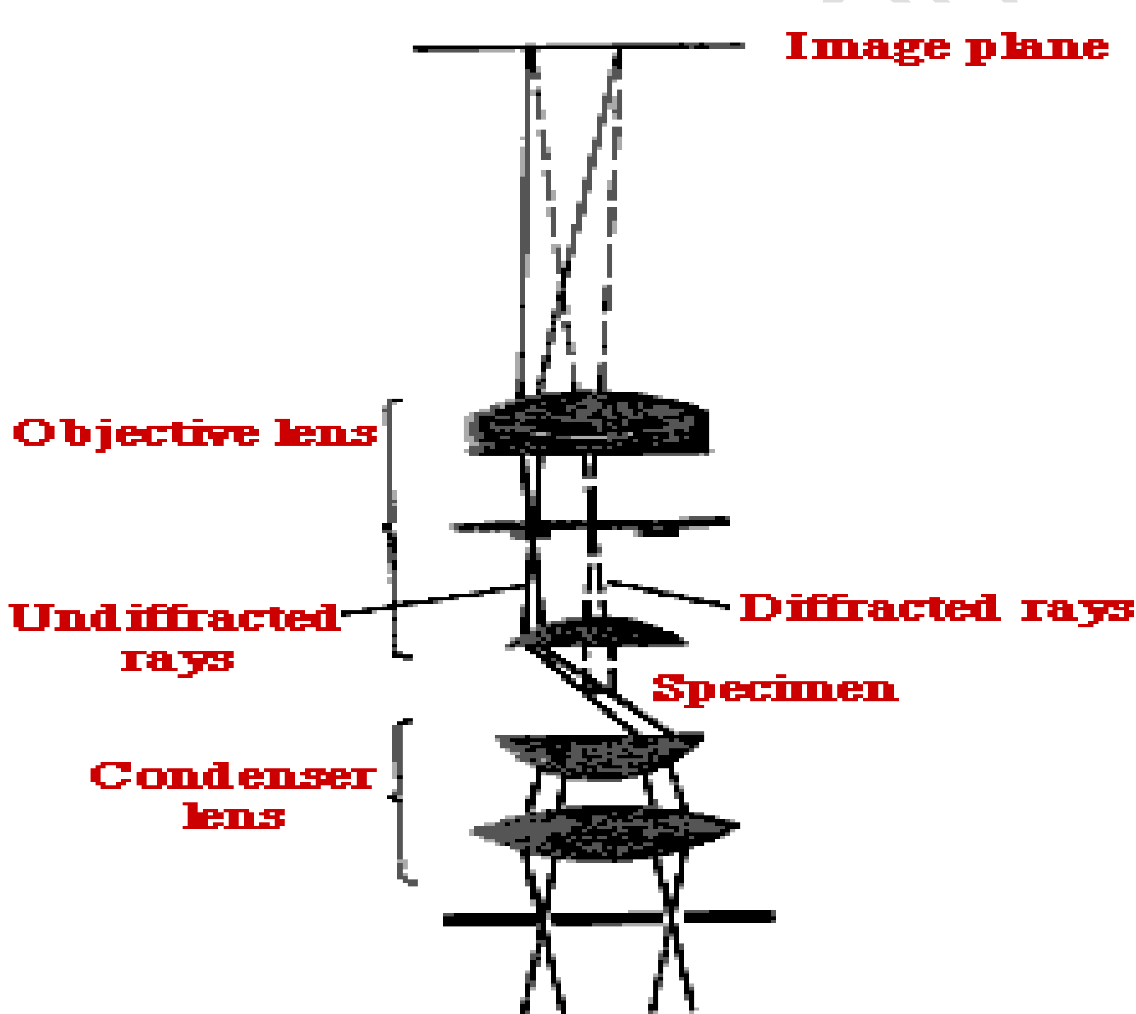
Experiment- 3

Aim: - To study phase contrast microscope.

Theory: - It was invented by Dutch scientist “Fredric Zernike” in 1935. The source of illumination is ordinary visible light. Thus its resolving power is same as that of light microscope. It makes highly transfer object much better visible. It is based on the principle that the light transmitted by region of high refractive index in the velocity with respect to light transmitted by region of lower refractive index . This phase contrast is produced deviated and un deviated light rays. So a transparent object appears in various shape of depending upon the thickness of object and difference between object and medium.



Phase plate: - It is present between objective lens amd eye peice. It has thin and thick region so that ray emerging through the thinner region will be out of phase with rays emerging to the outer thick region of phase plate. This results in grester resolution. Image seen in phase contrast microscope and contrasted between object and background is increased

Structure: - It has an annular diaphragm before condenser and phase plate after the objective lens. Yhe phase contrast microscope has eye piece, objective lens and a condenser comparable to one that is compound microscope. In addition n, the lens contains an annular diaphragm. The annular diaphragm permits light to pass through the condenser as a hollow cone and remaining light is focussed on the object to be seen. Phase plate is useful as it gives deviated path of light instead of direct path of light.

Uses: -

1. It is used for the study of organelles in the living cell.
2. To study movement of chromosome during cell division.
3. To study the streaming movement of cytoplasm.
4. It is used to distinguish between normal cell and cell which are fixed and stained.
5. It is valuable for observing cells, cultured in vitro during mitosis.

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Experiment -4

Aim: - To study prokaryotic cell by carrying out gram staining.

Requirements: - Sample slide, cover slip, crystal violet, gram iodine solution, 95% alcohol, safranine, inoculated needle, spirit.

Principle: - Gram staining is most important differential staining technique given by Dr. Chrisom Gram divide the bacterial cell into two major group gram positive and gram gram negative. It is essential for classification of microbes gram staining reaction is based on different cell wall gram positive have thick peptidoglycan and gram negative have thin. The crystal violet binds to cell wall in combination with gram iodine which is important intensity. The colour of stain and cell appear purple. When discolouring with 95% of alcohol that removes from thinner and less cross linked peptidoglycan layer in gram positive cell wall reaction the complex will remain purple.

Procedure: -

1) Make smear of bacteria on separate glass slide.
2) Allow smear to air dry.
3) Heat fix the smear.
4) Add crystal violet solution on the smear gently and wait for 1 minute.
5) Drain the crystal violet solution and wash gently with water.
6) Add gram iodine solution and wait for 1 minute, drain it.
7) Wash the smear with water gently.
8) Add ethanol drop by drop until no more colour flows from smear.
9) Gently wash slide with water.
10) Apply counter stain (saffaranin) to smear for 45 sec, drain it.
11) Wash with water gently.
12) Blot dry with blotting paper and examine under oil immersion objective.

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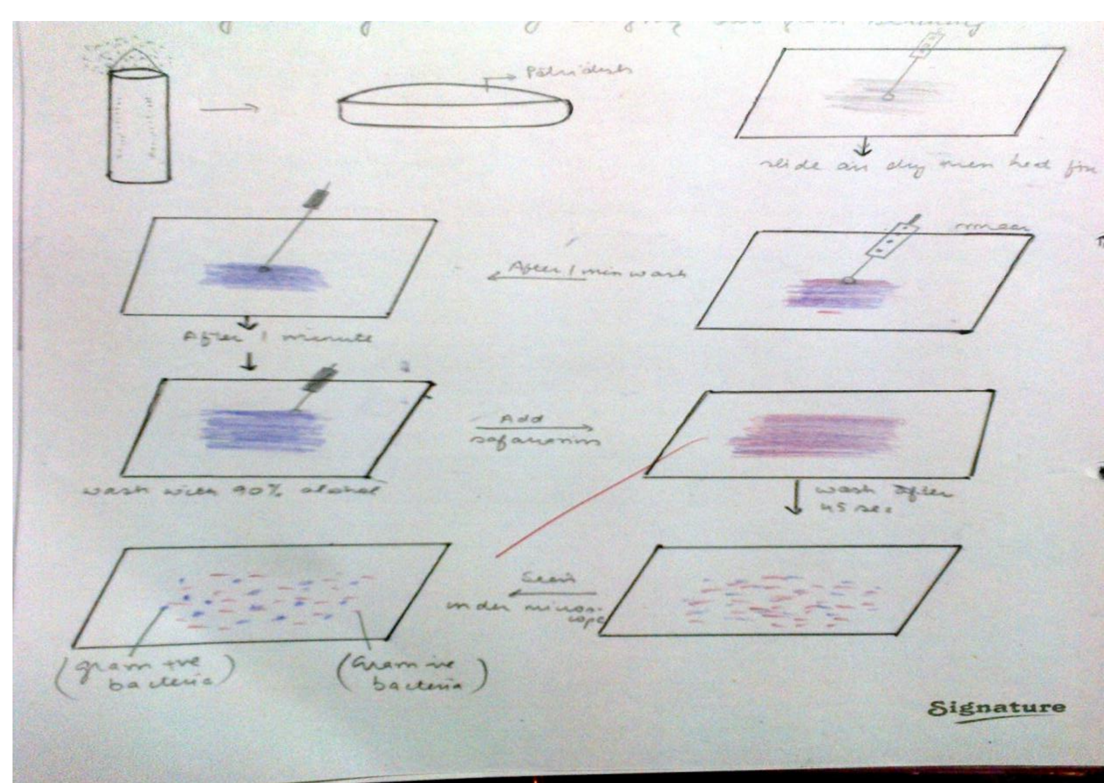

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Experiment – 5

Aim: - To study Lactobacillus by gram staining.

Identification Points: -

- Lactobacillus has hydrophilic and electrostatic cell surface property.
- Lactobacillus is gram positive facultative anaerobic bacteria.
- They convert lactose and other sugar to lactic acid.
- The Lactobacillus currently consist 125 species and comprises a wide variety of organism.

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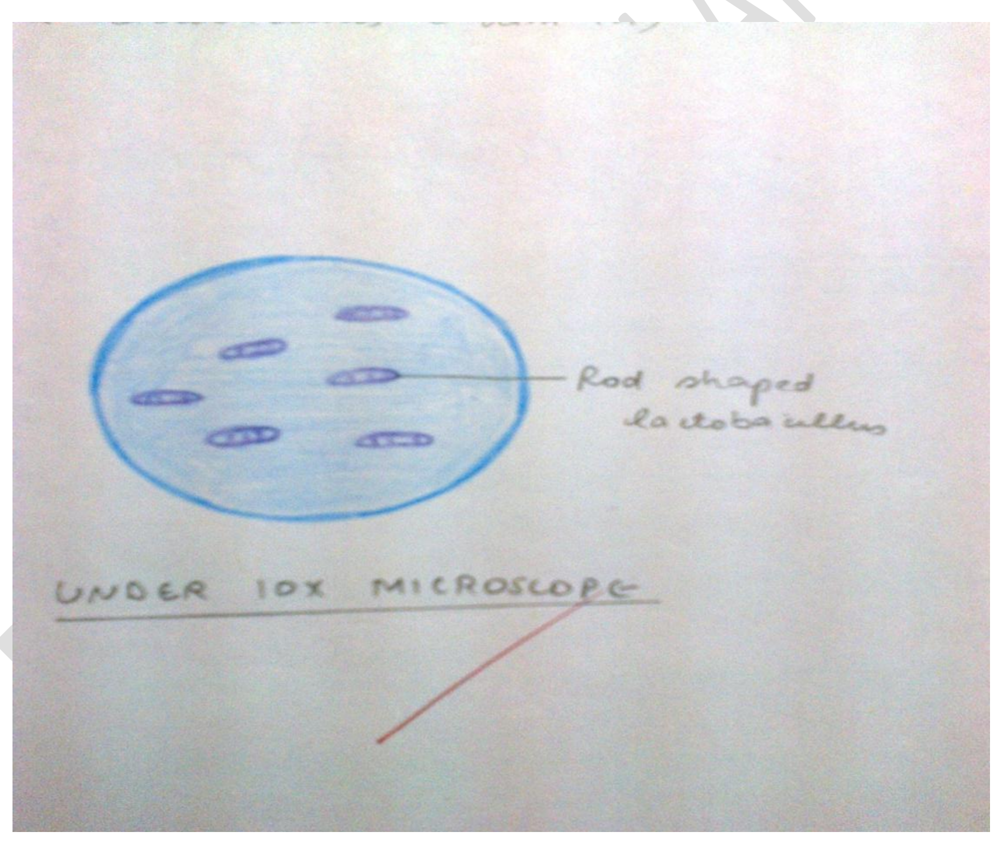
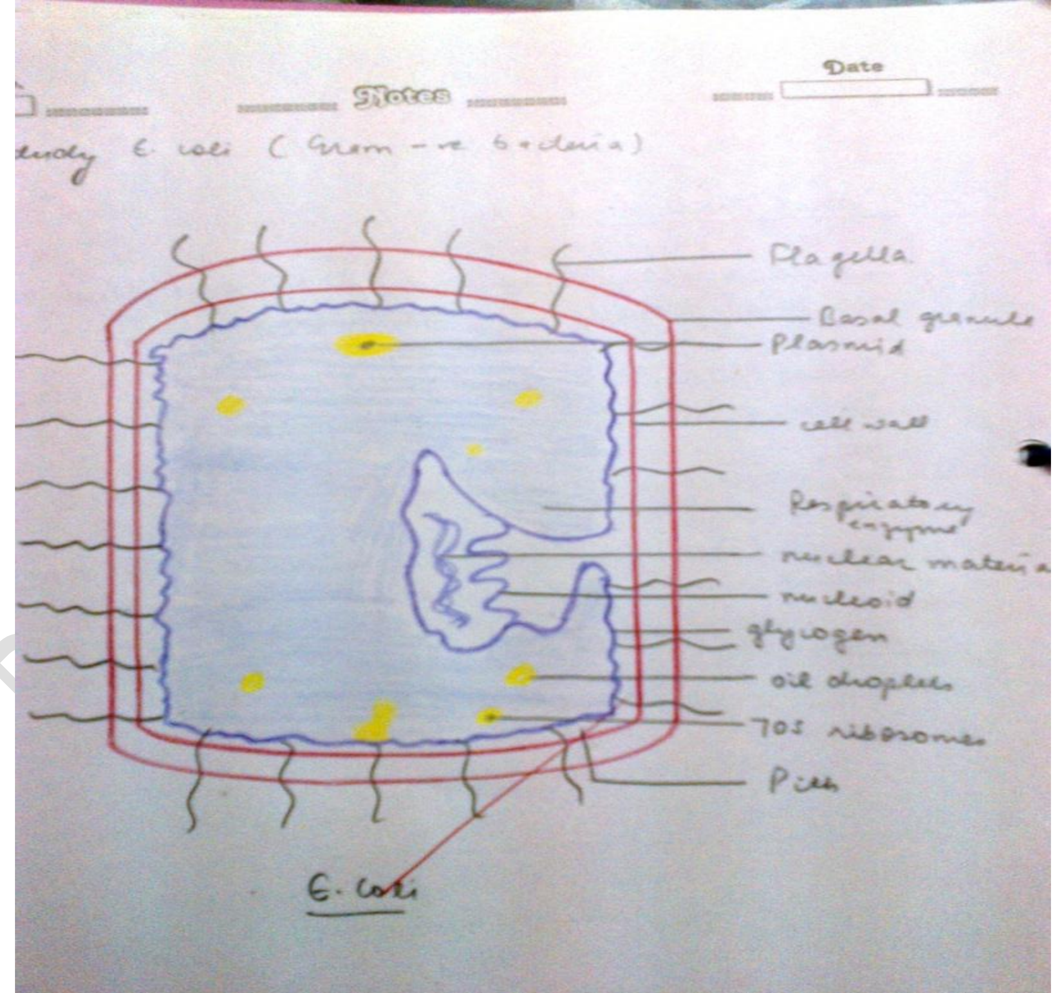


Experiment -6

Aim: - To study *Escherichia coli* by gram staining.

Identification Points: -

- Slime is the gelatinous substance sec by the protoplasm and deposited on cell wall.
- In case of some virulent bacteria slime becomes thicker called as capsule.
- Bacteria have a rigid wall which protect the protoplasm and provides definite shape.
- Each cell has plasma wall situated next to cell wall.



- It is thin elastic of selectively permeable membrane.

- Cytoplasm is semi fluid round containing ribosome a RNA.
- Nucleus with nuclear membrane is absent.
- Nuclear material consists of DNA material.

Experiment -7

Aim: - To study blue green algae by gram staining.

Identification Points: -

- It is unicellular and colonial species.
- Cyanobacteria blue green algae is phylum of bacteria which obtained their energy by photosynthesis.
- Body is rounded in shape and resembles bacterial cell in many aspects.
- They lack flagella as present in bacteria.
- Beneath the cell wall there is plasma membrane composed of lipoprotein and lipopolysachharides.
- DNA is circular and double helix called nucleoid cell contain photosynthetic pigment such as chlorophyll.

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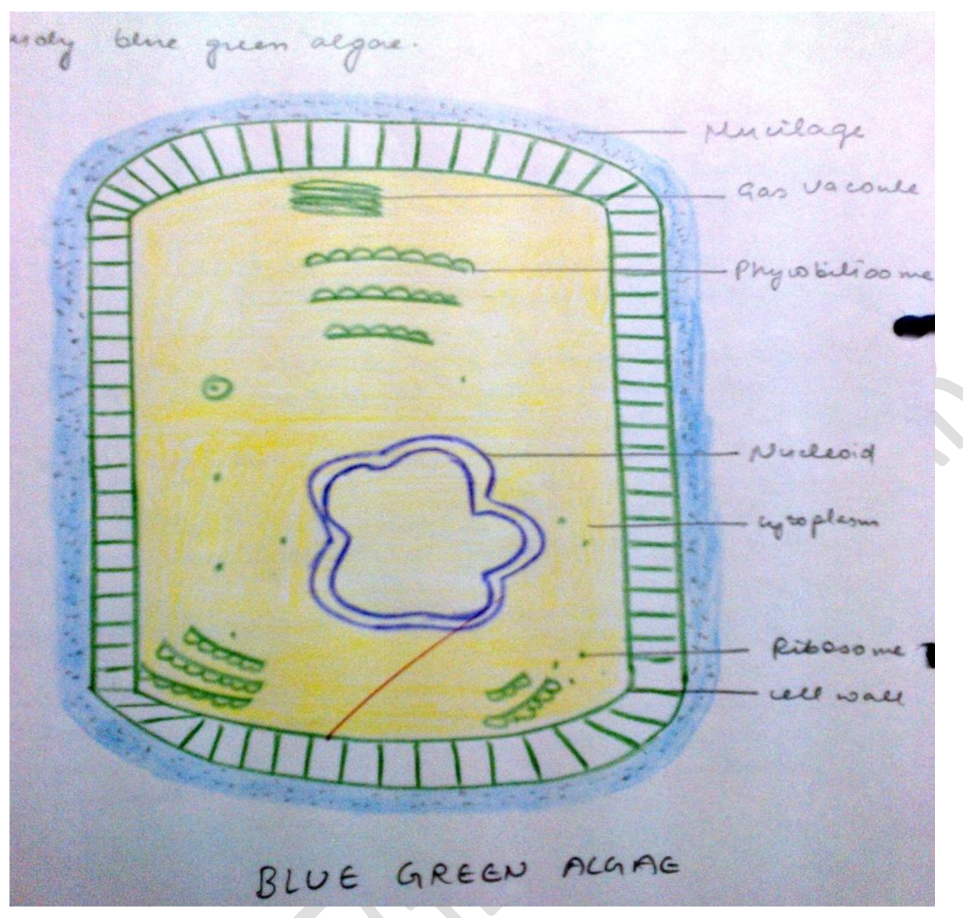
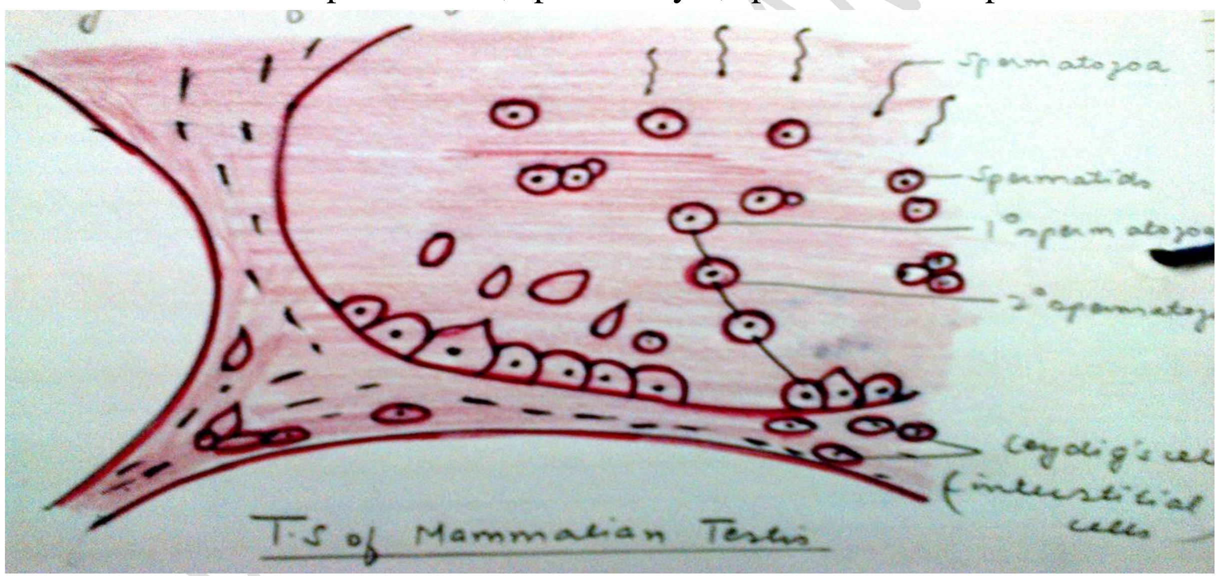


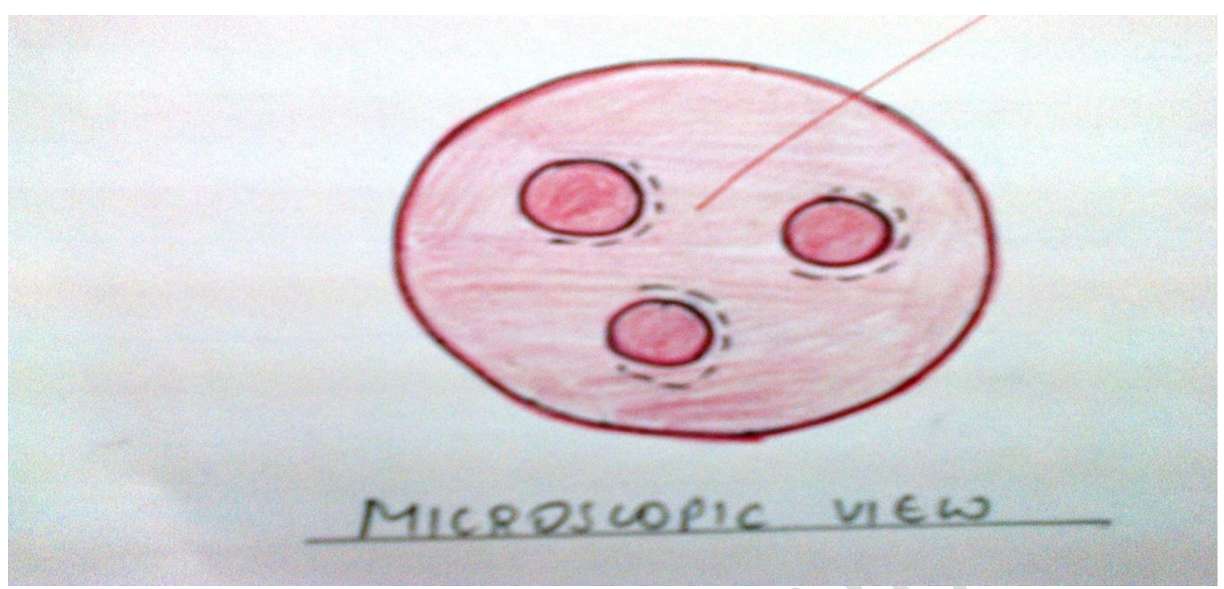
<u>Experiment</u> – 8

<u>Aim</u>: - To study testicular material of mammals.

<u>Characteristic Features</u>: -

1. Testis are somewhat rounded or oval shape and surrounded by peritoneum followed by a layer of fibrous connective tissue. The tunica albugenia.
2. Histologically each testis is composed of a mass of coiled seminiferous tubules.
3. Seminiferous tubules are separate from one another by intertubular tissue.
4. In between germinal cell contain larger cells contain larger cells called sertolli cells. These cells have role of supplying nourishment to the developing sperm.
5. Various development stages of sperm formation are seen they include spermatozoa, spermatocyte, spermatids and spermatozoa.





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Identification points: -

- Each tubule is composed of mass of smaller tubules called seminiferous tubules.
- These seminiferous tubules are seen to be sep to one another by intercellular tissues.
- Various stages of sperm development can be seen in the slide like 1degree spermatocyte spermatids on complete sperm.
- Sertoli cell and intestine cell on the leydig cell are also visible.

Experiment – 9

Aim: - To study the electron micrograph of mitochondria.

Comments: -

- The mitochondria are formed in eukaryotic cell except some parasitic cell.
- The mitochondria are absent in mature cell etc.
- The mitochondria consist of membrane that is outer and inner membrane, cisternae and matrix.
- Matrix consist of – protein, ribosome's, DNA, RNA, oxysomes are present in inner mitochondria membrane.
- Mitochondrion is after described as power house of cell some amino acid are found in mitochondria.
- Mitochondria provide intermediate for synthesis of biomolecules such as chlorophyll, steroid.

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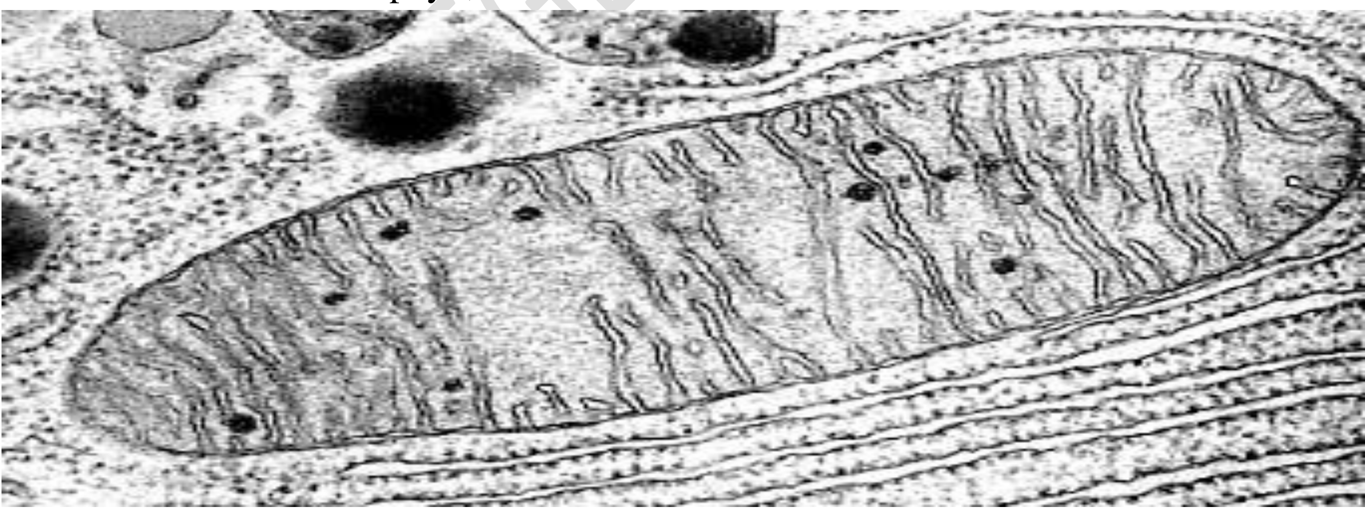

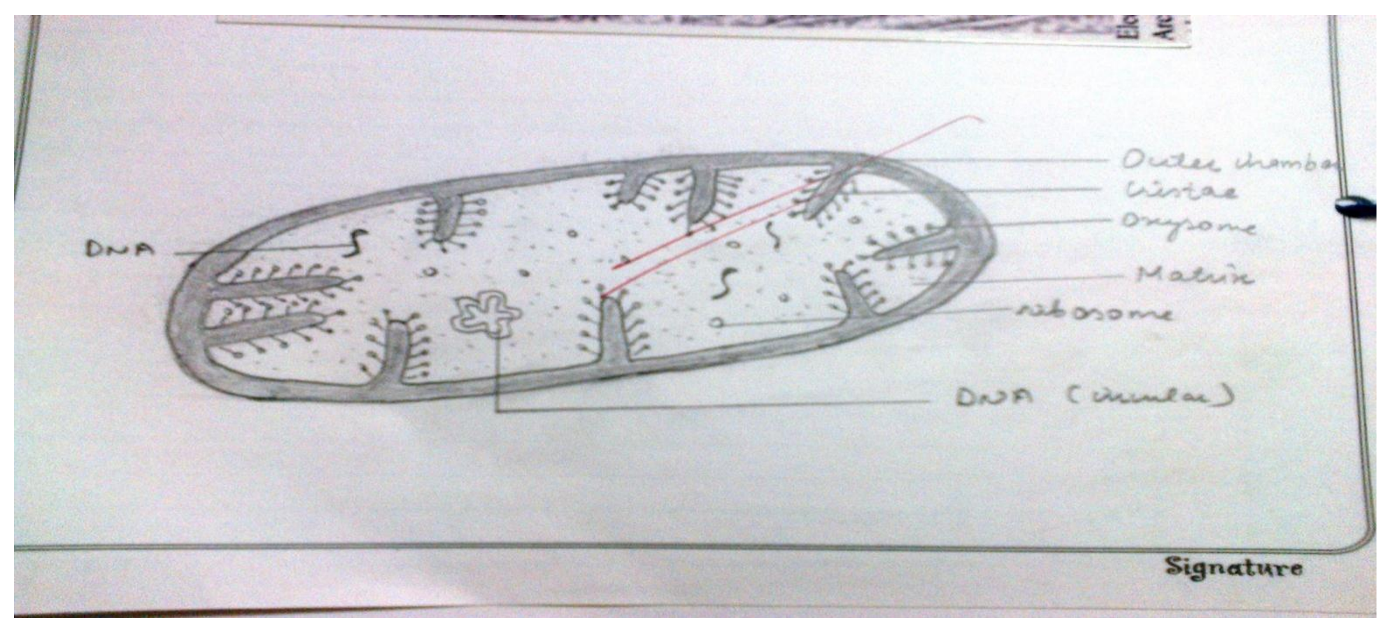

Experiment – 10

Aim: - TO study electron micrograph of Golgi apparatus.

Comments: -

- Golgi apparatus is present in all eukaryotic cells except of mammalian RBC's, sperm cell of bryophyte and sieve tube of plant. It is absent in prokaryotic cells.
- Golgi apparatus consist of cisternae, tubules and vesicles.
- Vesicles lies near the end and concave side of Golgi complex.
- They are pouched off from tubules of cisternae.
- They are of two types – coated vesicles and smooth vesicles.
- Golgi apparatus originates from ER.

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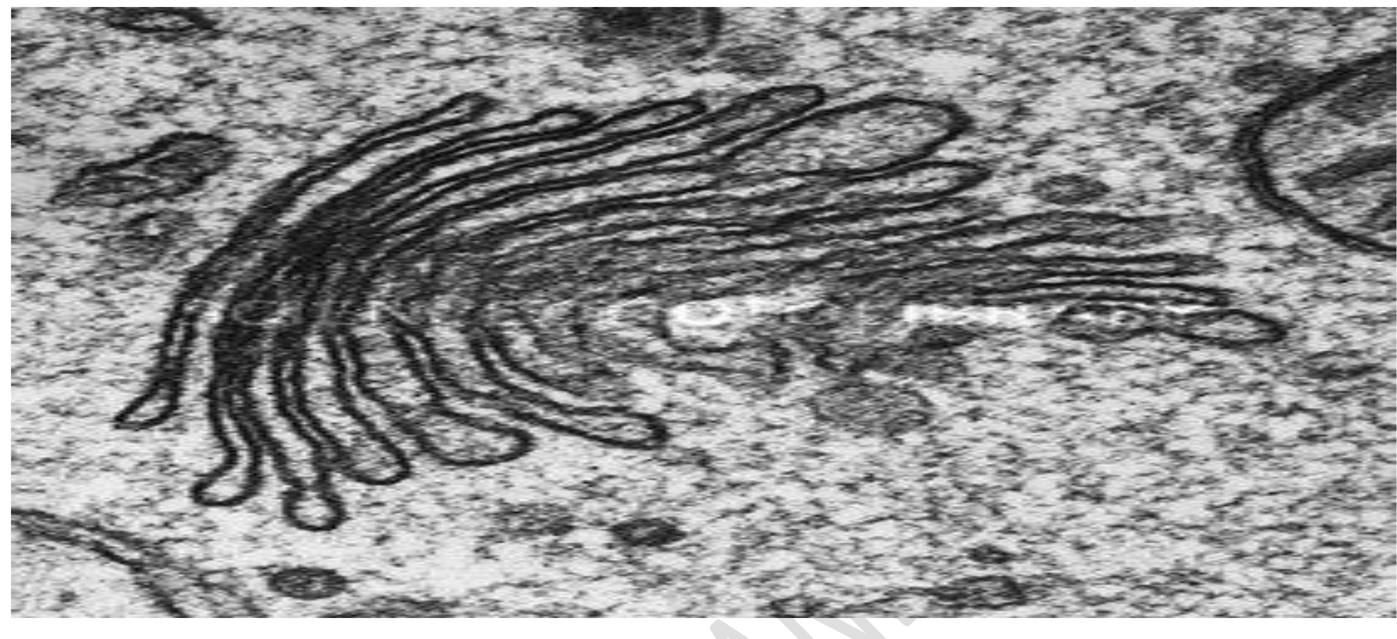
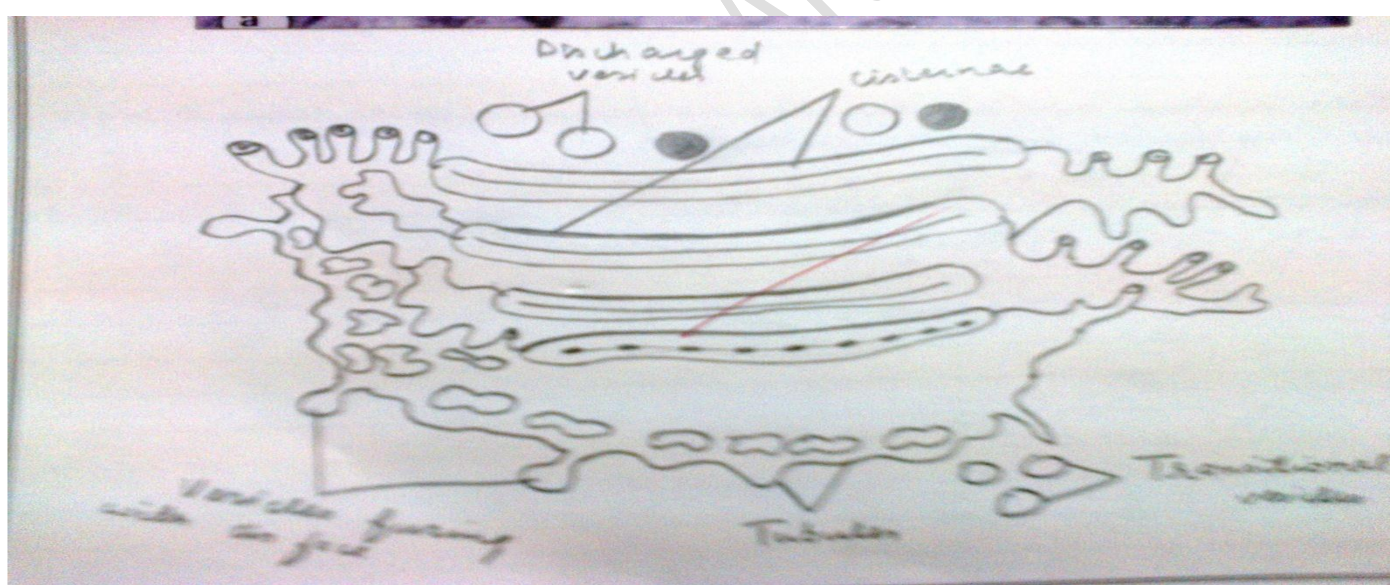




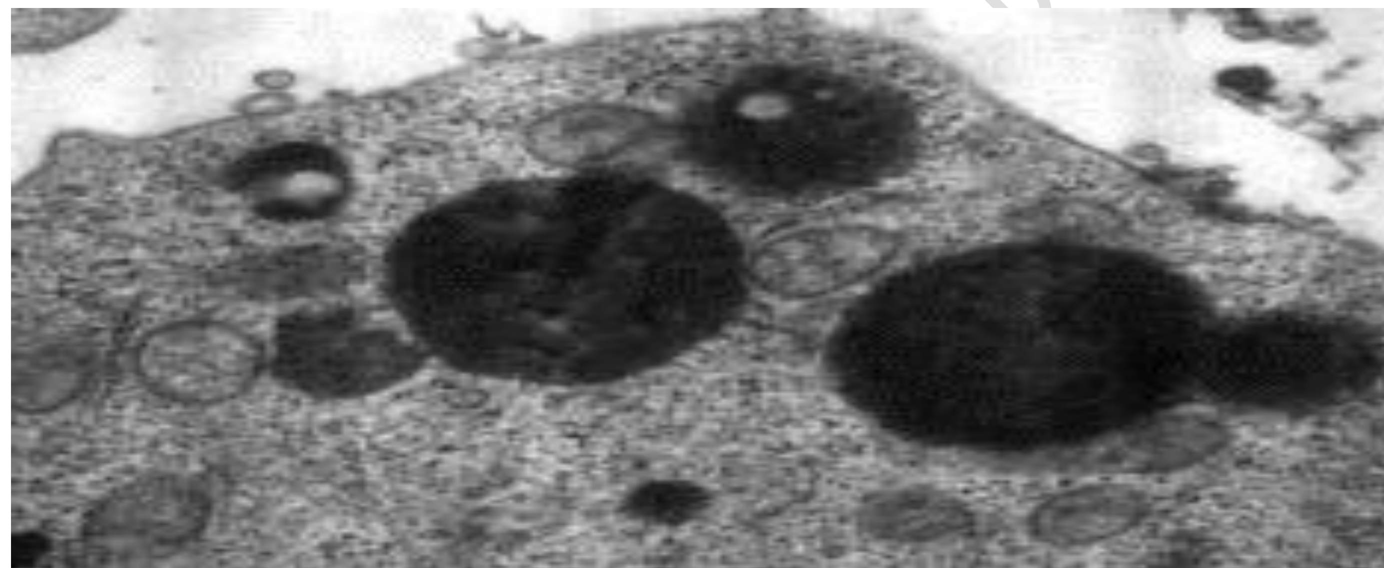
Experiment - 11

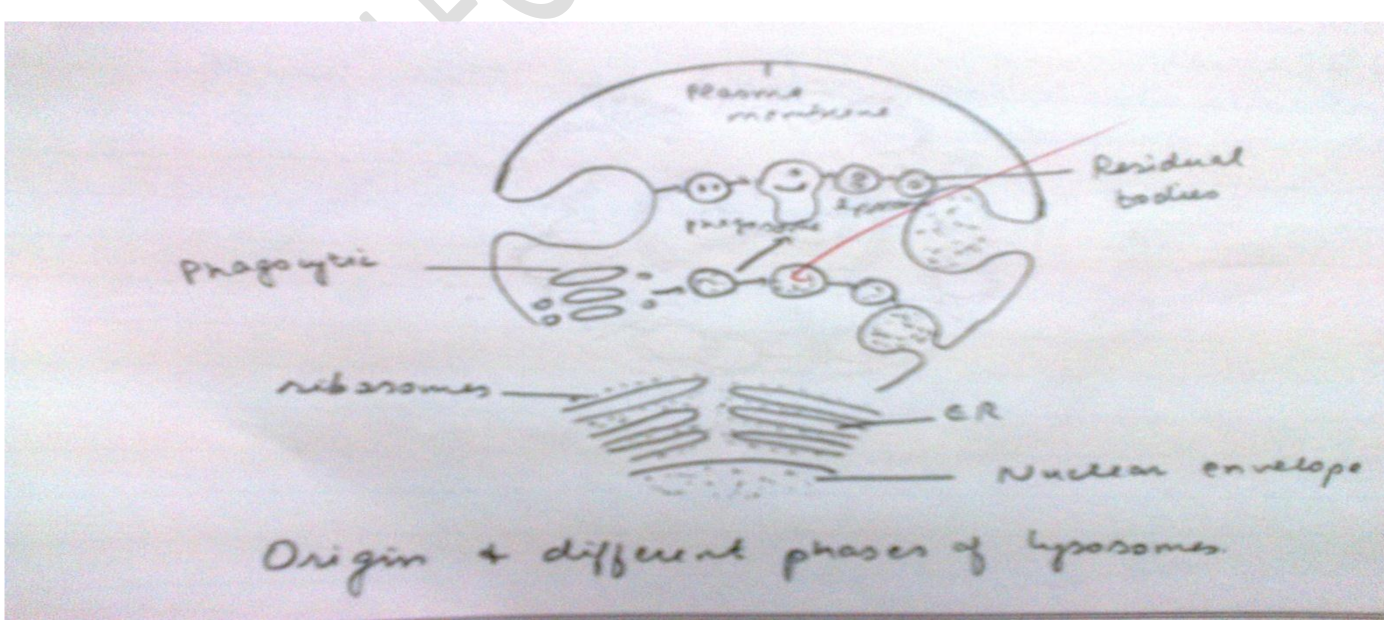
Aim: - To study the electron micrograph of Lysosomes.

Comments:

- Lysosomes are cytoplasmic organelles of eukaryotic cells.
- Lysosomes are found in proteston protozoan's and animal cells.
- Phagocytosis of microbes is assisted by lysosome contained in the lysosome.
- Typically lysosomes have four types of forms.

- They are divided into primary and secondary lysosomes.
- Secondary lysosomes are of three kinds-heterophagosome autophagosomes and residual bodies.
- In eggs lysosomes take part in digestion for stored food.
- During starvation the stored proteins fats and polysaccharides are broken down with the help of lysosomes.
- Lysosomes are regarded for initiation of mitosis.





<u>Experiment</u> – 12

Aim: - To study electron graph of Endoplasmic reticulum.

Comments: -

- ER is present in all most all eukaryotic cells except in ova, embryonic cells, mammalian RBC.
- It comprises of – cisternae, tubules and vesicles.
- ER is of two types – SER and RER. Smooth ER is free of ribosome.
- It lies in adipose tissue, interstitial cells, glycogen, liver cells, adrenal cortex cells.
- Rough endoplasmic reticulum contains ribosomes and it occurs deep in cytoplasm. These occur liver cells, pancreatic cells, salivary gland cells.

Functions: -

- Surface for synthesis.
- Glycogen metabolism.
- Skeletal muscle contraction.
- Fat oxidation.

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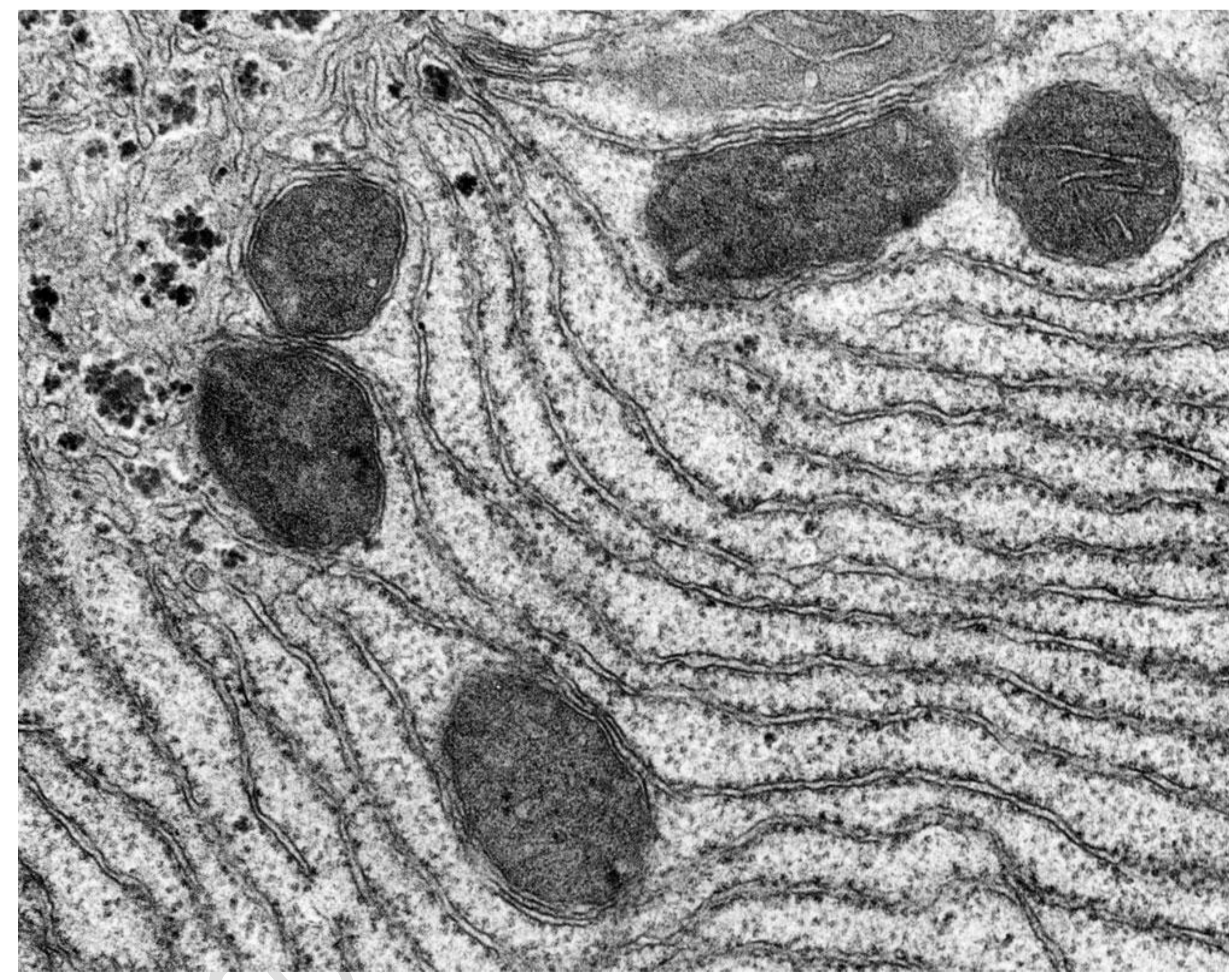

Experiment – 13

Aim: - To study electron micrograph of Cell membrane.

Comments: -

- Cell membrane consist of complete sheath present in form of intrinsic and extrinsic proteins.
- Lipid bilayer is continuous.
- It is made of phospholipids molecules. The hydrophobic head towards outer side and hydrophilic tail towards inner side like mosaic in sea of lipids 70% of membrane protein are intrinsic and 30% of extrinsic in nature.

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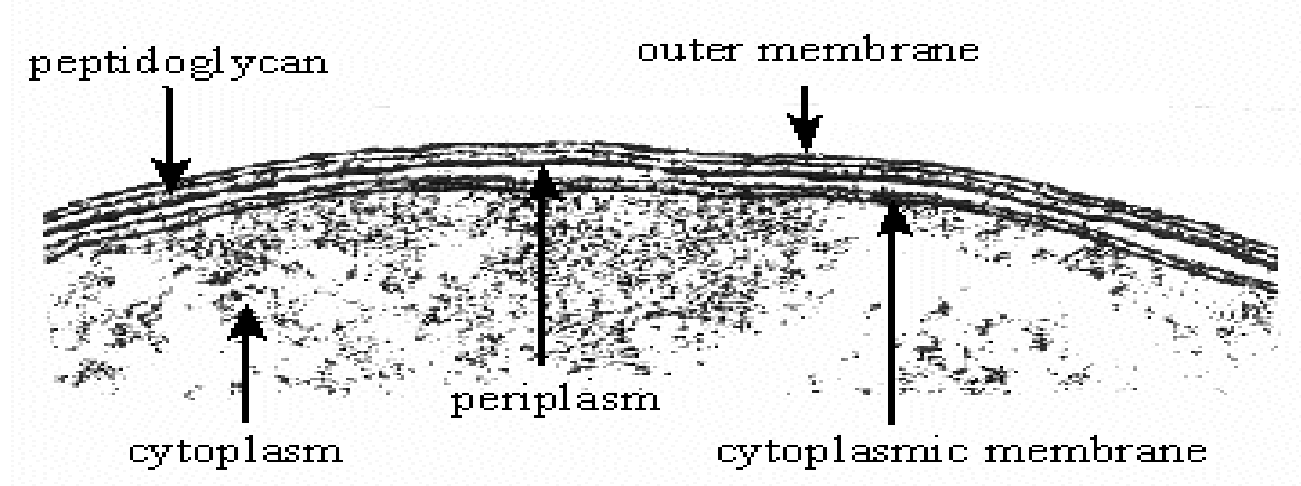
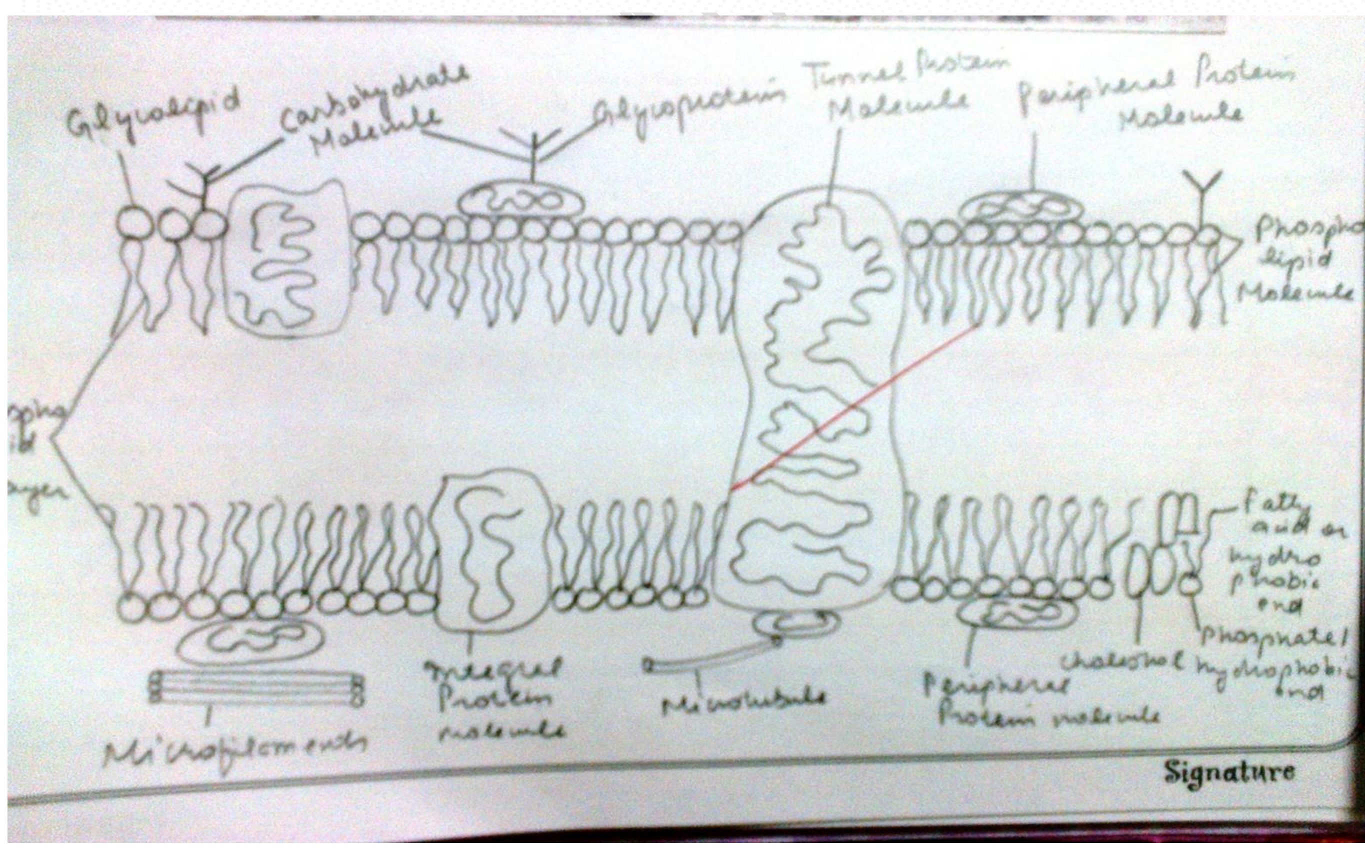
- The external surface of cell membrane posses' carbohydrates molecule. They are attached to glycoprotein, glycolipids called cell coated or proglycans. They form a 100 carbohydrates called glycolipids.





Experiment-14

Aim: - To study electron micrograph of centriole.

Comments:

- The centriole are formed in all eukaryotic cell which have mobile cells in their life cycle organism lack centrioles include amoeba, yeast, diatoms, unicellular red algae.
- Centriole consist of a set of microtubules tripods which lies evenly spaced in ring.
- The centrioles appear as two minute, dark staining granules with the light microscope.
- There are no microtubules at centre of ring giving 9+0 pattern for centrioles.
- Each microtubules in a triplet is about 250degree a wide.
- They provide basal bodies which gives size to cilia and flagella.
- Peri centrioles material act as the MTPC for the cytoplasmic microtubules.

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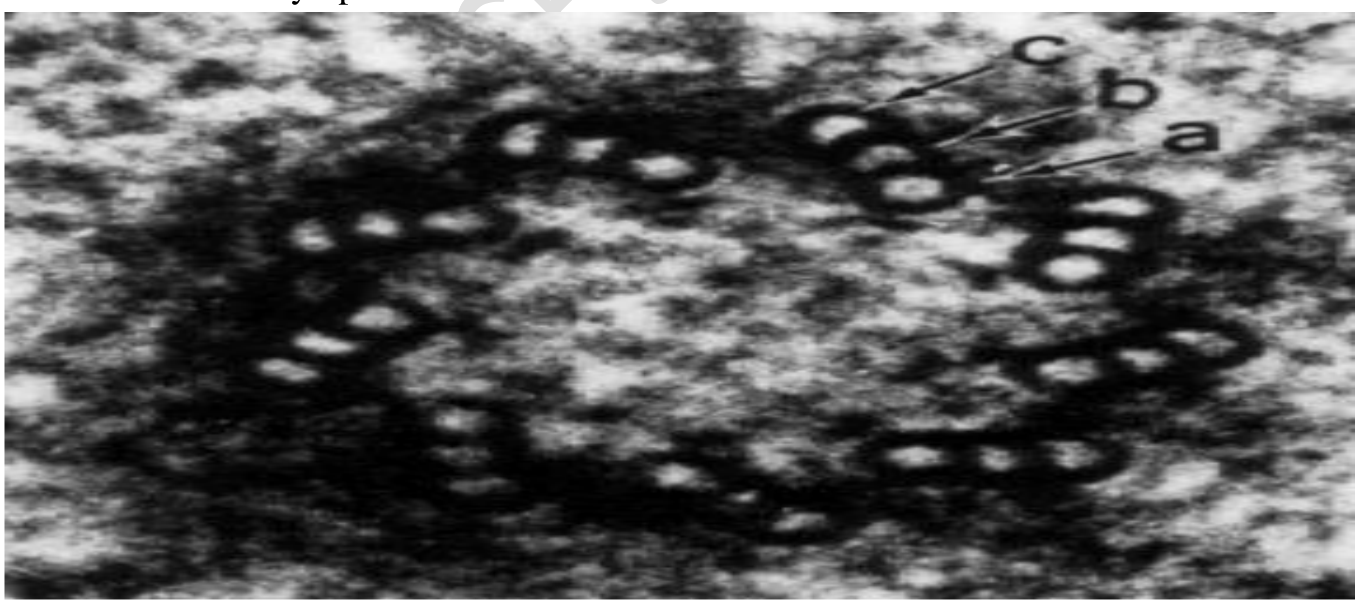

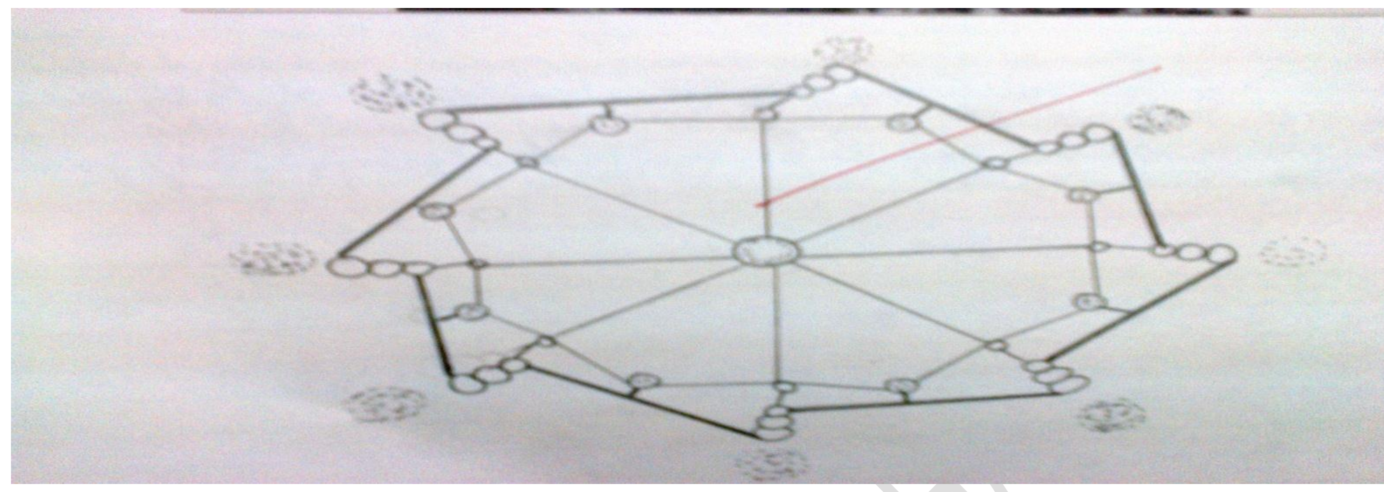

Experiment -15

Aim: - To study the electron micrograph of cilia.

Comments: -

- The ileum which is the slender and cylindrical process that projects forms free surface of cell.
- The basal body or granule the intercellular organelle similar to centriole forms which it originates.
- Cell fibres called ciliary rootlets that arise from basal granules and coverage into conical bundle the pointed extremely of which.
- The cilia created food currents in aquatic animals cilia provided locomotion to cell organism.

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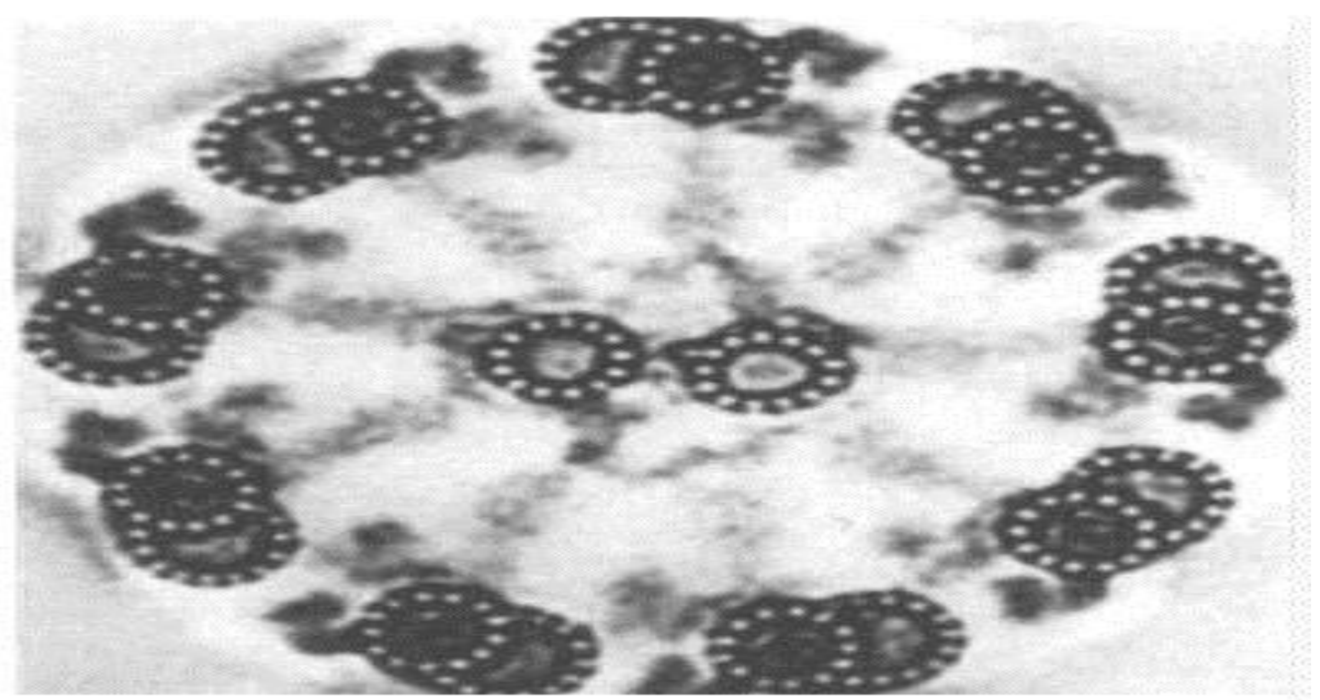

CILIA

IMMUNOLOGY PRACTICAL MANUAL

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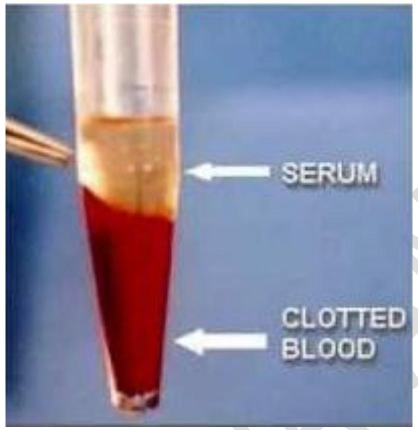
EXPERIMENT NO-1

AIM:- Separate serum from blood.

REQUIREMENTS:- centrifuge test tube, test tube stand, eppendorf, alcohol, cotton and blood sample.



THEORY:- the separated serum from blood contains particles or solute either in the form of protein or cell which are present in different states. When the centrifuge force is applied the particles in the sample starts settling down and finally settle at the bottom in the form of pellet. This occurs when force of gravity overcomes the force of diffusion.

PROCEDURE:-

i. Take blood sample in centrifuge tube and keep it at room temperature for 30 minutes.
ii. Centrifuge the tube at 300 rpm for 15 minutes.
iii. Take out the tube along with supernatant.
iv. Separate the supernatant (yellow coloured serum from blood).
v. Collect serum in a separate tube.

PRECAUTIONS:-

i. Balance the centrifuge carefully and set the rotation accordingly.

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EXPERIMENT-2

AIM:- To separate plasma from blood sample.

REQUIREMENTS:- Centrifuge test tubes, test tube stand, alcohol, cotton, syringe, EDTA as anticoagulant, blood.

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THEORY:-Plasma is the colourless watery liquid of the blood and lymph that contains no cells but in the blood cells i.e. erythrocytes, leucocytes and thrombocytes are suspended. It makes up approx. 55% of total blood vol.it is made primary of water with small amount of minerals, salts, ions, nutrients and protein in soln. the large variety of protein including albumin, immunoglobins and clotting proteins such as fibrinogen are present in it. It is the main contribution to osmotic pressure in it. the blood coagulated, collected and is centrifuge by adding few drops of anticoagulant i.e. EDTA at room temp and plasma is separated to anticoagulant to blood doesn't clot the cells including RBC's, WBC's, platelets and other clotting factors. It is the clot that makes the difference between serum and plasma.

PROCEDURE:-

1. The blood sample was taken by 5ml syringe through the vein. The blood is now put in centrifuge tube, add few drops of EDTA.

2. The centrifuge tube that were put in the centrifuge and tubes were centrifuged at 2000 rpm for 20 mins.
3. The tubes were then taken out and clearly visible out light yellow fluid were taken in another tube with help of auto pipettes.
4. The supernatant separated from blood settle at bottom.

PRECAUTIONS:-

Centrifuge should be properly balanced and rotations should be set accordingly.

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EXPERIMENT-3

AIM:- To determine the blood groups of human by A, B, O blood group system.

REQUIREMENT:- Sterilized needle, cotton, spirit, microscope, pipettes, tooth pick.

CHEMICALS REQUIRED:- Alcohol, antiserum-A, antiserum-B and Antiserum-D.

	Group A	Group B	Group AB	Group O
Red blood cell type	A	B	AB	O
Antibodies in Plasma	Anti-B	Anti-A	None	Anti-A and Anti-B
Antigens in Red Blood Cell	A antigen	B antigen	A and B antigens	None

THEROY:- Carl Landsteiner discovered ABO blood in 1900. Landsteiner described A, B and O and two of his students and Adriana discovered the fourth type 'AB' in 1902. Human blood is differentiated into four defined blood groups and the surface of RBC,s. There are two different types of antibodies 'a' and 'b' and two different types of antigens 'A' and 'B' in the blood. Different types of blood groups have following antigens and antibodies as given in table. When two different types of blood are mixed and RBC's come together and clumping of RBC's is known as agglutination. This is due to the interaction of antibodies-antigens which is due to the presence of RBC's on blood plasma.

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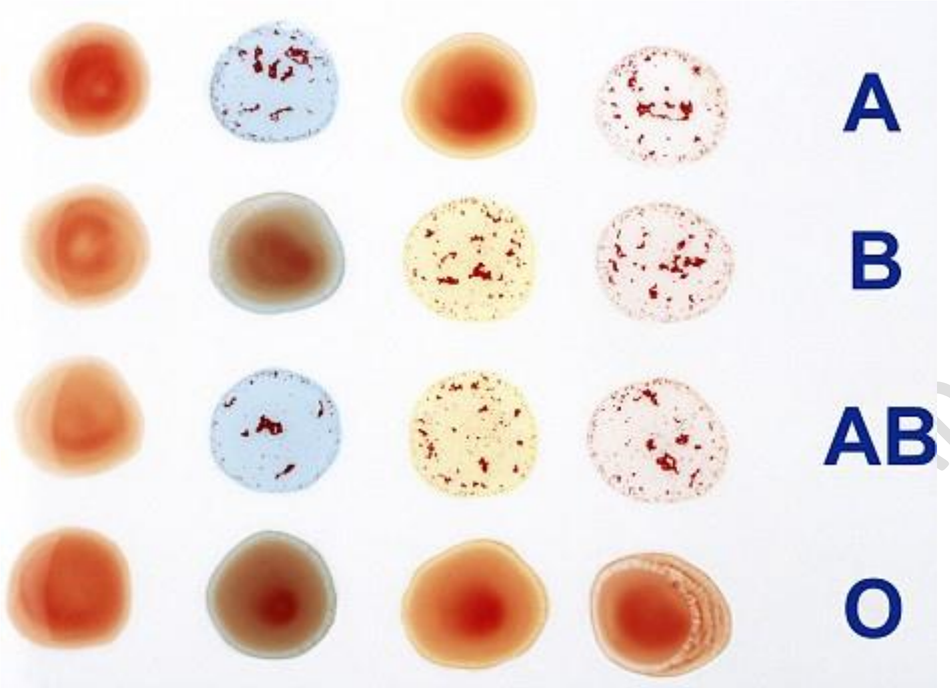

PROCEDURE:-

1. Take a clean slide and mark the point A, B, and D.
2. Sterilize the finger with alcohol with a bold prick with the help of sterilize needle.
3. Put one drop of blood on each and mark the position on slide.
4. Put a drop of antiserum A on mark A, antiserum B on mark B and antiserum D on mark D (Rh-factor).
5. Thoroughly mix the drop of blood with the antiserum by using tooth pick. Allow 2-5minites for the agglutination to take place.
6. Observe the slide for agglutination with naked eye and then under microscope.

PRECAUTIONS:-

1. Slides should be neat and clean.
2. Sterilized prickle should be used.
3. Separate tooth-pick should be used for mixing blood with antiserum.
4. The nozzle of antiserum should not be touched with blood sample.

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EXPERIMENT-4

AIM:- To separate lymphocytes for culturing.

MATERIALS REQUIRED:-

Two syringes of 5ml each, blood sample (5ml), anticoagulant , centrifugation machine, test tube and ficoll histopaque (150 lymph) solution (specific gravity=1.0777 g/l).

THEORY:- The most commonly used method used for the separation of lymphocytes is the sedimentation through high density medium of a mononuclear cells can be obtained by centrifuge whole blood or the ficoll plaque and the commercial preparation are from ficoll.

PROCEDURE:-

1. Collect peripheral blood in the vial containing anticoagulant.
2. Dilute blood 1:2 with phosphate buffer saline (PBS) not more than 3 times the volume of the distilled water into 3 times the layer of ficoll.
3. Always hold a tube at an angle with carrying out the layering of the ficoll and the blood do not get mixed.
4. Centrifuge at room temp at 400 rpm for 30 minutes.
5. Take care that the centrifuge doesn't accelerate so rapidly.
6. PBMC's were separated by density gradient centrifugation method.
7. 5 ml of blood was mixed with equal volume of PBS (phosphate buffer saline)
8. PBS consists of NaCl (0.8g) disodium hydrogen phosphate chloride.
9. The supernatant over interphase was removed and interphase containing population of PBMC's
10. The contents were mixed well and centrifuged at 1500 rpm at 25°C for 15 minutes.
11. Again the supernatant was discarded and the pellet suspended in 10ml PBS and centrifuged at 1200 rpm for 12 mins.
12. Another washing was given with PBS at 1000 rpm for 10 mins and supernatant was discarded.

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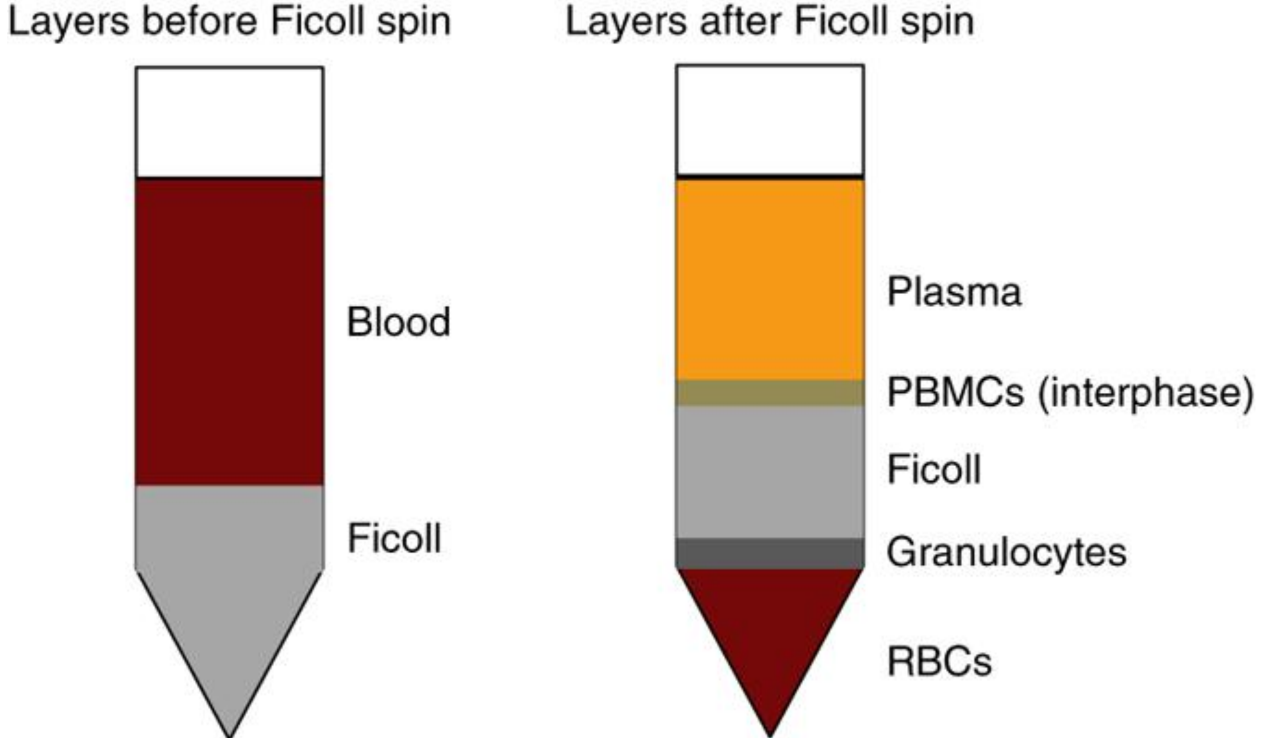
13. In the last, we obtained population of PBMC's these can be counted in member's chambers.



PRECAUTION:-

1. Layering should be done carefully.
2. Carefully dilute the blood.
3. Take care that centrifuge should not decelerates.
4. Observe the white blood carefully.

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EXPERIMENT-5

AIM:- Separation of peritoneal macrophages from rat.

REQUIREMENTS:- 10ml syringe, Ice cold PBS, 20% BSA, rat, centrifuge machine, cotton etc.

PROCEDURE:-

1. The rat was unconscious with the help of chloroform. The skin was drenched in 70% alcohol to sterile and was placed on its back.
2. A small incision was made rejected carefully without puncturing the peritonium.
3. 8ml ice cold solution of PBS and BSA was taken in a syringe and carefully injected by lifting the peritonium cavity.
4. The peritoneum was agitated slowly to withdraw as much as possible fluid out.
5. Centrifuge the fluid for 10 mins at 2000 rpm. Discard the supernatant and suspended the pellet in 10% RRMI medium.
6. Discard supernatant and suspended the pellet in 10% RPMI, use cells for further use.

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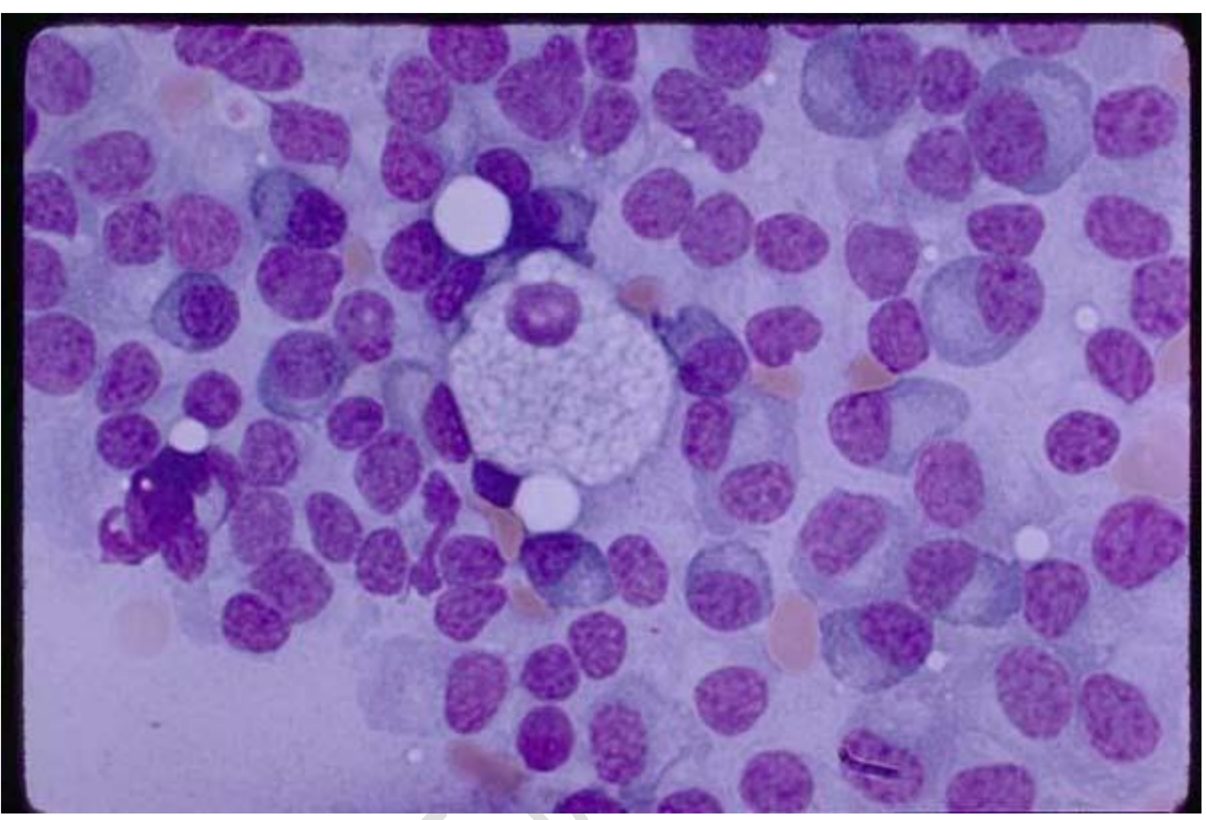

PRECAUTION:-

1. The experiment should be performed under aseptic conditions.
2. Insect the cold soln of PBS and BSA in peritoneum cavity carefully without disrupting it.
3. Sterile the rat before incision to avoid contamination.
4. Carefully collect the cells from peritoneal cavity.

OBERVATIONS:- When collected solution from peritonium cavity is seen under inverted microscopes, amoeboid and non-amoeboid macrophages are seen.

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GENETICS

PRACTICAL MANUAL

Semester- III

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Gregor Johann Mendel

Life history of Gregor Johann Mendel: Gregor Johann Mendel (Austrian 1822 to 1884) was born in a peasant family of Moravia. He received school education mainly by his own effort. Due to poverty he became a monk (religious man) in 1843 .He was later, in 1847 made an abbot of Austrian Monastry of St. Thomas at brunn Austria , now Bruno in Czech republic. From here, he went in 1851 to university of Vienna where he studied natura history and mathematics for two years. Whilst in Vienna, he became interested in the process of hybridization. His choice of the subject and his aptitude influence on his later work on inheritance on pea plant. Mendel returned to the monastery in 1853. Here, he also worked as a teacher of physics and natural history in a school. From 1865, Mendel conducted breeding experiment on garden peas in the garden of his monastery. He was not the first to experiment in the field of inheritance, but was luckily principles or law of heredity. He published his findings in 1866 in the "Annual proceedings of the natural history society of Brunn" His laws from the basis of genetic even today, and he is remembered as the "Father of genetics"

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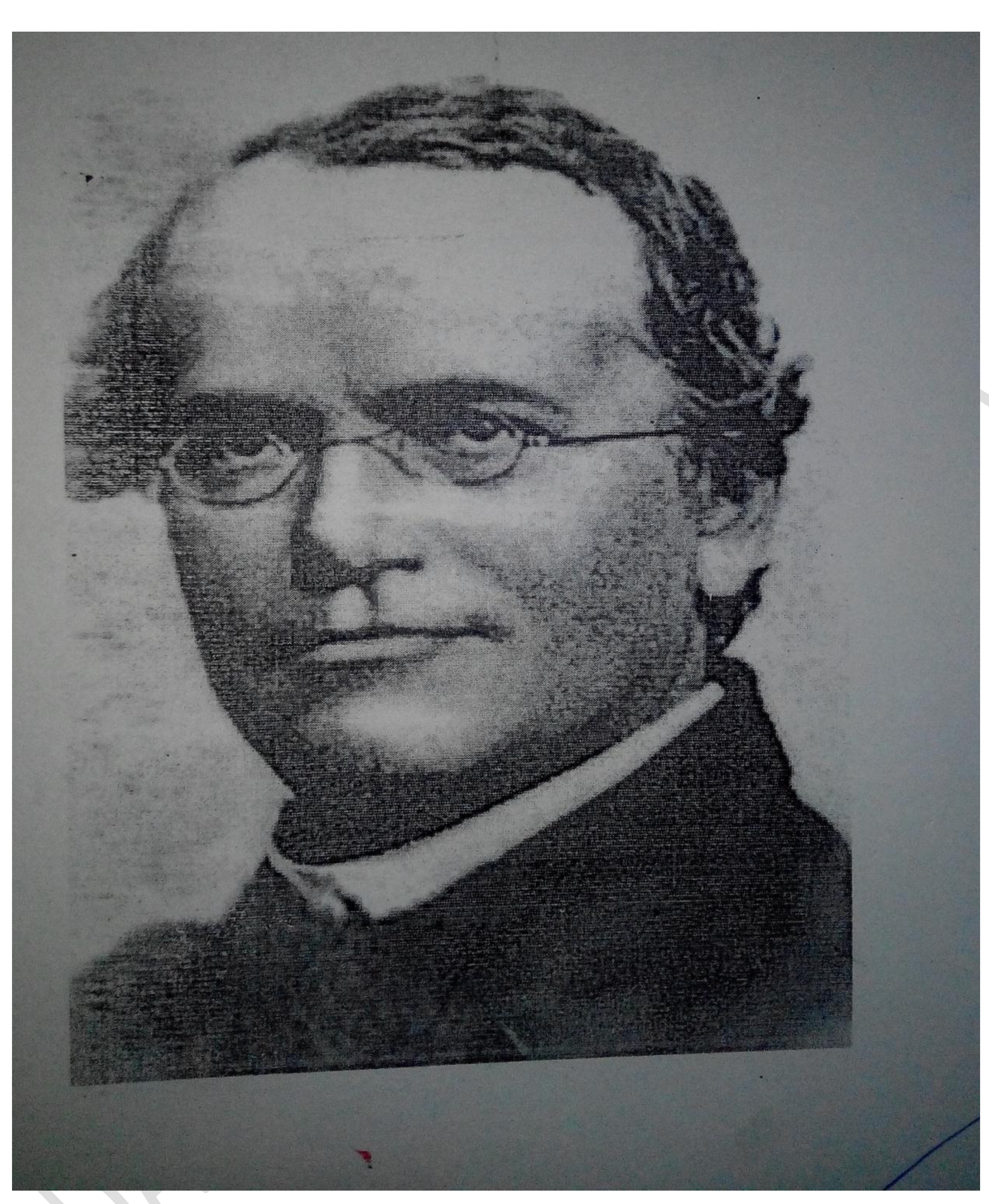

EXPERIMENT-1

Aim- Mendel's work on Garden Pea.

Theory- Seven pairs of contrasting traits in pea plant

Mendel worked on the garden pea plant because of the following special advantages :

1) Garden pea's were found to differ in certain certain definite and easily detectable traits. Some plants are *tall* and *climbing,* whereas others were *short* and *bushy*. Some had *coloured* flowers*, some white*. Some had *round* seeds, some *wrinkled* Mendel noted 7 pairs of such contrasting traits.

S. no	Characters	Dominant Traits	Recessive Traits
1.	Height	Tall , T	Dwarf , t
2.	Position of flower	Axillary , A	Terminal , a
3.	Colour of unripe pod	Green , G	Yellow , g
4.	Form of unriped pod	Inflated , I	Constricted , i
5.	Colour of seed coat/flower	Coloured , C	White , c
6.	Colour of cotyledons	Yellow , Y	Green , y
7.	Form of seeds	Round , R	Wrinkled , r

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The traits which always appear in two opposing directions / conditions , one dominant and other recessive , are called the contrasting traits.

2) The traits of each kind of pea plant were preserved on generation after generation because the plants had bisexual flowers and normally resorted to self pollination .

3) Pea plant , being annual ,had a short life cycle so that results could be had within a year.

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4)

Serial No.: 2

Seven pairs of Contrasting Traits in pea.

S.N	Characters	Dominant Traits	Recessive Traits
1.	Height	Tall	Dwarf
2.	Position of flower	Axillary	Terminal
3.	Colour of unripe pod	Green	Yellow
4.	Form of Unripe pod	Full	Constricted
5.	Colour of Seed Coat / flower	Coloured	White
6.	Colour of Cotyledons	Yellow	Green
7	Form of Seed	Round	Wrinkled

EXPERIMENT-2

AIM: To demonstrate the law of segregation.

LAW OF SEGREGATION : The law of segregation states that the two alleles controlling each character maintain identity in the organism but during the formation of gametes or spores by meiosis move apart due to separation of the homologous chromosomes which bear them , so that each gamete or spore receive only one allele of each character on random basis Since the gametes or spores posses one allele of each character , they are always pure .The law of segregation is therefore , also called the “law of purity of gametes/ spores.

EXPLAINATION:

CROSS BETWEEN PEA PLANT: Select pure tall pea plant and cross it with a pure dwarf pea plant . Here, the tall size is dominant over the dwarf size. Since each character is controlled by two alleles , and is a pure individual both the alleles of a trait are similar , the allele equipment of these parent plants may be represented by *TT* and *tt* , where T stands for the allele of dominant tall size and *t* stands for the allele for recessive dwarf size. During gamete formation, meiosis separates the alleles of tallness and dwarfness so that the gametes get the allele T and *t* respectively.

The F1 offspring formed by the fusion of two gametes with alleles T and *t* will have two unlike alleles Tt for size. The allele for tallness, being dominant, will express itself in the F1 plants, and the allele for dwarfness, being recessive, will remain unexpressed. All the F1 plants are,therefore , tall . They resemble tall parent to look at , but genetically they differ from both parents as they are hybrids .

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Now cross the two hybrid plants of F1 generation . The alleles for tallness and dwarfness seperate at the time of gametes formation due to meiosis . Half of the gametes receive the allele T for tallness and the half get the allele t for dwarfness in each parent. A gamete with allele T has an equal chance of fusing with the gamete having T or t . Therefore, F2 plants have 3 kinds of genotypes : TT , Tt and tt . These genotypes appear in the ratio of 1 TT : 2 Tt : 1 tt . The ratio of phenotypes is 3 tall pea plants to 1 dwarf pea plant because TT and Tt plants are tall of the tall plants , one third are pure tall (TT) and pure dwarf (tt) . And two thirds are hybrid tall (Tt). In other words , the pure tall , hybrid tall, pure dwarf are in the ratio of 1:2:1 .

In F2 generation the phenotype ratio and the genotype ratio are different. The phenotype ratio is 3:1 , whereas the genotype ratio is 1:2:1. These ratios are possible only if :

1) The two alleles of a trait do not affect each other when together ,
2) Segregate during gametogenesis ,
3) Gametes contain only a single allele of a trait ,
4) The alleles come together in the offspring by random fusion of gametes and
5) F1 plants are hybrids , having contrasting alleles (Tt).

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AIM → To demonstrate the law of Segregation

Characters studied → Stem Height (Tall, Dwa

Symbols used → TT for tallness

tt for dwarfness

Parents Phenotype Tall × Dwarf

Genotype TT × tt

Gametes T t

F1 generation Tt

Hybrid tall

F2 generation

♂ \ ♀	T	t
T	TT Homozygous Tall	Tt Heterozygous Tall
t	Tt Heterozygous Tall	tt Homozygous dwarf

Phenotypic ratio – 3 : 1

Tall : Dwarf

Genotypic ratio – TT : Tt : tt

1 : 2 : 1

Tall : Tall : Dwarf

(pure) (Hybrid) (pure

CAPSULE METHOD: It can be alo explained by capsule method. According to this, when homozygous tall and homozygous dwarf are crossed then the resulting F1 generation give tall plants and on selfing of F2 plants, tall and dwarf plants are obtained in the ratio 3:1.

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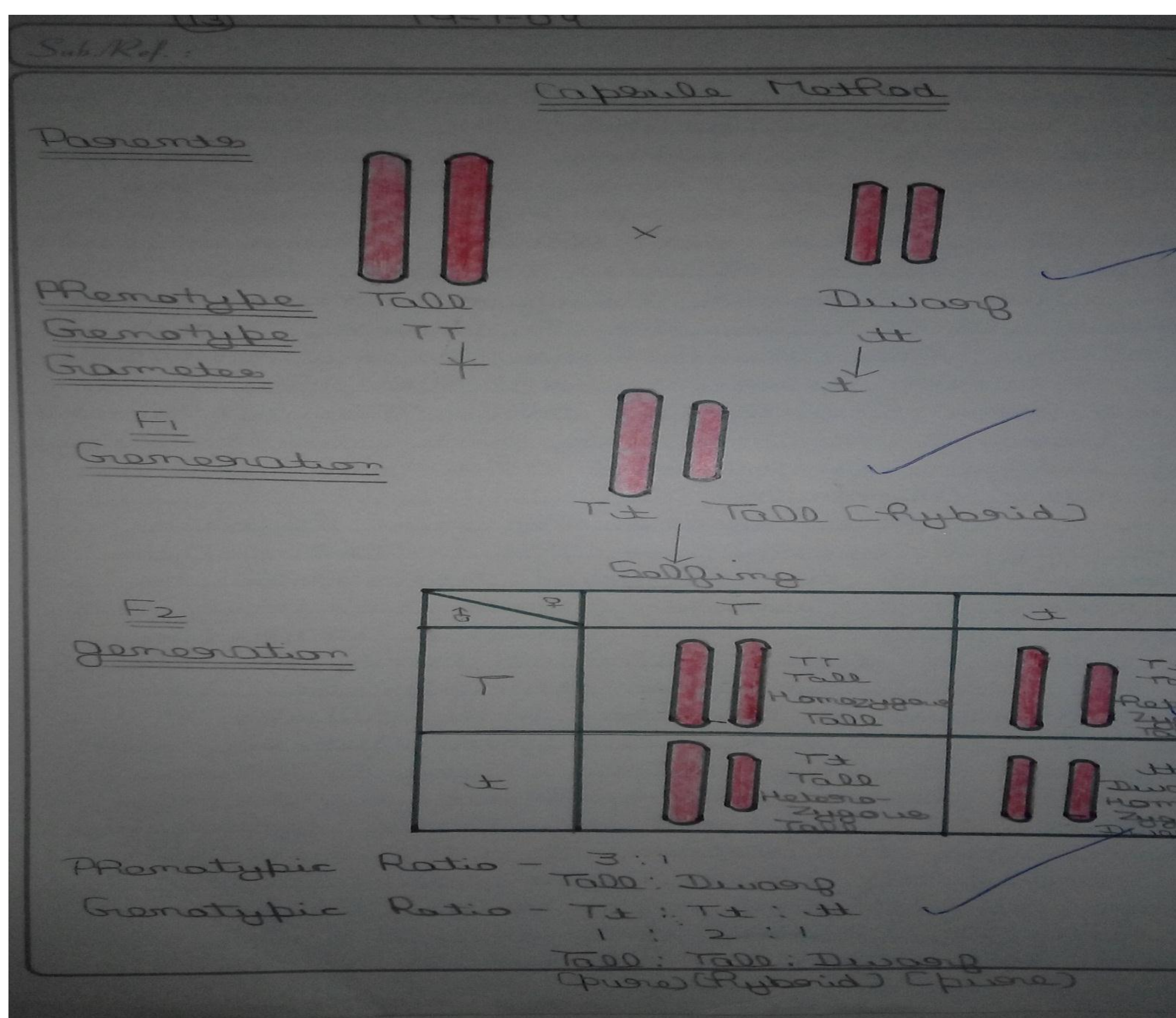
Sub./Ref. :
Capsule Method
Parents
Phenotype
Genotype
Gametes
Tall
TT
T
Dwarf
tt
t
F1
Generation
Tt Tall (hybrid)
Selfing
F2
generation
T
t
TT
Tall
Homozygous
Tall
Tt
Tall
Hetero-
zygous
Tall
Phenotypic Ratio - 3 : 1
Tall : Dwarf
Genotypic Ratio - Tt : Tt : tt
1 : 2 : 1
Tall : Tall : Dwarf
(pure) (hybrid) (pure)

EXPERIMENT-3

AIM: To demonstrate the law of independent assortment.

STATEMENT : This law states that the alleles of different characters located in different pairs of homologous chromosomes are independent of one another in their segregation during gamete formation and in coming together into the offspring by fertilization , both processes occurring randomly.

EXPLAINATION :

CROSS BETWEEN PEA PLANT : Cross a pure pea plant having round seeds and yellow cotyledons with a pure pea plant having wrinkled seeds and green cotyledons. Here , the round form of seeds and yellow colour of cotyledons are dominant over the wrinkled form of seeds and green colour of cotyledons. Since each trait is controlled by two allele and both the allele of a trait are similar in a pure individual, the allele equipment of the parent plant may be represented by RRYY and rryy, where R stands for the allele of dominant round seed form, r for the allele of recessive wrinkled seed form, Y for the allele of the dominant yellow cotyledon colour, and y for the allele of recessive green cotyledon colour. During gamete formation, meiosis separates the two alleles of each trait so that the gametes get only the allele of each trait. With the result, the allele equipment of the gametes formed by the parents in the cross will be RY and ry.

The F_1 plants formed by the fusion of gametes with alleles Ry and ry will have two unlike alleles for cotyledon colour, i.e., they have RrYy. They are all hybrids with round seeds and yellow cotyledons.

Now cross the two hybrid plants of F_1 generation. The alleles of seed form and cotyledons colour again segregate during gamete formation . Each hybrid forms four types of gametes RY, Ry, rY, and ry. These gametes on fertilization produce four types of plants in F2 generation .They are in the ratio of 9 with round seeds and yellow cotyledons , 3 with round seeds and green cotyledons , 3 with wrinkled seeds and yellow cotyledons and 1 with

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wrinkled seeds and yellow cotyledons. The plants having round seeds and wrinkled seeds are in the ratio of 3:1 and the plants pure seeds , hybrid for round seeds and pure for wrinkled seeds are in the ratio of 1:2:1 . Similarly , the plants with yellow cotyledons and green cotyledons are in the ratio of 3:1 and the plants pure for yellow cotyledons hybrid for yellow cotyledons are in the ratio of 1:2:1.

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Aim → To demonstrate the Law of independent assortment

Characters Studied → Seed Shape and Seed Colour

Symbols used → Seed Shape →
RR , rr
(Round), (wrinkled)

Seed colour →
YY, yy
(Yellow), (Green)

Parents

Phenotype – Round Yellow X Wrinkled Green

Genotype - RRYY X rryy

Gametes RY ry

F_1 generation RrYy

Round yellow

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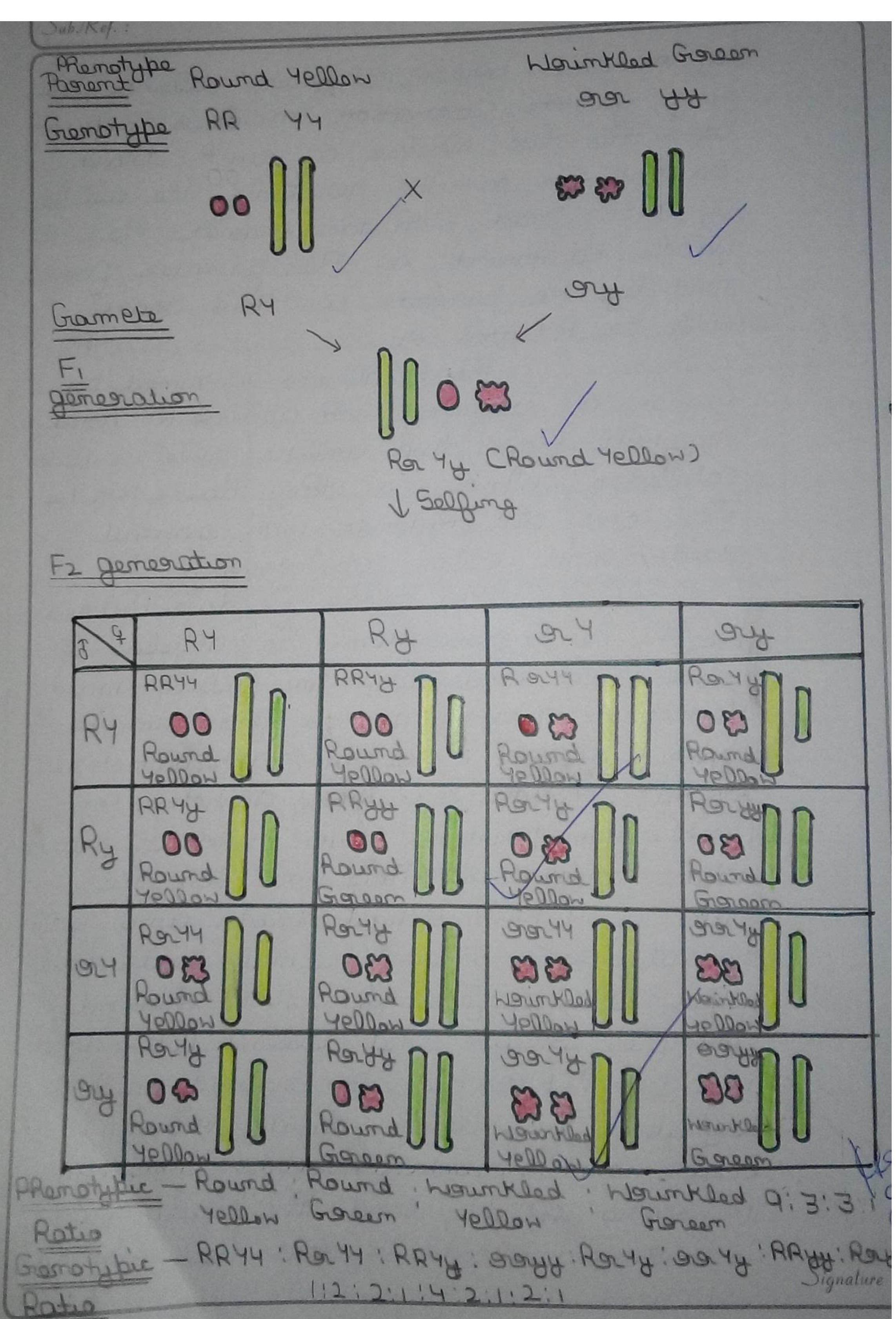
F1 generation → RrYy
Round yellow
↓
Selfing

♂ \ ♀	RY	Ry	rY	ry
RY	RRYY Round Yellow	RRYy Round Yellow	RrYY Round Yellow	RrYy Round Yellow
Ry	RRYy Round Yellow	RRyy Round Yellow	RrYy Round Yellow	Rryy Round Green
rY	RrYY Round Yellow	RrYy Round Yellow	rrYY Wrinkled Yellow	rrYy Wrinkled Yellow
ry	RrYy Round Yellow	Rryy Round Green	rrYy Wrinkled Yellow	rryy Wrinkled Green

Phenotypic ratio – 9 : 3 : 3 : 1
Round Yellow : Round Green : Wrinkled Yellow : Wrinkled Green

Genotypic ratio – RRYY : RRYy : RrYY : RrYy : RRyy : Rryy : rrYy : rrYY : rryy
1 : 2 : 2 : 1 : 4 : 2 : 1 : 2 : 1



Phenotype Parent — Round Yellow ... Wrinkled Green

Genotype — RR YY ... rr yy

Gamete — RY ... ry

F1 generation — RrYy (Round Yellow)

↓ Selfing

F2 generation

♂ \ ♀	RY	Ry	rY	ry
RY	RRYY Round Yellow	RRYy Round Yellow	RrYY Round Yellow	RrYy Round Yellow
Ry	RRYy Round Yellow	RRyy Round Green	RrYy Round Yellow	Rryy Round Green
rY	RrYY Round Yellow	RrYy Round Yellow	rrYY Wrinkled Yellow	rrYy Wrinkled Yellow
ry	RrYy Round Yellow	Rryy Round Green	rrYy Wrinkled Yellow	rryy Wrinkled Green

Phenotypic Ratio — Round Yellow : Round Green : Wrinkled Yellow : Wrinkled Green 9 : 3 : 3 : 1

Genotypic Ratio — RRYY : RrYY : RRYy : rryy : RrYy : rrYy : RRyy : Rr[illegible]

1 : 2 : 2 : 1 : 4 : 2 : 1 : 2 : 1

Signature

The dihybrid cross yields the same results as does a monohybrid cross normally.

1) In F1 generation raised from two pure parents , all offsprings are alike and resemble the parent with dominant traits respectively.
2) In F2 generation raised by inbreeding of F1 hybrids , the dominant and recessive traits appear in the ratio 3:1.

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BLOOD GROUP ANALYSIS

EXPERIMENT - 4

AIM : Determination of blood group by RBO system

REQUIREMENTS : Alcohol , cotton , sterilized pricking needle , glass slides , antiserum A , antiserum B , blood sample, etc.

THEORY : Landsteiner in 1900 discovered that blood of different could be distinguished into distinct group because there are RBC's posses specific surface molecules . There are two antigens present on surface of red blood corpuscles i.e, antigen A and antigen B . Based on the presence and absence of these antigens , there are fur blood groups :

Person with blood group A having - antigen A
Person with blood group B having - antigen B
Person with blood group AB have - antigen A and B
Person with blood group O have - no antigen

If a person has an antigen on his RBC's , his plasma has normal antibodies against antigen . Person having antigen A have antibody "b" in plasma , antigen B have antibody "a" , person having antigen A and B have no antibodies . Persons have no antigen on RBC's but have antibodies "a" and "b" in plasma.

PROCEDURE :

1) Give a massaging motion to the fleshy part of finger.
2) Clean the tip of finger with cotton soaked in alcohol.
3) Make a bold prick to the finger .
4) Put two drops of blood an two edges of the glass slide.

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5) Now put antiserum A to one drop and antiserum B to other drop.
6) Mix them separately with toothpicks .
7) If agglutination occurs in “A” , then blood group is A , if agglutination occur with antiserum B , then blood group is B . If there is no agglutination then blood group ‘O’ . If agglutination occurs in both , then ‘AB’ blood group.

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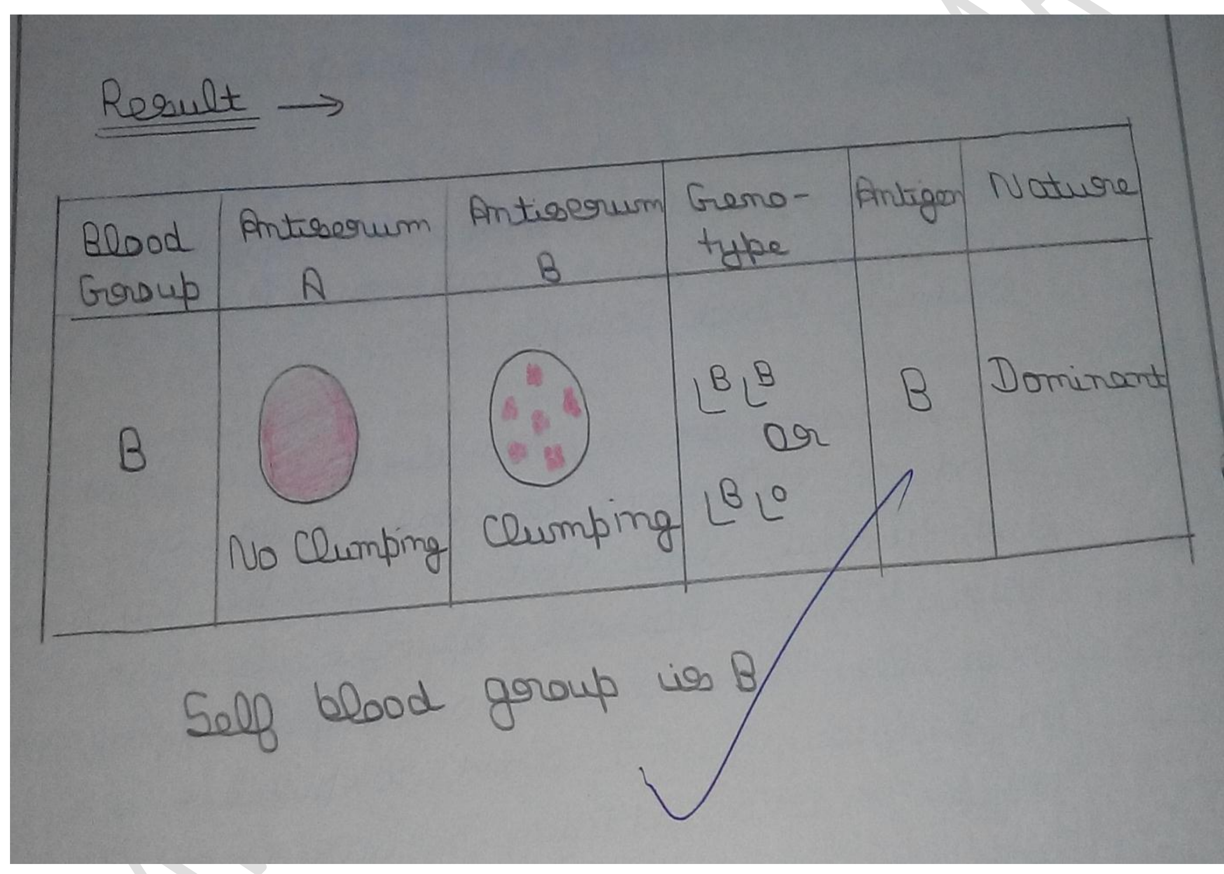
AIM → Determination of blood group by ABO System

Requirements → Cotton, alcohol, sterilized pricker, glass slides, Antiserum A, Antiserum B, blood sample.

Blood Group	Antiserum A	Antiserum B	Genotype	Antigen present	Nature
A	Clumping	No Clumping	$I^A I^A$ or $I^A I^o$	A	Dominant
B	No Clumping	Clumping	$I^B I^B$ or $I^B I^o$	B	Dominant
AB	Clumping	Clumping	$I^A I^B$	AB	Codominant
O	No Clumping	No Clumping	$I^o I^o$	None	Recessive

PRECAUTIONS :

1) Always use the sterilized pricker .
2) Discard the first drop of blood .
3) Always give a bold prick to finger.
4) Mix the blood and antiserum with separates toothpicks.



Result →

Blood Group	Antiserum A	Antiserum B	Geno-type	Antigen	Nature
B	No Clumping	Clumping	$I^B I^B$ or $I^B I^O$	B	Dominant

Self blood group is B

EXPERIMENT - 5

AIM: To determine the Rh-factor of blood.

REQUIREMENTS : Cotton , alcohol, pricking needle, antiserum D , glass slides , blood sample.

THEORY : In 1940, Landsteiner and Weiner discovered Rh-factor in the blood of rhesus monkey . Rh- factor is an antiserum present on the surface of RBC's . There is no antibody against it in the plasma . The person which have Rh factor on their RBC's are Rh positive and those who do not have Rh antigen on surface of RBC's are Rh negative . Rh^+ is dominant and Rh^- is a recessive character . Rh^- persons do not have natural antibodies against Rh^- antigen but when exposed to Rh antigen, they develop antibodies.

PROCEDURE :

1) Give a massaging motion to the fleshy part of the finger .
2) Prick the finger tip with sterilized needle .
3) Put the drop of blood on the glass slide .
4) Now put antiserum D on the drop of blood and mix it properly.
5) If agglutination takes place, then the person is $Rh^{+.}$
6) If agglutination does not take place , then the person is Rh^-.

PRECAUTIONS :

1) Always use sterilized needles.
2) Discard the first drop of blood.
3) Mix blood and antiserum properly.
4) Always give bold prick to the finger.

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Requirements → Cotton, alcohol, sterili[illegible] pricker, glass slide, antiserum D, bl[illegible] sample

Observations →

Rh System	Blood + Antiserum D	Genotype
Rh+	Clumping	RR or Rr CDE / cDE / Cde / cDe
Rh-	No Clumping	rr / cde

Result →

Self Rh- factor is Rh+

Rh+	Blood + Antiserum	Genotype
Self Rh Factor	Clumping	RR / Rr / CDE

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EXPERIMENT-6

AIM: Multiple inheritance of ABO blood groups.

THEORY : Berntstein in 1925 discovered that the inheritance of different blood groups in man is determined by a number of multiple allelic series. There are three alleles concerned with the determination of blood group of any person . The synthesis of antigens is controlled by these three alleles - L^A , L^B, L^O. Blood group O phenotype is wild type and recessive . It is controlled by genotype l^ol^o . Thus l^o is the wild gene and L^AL^B are its two dominant mutants and both the mutants alleles are codominant thus ,

1) Genotype of persons with blood group O = l^ol^o (homozyous recessive) .

2) Genotype of persons with blood group A = L^Al^O and L^Al^O (heterozygous dominant).

3) Genotype of person with blood group B = L^Bl^O or L^BL^B . (heterozygous / homozygous dominant)

4) Genotype of persons with blood group AB = L^AL^B . (co-dominant).

The gene A and B are dominant to gene l^o but are codominant to each other and both are expressed when pressed together .

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5)

AIM → Multiple Allelic Inheritence of ABO blood Groups.

SR No	Parents		Offspring's blood Group (Phenotype)				Genotype
	Blood Group Phenotype	Genotype	A	B	AB	O	
1	O×O	$l^ol^o \times l^ol^o$	—	—	—	—	l^ol^o
2	O×A	$L^OL^O \times L^AL^A$	All	—	—	All	L^AL^O
3	O×A	$L^OL^O \times L^AL^O$	1/2	—	—	1/2	L^OL^O, L^AL^O
4	O×B	$L^OL^O \times L^BL^B$	—	All	—	—	L^BL^O
5.	O×B	$L^OL^O \times L^BL^B$	—	1/2	—	1/2	L^OL^O, L^BL^O
6.	O×AB	$L^OL^O \times L^AL^B$	1/2	1/2	—	—	L^AL^O, L^BL^O
7.	A×A	$L^AL^A \times L^AL^A$	All	—	—	—	L^AL^A
8	A×A	$L^AL^O \times L^AL^O$	3/4	—	—	1/4	L^AL^A, L^AL^O, L^O
9	A×A	$L^AL^A \times L^AL^O$	All	—	—	—	L^AL^A, L^AL^O
10	A×B	$L^AL^A \times L^BL^B$	—	—	—	—	L^AL^B
11.	A×B	$L^AL^A \times L^BL^O$	1/2	—	—	—	L^AL^O, L^AL^B
12	A×B	$L^AL^O \times L^BL^B$	—	1/2	—	—	L^BL^O, L^AL^B
13.	A×B	$L^AL^O \times L^BL^O$	1/4	1/4	1/4	1/4	L^AL^O, L^BL^O, L^AL^B,
14.	A×AB	$L^AL^A \times L^AL^B$	1/2	—	—	—	L^AL^A, L^AL^B
15	A×AB	$L^AL^O \times L^AL^B$	1/2	1/4	—	—	L^AL^A, L^AL^O, L^BL^O,
16	B×B	$L^AL^B \times L^BL^B$	—	All	—	—	L^BL^B
17	B×B	$L^BL^O \times L^BL^B$	—	All	—	—	L^BL^O, L^BL^B
18	B×B	$L^BL^O \times L^BL^O$	—	3/4	1/4	1/4	L^OL^O, L^AL^O, L^BL^O,
19	B×AB	$L^BL^B \times L^AL^B$	—	1/2	—	—	L^AL^B, L^BL^B
20	B×AB	$L^BL^O \times L^AL^B$	1/4	1/2	—	—	L^AL^O, L^BL^O, L^AL^B,
21	AB×AB	$L^AL^B \times L^AL^B$	1/4	1/4	—	—	L^AL^A, L^BL^B, L^A

DAV C

LAW OF SEGREGATION IN MAIZE

EXPERIMENT-7

AIM : To study law of segregation on maize.

REQUIREMENTS : Maize cob , pin , chi square analysis table , calculator , etc.

THEORY : Statistical analysis can be used for examining and verifying whether the observed data of a cross fits or differs from the predicted or expected concurrences. This is called goodness of fit. The goodness of fit of the data to the predicted or expected values can be determined by a simple statistical test known as chi square (X^2) test. By chi square test analysis it can be determined whether the data fits exactly with the predicted data or differs from it and have much deviation . It helps in determining the probability that the deviation of observed ratio from predicted ratio is due to chance.

1) If the probability (P) of the observed ratio is equal to or less than 5 in 100 i.e. 0.05 then it is considered to be significant.
2) If the probability is greater than 1 or 0.01 the deviation is highly

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significant.

3) If probability is greater than 0.05 the deviation is non-significant.

The formula used in X^2 analysis is $X^2 = \frac{\Sigma(o-e)^2}{e}$

Where, 'o' → observed value

'e' → ecpected value

(o-e) → deviation

1.Maize cob is taken of two different colours.

2.The rows of seeds on maize are counted. The rows are counted vertically or longitudinally.

3.After counting the rows the number of yellow seeds and brown seeds are counted.

4.Then after this the mean of total yellow seeds and brown seeds is calculated separately using calculator.

5.Mean value of yellow and brown seeds were added together to find the expected ratio.

6.The difference between yellow and brown seeds was calculated to find the deviations.

7.With the help of calculator X^2 for each yellow and brown seed was calculated.

8.The mean of X^2 values for yellow and brown seeds was calculated to find probabilty.

RESULT: Probability is 90-95%. So, it is a non-significant change. So, it is a good fit between observed and expected value.

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AIM → To study 'law of Segregation' on maize.

Requirement → Maize cob, calculator, pin, Chi- square analysis table etc

Number of Rows	Number of Yellow Seeds in each row	Number of black Seeds in each Row
1	36	9
2	30	12
3	24	12
4	29	11
5	31	10
6	34	7
7	30	10
8	35	9
9	24	14
10	29	14
11	33	10
12	33	10
13	36	9
	$\Sigma = 404$	$\Sigma = 137$

17/2/14

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Σ yellow + black seeds = 404 + 137 = 541

x^2 analysis →

Colour of Seed	observed 'o'	expected 'e'	o-e	$x^2 = \frac{(o-e)^2}{e}$
Yellow	404	$\frac{3}{4} \times 541$ = 406	-406+404 = -2	0.0048
Black	137 Σ = 541	$\frac{1}{4} \times 541$ = 135	137-135 = +2	0.0296 Σ = 0.0394

$\Sigma x^2 = 0.0394$

Degree of freedom = 2-1 = 1

Probability = 90-95%

Result → Probability is 90-95%. So, it is a non-significant change. So, it is a good fit between observed and expected value.

17/2/10

DERMATOGLYPHICS

EXPERIMENT-08

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AIM: To perform dermatoglyphics of hand and finger tip pattern.

REQUIREMENTS: Carbon paper, mustard oil, unglazed white paper, towel.

THEORY: Dermatoglypics is the study of epidermal ridges of thick skin on the regions like fingers, toes, palms and soles. It was studied by Perkins in 1823 and was systematized by Galton in 1892. It is a well known method for personal identification. It has only become considerable, clinical and of scientific interest particularly in relation to abnormalities and syndromes. Pattern of dermal ridges are laid down in the third month of pregnancy.

PRINCIPLE: The principle on which dermatoglyphics is based as follows→

1.The pattern of ridges separated by narrow groove present on epidermis and dermis.
2.Many genes elaborate in determining details of these pattern (polygenic

inheritance).
3.They remain constant for whole life of the individual accept when there is disruption of skin.
4.There can be no identical finger prints not even of twins.

FINGER TIP PATTERNS: There are ridges as well as triradii which fall into following categories→

I. Arches: In these patterns,ridges run from one side of finger to another without backward turn. Arches may be simple or straight. Arches are of two types :

A) Simple or Parallel.

B) Tented: There is marked upward thrust of ridges.

2. LOOPS: One triradii follows a loop pattern, ridges transverse from one side and riturn on same side. It is of two types:

A) Ulnar: Loop open towards ulnar side(towards little finger).

B) Radial: Loop open towards radial side(towards thumb).

3. WHORLS: Two triradii always follow a whorl,in whorl ridges make a series of circle around the central point or core. Core maybe single or double. Whorls can be clockwise or anti-clockwise.

4. COMPOSITE: It is a pattern having combination of two or more patterns. It maybe S shaped.

PATTERNS ON PALMS: An important dermal diagnostic feature of the palm is the triradii. Triradius is the meeting point of three spokes with almost parallel ridges on the palm. There are only four triradii,one at the base of each finger called a,b,c and d. Others known as axial triradius, t

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which is normally found near wrist crease near the base of fourth metacarpal bone. Sometimes more than one axial triradius is present in that case more distal one is used as a marker.

PROCEDURE:

1.Wash hands with soap and water and dry it with a clean towel.
2.Each finger is applied with mustard oil.

3.Then fingers are rubbed against carbon paper, so that ink is uniformly spread on fingers.

4.Press the finger tips generally on which unglazed paper.

5.Observe the pattern.

6.Record the findings of the pattern.

PROCEDURE FOR PALM PATTERNS:

1. Wash left hand with soap and water and dry it with clean towel.
2. The palm is applied with mustard oil.
3. Then the palm is rubbed against white paper so that ink is uniformly spread on palm.
4. Press the palm gently on white unglazed paper, keeping fingers equidistant to get proportionate palm print with all triradii.
5. Record the palm impression and measure angle . Then repeat the procedure for right and record the findings of the pattern.

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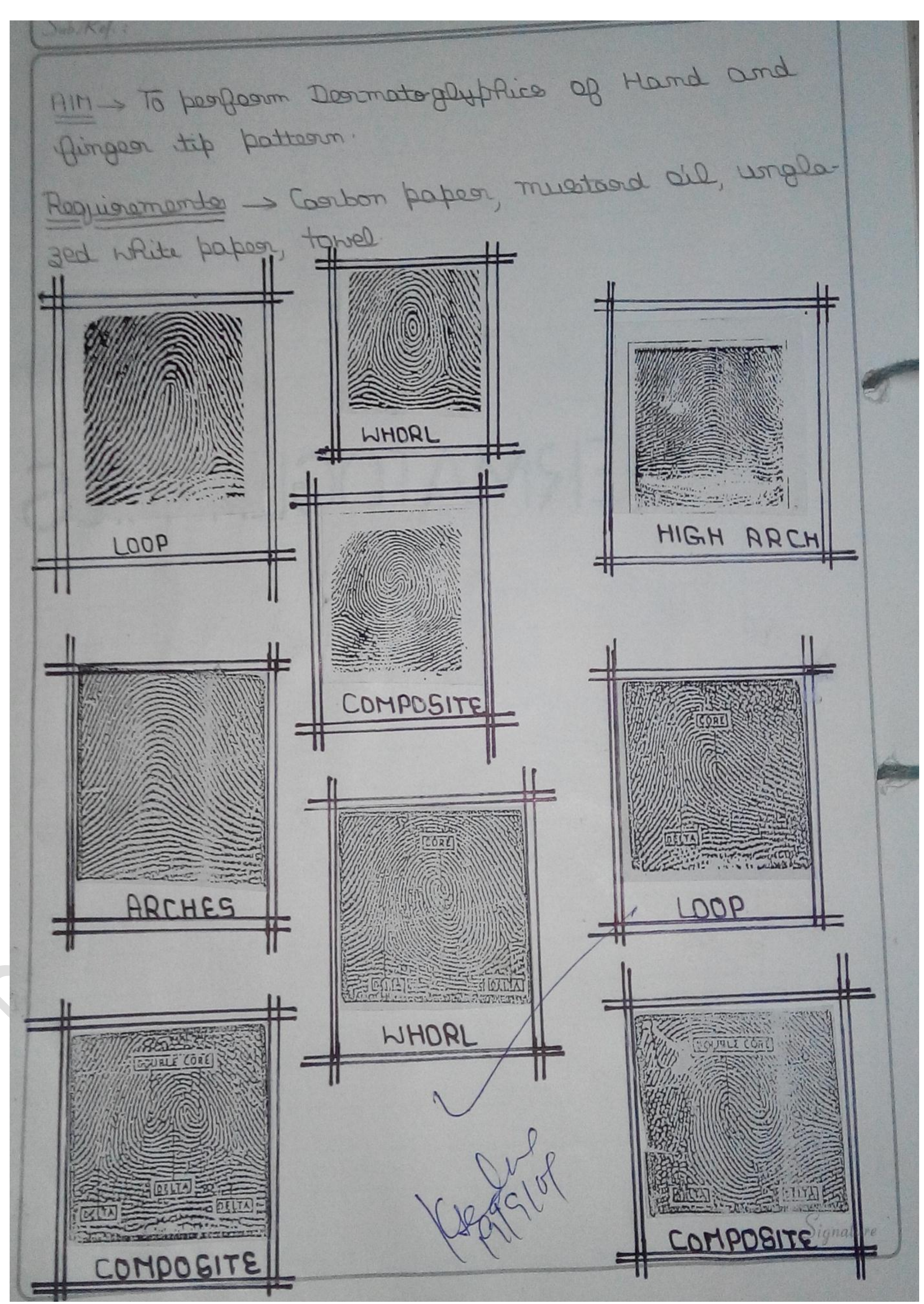
6.



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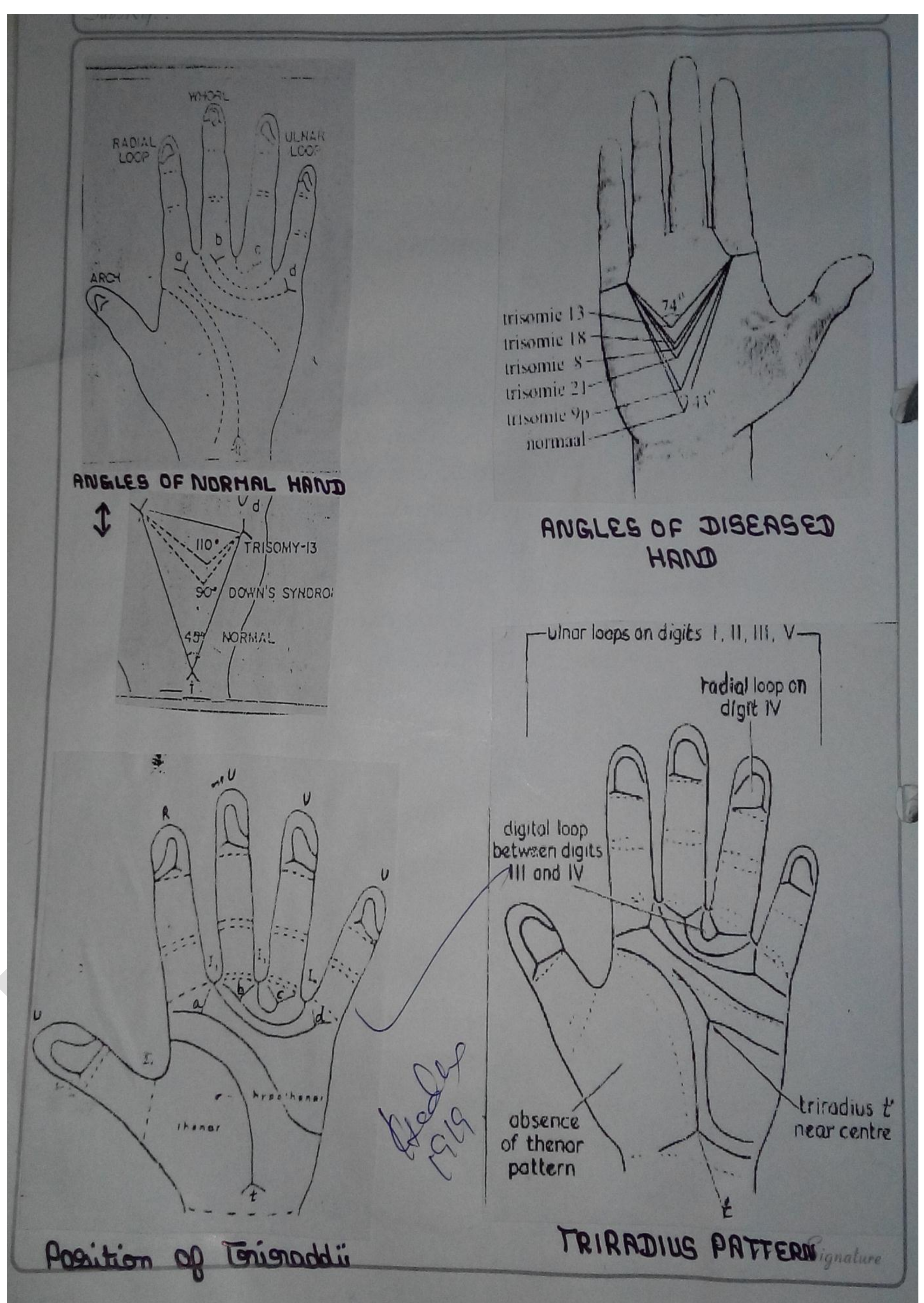
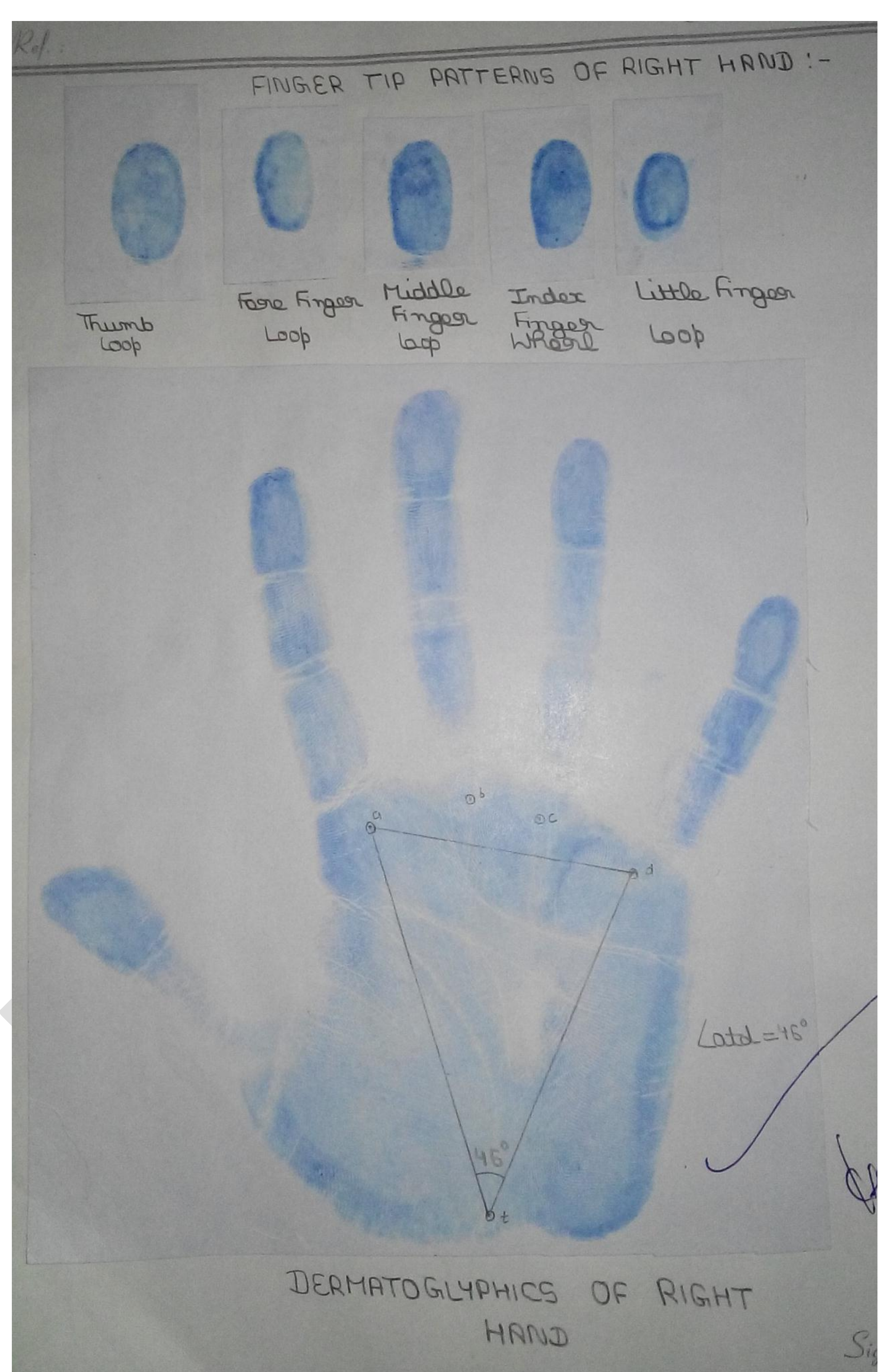


8. 

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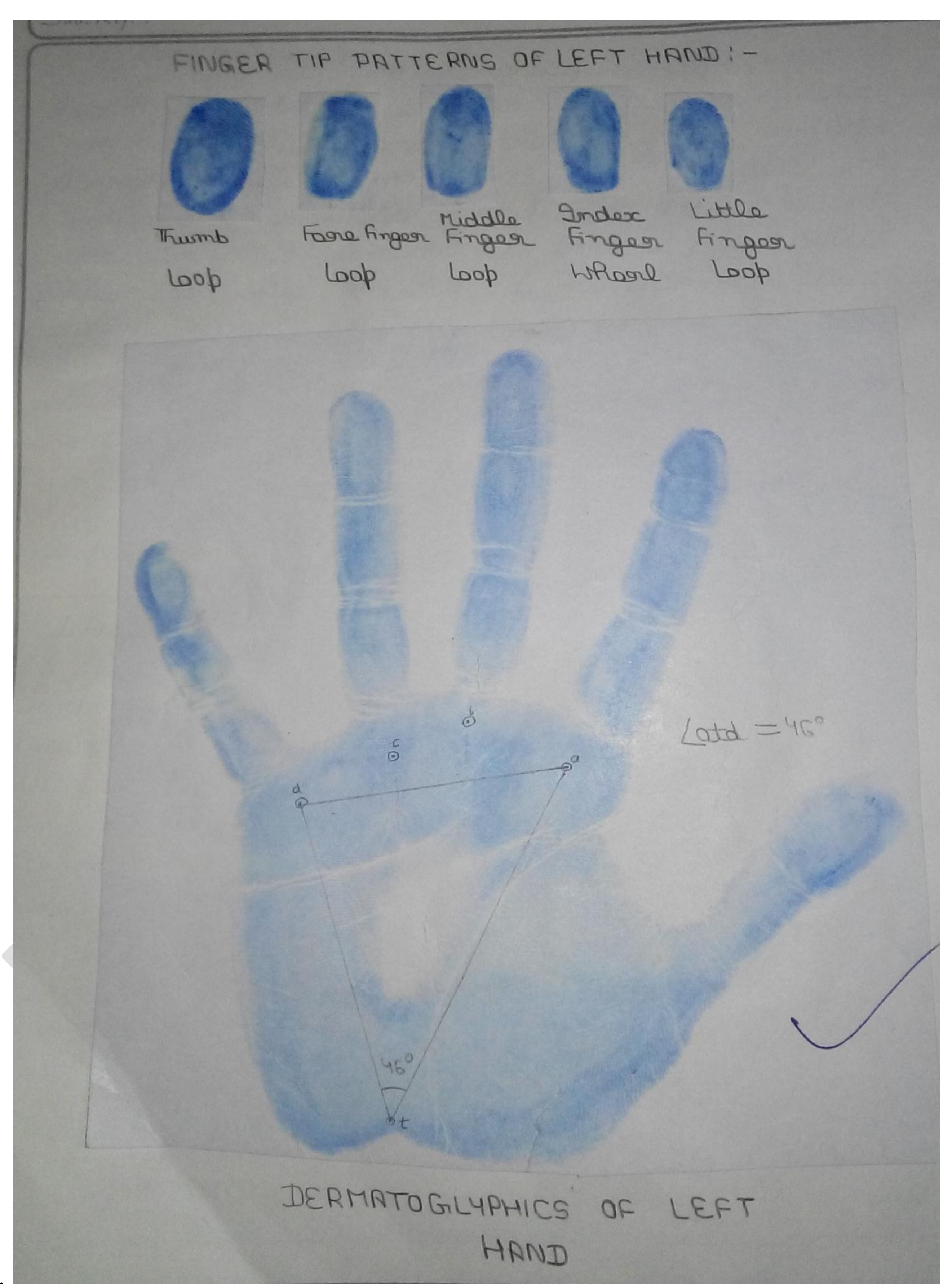

PRECAUTIONS :

1) Hands should be washed and dried properly.
2) Unglazed white paper should be used .
3) Carbon paper ink or ink from ink pads should be applied uniformly on palm and finger.
4) Hard palne surface should be used for obtaining prints.

SIGNIFICANCE : The ridges pattern are determined by many genes. Hence are inheritable and associated with many chromosomal abnormalities in the angle atd. Normal value of the angle is 45° to 48°.

1) DOHN'S SYNDROME : Angle atd = 81^0 . There is higher tendency of ulnar loops rather than whorl or an arch.
2) PATAU'S SYNDROME: Angle atd = 108^0 . In this abnormality there are two arches and whorls as well as loops on finger tips.
3) EDWARD'S SYNDROME: Angle atd = 106^0 . Finger tips almost always have their dermal patterns form of arches.
4) TURNER'S SYNDROME: Angle atd= 66^0. Whorls are very larger.
5) KLEINFELTER'S SYNDROME: In this case , abnormal person with XXX karyotype do not differ significantly in their finger tip and palm patterns from normal persons. The tendency towards pattern with low counts are mere grequent arches depends upon whether the individual are XXY, XXYY, XXXYY.
6) Significance for identification of criminal.
7) Significance for identification of dead body.
8) Significance for identification of some syndrome or genetic disorder.

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AGRO PRACTICAL MANUAL

Semester-3

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INDEX

S.No.	Name Of the Experiments
1.	Autoclaving
2.	Microbial cell counting by serial dilution technique
3.	Microbial cell counting by pour plate plate technique.
4.	Measurement of bacterial size.
5.	Metabolic characterization (e.g. IMViC test)
6.	One step growth of bacteriophage.
7.	Alcoholic and mixed acid fermentation.

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EXPERIMENT-1

AIM: Autoclaving

THEORY: Autoclave is a double jacketed steam chamber invented by Charles Chamberland in 1879. Heat in the form of saturated steam under pressure is the most practical and the same principle is used in autoclave.

PRINCIPLE: The killing action of heat on the organisms can be done by using increase in the steam in a closed system. The water molecules become aggregated resulting in increase in their penetration. The water boils at 100^0C and the steam accumulates in the closed container resulting in increase in pressure. This relationship between pressure and temperature is shown below:

PRESSURE (lb/sq.inch)	TEMPERATURE (^{0}C)
0	100
5	109
10	115.5
15	121.5
20	126.5
25	130.5
30	135.5
40	141.5

The autoclave is usually operating at 15lb/sq. inch steam pressure for 15-30 minutes which is equivalent to temperature 121.5^0C. This temperature for period of 15-30 minutes is sufficient to kill all the spores and vegetative cells of micro-organisms.

FEATURES:

- The autoclave is usually of pressure cooker type made up of gun metal sheets which is supported in an iron case.
- It is closed by swing door which is fastened by radicle bolts tightly.
- In microbiology labs, system jacketed horizontal type autoclave is necessary.

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- The steam passes from below at the base. The side walls are heated by steam jacket. It has a provision to record a pressure.
- There is a possibility to regulate the pressure using pressure meters. It consists of safety valves that guards against the accidents.
- It is based on moist heat that is used in sterilization.

AN AUTOCLAVE

PROCEDURE:

- Sufficient amount of water is placed inside the autoclave.
- The material is placed inside the autoclave for sterilization.
- The cotton plugs should be covered with a piece of butter paper or foil so that the plug does not wet.
- The lid of autoclave should be tightened with the help of screws, then switch on the plug.
- The steam outlet is kept open till we feel that the air inside the autoclave have been evacuated and then close the steam outlet. The pressure is allowed to remain at 15 lb/sq.inch for 15-30 mins. it is done by controlling the steam in the valve.
- After 15-30 mins., switch off the current and let the autoclave cool down and thus the pressure comes down to zero mark.

- When the autoclave is cooled down, the lid is opened and taken out the materials.

USES:

- The autoclave is used to sterilize usual non-carbohydrate media, growth and agar media, contaminated media, aprons, glass-wares etc.
- This type of sterilization is also used in the commercial canning for fruits and vegetables and also used in manufacturing sterilized milk.

PRECAUTIONS:

- The level of water should be checked before operating.
- The air should be completely evacuated and the steam must have access to the material to be sterilized.
- If a bacterial culture such as cotton, wool or glass beads are to be sterilized in a glass bottle closed with rubber stopper, the sterilization would not be complete as steam cannot pass through rubber stopper. So, the cotton or glass beads must be sterilized in glass container closed with either butter paper or foil.
- Too much loading must be avoided because this would prevent proper circulation of steam.
- The heat sensitizer substances should not be sterilized by autoclaving.
- Now-a-days, checking of complete autoclaving is possible by using strips containing red solution which turns green at 115^0C, if kept for 25 mins.
- The use of Bacilli spores after autoclaving is considered as spore fact, if no growth is obtained on thioglycollate medium or cooked meat medium.

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EXPERIMENT-2

AIM: Microbial cell counting by serial dilution technique.

REQUIREMENTS: test tubes, test tube stand, micropipette, tips, beakers, weighing balance, petri-plates.

CHEMICALS: Nutrient agar media, micro-organism source,Distilled water.

PRINCIPLE: Studies involving the analysis of materials such as food, water, milk and in some cases air required quantitative enumeration of micro-organisms in the substances. Many methods have been devised to accomplish this, including direct microscopic counts, use of electronic cell counter such as the coulter counter. Chemical methods for estimating cell mass or cellular constitutents, turbidimetric measurement for increase in cell mass and the serial dilution agar plate method.

THEORY: while all these methods may be used to enumerate the number of cells in a bacterial culture, the major disadvantage common to all is that the total counts include dead as well as living cells. Sanitary and medical microbiology at times require determination of viable cells. To accomplish this, the serial dilution agar plate technique is used. Briefly this method involves serial dilution of a bacterial suspension in sterile water blanks, which serve as diluent of known volume. Once diluted, the suspension are placed on suitable nutrient media. The pour plate technique is a procedure usually employed. Molten agar, cooled to 45^0C, is poured into a petri-dish containing a specified amount of diluted sample. Following addition of the molten than cooled agar, the cover is replaced and the plate is gently rotated in the circular motion to achieve uniform distribution of micro-organisms. Dilutions should be plated in duplicate for greater accuracy, incubated overnight and counted on a Quebec colony counter either by hand or by an electronically modified vision of the instrument.

PROCEDURE:

i) Liquefy deep tube in an autoclave or by boiling, cool the molten agar tube and maintain in the water bath at 45^0C.

ii) Label the *E.coli* culture tube as 1 and seven 9ml water blanks as 2-8. Place the labeled tube in a test tube rack. Label the petri-dishes 1a, 2a,2b, 3a and 3b-6a,6b.

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iii) Mix your *E.coli*culture (test tube1) by rolling the tube between the palms of your hands to ensure even dispersal of cells in culture.

iv) With a sterile pipette, aseptically transfer 1ml from the bacterial suspension tube (1) to water blank (2) and discard the pipette in the beaker of disinfectant. The culture has been diluted 10 times to 10^{-1}.

v) Mix tube (2) and, with a fresh pipette, transfer 1ml to tube (3). Discard the pipette. The culture has been diluted 100 times to 10^{-2}.

vi) Further made the dilution performing the same procedure, making the culture diluted to 10^{-7}.

vii) Mix tube (8) and with a fresh pipette, transfer 1ml of this suspension to plate 3b. Discard the pipette. The dilution procedure is now complete.

viii) Using the pour plate technique, pour the agar into plate 1a, and rotate the plate gently to ensure uniform distribution of cells in the medium.

ix) Repeat step (viii) for the addition of molten nutrient agar to plate 1b, 2a, 2b, 3a and 3b.

x) Once agar is solidified, incubate the plates in an inverted position for 24 hours at 37^0C.

DIAGRAM:

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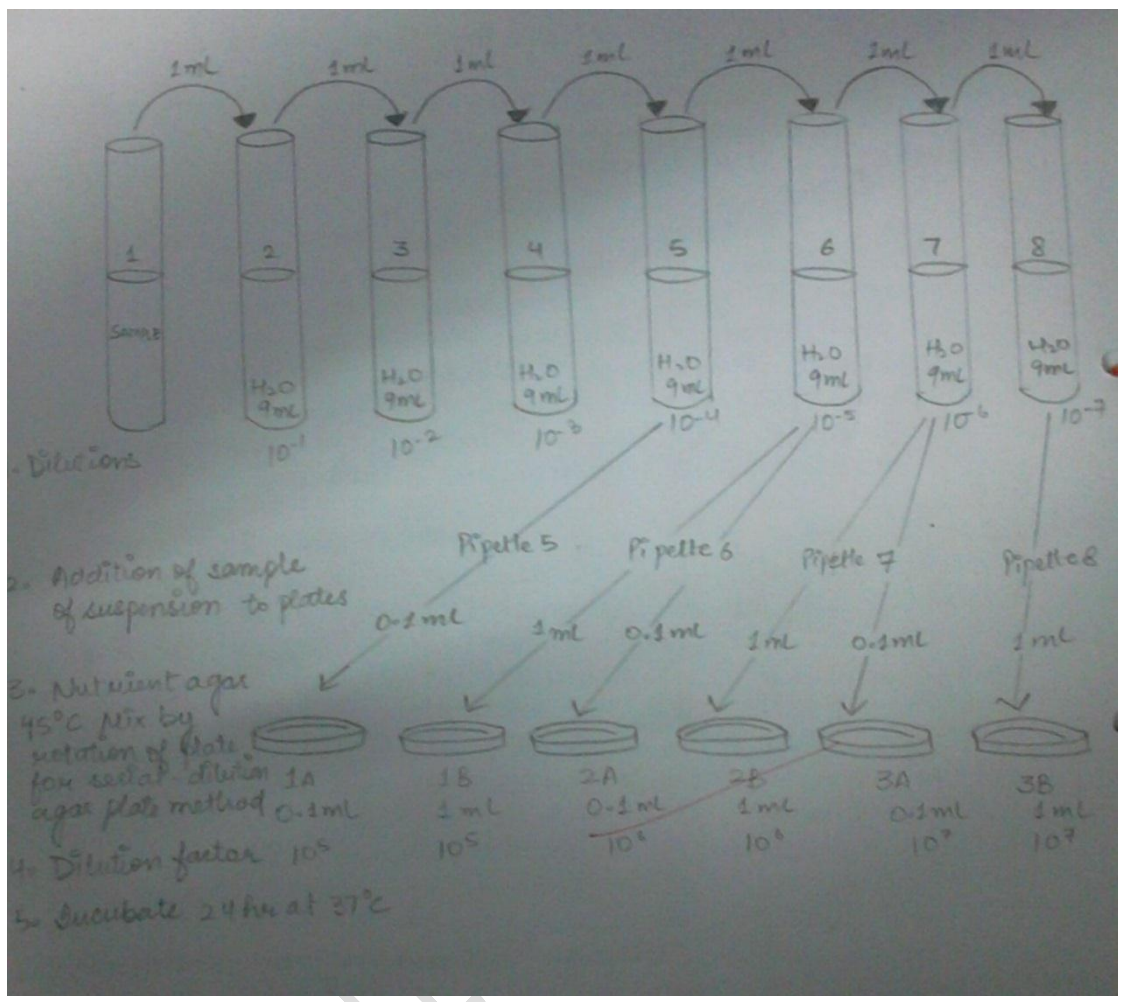

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CALCULATIONS:

a) Colonies per plate = 53

Dilution factor = $1:1x10^7$ = 1:1,00,00,000

Volume of dilution added to plate = 1ml

No. of cells =No. of colonies x dilution factor

= $53x\ 10^7$ (colonies forming unit)

b) Colonies per plate = 62

Dilution factor = $1:1 \times 10^6$

Volume of dilution added to plate = 0.1ml

No. of cells = No. of colonies x Dilution factor

$= 62 \times 10^6$ cells/0.1ml

$= 62 \times 10^6 \times 10$

$= 62 \times 10^7$ CFU/ml

PRECAUTIONS:

i) Plates with more than 300 colonies can't be counted, too numerous to count (TNTC), plates with fewer than 30 colonies is designated as too few to count (TFTC).

ii) Count only plates containing between 30-300 colonies.

iii) Apparatus should be completely clean and pipetting should be accurate.

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EXPERIMENT-3

AIM: Microbial cell counting by pour plate technique.

REQUIREMENTS: test tubes, test tube stand, micropipette, tips, beakers, weighing balance, petri-plates, colony counter.

CHEMICALS: Nutrient agar media, micro-organism source, Distilled water.

PRINCIPLE: Studies involving the analysis of materials such as food, water, milk and in some cases air required quantitative enumeration of micro-organisms in the substances.

Many methods have been devised to accomplish this, including direct microscopic counts, use of electronic cell counter such as the coulter counter. Chemical methods for estimating cell mass or cellular constitutents, turbidimetric measurement for increase in cell mass and the serial dilution agar plate method.

THEORY: while all these methods may be used to enumerate the number of cells in a bacterial culture, the major disadvantage common to all is that the total counts include dead as well as living cells. Sanitary and medical microbiology at times require determination of viable cells. To accomplish this, the serial dilution agar plate technique is used. Briefly this method involves serial dilution of a bacterial suspension in sterile water blanks, which serve as diluent of known volume. Once diluted, the suspension are placed on suitable nutrient media. The pour plate technique is a procedure usually employed. Molten agar, cooled to 450C, is poured into a petri-dish containing a specified amount of diluted sample. Following addition of the molten than cooled agar, the cover is replaced and the plate is gently rotated in the circular motion to achieve uniform distribution of micro-organisms. Dilutions should be plated in duplicate for greater accuracy, incubated overnight and counted on a Quebec colony counter either by hand or by an electronically modified vision of the instrument.

PROCEDURE:

i) Liquefy six deep agar tubes in an autoclave or by boiling, cool the molten agar tube and maintain in the water bath at 45^0C.
ii) Label the *E.coli* culture tubes as 1-8. Place the labeled tubes in a test tube rack. Label the petri-dishes 1a, 2a, 2b, 3a and 3b- 6a,6b.
iii) Mix your *E.coli* culture (test tube1) by rolling the tube between the palms of your hands to ensure even dispersal of cells in culture.
iv) With a sterile pipette, aseptically transfer 1ml from the bacterial suspension tube (1) to test tube (2) and discard the pipette in the beaker of disinfectant. The culture has been diluted 10 times to 10^{-1}.
v) Mix tube (2) and, with a fresh pipette, transfer 1ml to tube (3). Discard the pipette. The culture has been diluted 100 times to 10^{-2}..
vi) Further made the dilution performing the same procedure, making the culture diluted to 10-7.
vii) Mix tube (8) and with a fresh pipette, transfer 1ml of this suspension to plate 3b. Discard the pipette. The dilution procedure is now complete.
viii) Using the pour plate technique, pour the agar into plate 1a, and rotate the plate gently to ensure uniform distribution of cells in the medium.
ix) Repeat step (viii) for the addition of molten nutrient agar to plate 1b, 2a, 2b, 3a and 3b.
x) Once agar is solidified, incubate the plates in an inverted position for 24 hours at 37^0C.

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xi) Count all the submerged as well as surface colonies through Quebec Colony counter.

CALCULATION:

Colonies in plate of 10^{-5} dilution = 56

Dilution factor = $1:1x10^{5}$

No. of cells = No. of colonies x dilution factor

= $56x10^{5}$ CFUs/ml

PRECAUTIONS:

i) Plates with more than 300 colonies can't be counted, too numerous to count (TNTC). Plates with fewer than 30 colonies is designated as too few to count (TFTC).
ii) Count only plates containing between 30 and 300 colonies.
iii) Remember to count all subsurface as well as surface colonies.
iv) Apparatus should be completely clean and pipetting should be accurate.

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EXPERIMENT-4

AIM: Measurement of Bacterial Size.

REQUIREMENTS: Glass slides, bacterial culture, ocular micrometer, stage micrometer, microscope, immersion oil and lens paper.

PRINCIPLE: Determination of microbial size is not that simple. Before an accurate measurement of cells can be made, the diameter of the microscopic field must be

established by means of optical devices, namely, an ocular micrometer and a stage micrometer.

The ocular micrometer, which is placed on a circular shelf inside the eye piece, is a glass disc with graduations etched on its surface. The distance between these graduations will vary depending on the objective being used, which determine the size of the field. This distance is determined by using a stage- micrometer. A special glass slide with etched graduations that are 0.01 mm or 10μm apart.

The caliberation procedure for the ocular micrometer requires that the graduations on both micrometers be superimposed on each other. This is accomplished by rotating the ocular lens. A determination is then made of the number of ocular divisions per known distance on the stage micrometer. Finally, the caliberation factor for one ocular division is calculated as follows:

1 division on ocular micrometer in mm = known distance between 2 lines on stage

Micrometer / No. of divisions on ocular micrometer

Therefore, Length of organism = No. of ocular divisions occupied x caliberation

Factor for one ocular division.

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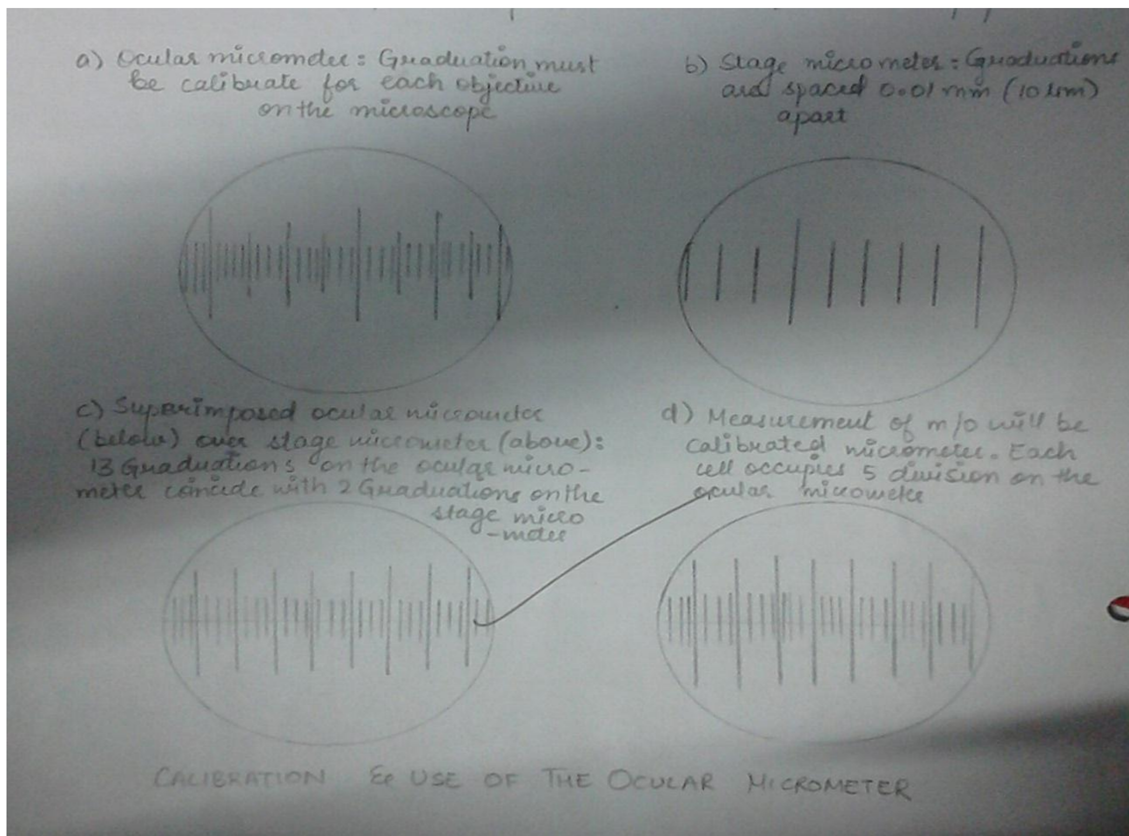
DIAGRAM:



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PROCEDURE:

i) Carefully place the ocular micrometer into the eyepiece.

ii) Place the stage micrometer on the microscope stage and centre it over the illumination source.

iii) With the stage micrometer in clear focus under the low far objective, slowly rotate the eyepiece to superimpose the ocular micrometer graduations over those of stage micrometer.

iv) Add a drop of immersion oil to the stage micrometer, bring the oil immersion objective into position and focus, if necessary, with the fine adjustment knob only.

v) Move the mechanical stage so that a line on the stage micrometer coincides with the line on ocular micrometer at one end. Find another line on the ocular micrometer that coincides with a line on stage micrometer. Determine the distance on stage micrometer (no. of divisions x 0.01mm) and the corresponding number of divisions on ocular micrometer.

vi) Determine the value of caliberation factor for oil immersion objective.

vii) Removed the stage micrometer from the stage.

viii) Determine and record the average of three measurements.

CALCULATION:

One ocular division = 0.02/20

= 0.001mm or 1µm

Length of organism = No. of division x caliberation factor for ocular Divisions.

= 3x1µm

= 3 µm

PRECAUTIONS:

i) Carefully place the ocular micrometer in eyepiece.

ii) Calculations should be done accurately.

iii) If the organisms are not round, both length and width measurements are required.

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EXPERIMENT-5

AIM: Metabolic characterization (e.g. IMViC test)

THEORY: Identification of enteric bacilli is of prime importance in controlling intestinal infection by preventing contamination of food and water supplies. The groups of bacteria that can be found in the intestinal tract of humans and lower mammals are classified as members of the family Enterobacteriaceae. They are short, gram negative, non-spore forming bacilli. Included in this family are:

1) Pathogens such as members of the genera *Salmonella* and*Shigella.*
2) Occassional pathogens such as members of the genera *Proteus* and *Klebsiella.*
3) Normal intestinal flora such as members of the genera *Escherichia.*and*Enterobacter,* which are saprophytic inhabitants of intestinal tract.

Differentiation of the principle groups of Enterobacteriaceae can be accomplished on the basis of their biochemical properties and enzymatic reaction in the presence of specific substrates. The IMViC series of tests (Indole, Methyl Red, Voges- Prokauer and citrate utilization) can be used.

PRINCIPLE:

- ❖ **<u>Indole Production Test:-</u>**

Tryptophan is an essential amino acid that can undergo oxidation by way of enzymatic activities of some bacteria. Conversion of tryptophan into metabolic products is mediated by the enzyme tryptophanase. This ability to hydrolyze tryptophan with the production of indole is not a characterization of all micro-organisms and therefore, serves as a biochemical marker. In this experiment, SIM agar, which contains the substrate tryptophan is used. The presence of indole is detectable by adding Kovac's reagent which produces a cherry red reagent layer. This colour is produced by reagent which is composed of p-dimethyl-aminobenzaldehyde, butanol and HCl and Indole is extracted from the medium into the reagent layer by the acidified butyl alcohol component and forms the complex with the p-dimethyl aminobenzaldehyde yielding Cherry Red colour. Producing a red reagent layer followed by addition of Kovac's Reagent areIndole positive.

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REQUIREMENTS:

1) 24-48 hours Trypticase Soy broth cultures of *E.coli.*
2) Media – SIM agar deep tubes.
3) Reagent- Kovac's reagent
4) Equipments- Bunsen burner, inoculation loop, test tube rack and glassware marker.

PROCEDURE:

1) Using sterile technique, inoculate organism into its labeled deep tube by means of stab inoculation.
2) Prepare another tube as a control.
3) Incubate tubes for 24-48 hrs at 37^0C.
4) Add 10 drops of Kovac's reagent to all deep tube cultures and agitate the cultures gently.
5) Examine the colour of the reagent layer in each colour.

CHEMICAL REACTION:

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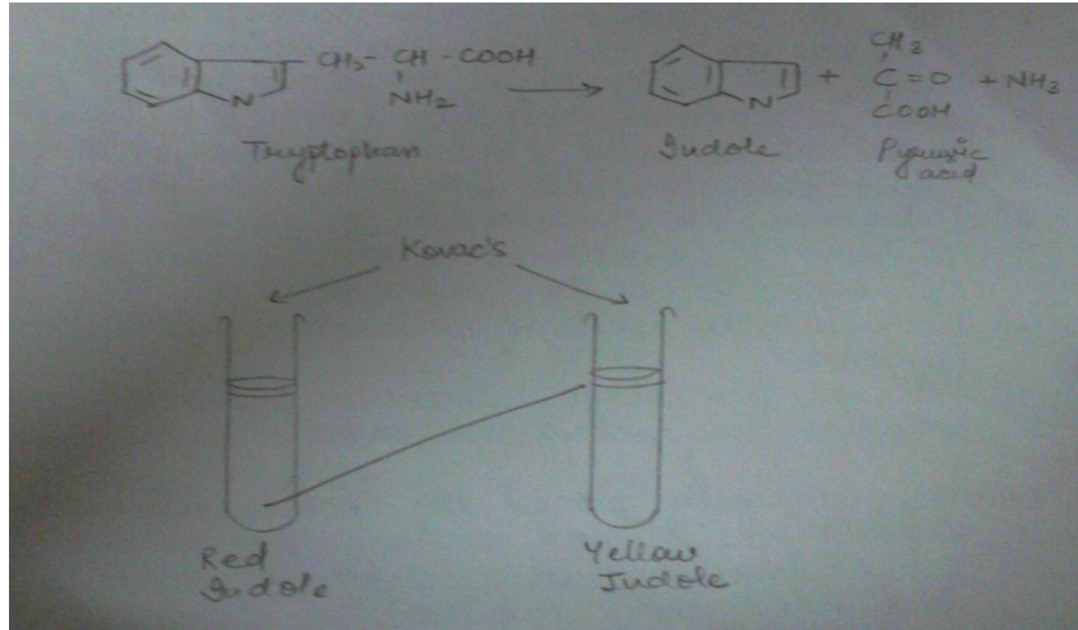

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- **<u>Methyl Red Test:-</u>**

The hexose monosaccharide glucose is the major substrate oxidized by all enteric organisms for energy production. The end products of this process will vary depending on the specific enzymatic pathways present in the bacteria. In this test, the pH indicator methyl red detects the presence of large conc. of acid end products. Although all enteric micro-organisms ferment glucose with production of organic acids, this test is of value in the separation of *E.coli* and *E.aerogenes.*

Both of these organisms initially produce organic acid end products during the early incubation period. The low acidic pH is stabilized and maintained by *E.coli*at the end period. *E.aerogenes*enzymatically convert these acids to non-acidic end products such as 2,3 butanediol resulting in elevated pH of approximately 6.

The Methyl Red indicator in the pH range of 4 will turn red, which is indicator of a positive test. At pH of 6, still indicating the presence of acid with a lower H^+ ion conc., the indicator turns yellow and gives negative test.

Materials: Broth cultures of *E.coli.*
Media: MR-VP broth

Reagents: Methyl red indicator

Equipments: Bunsen burner, inoculating loop, test tubes and glassware markers.

PROCEDURE: Using sterile technique inoculate organism into the appropriately labeled tube of medium by means of a inoculation loop. Incubate all cultures for 24-48 hours at 37^0C.

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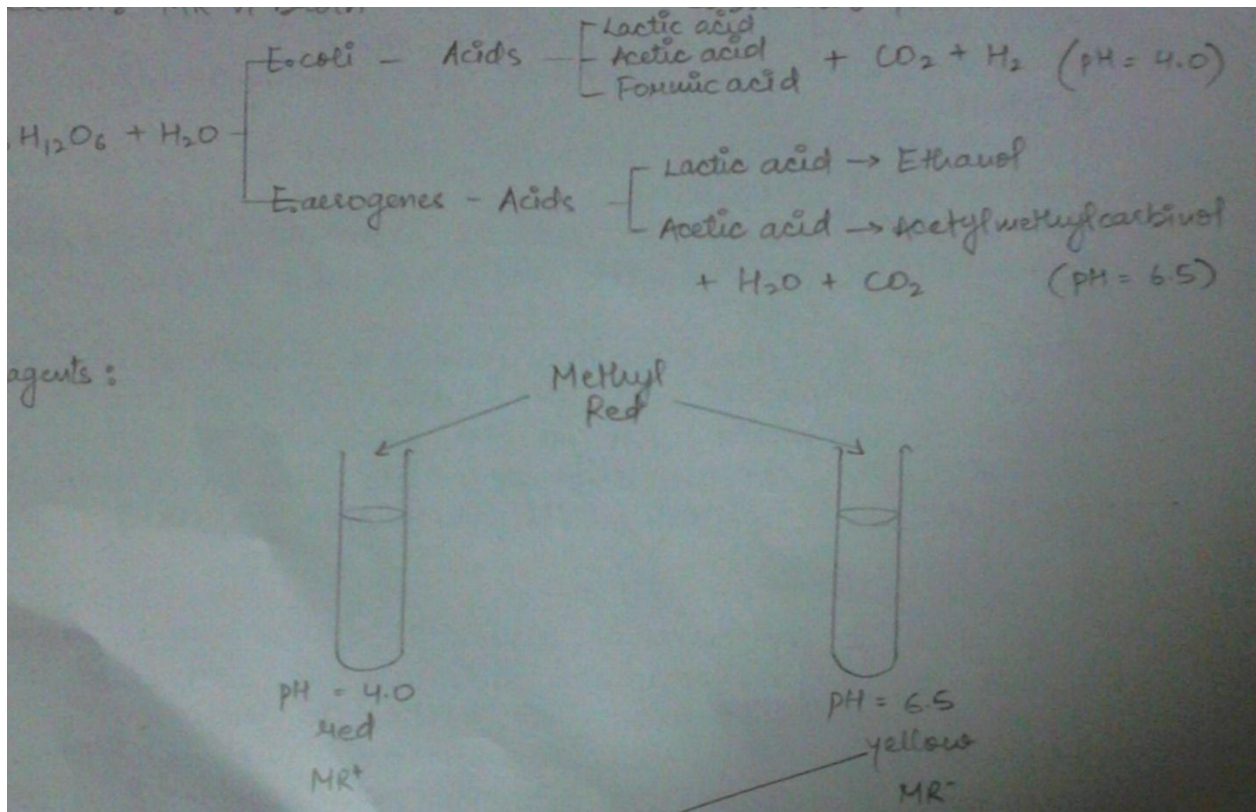
CHEMICAL REACTION:



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<u>Voges- Proskauer Test:-</u>

The VogesProskauer test determines the capability of some organisms to produce non-acidic or neutral end products such as acetyl methyl carbinol, from the organic acid that results from glucose metabolism. The reagent used in the test, Barritt's reagent, consists of a mixture of alcoholic α-nephthol and 40% potassium hydroxide solution.

Detection of acetyl methyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of the α-nephthol catalyst and a guanidine group that is present in the peptone of the MR-VP medium.

As a result, a pink complex is formed imparting a rose color to the medium. Development of a deep rose color in the culture of 15 min. following the addition of barritt's reagent is indicator of presence of acetyl-methyl carbinol and represents a positive result. The absence of rose colouration is α- negative result.

Material: Soy broth cultures of*E.coli*

Note:-Aliquots of this experimental cultures must be set aside from the methyl red , inoulation loop.

Procedure: 1) using sterile techniqueinoculating organism into the appropriately labeled tube of medium by meants of a loop inoculation loop.

2) Incubate all cultures for 24-48 hours at 37^0C.

- **Citrate Utilization Test:**

In the absence of fermentable glucose or lactose, some micro-organisms are capable of using citrate as a carbon source for their energy. This ability depends on the presence of citrate in the cell.

Citrate is the first major intermediate in the Krebs cycle and is produced by the condensation of active acetyl with oxaloacetic acid. Citrate is acted on by the enzyme citrase, which produced oxaloacetic acid and acetate.

These products are then enzymatically converted to pyruvic acid and CO_2. During this reaction the medium becomes alkaline- the CO_2 generated combines with Na and water to form Na_2CO_3, an alkaline product. The presence of Na_2CO_3 changes the bromophenol blue indicator incorporated into the medium from green to deep Prussian blue. Following incubation, Citrate positive cultures are identified by the presence of growth on the surface of slant which is accompanied by blue colouration. Citrate negative culture will show no growth and the medium will remain green.

Materials: Soy broth cultures of *E.coli.*

Media: Simmons citrate agar slants.

Equipments: Bunsen burner, inoculating needle, test tube rack, glassware marking pencil.

Procedure: 1) Using sterile technique, inoculate organisms into appropriately labeled tube by means of streak inoculation.

2) Incubate all cultures for 24**-**48 hours at 37^0C.

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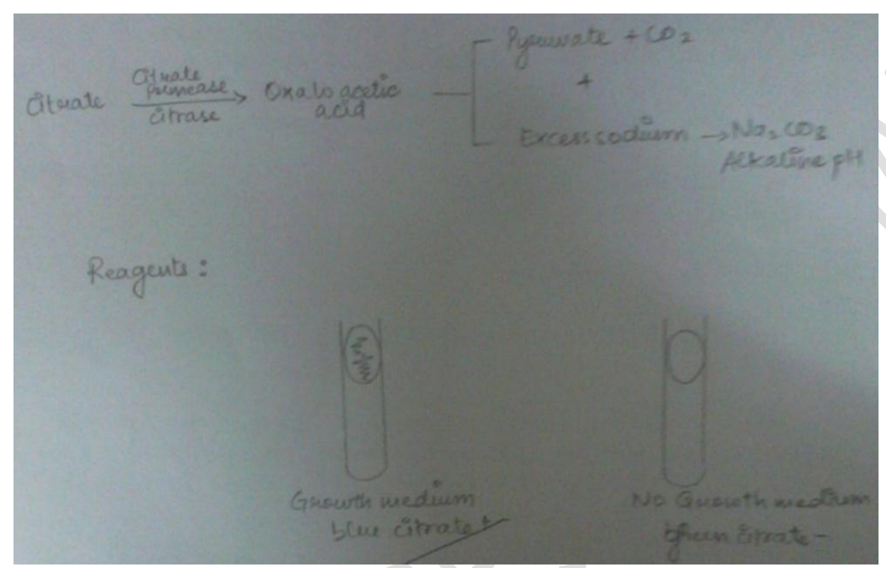
Chemical Reaction



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EXPERIMENT-6

AIM: One step growth of bacteriophage.

PRINCIPLE:

This exercise demonstrates all ability of viruses to replicate inside a susceptible host cell. This technique also enables to enumerate phage particles on the basis of plaque formation in solid agar medium. Plaque are clear area in an agar medium previously shedded with a diluted phage sample and a host cell culture. Each plaque represents the lysis of a phage infected bacterial cell. The procedure requires the use of a double layered culture technique in which the hard agar serves as the base layer and a mixture of phage and host cell in a soft agar forms the upper overlay. Susceptible *E.coli* cells multiply rapidly and produce a lawn of confluent growth on the medium. Each plaque can be designed as a Plaques- forming unit (PFU) and used to quantitate the number of infective phage particles in the culture.

The number of phage particles contained in the original stock phage culture is determined by counting the no. of plaques formed on the needed agar plate and multiplying this by the dilution factor.

REQUIREMENTS:

i) Cultures: 24 hr. nutrient broth cultures of *Escherichia coli* amd*T2 coliphage.*

ii) Media: Tryptone agar plates and tryptone soft agar, broth tubes.

iii) Equipment : Bunsen burner, H_2O broth, thermometer, 1ml sterile pipettes, sterile Pasteur pipettes, mechanical pipetting device, test tube rack and glassware marking pencil.

PROCEDURE:

1) Label all dilution tubes and media as follows:
   a) 5 tryptone soft agar tubes: 10^{-5}, $10^{-6,}$ 10^{-7}, 10^{-8}, 10^{-9}
   b) 5 tryptone hard agar tubes: 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}
   c) 9 tryptone broth tubes: 10^{-1} to 10^{-9}
2) Place 5 labeled soft tryptoneagar tubes into a H_2O bath. H_2O should be of a depth just slightly above that of the agar in the tubes. Bring the H_2O bath to 100^0C to melt the agar. Cool and maintain the melted agar at 45^0C.
3) With 1ml pipettes, aseptically perform a 10-fold serial dilution of the provided phage culture using the 9ml tubes of tryptone.

   To the tryptone soft agar tube labeled 10^{-5}, aseptically add 2 drops of the *E.coli*Culture with the Pasteur pipette and 0.1ml of the 10^{-4}tryptone broth phage dilution. Rapidly mix by rotating the tube between the palms of your

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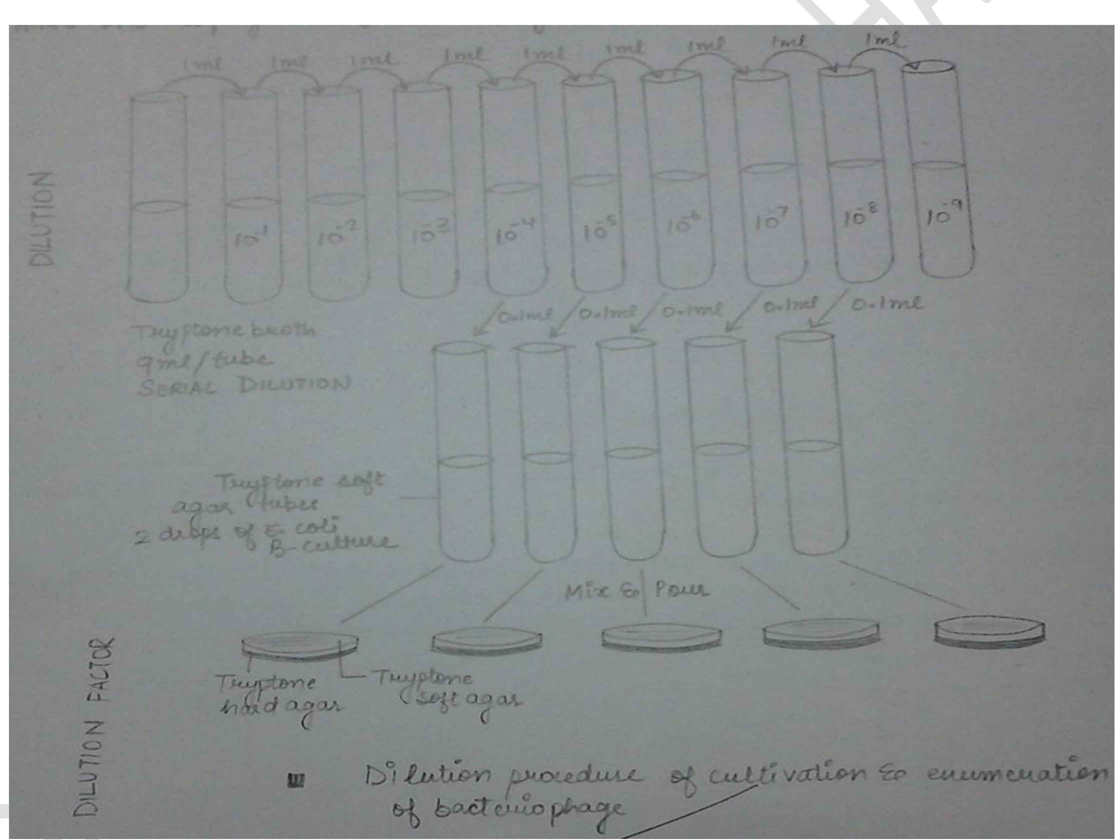
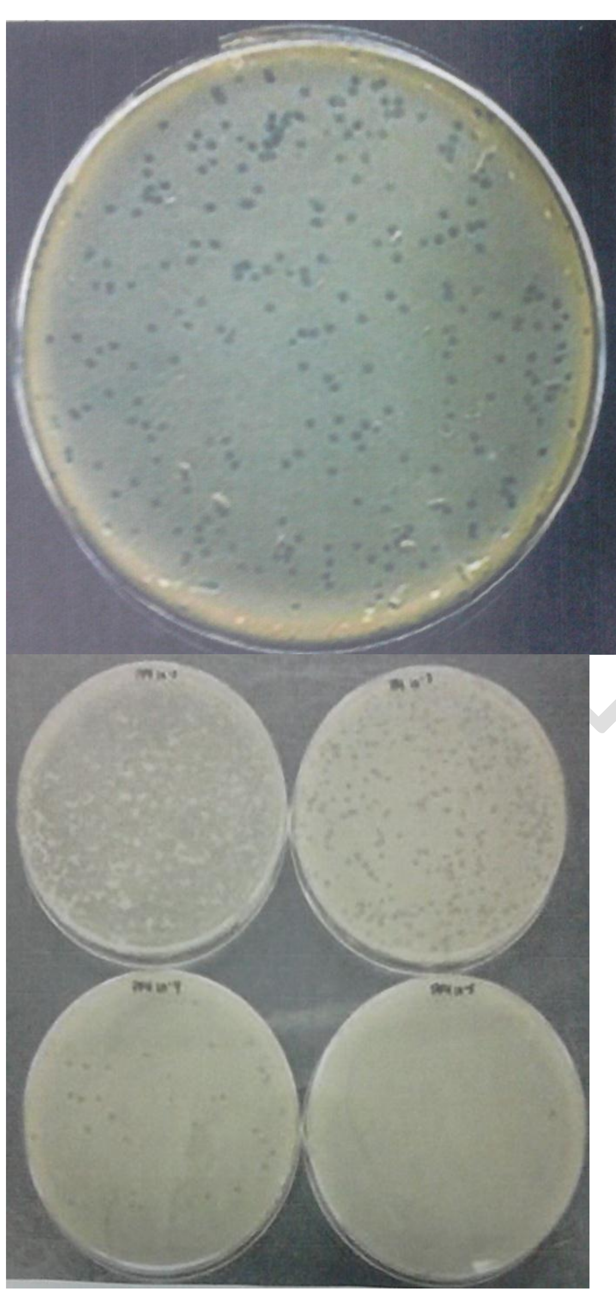
hands and pour the contents over hard tryptone agar plate labeled 10^{-5}, thereby forming a double layered plate culture preparation.

4) Using separate Pasteur pipettes and 1ml sterile pipettes, repeat step 4 for the tryptone broth phage dilution tubes labeled 10^{-5} through $10^{-8}$to effect the 10^{-6}.
5) Following solidification of the soft agar overlay, incubate all plate culture in an inverted position for 24 hrs. to 37^0C.

Diagram:



OBSERVATIONS:



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PRECAUTIONS:

1) Plates showing greater than 300 PFUs are too numerous to count (TNTC); plates showing fewer than 30 PFUs are too few to count (TFTC).

2) For phage count, the no. of plaques per plate should not exceed 300 nor be less than 30.

EXPERIMENT-7

AIM: Alcoholic and mixed acid fermentation.

THEORY: We will investigate alcoholic fermentation in yeast (single cell fungus) *Saccharomyces cerevisiae* or baker's yeast. When O_2 is low, some fungi including yeast and most plants switch from cellular respiration to alcoholic fermentation. The CO_2 produced can be used as an indication of the relative rate of fermentation taking place. The rate of fermentation, a series of enzymatic reaction can be affected by several factors. E.g. concentration of yeast, concentration of glucose or temperature. In this exercise, we investigate, the effects of yeast concentration. The alcoholic fermentation of glucose is described by following equation:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \xrightarrow[\text{enymes}]{} 2\underset{\text{Ethanol}}{CH_3CH_2OH}\,(aq) + 2CO_2\,(g) + \text{Energy (2ATP+ heat)}$$

Both alcoholic fermentation and aerobic respiration are multistep process, that involve the transfer of energy stored in the chemical bonds of glucose to bonds of ATP. The energy stored in ATP can be used to perform cellular work.

Ethanol is a byproduct of alcoholic fermentation since yeast doesn't have enzymes needed to metabolize ethanol (ethanol dehydrogenase). Much of the energy stored in molecules of glucose to CO_2 and H_2O in aerobic respiration yields much more energy than does the alcoholic fermentation. 36-38 ATP vs only 2 ATP molecules reduced by anaerobic respiration.

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REQUIREMENTS:

T1-83 Calculator, vernier gas pressure sensor, vernier lab-pro, test-tubes, 600ml beaker, rubber stopper assembly, 10% yeast solution, pipette pump, 5ml or 10ml glass pipette, vegetable oil, dropper bottle, 10% glucose solution, DI water.

PROCEDURE:

1) Take 4 test tubes, label them 1-4.
2) Add 5ml of glucose solution in test tube 1, 3 and 4.
3) Add 5ml of DI water in 1. Mix the water and glucose solution gently by pipetting the solution back into and out of the pipette. Then place enough vegetable oil to completely cover the surface of the fermentation solution. Be careful to not get oil on sides of test tube.
4) Insert the single rubber stopper to test tube.
5) Incubate the test tube for 10 min. at room temperature.
6) When incubation has finished, connect free end of the plastic tubing to the connector in the rubber stopper.
7) Plug in vernier lab-pro and connect to the calculator.
8) Turn calculator on 'select program' , select " Datamate". Press "enter" and then "enter" again.
9) Connect the plastic tubing to the value on the gas pressure sensor.
10) Connect the gas pressure sensor into channel "#1" on lab pro.
11) Start data collection, "#2". Data Collection starts.
12) Data collection after 15 mins. end.
13) After Data collection is completed, the unit will beep and the calculator will display a different screen on that screen. Simply press "Enter" and calculator will graph a time vs. pressure plot of all data, that was collected.
14) To obtain the equation of slope, simply return to the main menu by pressing "Enter".
15) Then select "Analyse" (#4), then select "curvefit" (#2) and finally on screen choose (#1).
16) Record the graph equation in the form of y= mx+b, where m is slope of fermentation.
17) Disconnect the plastic tubing connector from rubber stopper.
18) While you are collecting your data for test tube 1, prepare test tube 2. Gently swirl the yeast suspension to mix the yeast that settles to the bottom. Using the pipette, transfer 5ml of yeast into test tube 2. Allow the tube to incubate at room temperature for approx. 10 mins.
19) Repeat the data collection steps for test tube 3.

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20) While you are collecting data for tube 2, gently swirl to yeast suspension to mix yeast. Transfer 2ml of yeast and 3ml of DI water into test tube 3. Allow tube to incubate at room temperature for 10 mins.
21) Repeat the data collection steps for test tube 3.
22) While you are collecting data for test tube 3, prepare test tube 4, gently swirl the yeast suspension to mix yeast. Using pipette, transfer 2ml of yeast and 3ml of DI water into tube 4. Allow to incubate at room temperature for 10 mins.
23) Repeat the data collection steps for test tube 4.

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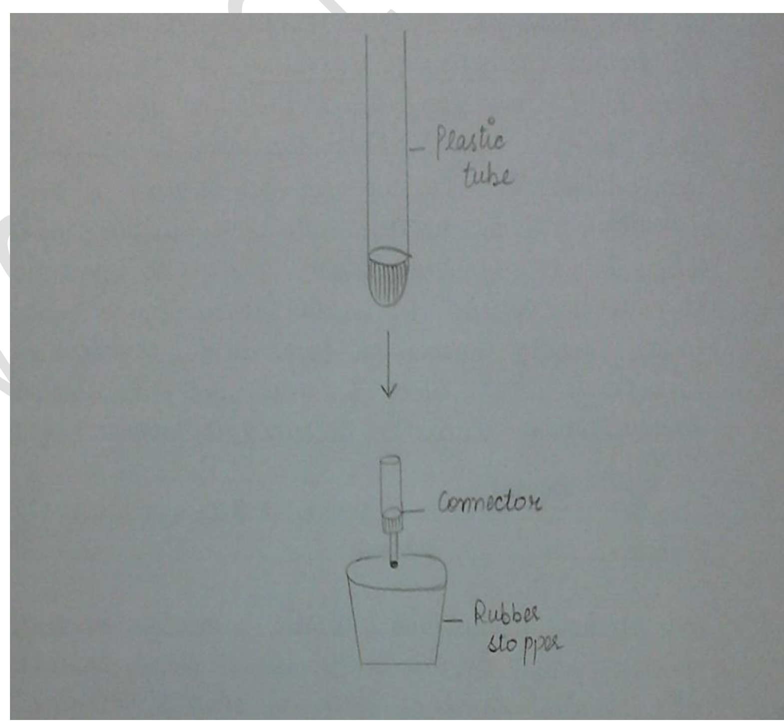
Diagram:



MIXED ACID FERMENTATION:-

THEORY: Mixed acid fermentation is an anaerobic fermentation when the products are the complex mixtures of acids particularly lactate, acetate, succinate and formate as well as ethanol and equal amount of H_2 and CO_2.
It is characteristic for the members of Enterobacteriaceae Family.
Methyl Red Test - *E.coli*and other members of low ratio ferment sugars by the mixed acid pathway resulting in low ratio of CO_2 to H_2 gas produced by fermentation. The large quantity of acids produced causes a significant decrease in pH of the culture medium. When the culture medium turns Red, after addition of Methyl Red, because of pH at or below 4.4 from the fermentation of Glucose. The culture has a positive result for the methyl red test. A negative methyl red test is indicated by Yellow colour in the culture medium, which occurs when less acid is produced (pH is high) from the fermentation of glucose.

REQUIREMENTS:
1) Materials: Soy broth cultures of *E.coli*.
2) Media: Simmon's Citrate agar slants.
3) Equipments: Bunsen burner, inoculating needle, glassware marker, test tube rack.

PROCEDURE: Using sterile technique, inoculate organisminto the labeled tube of medium by means of a inoculation loop. Incubate all cultures for 24-48 hrs at 37^0C.

Diagram:

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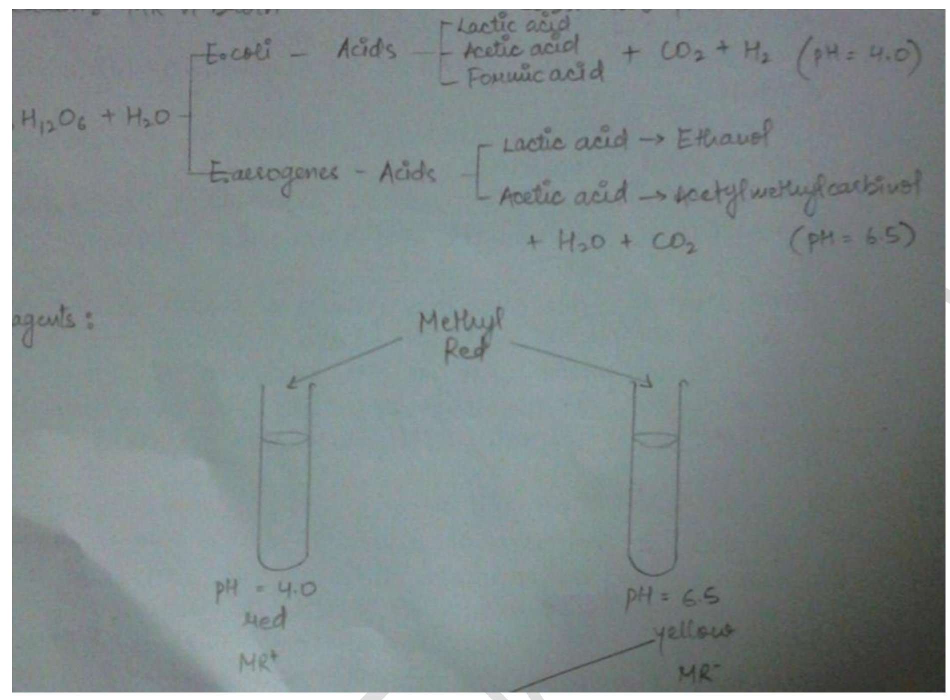

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PRECAUTIONS:

1) Handle the apparatus carefully.
2) Maintain sterile condition.

Agro and Industrial Applications of Microbes

Practical Manual

Semester-IV

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4.	Additive and synergistic effect of two drugs on the above micro-organisms.
5.	Plating the milk samples for microbial contamination.
6.	MBRT Test for determination of milk quality.

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EXPERIMENT-1

AIM- Screening of cellulose producing micro-organisms from wood degrading soil.

THEORY-Cellulose is a world's most abundant organic substance and comprises a major storage form of glucose. Microbial cellulose utilization is responsible for one of the largest material flow in the biosphere, therefore, the aim of the study is to isolate cellulose degrading from soil samples collected from different regions to identify cellulose degrading micro-organisms including bacteria and fungi.

Some examples of cellulose degrading bacteria are *Thermoactinomycetes* species and *Pseudomonas* species.

Some examples of cellulose degrading fungi are *Aspergillus* species and *Penicillium* species. Clear zones around the colonies were the indication of the cellulose degradation acting of the micro-organisms.

REQUIREMENTS- Soil samples from different regions,1% peptone, 1% carboxy -methyl cellulose, 2% K_2HPO_4, 1% agar, 0.3% $MgSO_4.7H_2O$, 25% Ammonium sulfate and 2% gelatin, Potato dextrose agar, Congo red solution(stain),1 molar NaCl, Weighing meter, Pipette, Petri-plate ,Beaker, pH meter, etc.

PROCEDURE:-

ISOLATION OF BACTERIA

1. Cellulolytic bacterial strains were isolated from soil by using serial dilution and pour plate technique.
2. Prepare CMC media and maintain its pH at 7.0 for 48 hours of incubation at 30 degree centigrade.
3. After inoculation bacterial colonies were purified by repeated streaking.
4. The purified colonies were preserved at 4 degree centigrade for further identification and screening for cellulose degrading media.

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ISOLATION OF FUNGI

1. The fungi were isolated by serial dilution methods and 1ml were plated onto the potato dextrose agar plates.
2. The plates were incubated for 7-8 days for 25-30 degree centigrade .
3. Different types of fungi were isolated.
4. These were sub-cultured on sterile PDA plates.

SCREENING OF BACTERIA FOR CELLULOLYTIC ACTIVITY

1. Pure cultures of bacterial isolates were individually transferred in CMC agar plates.
2. Incubate the plate for 48hours.
3. After incubation, CMC agar plate were flooded with 1% congo red and allow to stand for 15minutes at room temperature.\
4. 1M NaCl was thoroughly used for counter staining the plates.

SCREENING OF FUNGI

1. Isolated fungi were plated on PDA medium supplemented with carboxy- methyl cellulose(1.2% W/V)
2. After an appropriate incubation period of 5 days cellulolytic activity was detected by appearance of clear zones around colony.
3. The plates were flooded with 10ml congo red solution solution.
4. After 20 minutes, the congo red solution was poured off and re-flooded the plates with 10ml of 5ml/litre NaCl solution for termination of colorations.
5. After an additional 20 minutes the salt solution was discarded and CMC cellulose activity was revealed by the presence of clearing zones around the colony.

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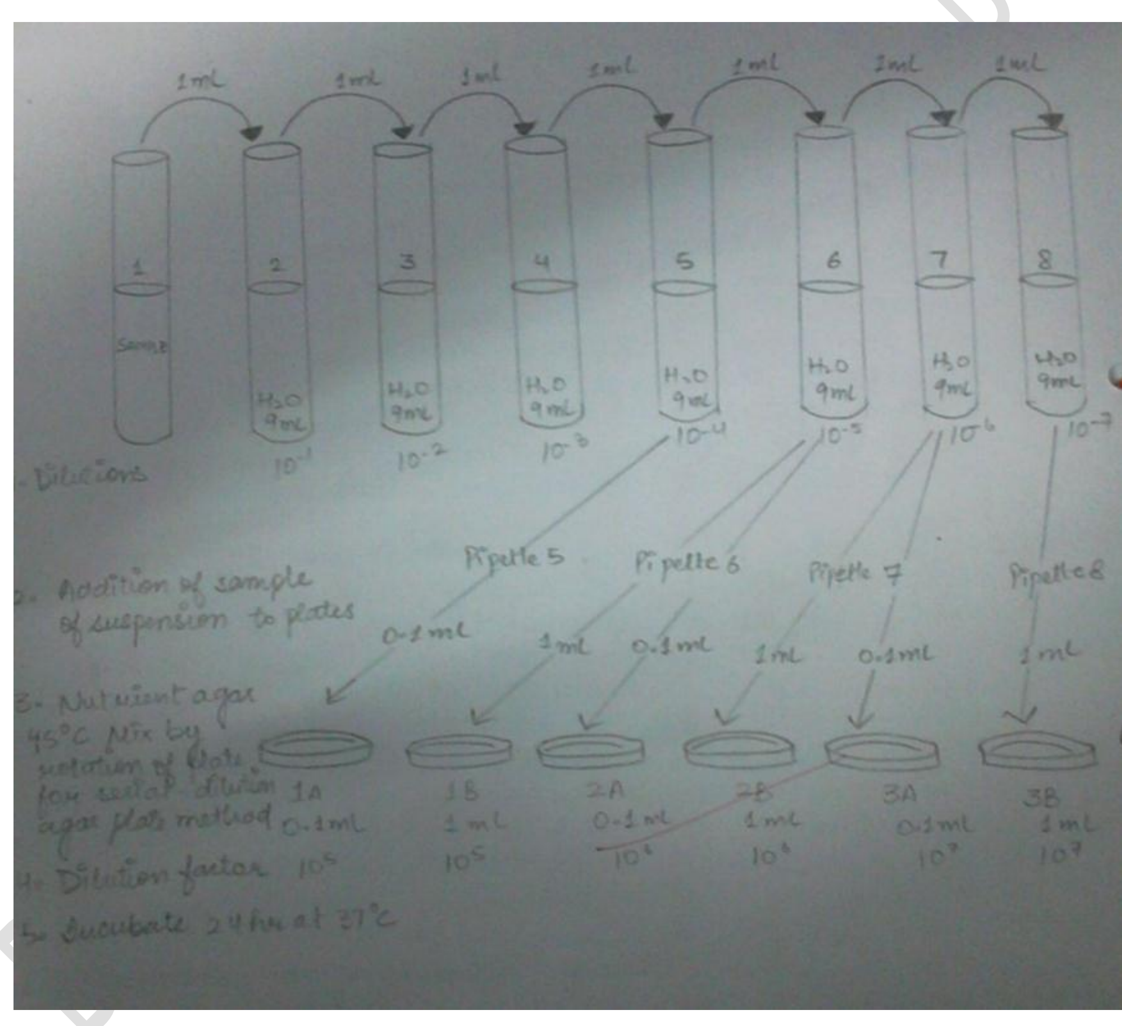
DIAGRAM



RESULT- clear zones appeared around growing bacterial colonies indicating cellulose hydrolysis.

EXPERIMENT-2

AIM: Antibiotic sensitivity of above micro-organisms.

THEORY: A true antibiotic is an antimicrobial chemical produced by micro-organisms against other micro-organisms. Bacteria respond in different ways to antibiotics and chemosynthetic drugs, even within the same species. For example, *Staphylococcus aureus* is a common normal flora bacterium found in the body. If one isolates this bacteria from different people, five isolates would likely be different strains that is slight genetically different. It is also likely that if antibiotic sensitivity test were run on these isolates, the results would vary against the different antibiotics used. The Kirby- Bauer test for antibiotic susceptibility called the disc-diffusion test, is a standard that has been used for years. First developed in the 1950s, it was refined by W. Kirby and A. Bauer.

This test is used to determine the resistance or sensitivity of aerobes or facultative anaerobes to specific chemicals which can then be used by the clinicians for treatment of patients with bacterial infections. The presence or absence of an inhibitory area around the disc identifies the bacterial sensitivity to the drug.

PRINCIPLE: The bacterium is swabbed on the agar and the antibiotic discs are placed on top. The antibiotic diffuses from the disc into the agar in the decreasing amount, the further it is away from the disc. If the organism is killed by the concentration of the antibiotic, there will be no growth in the intermediate area around the disc. This is called zone of inhibition. The zone size is looked up on a standardized chart to give result of sensitive resistant. Many charts have a corresponding column that also gives the MIC (Minimal Inhibitory Concentration) for that drug. The MIC is currently the standard test run for antibiotic sensitivity testing because it produces more pertinent information on minimal dosages.

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The Mueller-Hinton medium being used for the Kirby-Bauer test is very high in protein.

REQUIREMENTS: Agar plates, 24 hours old cultures, inoculation loop, antibiotic, ethanol, forceps.

PROCEDURE:

1. Aseptically inoculate the appropriately labeled agar plates with thin test organism by breaking each plate in horizontal vertical directions around the edge with a sterile inoculation loop.

2. Swab the surface of the agar completely.

3. Run the swab around the circumference of the plate before discarding it in the discard bag.

4. Allow the surface to dry for about 5minutes before placing antibiotic disc on agar.

5. Place the forcep in alcohol, flame the forcep until they catch on fire, let the flame to out sterile forcep.

6. Place each antibiotic disc approx. 2cm in from the edge of the plate.

7. Lightly touch each disc with your sterile inoculating loop. Make sure that it is in good contact with the agar surface.

8. Place each of the remaining antibiotic discs on the surface of each of the agar plate equidistant from each other around the periphery of the plate.

9. Incubate all plate cultures in an inverted position or 24-48 hours at 37degree centigrade.

ZONE DIAMETERS:

S.No.	ANTIBIOTICS (antimicrobial agent)	RESISTANCE (mm)	INTERMEDIATE (mm)	SUSCEPTIBLE (mm)

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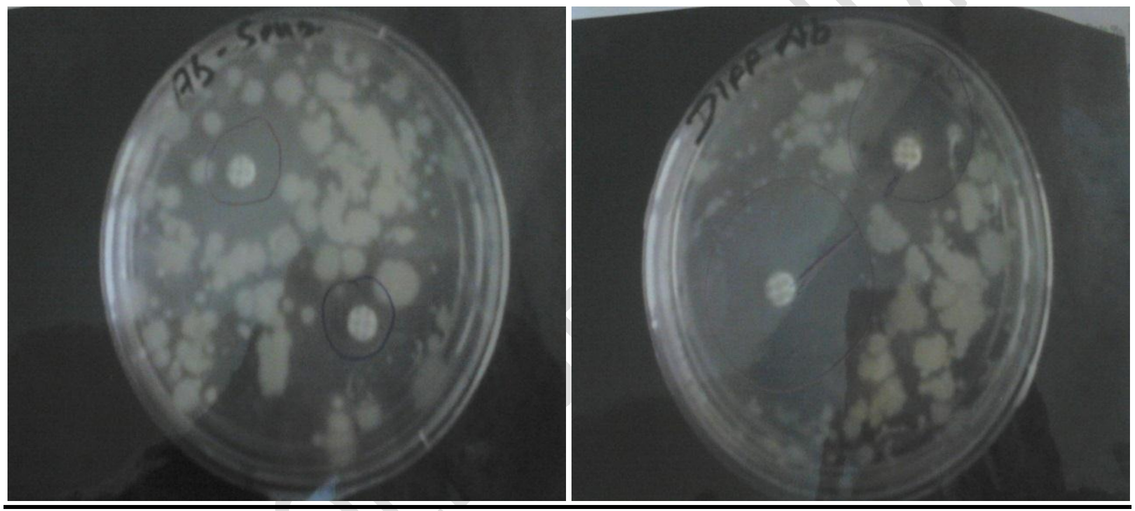
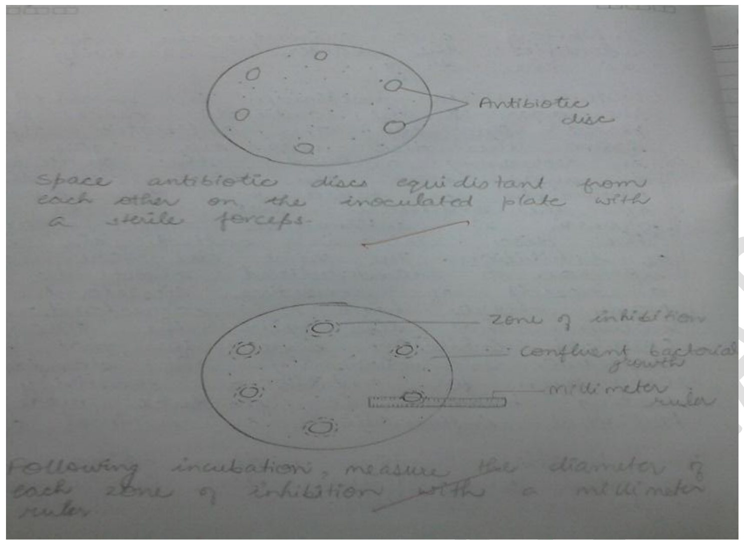
1.	Ampicillin(staph)	28	------	29
2.	Chloramphenicol	12	13-17	18
3.	Ciprofloxacin	15	16-20	21
4.	Kanamycin	13	14-17	18
5.	Penicillin G(staph)	28	-------	29
6.	Streptomycin	14	15-20	21
7.	Tetracycline	14	15-18	19



INTERPRETATIONS:

1. Place the metric ruler across the zone of inhibition at the widest diameter and measure from one edge of the zone to the other edge.
2. If there is no zone at all report it as 0—even though the disc itself is around 7mm.
3. Zone diameter is reported in mm, looked up on the chart and result reported as sensitive, resistant or intermediated.



PRECAUTIONS:

1. Maintain strict contamination free environment.
2. Do not leave any unswabed areas at all.

EXPERIMENT-3

AIM- Minimum inhibitory concentration of an antibiotic for above micro-organisms.

PRINCIPLE- In addition to the Kirby-Bauer paper discs agar diffusion procedures, the broth tube dilution method may be used to determine the susceptibility of an organism to an antibiotic. The latter procedure, in which dilutions of the antibiotic are compared in the broth medium, also permits the minimal inhibitory concentration (MIC). The (MIC) is the lowest concentration of an antimicrobial agent that inhibits the growth of the test micro-organism. Quantitative data of this nature may be used by a clinician to establish effective antimicrobial regimes for the treatment of a bacterial infection in a host. This data is of particular significance when the toxicity of the antibiotic is known to produce a major adverse effect in host tissues.

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THEORY- We need to establish the minimum inhibitory concentration (MIC) of an antibiotic which may inhibit the growth of micro-organism. Penicillin is effective against Gram – positive bacteria while *streptomycin* kills Gram – negative such as *E.coli*. *Penicillin* blocks the amino acid synthesis. Osmotic pressure exerts on the wall and cell breaks and lyse.

Streptomycin is used against the Gram – negative bacteria. It binds the protein of the 30S subunit of ribosome, blocking protein synthesis in the cell. The cell stops dividing due to check of new protein synthesis and lose viability.

REQUIREMENTS- Penicillin, Streptomycin, nutrient broth, culture tube, bacterial culture, spectrophotometer, incubator, inoculation loop.

PROCEDURE-

1. Prepare the solution of measuring 4 units/ ml of penicillin or 2 mg/ml of streptomycin.
2. Mix 2ml of the antibiotic solution in 2 ml of nutrient broth in test tube and shake well.
3. Transfer 2 ml in test tube 2 and subsequently transfer in rest of the test tube containing 2 ml of nutrient broth except the last that does not contain any antibiotics solution. It is possible to calculate the concentration of antibiotic in each tube.
4. Inoculate each tube with one drop of culture and incubate them at 37^0 C for 48 hours.
5. Measure the turbidity in term of optical density (OD) by spectrophotometer and prepare a table or plot a graph between antibiotic concentration and turbidity.

OBSERVATION TABLE:

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S.No.	Concentration (µg/ml)	Absorbance (500nm)
1.	5	0.800
2.	10	0.760
3.	15	0.650
4.	20	0.630
5.	40	0.570
6.	50	0.400
7.	60	0.270
8.	70	0.100
9.	80	0.00

RESULT- low concentration of antibiotic will show maximum optical density due to less inhibitory effect; whereas high concentration will reveal minimum optical density.

PRECAUTIONS:

1. Concentrations of antibiotics should be prepared carefully.
2. Spectrophotometer should be switched on 20 minutes before use.

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EXPERIMENT-4

AIM: Additive and Synergistic effects of two drugs.

PRINCIPLE: Combination chemotherapy, the use of two or more antimicrobial or antineoplastic agents, is being employed in medical practice with ever increasing frequency.The rationale for using drug combinations is the expectation that affective combinations might lower the incidence of

bacterial resistance, reduce host toxicity of the antimicrobial agents(because of decreased dosage requirements),or enhance the agents bactericidal activity. Enhanced bactericidal activity is known as synergism. Synergist activity is evident when the some of the effects of the chemotherapeutic agents used in combinations is significantly greater than some of their effects when used individually. This result is readily differentiated from an additive (indifferent) effect, which is evident when the interaction of two drugs produces a combined effect that is no greater than some of their separately measured individual effects.

A variety of in-vitro methods are available to demonstrate synergistic activity. In this experiment, a disc-agar diffusion technique will be performed to demonstrate this phenomenon. This technique uses the Kirby-Bauer antibiotic susceptibility test procedure. It requires both Mueller-Hinton agar plates previously seeded with the test organism and commercially prepared, antimicrobial-impregnated discs. The two discs representing the drug combinations are placed on the inoculated agar plate and separated by a distance (measured in mm) that is equal to or slightly greater than one half the sums of their individual zones of inhibitions when obtained separately. Following the incubation period and addictive effect is exhibited by the presence of two distinctly separate circles of inhibition. If the drug combination is synergistic, the two inhibitory zones merge to form a “bridge” at their juncture.

REQUIREMENTS: Bunsen burner, forceps, sterile cotton, swabs, millimeter ruler and glassware marking pencil, different antibiotics.

PROCEDURE:

1. Nutrient agar plates were prepared and pour plated with culture.
2. Centre of each plate was determined using ruler. 2 dots were marked at center of two areas.
3. Two wells were made at two sides of that dots.
4. Different combinations of antibiotics were filled into each well.
5. Each plate was incubated at 37^0C for 24 hours.

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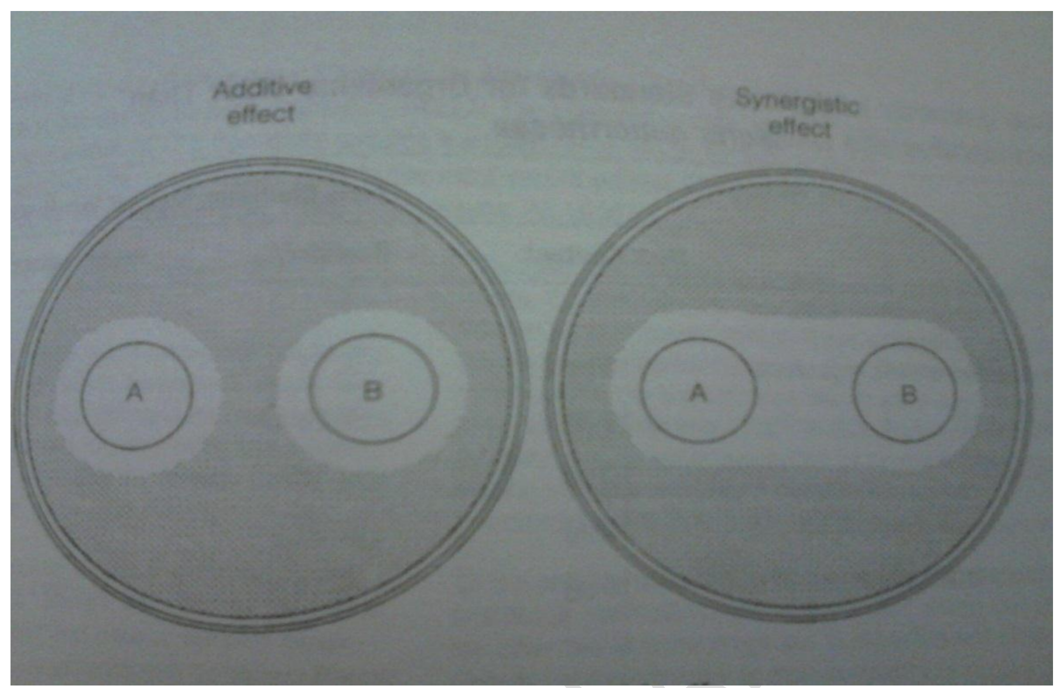

PRECAUTIONS:

1. Don't incubate the plates in inverted position.
2. Make the wells only at the center of halves.

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EXPERIMENT-5

AIM: Plating the milk samples for microbial contamination.

PRINCIPLE: Milk and food products are important sources of transmissions of various pathogens in human beings. Milk provides an excellent growth media for bacteria at room temperature. These products may be contaminated either by contaminated hands of workers, unhygienic utensils, flies and use of polluted water.

The sanitary quality of milk can be checked by bacterial count in the milk. American public health association (APHA) recommends the standard plate count as the official method in its ordinance.

Actually the presence of human pathogenic microbes may arise due to unsanitary holding of milk, diseased fodder, improper storage condition. The increase in number of bacterial count has the more possibility of transmission of disease.

REQUIREMENTS: Nutrient agar culture medium, milk sample, micropipette, petri-plate, distilled water, test tube stand, ethanol, autoclave, incubation, laminar air flow, MacConkey Agar medium.

PROCEDURE: Preparation of Nutrient agar Medium:

1) Weighed accurately ingredients and put them in flask containing distilled water.

COMPOSITION OF NUTRIENT AGAR CULTURE MEDIUM:

S.NO.	INGREDIENTS	QUANTITY (for 250 ml)
1.	Beef extract	0.75gm
2.	Peptones	1.25 gm
3.	NaCl	1.25gm
4.	Agar	3.75gm
5.	Distilled Water	250ml
6.	pH	7

2) Gently heated the contents with slight agitation to dissolve the ingredients. pH was adjusted at 7 using pH strip and autoclave the media at 15 lb/inch^2 at 121.5^{0}C for 15-30 minutes.
3) After autoclaving, poured the media into the petri-plates which are pre-sterilized.
4) Then, petri-plates are incubated after solidification at 37^{0}C to check the contamination for 24-48 hours.

PreparationOf MacConkey Agar Medium:

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1. Chemical ingredients of MacConkey agar medium were weighed accurately and then transferred into flask containing 250ml distilled water.

Composition of MacConkey Agar Medium:

S.No.	INGREDIENTS	QUANTITY (For 250ml)
1.	MacConkey	10gm
2.	Agar	3.75gm

2. Gently heated the contents with slight agitation to dissolve the ingredients, pH was adjusted at 7 using pH strip and autoclave the media at 15lb/inch^2 at 121.5^{0}C for 15-30 minutes.
3. After autoclaving, pour the medium into pre-sterilized plates.
4. Then, the petri-plates are incubated for solidification in the incubation at 37^{0}C for 24-48 hours.

Serial Dilution Method:

1. 1ml of milk was transferred into test tube containing 9ml of water to prepare dilution 10^{-1}.
2. Thus, 1ml of suspension from 10^{-1} dilution was transferred into 2nd test tube containing 9ml of distilled water to make 10^{-2} dilution.
3. Similarly, we can prepare 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and so on dilutions.

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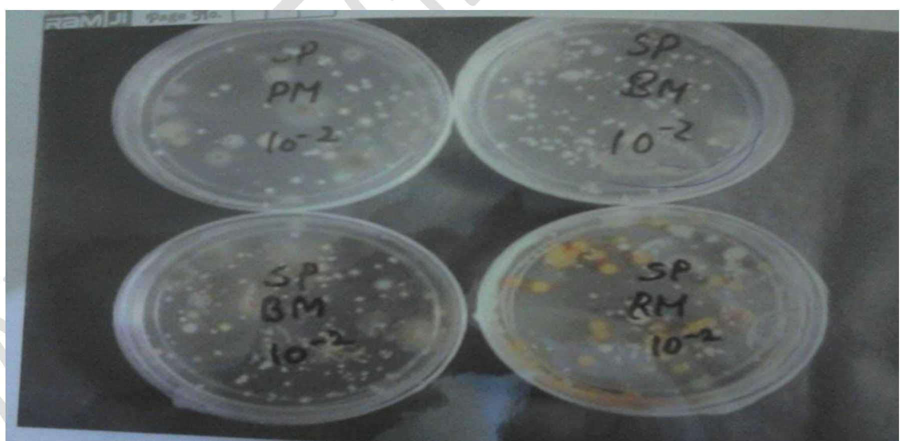
Pour Plate Method:

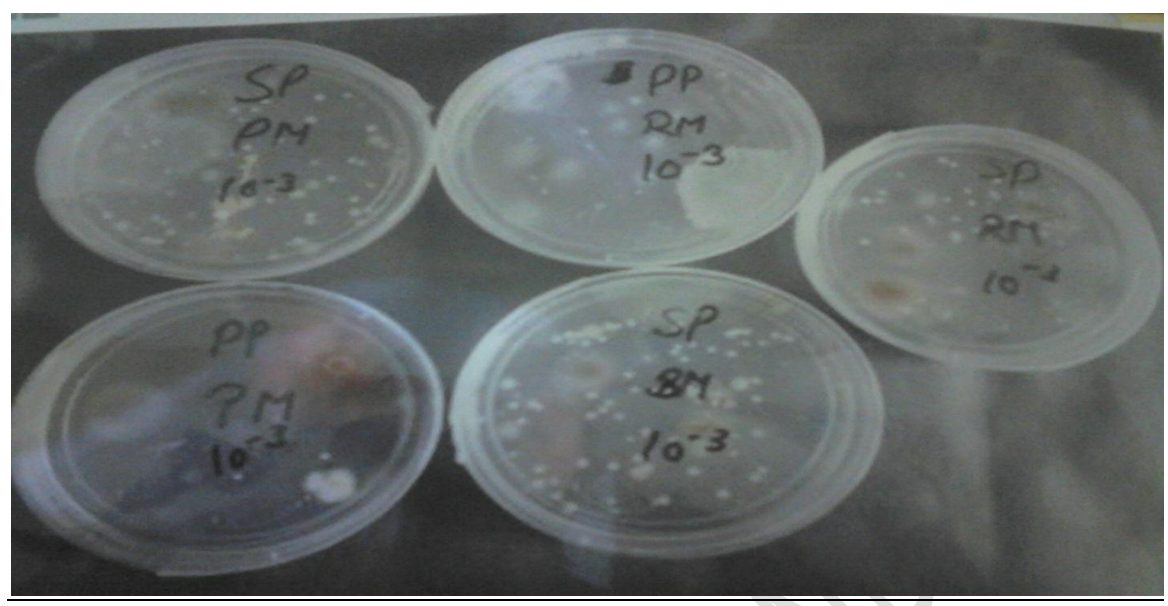
1. Petri-plates should be marked properly.
2. 0.1ml of the inoculum is taken in a petri-plate and then added known (15-20ml) amount of molten media in it and both are mixed well.
3. Petri-plates were allowed to solidify and incubated at 37^{0}C for 24 hours in an inverted position.

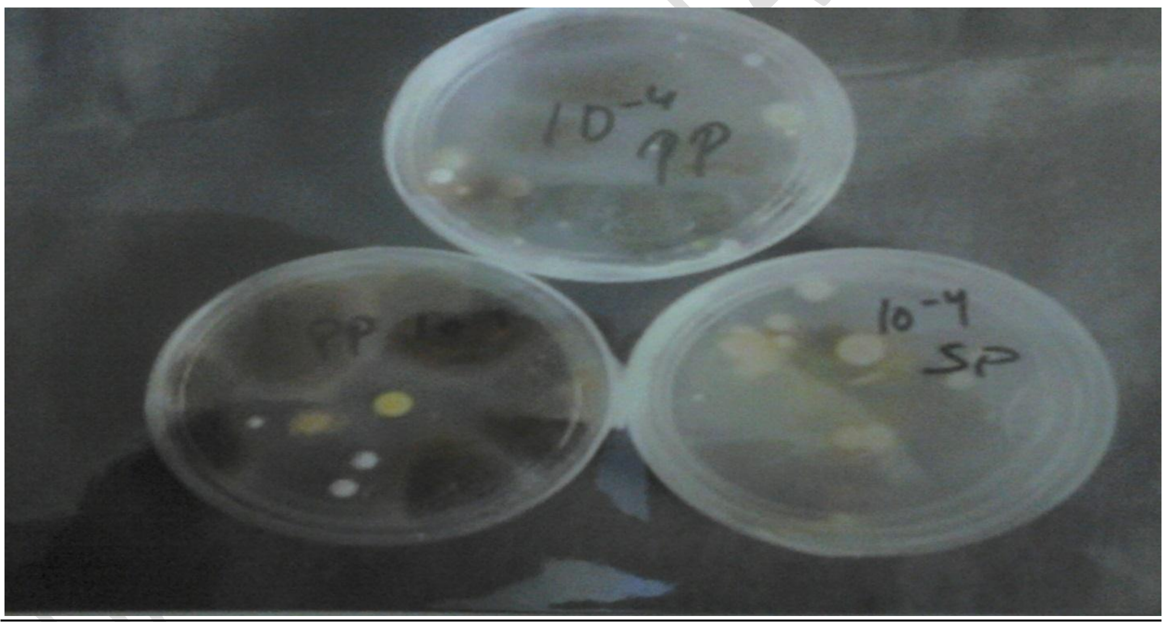
4. After incubation, observe the colonies and record the results.

Spread Plate Method:

1. Sterilize the spreader by putting it in ethanol and then on flame. Then added 1ml of suspension on the top of agar medium with the help of micropipette. Then spread it with the help of spreader so that it completely spread on the agar medium.
2. Then all the petri-plates were incubated in incubator at 37^0C for 24-48 hours.







PRECAUTIONS:

1. Placed the petri-plates in inverted position.
2. Incubation should be done aseptically.
3. Apparatus used should be sterilized.

EXPERIMENT-6

AIM: Methylene Blue Reductase Test (MBRT) for determination of milk quality.

PRINCIPLE: A milk sample that contains a large population of actively metabolizing micro-organisms will contain a markedly decreased concentration of dissolved oxygen because of the vigorous growth of the organisms. In other words, the oxidation-reduction potential of the sample is greatly lowered. The dye methylene blue (MB), a redox indicator, loses its color in anaerobic environment and is then said to be reduced.

The methylene blue reductase test is designed to screen the quality of raw milk, which may contain large populations of enteric organisms and *Lactococcus lactis*, which are potent reducers of the dye. The speed at which reduction occurs following addition of MB to a sample of milk indicates the milk's quality. This determination is made as follows:

1. Reduction within 30 minutes is indicative of very poor quality.
2. Reduction occurring between 30 minutes and 2 hours is indicative of poor quality.
3. Reduction occurring between 2 and 6 hours is indicative of fair quality.
4. Reduction occurring between 6 and 8 hours is indicative of good quality.

REQUIREMENTS: water bath, sterile test tubes, 10- ml or 1-ml glass pipette, stop watch, standard methylene blue solution, milk sample, beaker, test tube stand, marker etc.

PROCEDURE:

1) Label the test tubes as raw milk and pasteurized milk.
2) Using a different 10-ml pipette each time, transfer 10ml of each type of milk into its appropriately labeled test tube.
3) Add 1ml of methylene blue dye to each test tube.

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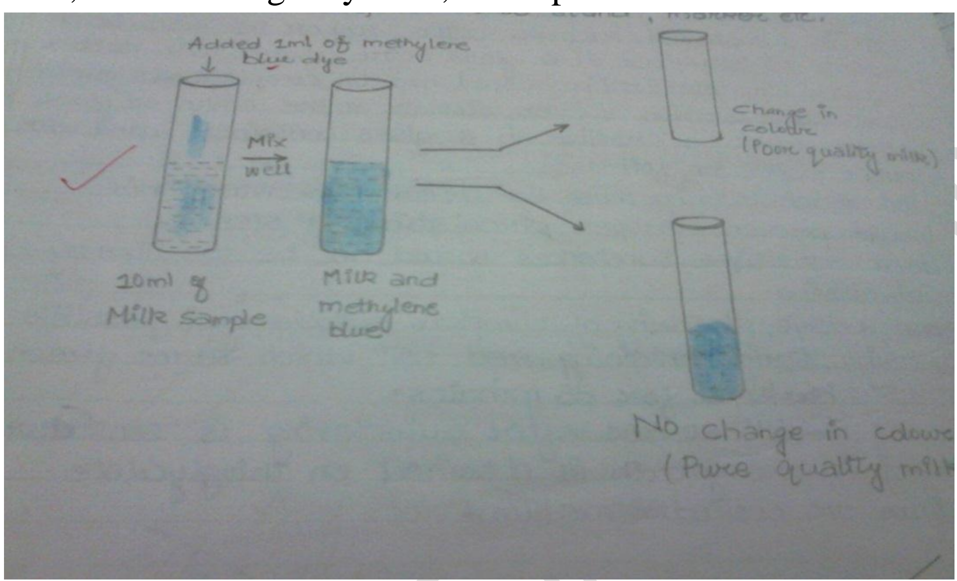
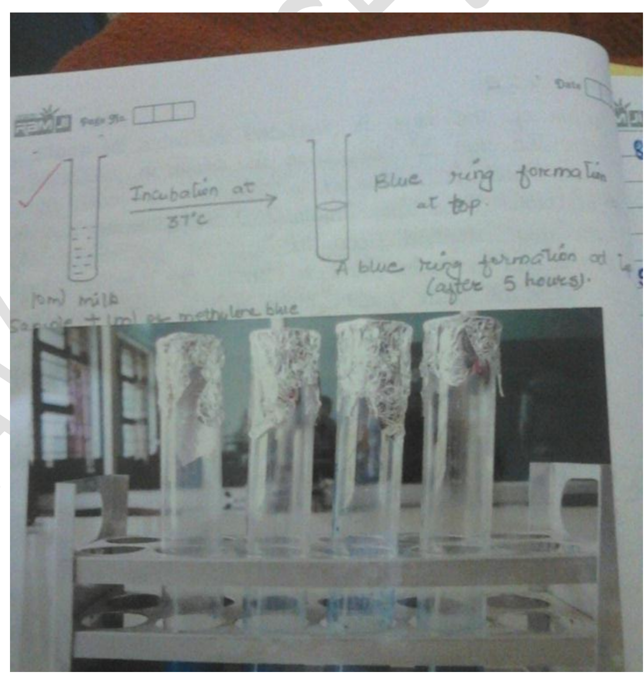
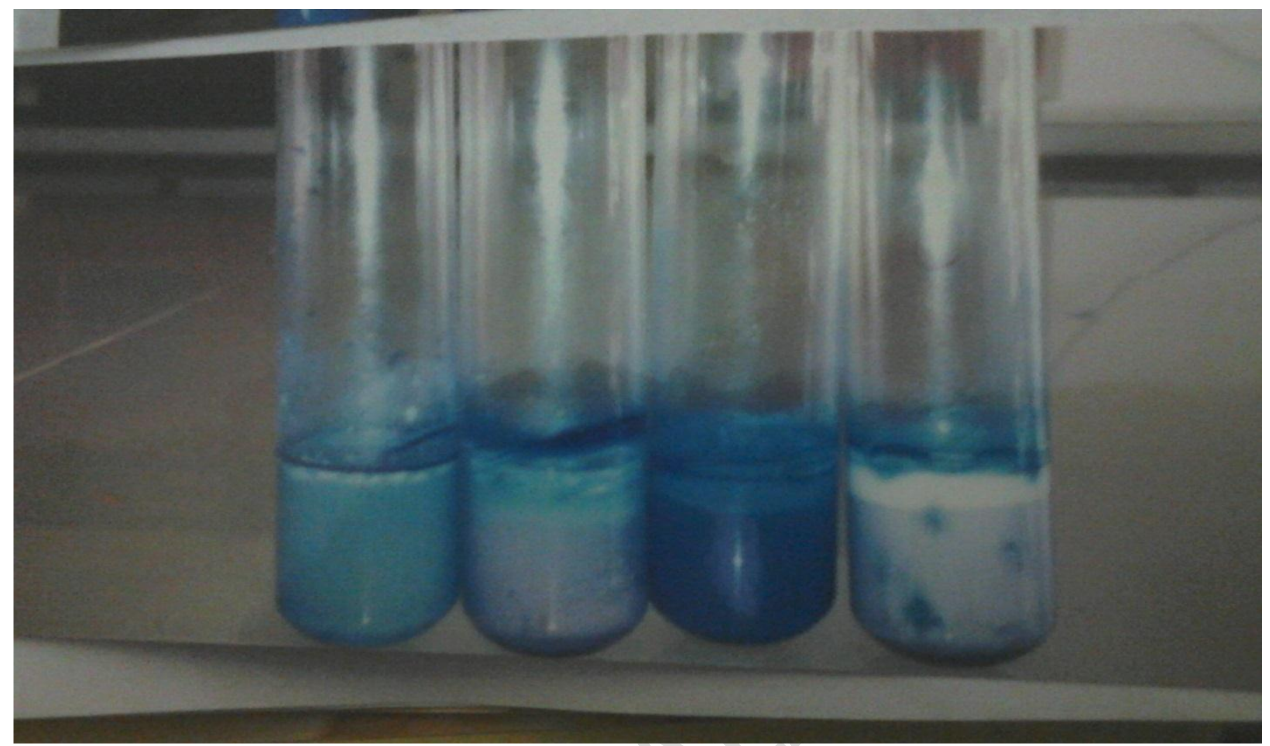
4) Invert the tubes gently about four times, and place in water bath. Record the time of incubation, i.e., record the amount of time elapsed for the color to turn white.
5) Allow the tubes to stabilize for 5 minutes, remove them for the water bath, invert them gently once, and replace them in the water bath.







PRECUTIONS:

1. Milk sample should be taken in sterile tubes.
2. Stock solution of dye should be sterile.
3. Appropriate ratio of dye and milk sample should be taken.
4. There should be minimum air contact with the milk sample and dye.

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