DBT-STAR COLLEGE SCHEME

Revised Practical Protocol Manual

Volume 5: ZOOLOGY

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TIRUCHIRAPPALLI-620 020

1. ISOLATION OF INSECT-GUT ASSOCIATED MICROBES

1.AIM

To isolate insect gut associated microbes

2.MATERIAL REQUIRED

Laminar Flow Chamber,Petriplates, Nutrient agar, Inoculation loop.

3.BACTERIAL ISOLATION, IDENTIFICATION, AND STORAGE

Bacterial isolation, purification and identification are the first steps to bacteriological studies. Isolation is done to obtain pure bacterial cultures. Bacteria are usually isolated from Insect gut .These tissues are monitor organs that usually harbor the disease-causing bacteria during infection.

To obtain a pure bacterial culture is the first step to bacterial identification. Pure culture is essential in the study of the morphology, physiology, biochemical characteristics, and susceptibility to antimicrobial agents of a particular bacterial strain.

Pure cultures are best obtained by using solid media, by streak plate or pour plate method. Streak plate, if properly done, is the most practical method. In the streak plate method, a loopful of the inoculum is placed near the periphery of the plate with agar medium and spread or streaked on the upper portion of the plate with parallel overlapping strokes. The inoculum is streaked over other portions of the plate so that isolated colonies could be observed in the last streaked area.

The identification of a bacterial pathogen is important in insect gut diagnosis. Treatment could be implemented only after the causative agent or the bacterium has been identified. Bacterial species differ in morphological, physiological and biochemical characteristics and those can be used when coding or labelling them. Therefore, identification is accomplished by performing several morphological, physiological and biochemical tests. Results of these tests are compared to established taxa or identification schemes.

Bacterial cultures should be preserved for future study. Storing in appropriate medium preserves bacterial cultures. The simplest method is by sub-culturing or by transferring the organism to fresh solid medium that has a minimal nutrient content to prevent bacterial overgrowth. The bacteria are allowed to grow before storing in the refrigerator or are covered with paraffin oil and stored at room temperature in the dark. Another simple method is by deep-freezing of the bacterial culture, stocked in a broth medium with glycerol. Glycerol is added to prevent the dessication of bacterial cells. Bacterial cultures may also be preserved by freeze-drying or lyophilization. In this method, water is removed from the frozen bacterial suspension by sublimation under vacuum.

Bacterial cultures should be properly labeled or coded before storage. It is important to label the tube or vial for storing bacterial cultures with an indelible ink. The label or code should include the reference number and other pertinent information such as source of sample (host animal, location), date of isolation, special properties, identification, name of the person who isolated the organism and the date of preparation of the stock culture.

MEDIA

Bacterial isolation can be done using a general medium, wherein various bacteria grow, and selective media that allows growth of specific genera. Examples of general media are nutrient agar (NA), tryptic soy agar (TSA), and brain heart infusion agar (BHIA). Examples of selective media are thiosulfate citrate bile sucrose agar (TCBS) for vibrios, and glutamate starch phenol red agar (GSP) for aeromonads and peudomonads. Media are supplemented with 1-2% sodium chloride (NaCl) if to be used for marine species. Adjust the pH of the culture media to 7.2-7.4 by adding 0.1 N NaOH.

STREAKING

1. Using inoculating loop, get samples of shrimp (such as the hepatopancreas and muscle) and fish (kidney, spleen) tissues and streak onto the upper one-fourth portion of an agar plate with parallel overlapping strokes. Use one agar plate for each animal sample. The plate can be divided into half and streaked with two different tissues from the same sample. Be sure to label the plate.

2. Flame the loop and allow it to cool. Turn the plate at right angle. Overlap the previous streak once or twice and repeat the streaking process on one-half of the remaining area.

3.Repeat procedure 2.

4. Incubate plates overnight at 30· C. Photo at right shows a streaked plate after incubation.After incubation for 16-20 hours, check for bacterial growth. Check for luminescence under dark conditions, marking the luminous colonies on the plate with a pentel pen. Isolated colonies should be observed in the last streaked area.

5. Select representative bacterial colonies based on the difference in shape, size and color. Mark selected colonies from each plate. Subculture onto trypticase soy agar (TSA) plate and incubate overnight.

6. Observe the colonies on the agar plate to determine the purity of the culture. Pure cultures should show the same colony characteristics and not overlapping.

7. Select a pure well- isolated colony.Stabeach strain into 2 tubes of 1.2% TSA, label and incubate. These will serve as stock cultures.

8. Keep the stock cultures in the lowest compartment of the refrigerator (8-12°C) or at room temperature until use. Do not stock cultures in these conditions for over 6 months.

COLLECTION OF INSECTS:

The first instar Bombyxmori larvae were purchased from the Tamil Nadu Sericulture Centre, Trichy. The larvae were reared from first to fifth instar in sterile cages at room temperature (32 ± 1°C) at a humidity of 82-90% (Upadhyayay and Mishra 2002). Larvae were fed mulberry leaves that had been sterilized by exposure to UV light. The sterilization was done in precaution to reduce external bacterial contamination. No antibiotics were used in the experiment, and none were used by the breeder. The experiments were repeated three times using separate batches of larvae purchased from the same breeder.

ISOLATION AND CHARACTERIZATION OF CULTIVATABLE BACTERIA:

Isolation and characterization of cultivatable bacteria with the property of utilizing cellulose, xylan, pectin and starch from larval digestive tract

- Five B. mori 5th instar larvae (approximately of 10 gm) were used in this experiment. The entire digestive tract was aseptically isolated in a UV laminar flow hood.
- The isolated digestive tract was washed with sterile ice-cold NaCl (0.85%) solution, chopped with a sterile blade, homogenized and incubated for 30 minutes at 37ºC. The supernatant was taken and serially diluted 1000-10,000 times.
- The pour plate method was used to estimate total bacterial count on lysogenic broth agar plates and on Berg's agar plates containing different substrates. The ability of the bacteria to degrade a substrate was checked using 0.1% carboxy methyl cellulose (CMC), 1% citrus pectin, 1% oat spelt xylan or 1% starch, as respective substrates.
- Anaerobic cultures were made to screen obligative anaerobic bacteria on these substrates. The total viable count was expressed as the number of colony forming units (CFU) in 1 ml of sample from substrate agar plates and lysogenic broth agar plates.
- Cellulolytic activity of cellulose-degrading bacteria in CMC medium was assayed using degradation of Whatmann No. 1 filter paper in Berg's broth. As a control, a single agar plate from each batch was opened in the UV laminar flow hood for 15 minutes. This was done to check the contamination from within the hood.

CULTIVATABLE TOTAL BACTERIA AND CELLULOLYTIC BACTERIA FROM 1ST TO 5TH INSTAR LARVAE OF *BOMBYXMORI*:

The entire digestive tract was isolated from larvae of each instar for a total of approximately 10 gm, just prior to the change to the next instar. The isolation procedure was carried out as given above. The cellulose degrading bacteria were enumerated by serial dilution in Berg's agar plates containing CMC, while the total bacteria were enumerated on lysogenic broth agar plates. The total viable count of cultivatable total bacteria and cellulolytic bacteria were expressed as the number of CFU in 1 ml of sample. The experiments were repeated with different batches of larvae purchased at three different times from the same breeder.

SCREENING AND IDENTIFICATION OF BACTERIA:

Colonies showing degradation capacity was assayed by plate screening using the Congo red overlay method and the iodine method for each substrate. Selected isolates were plated on respective agar plates for subsequent work and maintained as pure cultures. The selected colonies with degradation capacity were identified using the Congo red overlay method and the iodine method according to Bergey's Manual of Systemic Bacteriology.

For the Congo red method, plates were flooded with 0.1% aqueous Congo red for 10 minutes and then washed with 1M NaCl solution. Congo red interacts with (1-4)-β-D-glucans, (1-4)-β-D-xylan and (1-4)-α-D-polygalactopyronosyluronic acid. A clearing zone around the colony indicates the hydrolysis of polysaccharides namely CMC, xylan and pectin respectively.

For the iodine method starch plates were flooded with iodine solution resulting in dark blue plates with uncoloured zones where the starch had been degraded.

4.PREPARATION OF CULTURE MEDIUM:

- Lysogenic broth agar was prepared using 10 g peptone, 5 gm yeast extract, 5 gmNaCl and 2% agar per liter. The pH was adjusted to 7.0 with NaOH, before adding agar to the medium and autoclaving.
- Isolated bacteria on plates were screened for ability to degrade various carbohydrates, using standard dyes: Congo red for cellulolytic, xylanolytic (Ruijssenaars and Hartsmans 2000) and pectinolytic activity, and iodine for amylolytic.
- The following ingredients were used for the preparation of Berg's agar minimal medium without changing its composition (in g/100 m1) of 0.2 gm $NaNO_3$, 0.05 gm $MgSO_4$, 0.005 gm K_2HPO_4, 1 mg $FeSO_4$, 2 mg $CaCl_2$, 0.2 mg $MnSO_4$, and 2% agar.
- Berg's agar with 0.1% CMC, 1% oat spelt xylan, 1% citrus pectin and 0.1% starch on respective plates as carbohydrate substrates. Except agar, all other requirements of Berg's agar minimal medium were added in the preparation of Berg's broth.

5.RESULT

The Bacterial colony Count was _____

6. PRECAUTIONS

i. Ensure that the bacterial culture used for this study is a pure culture.

ii. The Laminar Flow Chamber should not have any air contamination.

iii. Precaution should be taken for the accurate counting of the bacterial colony.

7.EXTENSION ACTIVITIES

i. Find out the microbial load in the selected species.

ii. List out the gut associated microbes in the case of different insect species.

iii. Find out the Probiotic and Pathogenic microbes associated to different group of insects.

SUGGESTED READING(S)

i. Lee, P. Y., Costumbrado, J., Hsu, C. Y., & Kim, Y. H. (2012).Agarose gel electrophoresis for the separation of DNA fragments.*J. Vis. Exp.* (62), pii:3923. doi: 10.3791/3923.

ii. Sambrook, J., Russell, D. W. (2001). *Molecular Cloning.*(3rd ed.). New York:Cold Springer Harbor Laboratory Press.

iii. Kirkpatrick, F. H. (1991). *Overview of agarose gel properties. Electrophoresis of large DNA molecules: theory and applications*. pp. 9-22.

2. QUANTITATIVE ESTIMATION OF FRESH WATER PLANKTON

1.AIM

To analyze quantitatively the plankton sample collected from the fresh water pond.

2.MATERIALS REQUIRED

Plankton sample, 100 ml measuring jar, pipette, distilled water, Sedgewick-Rafter, cover slip, glass slide, brush and compound microscope.

3.PROCEDURE

The preserved plankton sample (collected from 200 litres of water from pond) was diluted to 100 cc of distilled water and from this 1 ml of plankton sample transfers to plastic slide (Sedgewick-Rafter). The Sedgewick-Rafter has a counting chamber at its centre with 1 ml capacity. This counting chamber is divided into 100 small squares. After transferring 1 ml of sample to the counting chamber cover slip was placed over it and observed under the low power of the microscope. The different plankton components counted in all the 100 small squares are tabulated (Table 1). The numbers counted in I ml of sample are computed for 1000 litres (M3).

4.RESULT

The Total number of plankton in 1000 litres = …… M^3.

5.DISCUSSION

Among Zooplankton components of pond ……were found in abundant measure. Next to……., ……were numerically abundant. This shows that they are found more adapted to the fresh water environment, other plankton such as ……. ,…….and……. were least in number.

Plankton plays an important role in food chain. Zooplanktons are grazing on phytoplankton. The Zooplankton form the foods of fishes are directly proportional to the quantity of plankton. Thus plankton is considered as an index of fertility.

Table 1. The numbers counted in 1 ml sample are computed for 1000 litres (m^3).

S. No.	Name of the Plankton	Tally marks	Number in 1 ml	Number per 1000 litre (M^3)
				….. x 100 x 1000 / 200
1				….. x 100 x 1000 / 200
2				….. x 100 x 1000 / 200
3				….. x 100 x 1000 / 200
4				….. x 100 x 1000 / 200
5				….. x 100 x 1000 / 200
6				….. x 100 x 1000 / 200
7				….. x 100 x 1000 / 200
8				….. x 100 x 1000 / 200
9				….. x 100 x 1000 / 200
10				….. x 100 x 1000 / 200
Total				

6.PRECAUTIONS

- **i.** Samples should be collected early in the morning to get maximum result.
- **ii.** Collected water should be clean and clear.
- **iii.** Try to immediately preserve the sample after collection.

7.EXTENSION ACTIVITIES

i. Compare the phytoplankton diversity of two aquatic habitats in your surroundings.
ii. Learn to prepare the permanent mounts of plankton samples.

SUGGESTED READING(S)

i. Asha Udayamali M. Lokuhewage, & Yasuhiro Naiki (2015). Quantitative Estimation of Phytoplankton Species in Freshwater by Two Step Linear Regression Analysis Using Spectral Absorption Method. Springer *Optical Review* volume 12, pages420–426
ii. http://www.drs.nio.org/drs/bitstream/handle/2264/95/Zooplankton_Manual.pdf?sequence=1

3. SCREENING OF NATURALLY OCCURRING RADIONUCLIDE POLONIUM 210 IN WATER SAMPLES.

1. AIM

To Screen the naturally occurring radionuclide Polonium – 210 in the water samples.

2. INTRODUCTION

Polonium – 210 (Po-210) is the naturally occurring alpha emitter radio nuclides and wide distribution in the environment potential for human radiation exposure through ingestion and inhalation.

3. MATERIALS REQUIRED

- **i.** Sample: water sample
- **ii.** Chemicals/ Reagent : HCL, Ferric Chloride, Ascorbic Acid
- **iii.** Equipment: Alpha counting system a scintillation principle of detection using Zn(Ag), Magnetic stirrer with thermostat control .
- **iv.** Glassware: Glass Beakers, measuring cylinder, Pipette stand .
- **v.** Miscellaneous: Silver planchette

4. PROCEDURE

The sample solution in 0.5N HCL was placed on a magnetic stirrer with thermostat control at a temperature of 90-95^{o}C. Ascorbic acid (100 mg) was added to reduce ferric ions to ferrous , thus eliminating interferences in electrochemical deposition of Polonium -210. A silerplanchette (0.8 mm thickness and 2.5 cm diameter) of predetermined background whose both sides were brightly polished with emery paper was suspended in the sample solution by means of nylon thread at the end of a rod, connected to the stirrer . Spontaneously deposition of Po-210 on both sides of the silver planchette took place under these conditions for period of 5-6 h. At the end on the plating period the planchette was taken out, rinsed with alchohol, dried under an inferred lamp for five minutes and counted for activity on both sides in an alpha counter. Deposition efficiency of Po-210 on water and biological samples spiked with uranium ore solutions containing equilibrium activity of Po-21o using the above method yielded recovery range varying from 95-100% with an average efficiency of 98±2. Activity measurements were always carried out within two weeks of sample collection and it is estimated that any additional error due to activity changes in this period does not exceed 0.5% result therefore applicable to the dates of sampling (Iyengar ,1983).

Fig : Alpha counting system

5.Calculation

$$\text{Cpm} \times \frac{100}{E} \times \frac{1}{60} \times \frac{1}{W} = \text{Bq/g}$$

6.RESULT

Polonium 210 activity in the water sample is _____ mBq/L.

7.PRECAUTIONS

i. Utmost precaution should be taken in handling with the Acid as it can easily injure the body.

ii. Care should be taken in collecting the radionuclide precipitate and transporting it to laboratory.

iii. After the electrochemical deposition is complete, turn off the main power supply to ensure complete voltage discharge.

8.EXTENSION ACTIVITIES

i. Detect the radionuclide concentration in various drinking water sources and other aquatic systems.

SUGGESTED READING(S)

1. Iyengar, M.A.R., (1983). Studies on the distribution of natural radioactivity in marine organisms. Ph.D. Thesis, University of Bombay, Bombay.

2. Kannan, V., 1983.Radioactivity and trace elements in marine macrophytes from Kalpakkam. M.Sc., Thesis, University Bombay

4. PREPRATION OF HERBAL EXTRACT AND STUDIES ON ANTIMICROBIAL ACTIVITIES

1.AIM

To prepare a herbal extract and study its antimicrobial activities.

2.MATERIALS REQUIRED

i. Herbal plant material :- Collect healthy herbal plant and transporte to the laboratory. Treat the leaves of plant to shed drying and crush to powder. The powder to be filtered using mesh 40 for further analysis.

ii. Test bacterial strains :- Clinical isolates of bacterial strains can be obtained from Doctors Diagnostic Centers. Test strains of *Acinetobacter*sp, *Klebsiellapneumoniae*, *Pseudomonas aeruginosa* and Methicillin resistant *Staphylococcus aureus*were can be isolated from hospitalised Intensive care unit patients.

iii. Soxhlet apparatus

3.PROCEDURE

Phytochemical analysis

Phytochemicals screening can be performed using according to the previously described methods (Harborne (1984), Brunton (1995), Wagner *et al*. (1984). These tests used to carried out to find out the presence of tannin,flavonoids,alkaloids,carbohydrates, terpenoids and steroids.

Figure : Soxhlet extraction

Antibacterial activity

Disc diffusion assay

Antimicrobial activity can be tested for all extracts against drug resistant *test* bacterial strains on Luria - Bertani (LB) agar plate using a disc diffusion method. Determine the zone of inhibition (ZoI) to swot up the growth inhibition of test strains measured by a vernier caliper. Spread plate LB agar about 10^6 CFU/ml of test pathogens, impregnate with the sample load disks incubate at 37 ˚C for 18 h and measure ZoI after incubation. Analyze each extract in triplicate and represent the mean values.

Minimal Inhibitory concentration (MIC) test

Inoculate I ml of the sterilized Lauria Bertani in the first tube. Add 1 ml of the compound solution to it and thoroughly mix to a concentration of 25 µg/ml. Dilute this solution further by inoculating 1 ml from the prior test tube into subsequent tubes. Kept the inoculated tubes at 37˚C±1˚C for 24 hrs. After the incubation period, observe the tubes for any deposits or turbidity to determine the MIC.

Agar well diffusion method

Sterilized All the glassware, media, and reagents in an autoclave at 121°C for 20 minutes. Prepare Escherchia coli (MTCC 45), Salmonella typhimurium (MTCC98) and S.aureus(ATCC29122) bacterial suspension by growing a single colony overnight in a nutrient broth. Inoculate the nutrient agar plates with this bacterial suspension and add 50 µg (100 µL) of Ag nanoparticle to the well. Use de ionized water as the control. Incubate these plates at 37°C for 24 hours in a bacteriological incubator, and measure the zone of inhibition(ZOI) by subtracting the well diameter from the total inhibition zone diameter.

1. CALCULATION

% Inhibition = 100 – (OD of sample/OD of Control) x100

2. PRECAUTIONS

i. Sterilize all the glassware, media, and reagents in an autoclave at 121°C for 20 minutes prior to any test.

ii. Handle the soxhlet extraction apparatus with utmost care.

3. EXTENSION ACTIVITIES

i. Prepare the herbal extract of plants belonging to same species but in different bio geographical areas and study the difference in its antimicrobial activities.

ii. Do the test for a wide number of pathogenic bacteria to find out suitable control measures.

SUGGESTED READING(S)

i. Hwei SanLoh & Christophe Wiart (2011).Optimal methods for evaluating antimicrobial activities from plant extracts. Journal of Microbiological Methods

ii. Volume 84, Issue 2, February 2011, Pages 161-166.

iii. S. Arunkumar and M. Muthuselvam(2009). Analysis of Phytochemical Constituents and Antimicrobial Activities of Aloe vera L. Against Clinical Pathogens. World Journal of Agricultural Sciences 5 (5): 572-576, 2009.

5. SQUASH PREPARATION OF SALIVARY GLAND OF DROSOPHILA LARVAE FOR POLYTENE CHROMOSOME

1. AIM

To demonstrate the giant polytene chromosomes of the larvae of the fruit fly *Drosophila melanogaster* using the squash technique and to study its morphology.

2. INTRODUCTION

The salivary glands of Dipteran insect larvae have nuclei which are in perpetual interphase. They were described by Balbiani in 1881. These nuclei have chromosomes which are abnormally large as compared to the chromosomes of the other body cells. Because of their size they come to be known as giant chromosomes. The giant chromosomes when fully extended are about 100 times longer than those normally found at the metaphase of mitosis. The DNA of the giant chromosomes repeatedly replicates but the daughter strands do not separate. The replication occurs nearly 10 times resulting in 1024 parallel strands held together. The fully developed banded chromosomes are nearly 0.25 to 0.55 mm long. The chromosomes show alternating dark and light bands and each band represents a gene. The amplification of DNA of salivary gland chromosomes in the insect larvae is also known as polytenisation and the chromosomes are referred to as polytene chromosomes. In this lab exercise you will learn to prepare the giant polytene chromosomes of the larvae of the fruit fly *Drosophila melanogaster* using the simple squash technique and to study its morphology.

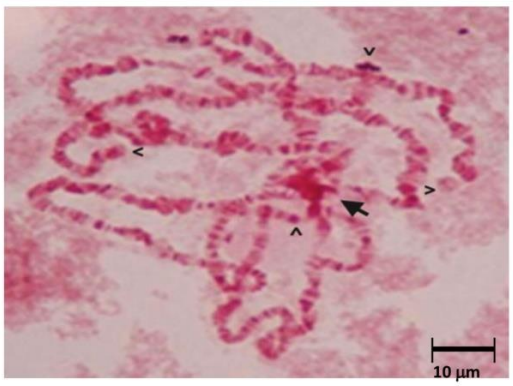

Figure 1. Squash of Polytene Chromosomes from *Drosophila* larval salivary gland. Note the light and dark banding pattern along each chromosome arm, the darkly staining chromocenter containing centromeric heterochromatin in the center of the squash (large arrow), and the telomeres (small arrowheads).

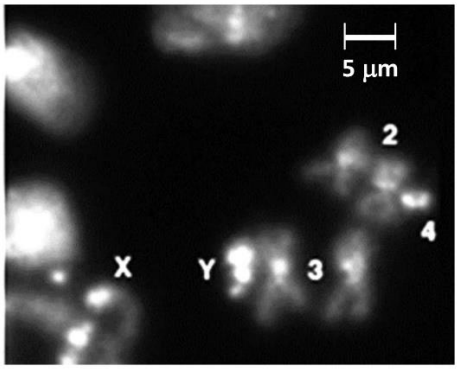

Figure 2. Example of a High Quality Squash of Chromosomes from the Mitotically Active *Drosophila* Larval Brain. The individual chromosomes were identified on the basis of their characteristic shapes and sizes.

3. MATERIALS REQUIRED

i. 3rd Instar Drosophila larvae
ii. Dissection kit (dissection needle, forceps)
iii. Dissection microscope and compound microscope
iv. 1% Acetoorcein
v. 45% Acetic Acid
vi. Slides
vii. Coverslips
viii. Pasteur Pipettes
ix. Filter Paper
x. H2O wash bottle

4.PROCEDURE

i. Obtain a few late 3rd instar larvae of Drosophila. The 3rd instar larvae can be identified by their larger size.
ii. Gently transfer a larva onto a slide and a drop of insect Ringer solution. Place this slide under the dissection microscope. Note that the larva has a blunt rear end and a pointed head end that contain the mouthparts.
iii. Hold down the larva firmly on the slide with the help of a needle placed at the thorax and pull the head away from the thorax with another needle.
iv. The salivary glands are now released and can be seen attached to the head but floating in the Ringer solution.
v. Observe the salivary glands under the dissection microscope and remove any fat tissue adhering to it.
vi. Transfer the glands onto a watch glass and add a few drops of acetic methanol (methanol: acetic acid 3:1). You may use a small brush or the dissecting needle for the transfer. Fix the acetic methanol for only a minute.
vii. Using a Pasteur pipette remove the fixative completely and place a few drops of acetoorcein stain on the glands. Stain the glands for about 10 to 15 minutes.
viii. Transfer the glands onto a slide, add a drop of 45% acetic acid and place a coverslip over it.
ix. Remove the excess stain by continuously adding acetic acid drop by drop from one side of the coverslip and drawing the excess fluid on the other slide using the edge of a filter paper.
x. Then place the slide between the folds of a filter paper, gently press the coverslip down and tap with the flat bottom of a pencil to obtain a good spread.
xi. Seal the edges of the coverslip with nail polish and observe under a compound microscope.

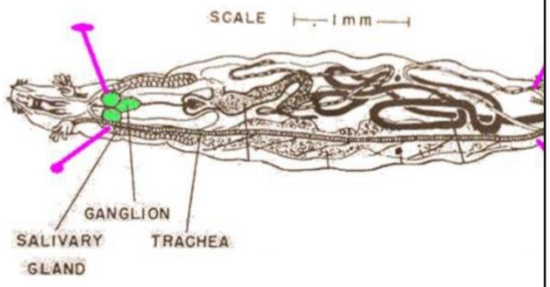

Figure 3. Diagram of Drosophila larva, showing the positions of the tracheal tubes, salivary glands ventral ganglion (brain).

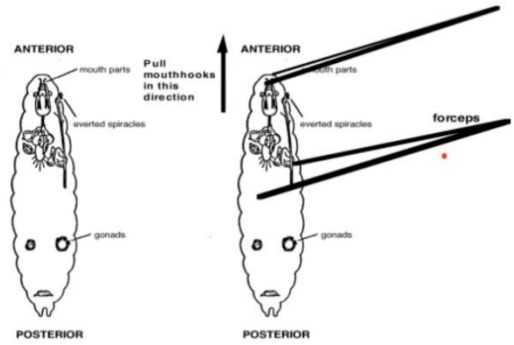

Figure 4. *Drosophila* larval dissections

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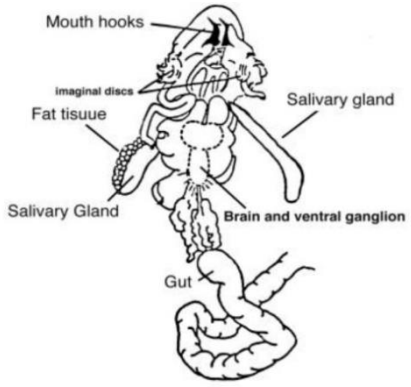


Figure 5. Diagram of head with attached salivary glands and ventral ganglion remaining

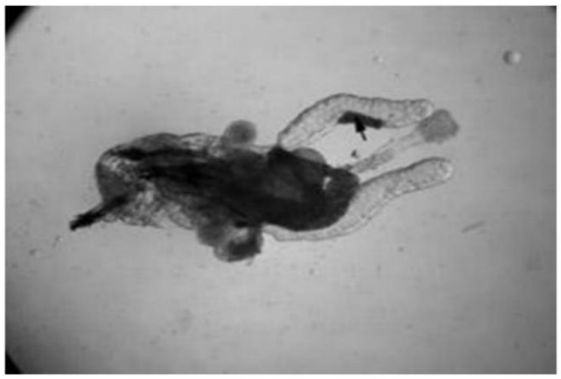


Figure 6. Photograph of head with attached salivary glands and ventral ganglion remaining after clean dissection.

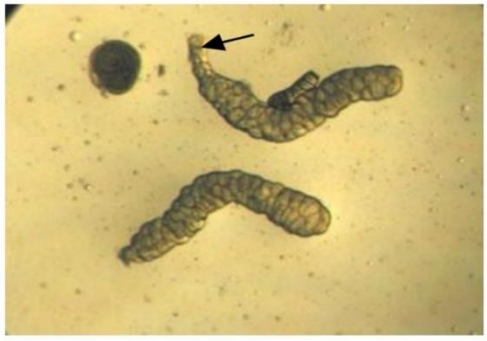


Figure 7. Photograph of salivary glands after clean dissection from the larval body and head regions.

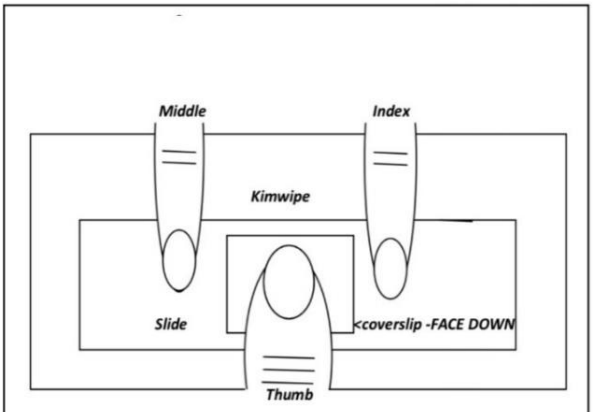


Figure 8. Diagram of squashing procedure.

5.OBSERVATIONS AND RESULT

If the salivary gland is sufficiently squashed, the cells would separate from each other, and the polytene chromosomes would spread well. If staining is appropriate, you should be able to see the banded pattern along the length of the polytene chromosome. In a good preparation of salivary gland chromosomes four pairs of chromosomes can be observed. The fourth pair of chromosomeis very small but the other three pairs are long.

6.PRECAUTIONS

- **i.** You may not successful in removing the salivary glands in one or two attempts. The dissection procedure requires practice and patience. You may have to try a number of times before getting intact glands. Reasonable results can be obtained with a little practice.
- **ii.** In handling the salivary glands, either grab them from their less fragile smallest diameter base or use your forceps to scoop them underneath.
- **iii.** The coverslip should rotate somewhat freely on the surface of the microscope slide; this is needed to burst the membranes of the polytene nuclei and allow the chromosome arms to spread.
- **iv.** For squashing action, use your thumb to apply pressure to the coverslip while using the middle and index fingers of your other hand to secure the position of the slide and coverslip.

7.EXTENSION ACTIVITIES

- **iii.** Draw a sketch of your best polytene chromosome spread. Identify all five major chromosome arms (X, 2L, 2R, 3L, 3R). Find out the small 4^{th} chromosome. Locate the chromocenter in your spread. Track along a single chromosome arm from its centromere and telomere.
- **iv.** Compare the scale bars for the photomicrographs of the polytene chromosomes in Figure 1 and the metaphase chromosomes from a mitotically active cell in Figure 2. What can you conclude about their relative sizes from this?

SUGGESTED READING(S)

- **i.** Tatyana Yu Zykova&Victor G. Levitsky,(2018). Polytene chromosomes – A portrait of functional organization of the *Drosophila* Genome. Curr Genomics. 2018 Apr; 19(3): 179–191.
- **ii.** Benjamin M. Stormo and Donald T. Fox. (2017). Polyteny : Still a giant player in chromosome research. Chromosome Res. 2017 Oct; 25(3-4): 201–214.

6. ISOLATION OF DNA FROM BLOOD SAMPLES

1. AIM

To isolate the genomic DNA from blood samples

2. INTRODUCTION

Blood is a major connective tissue of biosystem which interacts with other major system. Blood cells (lymphocytes) contains genomic DNA considered an ideal genomic source for gene amplification, and chromosomal studies.

3. MATERIALS REQUIRED

Equipments

i. SARSTEDT tubes (50ml)
ii. Nunc tubes (4.5ml)
iii. Centrifuge
iv. An aspiration system (a pump, a vacuum trap, a bottle).
v. 2-3 DONASET tubules and 1-2 needles (lumbar puncture type)
vi. Disposable gloves

Chemicals

i. TRIS Base PM - 121.1g,
ii. Magnesium Chloride PM - 203.3 g,
iii. NaCl PM - 58.44 g,
iv. EDTA PM - 372.4 g,
v. HCl (37%) - 1L,
vi. NaOH (PASTILLE) -500 g,
vii. SDS (20%) - 500 ml,
viii. Absolute ethanol – 1L,
ix. Phenol – 1L,
x. Chloroform - 1l,
xi. Propanol - 1l
xii. Proteinase K - 500 mg

STOCK preparations

i. **TRIS pH 7.6**
For 2M Tris Basepreparation, 242.2g of Tris Base dissolved in 800ml distilled water. HCl was added to adjust the pH (7.6) and make upto 1000ml

ii. **MgCl2**
For 1M MgCl2 preparation, 203.3g of $MgCl_2$ dissolved in 1000 ml of distilled water

iii. **NaCl(3M)**
For 3M NaCl preparation, 175.32g of NaCl dissolved in 1000 ml of distilled water

iv. **EDTA (0.4M)**
For 0.4M EDTA preparation, 148.96 g of EDTA, 18 g of NaOH were dissolved in 800 ml of distilled water. pH was adjusted to 8 by adding NaOH and finally solution make upto1000 ml.
Stock solutions are stored at +4°C and are used for the preparation of working solutions.

Working Solutions Preparation

Working solutions are prepared in sterile graduate cylinders using sterile distilled water (cover with parafilm to mix) and stored at 4°C (Anandika 2013).

i. **SLR**
- TRIS (2M, pH 7.6) 10 ml
- MgCl2 (1M) 10 ml
- NaCl (3M) 6.6 ml
- Distilled water to 2000 ml

ii. **SLB**
- TRIS (2M, pH 7.6) 10 ml
- EDTA (0.4 M, pH 8) 50 ml
- NaCl (3M) 34 ml
- SDS (20%) 20 ml
- Distilled water to 2000 ml

iii. **SLB Solution / Proteinase K**
500 mg of proteinase K powder dissolved in 10 ml of SLB solution. This solution was added into a graduated cylinder (2L) containing 500 ml of SLB, rinse the flask with 10 ml of this mixture, and adjust the volume of SLB to 1250 ml. The solution was stored at –20°C.

iv. **TE (10^{-1})**
- TRIS (2M, pH7.6) 5ml
- EDTA 0.4 M pH8 2.5 ml
- Distilled water to 1000 ml

v. **Saline**
- NaCl(3M) 50 ml
- Distilled water to 1000 ml

vi. Ethanol Solution (70 %)

- TE 10-¹ 600 ml
- Absolute ethanol to 2000 ml
-

4. PROCEDURE

i. Whole PlasmaDiscarding

Blood samples were pooled in GREINER tube (50 ml) and centrifuged at 2000 rpm for 10min. Blood plasma was aspirated and finally the blood cells were mixed.

ii. RBC lysis

RBC were lysed in 50 ml final volume with a solution of SLR and centrifuged at 2000 rpmfor 10 min. 45 ml of lysed red blood cells were aspirated.The pellets were suspended by using a sterile pipette, and make up to 50 ml with SLR and again centrifuge at 2000 rpm for 5 min. Supernatant was discarded.Freeze the pellet at -80°C

iii. WBC Lysis

Pellets were defrosted in a water bath for 1minat 50°C. Leukocytes were lysed in one volume of SLB + proteinase K, the volume to use depends on the size of the pellet. Homogenize the tube with gentle rotation. Incubate overnight at 42°C with agitation

iv. Protein Extraction

i) Add one volume of phenol-chloroform equal to the volume of SLB-Proteinase K. Stir for 10 min.

ii) After stabilization,the lower phase was aspirated

iii) Above Step was repeated, with 5 minute-agitation until clear sample was obtained

5. OBSERVATIONS

DNA PRECIPITATION & WASH

i) Precipitate the DNA with 1 to 1.5 volumes of absolute isopropanol (propanol 2) in the presence of NaCl (60 mM). Homogenize with gentle rotation until the DNA precipitate (medusa) appears.

ii) Discard the liquid by transferring the medusa to a 5 ml Nunc tube. Wash the medusa twice with 3 ml of isopropanol.

iii) Wash the medusa three times with 3 ml of 70% ethanol in TE 10-¹. Remove the liquid between each wash.

iv) Dry the medusa at room temperature and DNA was observed

6. RESULT

DNA was precipitated in aliquots.

7. PRECAUTIONS

i. Depending on the size of the medusa, DNA suspended in 0.5 to 2 ml of TE 10^{-1}
ii. When the medusa is translucid and therefore dry, re suspend it in TE 10^{-1}.
iii. Incubate overnight at 37°C with rotation. Place it at 4°C for one week Then it can store the tube at –20°C for a long period of time

8. EXTENSION ACTIVITIES

i. Isolate genomic DNA from different biological materials such as Human cheek epithelium, Chicken liver etc.

SUGGESTED READING(S)

i. Anandika Dhaliwal. DNA Extraction and PurificationMATER METHODS 2013;3:191
ii. Debomoy K. Lahiri and Bill Schnabel .DNA isolation by a rapid method from human blood samples: Effects of $MgCl_2$, EDTA, storage time, and temperature on DNA yield and quality. Biochemical Genetics volume 31, pages321–328 (1993)

7. SEPARATION OF DNA SAMPLES USING AGAROSE GEL ELECTROPHORESIS

1.AIM

To perform agarose gel electrophoresis of isolated DNA.

2. INTRODUCTION

Agarose gels are a standard component of gel electrophoresis, an effective technique used in the separation and analysis of deoxyribonucleic acid (DNA) fragments. Agarose is a polysaccharide composed of agar (agarobiose), a natural product of seaweed. During gelation, agarose forms a porous matrix that allows diffusion of nucleic acids. The DNA fragments were resolved in 2% agarose gel under an applied electric field to the gel matrix. DNA molecules migrate towards the anode due to negatively charged phosphate along the backbone of DNA. The rate of migration of DNA is inversely proportional to the ratio of its molecular weight. Thus, the larger molecules travel at a much lower speed when compared to smaller one. TAE is best used if recovering DNA from gel slice, while TBE is better for smaller (<1kB) DNA strands. The gel loading dyes move at steady rate in the gel, so we can get estimation about how far DNA fragments have move in the gel.EtBr which bind perpendicularly with DNA helices via van der waals forces, when EtBr is exposed to uv light, electrons in the aromatic ring of the ethidium molecule get activated, which releases energy in the form of light and easy to visualize the band.

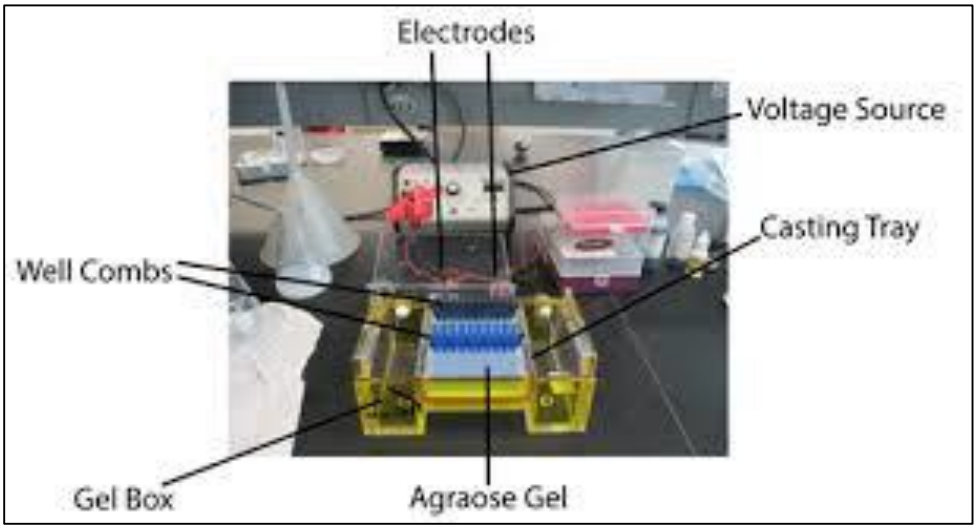


Fig :- Electrophoresis Apparatus

3. MATERIALS REQUIRED

i. **Biological Material:** Isolated DNA sample.

ii. **Chemicals/Reagents:** TBE buffer 1X (Tris, Boaric acid, EDTA) (pH 8.0))

iii. Agarose, bromophenol blue dye/ 6X gel loading dye, ethidium bromide, 100 kb/1 kb DNA size marker and distilled water.

iv. **Equipment:** Minigel electrophoretic system with power supply unit, UV transilluminator.

v. **Glassware and Materials:** Micropipettes, Microwave oven, Weighing balance, Microwave safe beaker or flask, tissue paper, Gloves, parafilm, cello tape etc.

4. PREPARATION OF REAGENTS

i. 10X TBE (stock solution): Prepare 10 X TBE solutions by dissolving 108 gmTris, 54.85 gm Boaric acid and 7.44 gm EDTA (pH 8.0) and and adjust the volume to 1000 mL by adding double distilled water. As EDTA does not dissolve easily, it is to be put in a magnetic stirrer.

ii. 1X TBE (working solution): Take 100 ml of 10x TBE (Stock solution) Add 900ml of distilled water.

iii. Agarose Gel: 2% Agarose gel is prepared by dissolving 2.0 gmAgarose in 100 mL 1X TBE buffer. Boil the solution to dissolve Agarose. Swirl gently to ensure all agarose gel has melted, and allow to cool somewhat so that vapour is not being emitted from the flask. Add 2µl of 1% EtBr and casted in an electrophoretic casting plate and Set up the gel caster with a well coamb placed about 1-2 cm from the top of the casting tray. It is then allowed to solidify.

iv. DNA sample for loading:10µL DNA sample is mixed with 2 µL (approx.) 6X Gel Loading dye and loaded in the gel.

5. PROCEDURE

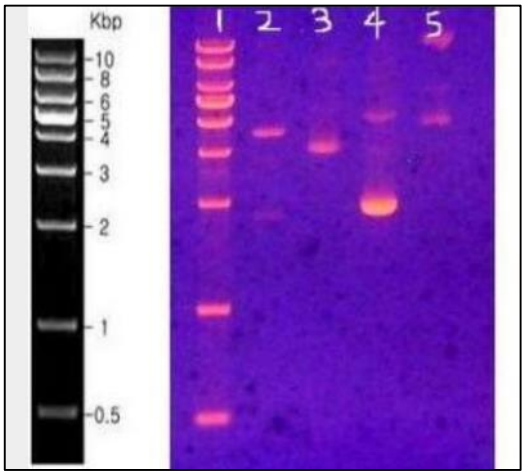
Placing the gel and loading the sample:

i. After 15 min. the comb was removed tacking care that the sample wells were not teared. The platform was then placed into the gel running tank keeping the wells towards the cathode.

ii. Fill the electrophoretic tank with 1X TBE buffer until it covered the gel, making sure that no air is trapped within the wells.

iii. Load the DNA samples along with molecular size markers into the wells with the help of micropipette.

iv. Stop the run when the dye marker is approximately % of the way down the gel.



- Lane 1: 1 kb DNA ladder
- Lanes 2- 5 test samples

Fig.1 Visualizing the DNA

6. PRECAUTIONS

i. Generally 2% agarose gel Provide good resolution for small 0.2–1kb fragments.

ii.1 X TBE is better for smaller (<1kB) DNA strands.

iii. Make sure power is off,be sure that leads are fully seated, make sure buffer has not splashed onto power unit.

iv. Make sure gel is oriented correctly - DNA is charged negatively and will run towards the positive (red) electrode.

v.Gel should be carefully transferred from the chamber/tray on to the transilluminator to avoid dropping the gel or breaking in between.

vi. Care should be taken not to run the gel for too long as it can exhaust the buffering capacity of the solution.

vii. If electrophoresis buffer is spilled or leaks from the gel box, STOP the run and clean up the bench top.

viii. Once run is complete, turn off the main power supply to ensure complete voltage discharge.

ix. Ethidium bromide is a mutagen and likely carcinogen. EtBr should be handled with appropriate caution.

xi. Wear hand gloves during the entire course of operation.

7.EXTENSION ACTIVITIES

i. Conduct the same experiment using the DNA samples isolated from different biological materials.

SUGGESTED READING(S)

i. Lee, P. Y., Costumbrado, J., Hsu, C. Y., & Kim, Y. H. (2012).Agarose gel electrophoresis for the separation of DNA fragments.*J. Vis. Exp.* (62), pii:3923. doi: 10.3791/3923.

ii. Sambrook, J., Russell, D. W. (2001). *Molecular Cloning.*(3rd ed.). New York:Cold Springer Harbor Laboratory Press.

iii. Kirkpatrick, F. H. (1991). *Overview of agarose gel properties. Electrophoresis of large DNA molecules: theory and applications*. pp. 9-22.

8. EFFECT OF PH ON ENZYME ACTIVITY OF ACID PHOSPHATASE

1. AIM

To determine the effect of pH on the activity of acid phosphatase.

2. INTRODUCTION

The pH of a solution can have several effects on the structure and activity of enzymes .For example ,pH can have an effect on the state of ionization of acidic or basic aminoacids. Acidic aminoacids have carboxyl functional groups in their side chains .Some basic aminoacids have amine functional groups in their side chains. If the state of ionization of aminoacids in a protein is altered then the ionic bonds that help to determine the 3-D shape of the protein can be altered. This can lead to altered protein recognition or an enzyme might become inactiveate extreme of pH.

Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrates that either the substrate cannot bind to the active site or it cannot undergo catalysis. In geneal enzyme have a optimum pH for their folding in such a manner that they are active in that optimum pH. However the optimum pH is not the same for allenzymes.

Optimum pH level:

Changes in pH have influence on enzyme activity. The pH at which enzyme shows maximum activity is called as optimum pH. This is the point that the enzyme is most active. Extremely high or low pH values generally result in complete loss of activity for most enzymes.

3. MATERIALS REQUIRED

i. **Biological Material:** Mung beans sprouts extract (contains acidphosphatase).
ii. **Chemicals/Reagents:** 1 M Acetate buffer (stock) and 0.1 M (working) pH 5.5, p-nitrophenylphosphate(PNPP),
iii. 0.5MKOHsolution,citratebuffer (pH2,2.5,3,3.5,and4), acetate buffer (pH4.5,5,5.5,and6) and Tris buffer (pH 6.5,7.8).
iv. **Equipment:** UV-Vis spectrophotometer, centrifuge ,micropipette.
v. **Glassware/Plastic ware:** Cuvettes, test tubes, test tube stand.
vi. **Miscellaneous:** Mortar and pestle.

4. PROCEDURE

i. Prepare the enzyme extract in acetatebuffer.
ii. Prepare the citrate buffer pH(2,2.5,3,3.5,and4), acetate buffer pH(4.5,5,5.5,and6) and Tris buffer pH (6.5,7.8).
iii. Add the required solutions in required volumes of enzyme extract, substrate PNPP, acetate buffer, citrate buffer and tris of respective pH in labeled test tubes and also make control tubes as given in the observation table.
iv. Add2mLof0.5KOHtothecontroltubespriortoadditionofsubstrate.
v. Post incubation; add 2 mL of 0.5 M KOH to the reactiontubes.
vi. Note the absorbance for each reaction, plot the graph and calculate the enzyme activity.

5. OBSERVATION TABLE

Blank solution = 2 mL KOH + 3 mL acetate buffer

λmax for PNP = 405 nm

Dilution of enzyme =1:10

Molar extinction coefficient (ϵ) of PNP = 1.47×10^4

* T = Test, C = control

Sample	pH	Buffer (2.7 mL)	PNPP (mL)	Enzyme (mL)	Incubation for 10 min	0.5 M KOH 2 mL	Abs at 405 nm (OD)	Abs. due to product (OD) (T-C)	Enzyme activity (µmoles mL^{-1} min^{-1})
	2	citrate							
	2.5	citrate							
	3	citrate							
	3.	citrate	0.2	0.					

Add KOH in all cont

	5			1					
	4	citrate							
	4.5	acetate							
	5	acetate							
	5.5	acetate							
	6	acetate							
	6.5	Tris							
	7.8	**Tris**							

6. RESULT

Thus,the effect of pH on enzyme activity was successfully studied for acid phosphatase enzyme and optimum pH of acid phosphatase was found to be pH____.

7. DISCUSSSION

From the graph, it can be clearly seen that enzyme activity rises with increase in pH, reaches an optimal value of pH and decreases hence forth with further increase in pH. The enzyme activity of acid phosphatase is maximum at a pH of 5.5. This illustrates the fact that the enzyme is active under acidic pH.

Moreover, decline in enzyme activity due to increase in pH beyond optima may be explained by virtue of the fact that changing $[H^+]$, affects the charge stabilization due to electrostatic interactions between charged amino acid residues.This affects the 3-dimensional conformation of the enzyme,which may even be lost under extreme pH changes.

9. EFFECT OF TEMPERATURE ON ENZYME ACTIVITY OF ACID PHOSPHATASE

1. AIM

To determine the effect of temperature on the activity of acid phosphatase.

2. INTRODUCTION

Temperature Effects:

Every enzyme has an optimum temperature at which it shows maximum activity. If the temperature is too low,there can be no noticeable reaction rate since the enzyme is operating at a temperature too below its optimum. If the temperature at which the enzyme is operating at is well above 100 °C, then thermal deactivation can occur. This occurs because as the high temperature produce enough thermal energy to break some of the intramolecular interactions between polar groups (Hydrogen bonding, dipole-dipole attractions, ionic interactions) as well as the hydrophobic forces between the non-polar groups within the enzyme structure. When these interactions are disturbed, the secondary and tertiary levels of the enzyme structure change to a random coiled form that alter the active site's confirmation beyond its ability to bind the substrate molecule as it was - proposed to catalyze. The overall phenomenon is called "Thermal deactivation or Denaturation".

Like most chemical reactions, the rate of an enzyme- catalyzed reaction increases as the temperature is raised. A 10°C rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1or2 degrees may introduce changes of 10 to 20% in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures.

As shown in Fig. the reaction rate increases with temperature to a maxi- mum level, then abruptly declines with further increase of temperature. Because most enzymes rapidly become denatured at temperatures above 40 °C, most enzyme determinations are carried out some- what below that temperature. Over a period of time ,enzymes will be deacti- vated at even moderate temperatures.

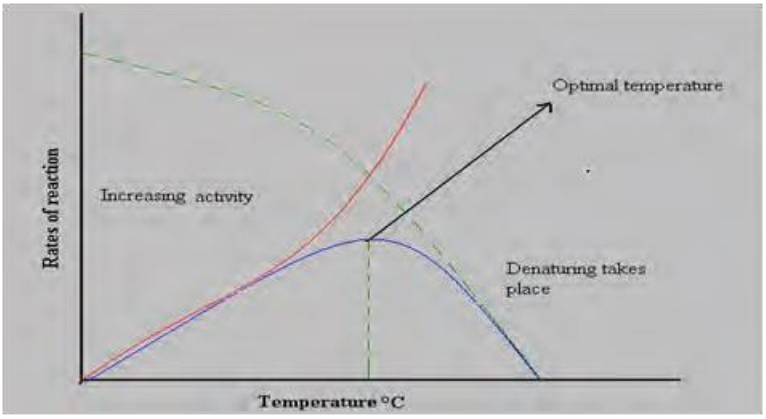


Fig : Effect of Temperature On reaction rate

Storage of enzymes at 5 °C or below is generally the most suitable. Some enzymes lose their activity when frozen.Rate of reactions including those catalyzed by enzymes rises with increase in temperature based on the Arrhenius equation $k = Ae^{-Ea/RT}$ where k is the kinetic rate constant for the reaction, A is the Arrhenius constant also known as the frequency factor.

ΔG is the standard free energy of activation which depends on entropic and enthalpic factors, R is the universal gas constant and T is the absolute temperature. Kinetic energy (K.E.) of the system increases with increase in temperature of a system results from the increase in the kinetic energy of the system.When a molecule collides, the K.E. of the molecules is converted in to chemical potential energy of the molecules. Thus greater K.E of the molecules in a system automatically increases the resulting chemical potential energy. As the temperature increases it is possible that more molecules per unit time will attain the activation energy. Thus the rate of the reaction may increase.In order to convert substrate into product, enzymes must collide with and bind to the substrate at its active site.Increase in temperature of a system will increase the number of collisions of the enzyme and substrate per unit time. Thus within the limits, the rate of the reaction will increase.

OPTIMUM TEMPERATURE

Each enzyme has a temperature that it works optimally in, which in humans is around 98.6ºF/37ºC i.e. the normal body temperature for humans. However, some enzymes work really well at lower temperatures like 39ºF/4ºC, and some work really well at higher temperatures. For instance, animals from the Arctic have enzymes adapted to have lower optimum temperatures while animals in desert climates have enzymes adapted to higher temperatures. While higher temperatures do increase the activity of enzymes and the rate of reactions, enzymes are still proteins, and as with all proteins, temperatures above 104 ºF / 40 ºC, will start to break them down. So, the two ends of the activity range for an enzyme are determined by what temperature starts the activity and what temperature starts to break down theprotein.

TEMPERATURE VS. ENZYME ACTIVITY

Collisions between all molecules increase as temperature increases. This is due to the increase in velocity and kinetic energy that follows temperature increases. With faster velocities, there will be less time between collisions. This results in more molecules attaining the activation energy, which increases the rate of the reactions. Since the molecules are also moving faster, collisions between enzymes and substrates alsoincrease.

3. MATERIALS REQUIRED

i. **Biological Material:** Mung bean sprouts extract (source of acidphosphatase).

ii. **Chemicals/Reagents:** 1 M Acetate buffer (stock) and 0.1 M (working)pH5.5,p-nitro phenyl phosphate (PNPP), 0.5 M KOHsolution.

iii. **Equipment:** UV-Vis spectrophotometer, centrifuge,micropipette.

iv. **Glassware/Plastic ware:** Cuvettes, test tubes, test tubestand.

v. **Miscellaneous:** Mortar and pestle.

4. PROCEDURE

i. Prepare the enzymeextract.

ii. Prepare the 0.1 M acetate buffer pH 5.5 as described earlier.

iii. Add acetate buffer, substrate PNPP, enzyme extract in labeled test tubes and also make control tubes.

iv. Add 2Ml of 0.5KOH to the control tubes prior to addition of substrate for each reaction mixture.

v. Set the test tubes at various temperatures (4,10,25,37,50,60and70)° Can incubate for equal time duration of 10 min for each test tube.

vi. Post incubation; add 2 mL of KOH to the reaction tubes.

vii. Note the absorbance for each reaction; calculate the enzyme activity and plot the graph between temperature and enzyme activity.

5. OBSERVATION

Observation Table

Blank solution = 2 mL KOH + 3 mL acetate buffer

λmax for PNP = 405 nm

Dilution of enzyme =1:10

Sample	Acetatebuffer (mL)	PNPP (mL)	Enzyme (mL)	Temp. for incubation (°C)	Incubation for 10 min	0.5 M KOH 2 mL	Abs. at 405 nm (OD)	Abs. due toproduct (OD) (T-C)	Enzyme activity (µmoles mL^{-1} min^{-1})

6. RESULT

The effect of temperature on enzyme activity was successfully studied for acid phosphatase and maximum enzyme activity was observed at____°C.

7. DISCUSSION

From the graph it can clearly be determined that enzymatic activity increases with increase in temperature. This can be mainly because, on increasng temperature thermal energy increases and the kinetic energy which imparts greater tendency among molecules to undergo effective collision. Thus, increasing kinetic energy imparts chemical potential to overcum activation energy barrier, enabling facilitated product formation, and hence marked by increased enzyme activity. At low temperature kinetic energy is reduced which reduces collisions, thus reducing enzyme activity. Under physiological conditions, a temperature of 37°C is optimum and high temperature in a range of 70°C rarely exist.

Further increase in temperature would result in loss of enzyme activity. This is because at extremely high temperature, enzymes (being protein in nature) get denatured and lose their conformation in an irreversible manner.

8. PRECAUTIONS

i. Chemical preparations should be accurate as it can directly alter the result.

ii. Handle the glass wares and other laboratory equipments with utmost care.

iii. Ensure that the incubation time is uniform for all the test samples.

SUGGESTED READING(S)

i. E. Vasileva-Tonkova, D.M. Balasheva, & D. Galabova (1996). Influence of growth temperature on the acid phosphatase activity in the yeast Yarrowia lipolytica. FEMS Microbiology Letters 145 (1996) 267-271.

ii. Jumpen Onthong , Sayjai Gimsanguan & Ashara Pengnoo (2007). Effecf of pH and some cations on activity of acid phosphatase secreted from Ustilago sp. isolated from acid sulphate soil. Sci. Technol., 2007, 29(2) : 275-286.

iii. Sadia nadir and Asma saeed(2012). Isolation, Purification and Characterization of Acid Phosphatase fromGerminating Vigna radiata Seed.J.Chem.Soc.Pak. Vol.34.No.3,2012

9. PAPER CHROMATOGRAPHY

1 .BACKGROUND INFORMATION:

Paper Chromatography is used for separating chemicals based on their different properties (ex: solubility, size, mass, etc.) and thus, allows scientists to distinguish various organic and inorganic materials. This technique is commonly used in crime scenes or in laboratories to identify an unknown compound by comparing it to known compounds.

Paper Chromatography is divided into two phases: *Stationary Phase* and *Mobile Phase.* Stationary Phase describes the paper before the solvents start to move up and Mobile Phase is when the solvents travels up the chromatography paper while carrying the solute (e.g. pigments).

2. OBJECTIVES

In this experiment, we will observe black ink consists of other pigments and how different pigments in black ink separate based on their solubility. Capillary action allows solvent to travel up the paper and different pigments in black ink will separate based on their solubility. Pigment that is more soluble in polar solvent will travel up the paper along with the solvent, but pigments that are less soluble in polar solvent will travel more slowly because of their interaction with the chromatography paper.

3. MATERIALS REQUIRED

- **i.** Chromatography Paper (White Paper Towels can be used as asubstitute)
- **ii.** Solvents: must be polar (Ex: distilled water & rubbingalcohol)
- **iii.** Ruler
- **iv.** Pencil
- **v.** 3 Black Ink Pens/Markers
- **vi.** Cup (preparably clear, plastic cup but notnecessary)
- **vii.** Tape

4. PROCEDURE:

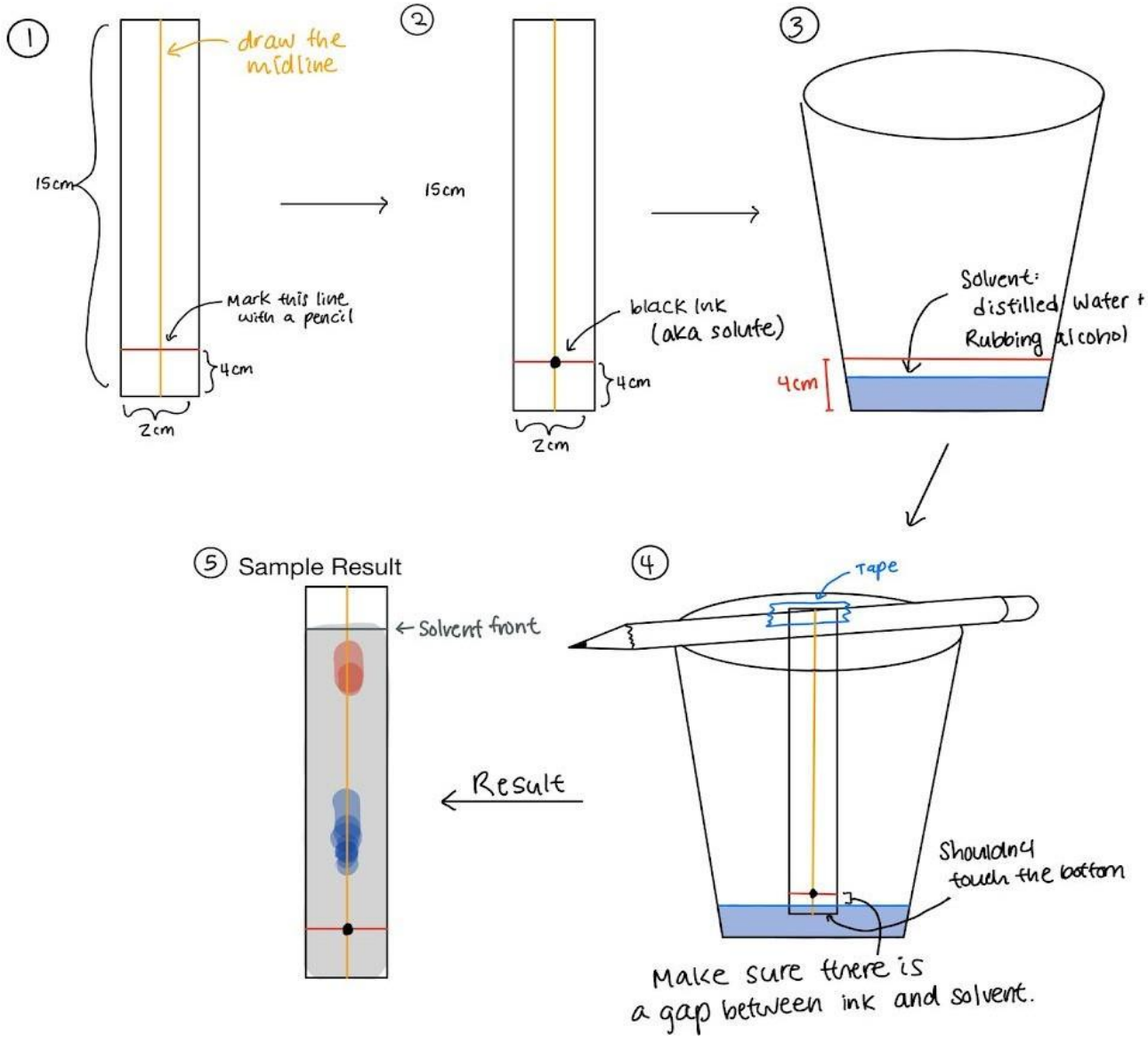
- **i.** Preparingchromatographypaper:Cutthepapertowel:2cmby15cm.Then,measure4 cm from one end and draw a line using a pencil and also a midline dividing the 4 cm line you just drew (as shown in the picture below). This is the *baseline.* It's important to use *pencil* because lead will not dissolve in water, preventing experimental error in chemical separation.
- **ii.** Usingblackinkpenormarker,drawasmalldotatthecrosssectionoftwolinesyou have drawnpreviously.
- **iii.** Preparing Solvent:Mix equal amountsof water and rubbingalcoholinthecup(height~ 2 cm). Rubbing alcohol by itself is also fine. Make sure the height of the solvent is less than 4cm.

iv. Place the chromatography paper (aka. Paper towel) inside the cup and make sure that the solvent Does Not reach where the ink is (there should be a gap between ink and solvent).Also,makesurethatthechromatographypaperdoesn'ttouchthebottomofthe cup.Tapetheotherendofchromatographypapertoapencilandplaceitonthecup.

v. Wait for about 15 minute. Then, take out the chromatographypaper.

vi. Usingthesamesetup,repeattheexperimentwithdifferenttypesofblackinkpen/ marker.

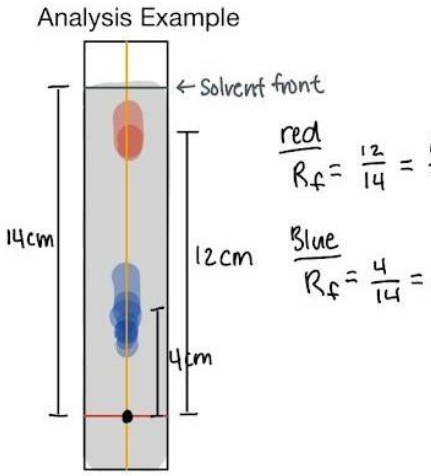
Fig :- Procedure



5.ANALYSIS:

4. Were you able to see separation of color from the black ink you used? What colors were in there? (Faster travelled pigment is more solublebecause)

5. Based on your experimental result, what pigmentdo you think was more polar? (Polar pigments travel faster in paper chromatography because they have higher solubility. “Like dissolveslike”)



6. Calculate the Rfvalue:

- Rf value describes how soluble a substanceis.

$$Rf = \frac{\textit{distance travelled by solute (ink) from baseline}}{\textit{distance travelled by solvent from baseline}}$$

6.PRECAUTION

i. Chromatographic paper should be handled neatly (Do not touch in the middle portions of the paper)
ii. Sampling mark should be proper to get the correct result.

6.EXTENSION ACTIVITIES

i. Try another experiment with different, same colored pens (such as three blue pens or three red pens) to see which pen’s ink is most soluble insolvent.
ii. Use Spinach extract (and any other plant extracts)to observe different chlorophyll held by plants. Hint: you will be able to observe more colors than justgreen.

SUGGESTED READING(S)

i. https://amrita.olabs.edu.in/?sub=79&brch=17&sim=124&cnt=4

ii. https://www.chemguide.co.uk/analysis/chromatography/paper.html

11. ANALYSIS OF WATER QUALITY PARAMETERS IN AQUACULTURE POND

i) .ESTIMATION OF CALCIUM

1.AIM:

To estimate the calcium level in aquaculture pond water

2.MATERIALS REQUIRED

i. Sodium hydroxide, NaOH, 1N.
ii. Murexide (ammonium purpurate) indicator: Mix 200 mg dye with100 g solid NaCl. Grind to 40 to 50 mesh size.
iii. Standard EDTA titrant, 0.01M: Weigh 3.723 g di-sodium salt of EDTA, EDTA dihydrate, dissolve in distilled water and dilute to 1000 mL. Store in polyethylene bottle, 1 mL = 400.8 µg Ca.Standardise EDTA against standard calcium solution periodically following the method described below.
iv. Standard calcium solution: Weigh 1.000 g anhydrous $CaCO_3$ in 500 mL flask (primary standard). Add 1 + 1 HCl in small amounts through a small funnel till all $CaCO_3$ is dissolved. Add 200 mL distilled water and boil for a few minutes to expel CO_2. Cool and add a few drops of methyl red indicator and adjust to intermediate orange colour by adding 3N NH_4OH or 1 + 1 HCl, as required. Transfer quantitatively and dilute to 1000 mL with distilled water, 1 mL = 400.8µg Ca.

3.PROCEDURE

i. Take 50 mL sample or an aliquot diluted to 50 mL such that the calcium content is not more than 10 mg. Samples which contain alkalinity greater than 300 mg/L should be neutralised with acid, boiled for 1 min and cooled before titration.
ii. Add 2 mL NaOH solution or a volume sufficient to produce a pH of 12 to 13. Start titration immediately after addition of the alkali. Add 0.1 to 0.2 g indicator mixture. Titrate with EDTA solution, with continuous mixing, till the colour changes from pink to purple. Check end point by adding 1 to 2 drops excess titrant to make certain that no further colour change occurs.

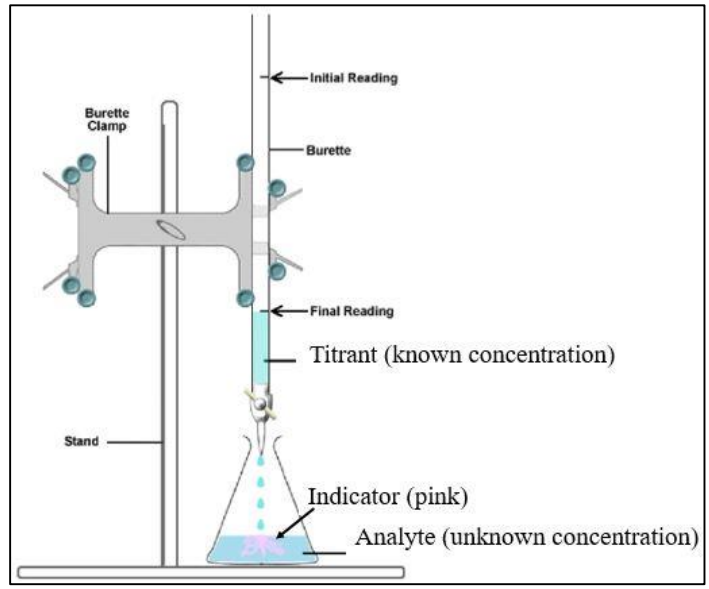


Fig : Experimental Setup

4.CALCULATION

$$\text{Calcium mg/l} = \frac{X \times 400.8}{\text{ml of sample}}$$

Where

X – volume of EDTA consumed

5.RESULT

Calcium level in the aquaculture pond water is _____ mg/L

ii) .ESTIMATION OF DISSOLVED OXYGEN

1.AIM:

To estimate the dissolved oxygen level in aquaculture pond water

2.MATERIALS REQUIRED

i. DO sampler, for collection of undisturbed samples from surface waters.
ii. BOD bottles, 300 mL, narrow mouth, flared lip, with tapered and pointed ground glass stoppers.
iii. A siphon tube, for laboratory use.
iv. Manganoussulphate solution: Dissolve 480 g MnSO4 .4H2O, 400 g MnSO4.2H2O or 364 g MnSO4.H2O in distilled water, filter and dilute to IL.
v. Alkali-iodide-azide reagent: Dissolve 500 g NaOH (or 700 g KOH) and 135 g NaI (or 150 g KI) in distilled water and dilute to IL. Add 10 g NaN3 dissolved in 40 mL distilled water.
vi. Sulphuric acid, conc
vii. Starch indicator: Dissolve 2 g laboratory grade soluble starch and 0.2 g salicylic acid as a preservative, in 100 mL hot distilled water.
viii. Standard sodium thiosulphate titrant, 0.025M (0.025N): Dissolve 6.205 g Na2S2O3.5H2O in distilled water. Add 1.5 mL 6 N NaOH or 0.4 g solid NaOH and dilute to 1000 mL.Standardise with bi-iodate solution.
ix. Standard potassium bi-iodate solution, 0.0021M (0.0126N): Dissolve 812.4 mg KH(I03)2 in distilled water and dilute to 1000 mL .

STANDARDISATION:

Take 100 to 150 mL distilled water in an Erlenmeyer flask. Add approximately 2g KI, dissolve. Add 1 mL 6N H2S04 or a few drops of conc H2SO4 and 20 mL bi-iodate solution. Dilute to 200 mL and titrate liberated iodine with thiosulphate titrant to a pale straw colour. Add a few drops of starch indicator. Continue titration to first disappearance of blue colour. Calculate

molarity, M of thiosulphate as:

where: V = mL of thiosulphate used

3.PROCEDURE

i. Drain any liquid in the flared lip of the BOD bottle containing the sample.
ii. Remove stopper and add 1 mL of MnSO4 followed by 1 mL alkali-iodide-azide reagent. Hold thepipette tip just below the liquid surface touching the side of the bottle. Wash the pipette before returning to the reagent bottles.
iii. Stopper carefully to exclude air bubbles. Mix by inverting the bottle a few times.
iv. Allow the brown manganese hydroxide floc (white floc indicates absence of DO) to settleapproximately to half the bottle volume, add 1.0 mL conc H2SO4 and re-stopper. Mix byinverting several times until dissolution is complete.
v. Titrate 201 mL with standard Na2S2O3 as for standardisation procedure described above.

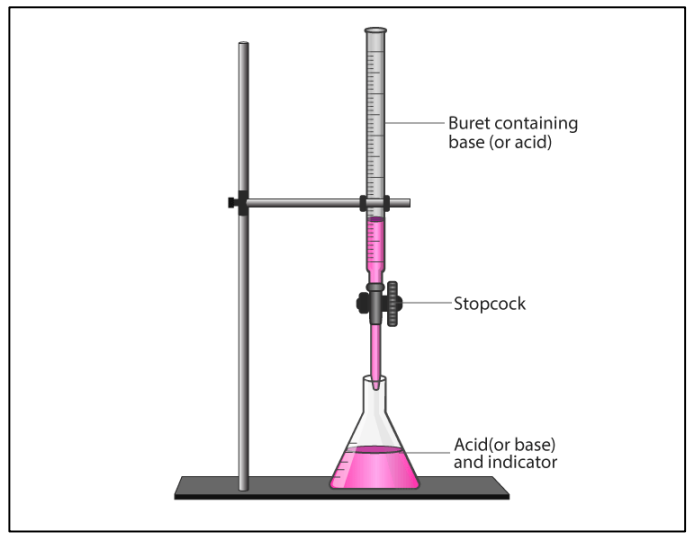


Fig : Experimental Setup

4.CALCULATION

Calculations O2 in ppm (mg/L) = K * 200 * Volume of 0.025 N thiosulfate used in titration

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Volume of sample titrated

K is a constant to correct for displacement of oxygen in the sample when manganous sulfate and alkaline iodide azide were added. (No correction needed for acid unless floc is displaced)

For a 300 ml bottle K = $\frac{\text{Volume of bottle}}{\text{Vol. Of bottle - Vol. of reagents}} = \frac{300}{(300\text{-}4)}$

5.RESULT

Dissolved oxygen level aquaculture pond water is _____ mg/L (e.g. 8.2 mg/L).

ii) .DETERMINATION OF SALINITY BY TITRATION (LOW PRECISION METHOD)

1.AIM

To determine the salinity of the aquaculture pond water.

2.MATERIALS REQUIRED

i. 10-ml pipette, 25-ml burette, tall-form beaker, burette stand

ii. SILVER NITRATE SOLUTION (APPROXIMATELY 0.28N):-Dissolve 49 g of good quality silver nitrate for each liter of final solution. Usedistilled water and store the solution in a dark bottle. The solution may need dilution with a little water to bring it to the desired strength, as described in Section H. Thesolution should be well mixed in the bottle once each day or prior to each batch oftitrations.

iii. INDICATOR-DILUENT SOLUTION:-Dissolve 3.5 g of analytical quality potassium chromate, K2Cr04, in each literof distilled water.

3.PROCEDURE

Add the sample of sea water to a 200-ml spoutless tall-form beaker by meansof the automatic 10-ml pipette and then add 15 ml of indicator—diluent solution.Titrate the solution from the 25-ml automatic burette. About 1 ml before it isjudged that the end point is reached, rinse the sides of the beaker and stirrer with alittle distilled water from a wash bottle.As the end point approaches, the localized red precipitate formed by the silversolution will begin to spread throughout the solution. At the end point the palegreenish-yellow colour of the contents of the beaker changes to a full yellow andthen becomes a definite pale red as the end point is exceeded. The exact point islargely subjective and errors in its estimation are allowed for to some extent in thestandardization procedure (see Sect. H). Record the burette reading to the nearest0.01 ml. Note the average temperature of the samples Tc, for each titration periodof 1-2 hr (say every 20 or 30 samples) by a thermometer placed in a tightly packedcase of bottles that has already equilibrated with the laboratory temperature. Notethe average temperature TAg of the silver nitrate solution during the same periodshown on a thermometer placed in a small tube through which the solution flowsbefore entering the burette. If possible, TAg should be kept greater than T01and thedifference between the two should not exceed 5 C.The sequence of operations described in Part 1.1can be used in the presentmethod when the maximum speed is required for routine analyses.

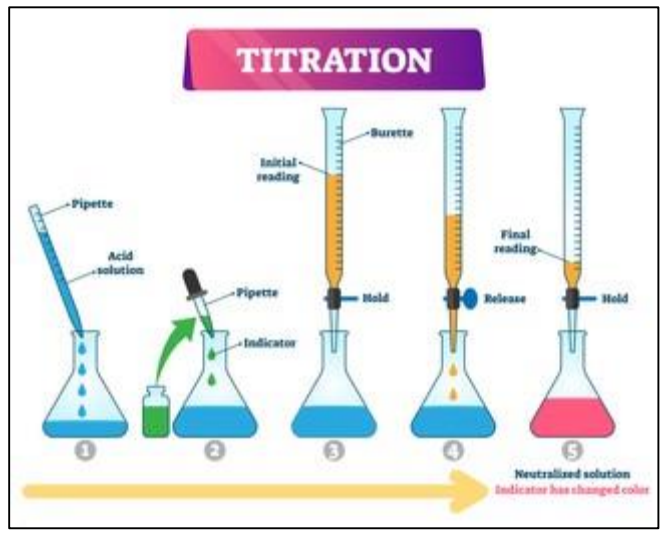

Fig : Experimental Setup

4.CALCULATIONS

Consumption of $AgNO_3$ x 1000 x 35.5

Chloride (mg/l) = -

ml of Sample

Salinity (ppt)= (1.80655) Chlorinity (ppt)

5.RESULT

Salinity of the given water sample is ……

6. PRECAUTIONS

i. Glass wares should be properly handled to avoid its breakage.

ii. Precision should be taken in the preparation of various solutions as it can alter the result.

iii. Repeat the experiment many times to get the concordant value.

7.EXTENSION ACTIVITIES

i. Compare the water quality parameters of two different habitats.

ii. Find out the optimum value of various minerals in an aquaculture pond.

SUGGESTED READING(S)

i. Bunce, N. J. Environmental Chemistry; Wuerz: Winnipeg, Canada, 1991; pp 118–120.

ii. Mitchell, M. K.; Stapp, W. B. Field Manual for Water Quality Monitoring; 9th ed.; Thomson-Shore: Dexter, Michigan, 1995; pp 27–33

iii. http://egyankosh.ac.in/bitstream/123456789/16340/1/Experiment-22.pdf