## BIOCHEMISTRY AND MICROBIOLOGY – PRACTICAL I

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

**Course Objectives:**

- To train the students of the subject on handling various experimental methods and techniques in order to analyze the given biological samples from biochemical stand points.
- To provide foundation of various methods to cultivate the microbes and maintenance of the microorganism.

**Course Outcomes:**

- Students of the subject will acquire skills to quantitatively estimate various biomolecules and as well to carryout enzyme kinetics.
- After completion of this course students should have out line knowledge on isolation, sub culture and maintenance of microbes.

**BIOCHEMISTRY**

1. Quantification of proteins – Lowry *et al*/ Bradford method
2. Quantification of sugars – Anthrone method
3. Total free amino acids
4. Quantification of lipids
5. Quantification of Ascorbic acid
6. Thin Layer Chromatography (Amino acids / fatty acids/ sugar/ nucleic acids)
7. Effect of pH, temperature, substrate concentration (any one enzyme - Catalase / SOD / amylase by OD method)

**MICROBIOLOGY**

1. Pure culture technique –pour spread, loop out technique and streaking, preservation,
2. Staining technique –grams and fungal.
3. Motility –Flagellar staining, hanging drop and soft agar analysis.
4. Growth curve (Bacteria and Fungi) and Biomass estimation

**REFERENCES**

- Boyer, Rodney. (2010). *Biochemistry Laboratory : Modern Theory and Techniques.* New Jersey: (3 rd ed.) Pearson Education, Inc.
- Palanivelu, P. (2001). *Analytical Biochemistry and Separation Techniques.* Madurai: Kalaimani Printers.
- Sadasivam. S., & Manickam, A. (2008). *Biochemical Methods.* (3 rd ed.) New Delhi: New Age International Private Limited Publishers.
- Keith Wilson, & John Walker (Eds.). (2010). *Principles and Techniques of Biochemistry and Molecular Biology.* New York, NY: Cambridge University Press.

## BIOCHEMISTRY

### 1. QUANTIFICATION OF PROTEINS

**Aim**

To estimate the protein levels from given samples by Lowry"s method.

**Principle**

The blue colour developed by the reduction of the phosphomolybdic- phosphotungstic components in the Folin – Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry"s method.

**Materials Required**

2% Sodium Carbonate in 0.1 N Sodium Hydroxide **(Reagent A)**

**0.5** % Copper Sulphate ($CuSO_4.5H_2O$) in 1%potassium sodium tartrate **(Reagent B)**

**Alkaline Copper Solution:** Mix 50 ml of A and 1 ml of B prior to use **(Reagent C)**

**Folin – Ciocalteau Reagent (Reagent D):** Reflux gently for 10 hours a mixture consisting of 100g sodium tungstate, 25 g sodium molybdate, 700 ml water, 50 ml of 85% phosphoric acid and 100 ml of concentrated hydrochloric acid in a 1.5 L flask. Add 150 g lithium sulphate, 50 ml water and a few drops of bromine water. Boil the mixture for 15 min without condenser to remove excess bromine. Cool, dilute to 1L and filter. The reagent should have no greenish tint. (Determine the acid concentration of the reagent by titration with 1N NaOH to a phenolphthalein end-point).

**Protein Solution (Stock Standard)**

Weigh accurately 50 mg of bovine serum albumin (Fraction V) and dissolve in distilled water and make up to 50 ml in a standard flask.

**Working Standard**

Dilute 10 ml of the stock to 50ml with distilled water in a standard flask. One ml of this solution contains 200 µg proteins.

**Procedure**

**Extraction of protein sample**

Extraction is usually carried out with buffers used for the enzyme assay. Weigh 500mg of the sample and grind well with a pestle and mortar in 5-10ml of the buffer. Centrifuge and use the supernatant for protein estimation.

**Estimation of protein**

1. Pipette out 0.2. 0.4, 0.6, 0.8 and 1 ml of the working standard into series of test tubes.
2. Pipette out 0.1ml and 0.2 ml of the sample extract in two other test tubes.
3. Make up the volume to 1ml in all he test tubes. A tube with 1ml of water serves as the blank
4. Add 5ml of reagent C to each tube including the blank. Mix well and allow standing for 10 min.
5. Then add 0.5 ml of reagent D, mix well and incubate at room temp in the dark for 30min. Blue color is developed.
6. Take the reading at 660 nm.
7. Draw a standard graph and calculate the amount of protein in the sample.

**Calculation**

Express the amount of protein mg/g or 100g sample.

## 2. QUANTIFICATION OF SUGARS

**Aim**

To estimate the sugar levels from given samples by Anthrone method.

**Principle**

Carbohydrates are the important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. The basic units of carbohydrates are the monosaccharides which cannot be split by hydrolysis into more simple sugars. The carbohydrates content can be measured by hydrolyzing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharides.

Carbohydrates are first hydrolyzed into simple sugar using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green colored product with an absorption maximum at 630nm.

**Materials Required**

2.5N – HCL

Anthrone Reagent: Dissolve 200mg anthrone in 100ml of ice cold 95% $H_2SO_4$. Prepare fresh before use.

Standard Glucose: Stock – Dissolve 100 mg in 100 ml water.

Working standard: 10 ml of stock diluted to 100 ml with distilled water. Store refrigerated after adding a few drops of toluene.

**Procedure**

1. Weigh 100 mg of the sample into a boiling tube
2. Hydrolyze by keeping it in a boiling water bath for 3 h with 5ml of 2.5 N- HCL and cool to room temperature.
3. Neutralize it with solid sodium carbonate until the effervescence ceases.
4. Make up the volume to 100 ml and centrifuge.
5. Collect the supernatant and take 0.5 and 1ml aliquots for analysis.
6. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard. „0" serves as blank.
7. Make up the volume to 1ml in all tubes including the sample tubes by adding distilled water.

8. Then add 4 ml of anthrone reagent
9. Heat for 8 min in a boiling water bath.
10. Cool rapidly and read the green to dark green color at 630 nm.
11. Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y- axis.
12. From the graph calculate the amount of carbohydrate present in the sample tube.

**Calculation**

Amount of carbohydrate present in sample

$$= \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

## 3. TOTAL FREE AMINO ACIDS

**Aim**

To assess the free amino acids levels from given samples.

**Principle**

The amino acids are colourless ionic compounds that form the basic building blocks of proteins. Apart from being bound as proteins, amino acids also exists in the free form in many tissues and are known as free amino acids. They are mostly water soluble in nature. Very often in plants during disease conditions, the free amino acids composition exhibits a change and hence, the measurement of the total free amino acids gives the physiological and health status of the plants.

Ninhydrin, a powerful oxidizing agent, decarboxylates the alpha – amino acids and yields an intensely colored bluish purple product which is calorimetrically measured at 570nm.

Ninhydrin + alpha –amino acids → Hydrindantin + Decarboxylated amino acid + Carbon dioxide + Ammonia

Hydrindantin + Ninhydrin + Ammonia → Purple coloured product + Water

**Materials Required**

**Ninhydrin:** Dissolve 0.8 g stannous chloride in 500 ml of 0.2 M citrate buffer (pH 5.0). Add this solution to 20 g of Ninhydrin in 500 ml of methyl cellosolve (2 methoxyethanol).

0.2 M Citrated buffer pH 5.0

**Dilute Solvent:** Mix equal volume of water and n – propanol and use.

**Standard**

Dissolve 50 mg Leucine in 50ml of distilled water in a volumetric flask. Take 10ml of this stock standard and dilute to 100ml in another volumetric flask for working standard solution. A series of volume from 0.1 to 1ml of this standard solution gives a concentration range 10 µg to 100 µg. Proceed as that of the sample and read the colour.

**Procedure**

**Extraction of amino acids**

Weigh 500mg of the plant sample and grind it in a pestles and mortar with a small quantity of acid – washed sand. To this homogenate, add 5 to 10ml of 80% ethanol. Filter or centrifuge. Save the filtrate or the supernant. Repeat the extraction twice with the residue and pool all the supernatants. Reduce the volume if needed by evaporation and use the extract for the quantitative estimation of total free amino acids. If the tissue is tough, use boiling 80% ethanol for extraction.

**Estimation**

1. To 0.1ml of extract, add 1ml of Ninhydrin solution.
2. Make up the volume to 2ml with distilled water.
3. Heat the tube in a boiling water bath for 20 min.
4. Add 5ml of the diluents and mix the contents.
5. After 15min read the intensity of the purple colour against a reagent blank in a colorimeter at 570nm. The colour is stable for 1h.
6. Prepare the reagent blank as above by taking 0.1 ml of 80% ethanol instead of the extract.

**Result**

Draw a standard curve using absorbance versus concentration. Find out the concentration of the total free amino acids in the sample and express as percentage equivalent of Leucine.

**Note**

Ninhydrin is carcinogenic. Wear gloves while handling it.

Since this estimation includes only alpha- amino acids, non- protein amino acids are not accounted.

## 4. QUANTIFICATION OF LIPIDS

**Aim**

To quantify the lipids level from given samples.

**Principle**

Fats are fatty acids esters of glycerol. Fat as liquid is called as oil. Seeds like gingely, ground nut, castor, sunflower etc. contain oil as reserve food material for the embryo.

Crude lipid from a known quantity of the seed is extracted with petroleum ether. It is then distilled off completely, dried, the crude lipid weighed and the% oil is calculated.

**Materials Required**

Petroleum ether (40-160$^0$C)

Whatmann No.2 Filter Paper

Absorbant Cotton

Soxhlet Apparatus

**Procedure**

1. Fold a piece of filter paper in such a way to hold the seed meal. Wrap around a second filter paper which is left open at the top like a thimble. A piece of cotton wool is placed at the top to evenly distribute the solvent as it drops on the sample during extraction.
2. Place the sample packet in the butt tubes of the Soxhlet extraction apparatus.
3. Extract with petroleum ether (150 drops/min) for 6h without interruption (For castor beans use hexane) by gentle heating.
4. Allow to cool and dismantle the extraction flask. Evaporate the ether on a steam or water bath until no odour of ether remains. Cool at room temperature.
5. Carefully remove the dirt or moisture outside the flask and weigh the flak. Repeat heating until constant weight is recorded.

**Calculation**

$$\text{Crude lipid in ground sample \%} = \frac{\text{Weight of lipid (g)}}{\text{Weight of sample (g)}} \times 100$$

$$\text{Crude lipid to dry weight basis} = \frac{\text{\% lipid in ground sample}}{\text{100\% moisture in whole seed}}$$

**Note**

**Sample Preparation**

Sample preparation varies depending upon the materials.

**Peanut**

Place about 50 g of kernels in a drying dish and dry at 130$^{o}$C for not more than 20 min in a forced – draft oven. Cool to room temperature and then pass through the nut slicer. Care is to be taken to prevent expressing of any oil while slicing. Mix the sliced sample well. Weigh accurately 2 g into the filter paper fold.

## 5. QUANTIFICATION OF ASCORBIC ACID

**Aim**

To quantify the ascorbic acid level from given samples.

**Principle**

Ascorbic acid is determined by colorimetric method. The dehydroascorbic acid alone reacts quantitatively and not the other reducing substances present in the sample extract. Thus this method gives an accurate analysis of ascorbic acid content than the dye method.

Ascorbic acid is first dehydrogenated by bromination. The dehydroascorbic acid is then reacted with 2,4 dinitrophenyl hydrazine to form osazone and dissolved in sulphuric acid to give an orange red colour solution which is measured at 540nm.

**Materials Required**

- 4% Oxalic acid solution
- 0.5N Sulphuric acid
- 2% 2,4 dinitrophenyl hydrazine (DNPH) reagent. Dissolve by heating 2 g DNPH in 100ml 0.5N $H_2SO_4$. Filter and use.
- 10% Thiourea solution
- 80% Sulphuric acid

**Bromine water**: Dissolve 1-2 drops of liquor bromine in approximately 100ml cool water.

**Ascorbic acid stock solution**: Dissolve 100mg ascorbic acid in 100 ml of 4% oxalic acid solution in a standard flask (1mg/ml).

**Working standard**: Dilute 10ml of the stock solution to 100ml with 4% oxalic acid. The concentration of working standard is 100 µg/ml.

**Extraction**

Grind 0.5-5g of sample material either mechanically or using a pestle and mortar in 25 - 50ml.4% oxalic acid solution. Centrifuge or filter and collect the liquid.

Transfer an aliquot (10ml) to a conical flask and add bromine water drop wise with constant mixing. The enolic hydrogen atoms in ascorbic acid are removed by bromine. When the extract turns orange yellow due to excess bromine, expel it by blowing in air. Make up to a known volume (25 or 50ml) with 4% oxalic acid solution.

Similarly, convert 10ml of stock ascorbic acid solution into dehydro form by bromination.

**Procedure**

1. Pipette out 10-100µg standard dehydroascorbic solution into a series of tubes.
2. Similarly, pipette out different aliquots (0.1ml – 2ml) of brominated sample extract.
3. Make up the volume in each tube to 3ml by adding distilled water.
4. Add 1ml of DNPH reagent followed by 1-2 drops of thiourea to each tube
5. Set a blank as above but with water in place of ascorbic acid solution.
6. Mix the content of the tubes thoroughly and incubate at $37^{o}$c for 3h.
7. After incubation dissolve the orange – red osazone crystals formed by adding 7ml of 80% sulphuric acid.
8. Measure absorbance at 540nm
9. Plot a graph ascorbic acid concentration versus absorbance and calculate the ascorbic acid content in the sample.

**Note**

Liquor bromine can cause burns. Prechill the sample containing bromine prior to cut opening it.

## 6. THIN LAYER CHROMATOGRAPHY

**Aim**

To identify the Amino acids / fatty acids/ sugar/ nucleic acids from given samples using thin layer chromatography.

**Principle**

The separation of the solutes (Amino acids / fatty acids/ sugar/ nucleic acids) is based on the liquid –liquid partitioning of amino acids in chromatography. The partitioning takes place between the water molecule (static phase) adsorbed to the cellulosic matter of the paper and the organic (mobile phase).

**Materials Required**

- Whatman No.1 filter paper
- Chromatography chamber
- Hair- dryer or spot lamp
- Atomizer
- Micro syringe or micropipette
- Mobile phase: Mix n-butanol, glacial acetic acid and water in the ratio 4:1:5 in a separating funnel and stand to equilibrate for 30min. drain off the lower aqueous phase into a beaker and place it inside to saturate the chromatography chamber. Save the upper organic phase and use it for developing the chromatogram.
- Dissolve different individual amino acids in distilled water at a concentration of 1mg/ml. use very dilute (0.05N) HCL to dissolve the free amino acids tyrosine and phenylalanine. Dissolve tryptophane in very dilute (0.05N) NaOH.

**Extraction of Sample**

Grind a known quantity of the sample material (dry/ wet) in a pestle and mortar with 10 – fold volume of 70% ethanol. Shake the contents at 55$^{o}$C for 30 min. centrifuge the contents at 10,000 rpm for 10 min. collect the supernatant. Repeat the extraction of the pellet at 55$^{o}$C at least twice. Pool the supernatant (for leaf extracts, treat with equal volume of petroleum ether 40 -60$^{o}$C) and shake vigorously. Discard the petroleum ether layer containing chlorophyll. Evaporate the alcohol fraction to dryness under vacuum using either a water – pump or rotary

evaporator at 40-50oc. Dissolve the residue in a known volume of absolute ethanol or water for analysis.

Ninhydrin Reagent: Dissolve 100 mg Ninhydrin in 100 ml acetone.

Elution Mixture: Prepare 1% copper sulphate solution. Mix ethanol and copper sulphate solution in the ratio 80:20 (v/v).

**Procedure**

1. Cut the chromatography sheet carefully to a convenient size (40 X 24cm). Draw a line with pencil across the sheet about 5cm away from one end. Mark a number of points at intervals of 3cm.
2. Apply a small volume (25µl) of each amino acid as a separate small spot using a micro syringe. A stream of hot air from a hair – dryer facilitates fast drying of spot. The spot should be as small as possible for better resolution.
3. Similarly spot different known aliquots of sample extract
4. After spotting, place the sheet in a steel trough in the chromatography chamber, firmly hold it by placing a long steel rod over the sheet. The spot-end of the sheet should be in the trough (decending chromatography). Otherwise, the sheet may be rolled as a cylinder, tied together with fine thread and placed upright with the spots as the bottom in a large petridish for upward movement of solvent (ascending chromatography)
5. Add the organic (phase) solvent to the trough/Petri dish and close the chamber airtight. Develop the chromatogram, preferably overnight or longer, until the solvent moves almost to the other end.
6. Note the solvent front and dry the chromatogram free of solvent in a fume chamber.
7. Spray the chromatogram with the Ninhydrin reagent using automizer. Dry the paper for about 5 min at room temp followed by at 100oc in an oven for 2-3min.
8. Amino acid appears as purple spots; hydroxyproline and praline give yellow coloured spots.

Mark all the spots and calculate their $R_f$ values by the formula

$$R_f = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}$$

The amino acids present in the sample are then identified by comparing the $R_f$ values with that of the authentic amino acids.

9. For quantitative estimation, cut each spot into several small bits and transfer to the bottom of the test tubes. Add 3ml of elution mixture. Shake the tubes vigorously for 15 min. Decant the liquid and elute the pieces with another 2ml of elution mixture. Repeat the elution with small aliquots until the bits are colourless. Combine and clear the elute by centrifugation at 10,000 rpm for 10min. Read the intensity of purple color at 570nm in a colorimeter. Use the spot of Leucine (50µg) run as standard for comparison.

## 7. EFFECT OF pH, TEMPERATURE, SUBSTRATE CONCENTRATION (any one enzyme - Catalase / SOD / amylase by OD method)

### Effect of pH on Catalase Activity Experiment

### Aim

To determine the effects of pH on catalase enzyme activity.

### Introduction

Catalase breaks down $H_2O_2$ (hydrogen peroxide) into water and oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

$H_2O_2$ is a toxic substance formed during anaerobic respiration.

### Materials and method

We used four different concentrations (pH values) of a buffer solution of sodium phosphate $Na_2PO_4$ between pH 4 and pH 8. $3cm^3$ of shredded potato (containing catalase) was placed in a flask, with $10cm^3$ of buffer solution at the lowest pH. A rubber bung equipped with both a syringe and a delivery tube was placed in the neck of the flask. The other end of the delivery tube was placed in a beaker containing water. The air in the flask was displaced by the injection of $H_2O_2$ solution. The air was allowed to bubble out through the delivery tube into the beaker. A measuring cylinder was filled with water and inverted carefully into the beaker over the end of the delivery tube. Timing was begun immediately after the oxygen bubbles began. After five minutes, the volume of oxygen present in the measuring cylinder as a result of the reaction in the flask was determined. This was repeated for all buffer concentrations. A control set-up was used in the exact manner as above - but instead of using buffer solution each time, the same quantity of distilled water was used.

**Results and discussion**

The relative reaction rate (volume of oxygen evolved during five minutes) was plotted against pH. The control showed the same enzyme activity each time the experiment was run. The relative reaction rate increased with pH - up to the optimum value (around 6.3) and then decreased at higher pH values.

**Effect of Temperature on Trypsin Activity**

**Aim**

To investigate the effect of temperature on the activity of trypsin - using casein as the substrate.

**Introduction**

When casein (a protein in milk) is hydrolysed, the milk turns from cloudy to clear. Trypsin is one of the enzymes able to do this.

**Materials and method**

A water bath was heated to 25°C. 5cm³ of 4% casein solution was put into one test tube and 5cm³ of 0.5% trypsin was added to another test tube. Both were stood in a water bath until they reached the correct temperature. The same quantity of casein solution was added to another test tube and 5cm³ distilled water in a fourth test tube. This acted as a control. Another control consisted of 5cm³ distilled water in a fifth test tube and 5cm³ casein in a final test tube. All of these were also placed into the water bath. Black card was placed behind the test tubes to help spot the clearing of the solution. The enzyme and substrate were mixed, a stopwatch started immediately, and the time for the suspension to clear noted. This was repeated for the controls, and the whole experiment repeated for different temperatures, ranging from 25°C - 65°C.

**Results and discussion**

The control showed no change in colour at any temperature. In the graph below, we plotted the relative reaction rate (1/time taken to see a colour change) against the temperature. As the temperature increased, the time for a colour change to be seen was less (1/t was bigger). At a certain point, the relative reaction rate started decreasing. This was the optimum temperature (~ 37°C).

**Possible errors**

The timing may have been inaccurate. The colour change is subjective. This means that each person will decide differently when they see a colour change and so everyone would have noted a different time.

**Effect of Substrate Concentration on Amylase Activity**

**Aim**

To investigate the effect of amylase concentration on its activity. The relative activity is determined by noting the time taken for the starch substrate to break down.

**Introduction**

Amylase is an enzyme present in saliva and pancreatic juice. It catalyses the hydrolysis of amylose and amylopectin (both starch components) to a mixture of products including maltose and dextrin**.**

**Materials and methods**

A water bath was heated to 35°C. 5cm³ of undiluted 0.1% amylase solution was pipetted into one test tube and 5cm³ starch into another. Both were stood in a water bath and left to reach the correct temperature. The solutions were then mixed, the test tubes returned to the water bath and at one minute intervals a drop of the mixture was removed and tested with iodine solution on a white tile. The starch had been broken down completely when the blue-black colour (obtained

by testing for starch with iodine) disappeared. This was repeated with the other enzyme concentrations of 0.05%, 0.025% and 0.01% respectively.

**Results and discussion**

A graph of the relative reaction rate (1/time taken for a colour change to occur) showed that, as the enzyme concentration increased, the reaction rate was faster. This was true up to the point at which the substrate was saturated with enzymes - at this point any higher enzyme concentrations gave no further increase.

**Possible errors:**

Because we only measured at one minute intervals, the colour change may have occurred between measurements. As explained before, the measuring of colour change by eye is subjective. A control could also have been implemented using $5cm^3$ starch solution with one concentration of amylase for all the experiments

## MICROBIOLOGY

## 1. PURE CULTURE TECHNIQUE –POUR, SPREAD, LOOP OUT TECHNIQUE AND STREAKING, PRESERVATION

### PURE CULTURE TECHNIQUE

**Aim**

To learn the techniques of pour, spread, loop out, streaking, and preservation of microbes.

**Background**

The microbial population in our environment - air, soil, water - is large and include many" species of bacteria, fungi and algae. A study of the microorganisms in these habitats requires knowledge of the specific microbes present. This requires techniques for preparing species as pure cultures from the mixed population.

Pure culture represents a population of organisms of a single species in the absence of living cells of any other species. There are various techniques whereby the different species in a natural population can be isolated and grown as pure culture. They are a) streak plate technique, b) pour plate technique and c) spread plate technique.

**Materials required**

Nutrient agar, Nutrient broth, Petriplates, Pipettes Dilution blank, L-rod, tubes, Vortex mixer.

**a) Pour Plate Technique**

1. Liquefy the nutrient agar in the tubes by heating them in water bath.
2. Cool the tubes to 45°C and hold at this temperature until ready to pour into the plate.
3. Label the tubes and corresponding Petridishes.
4. Serially dilute the given mixed culture and from each dilution take 1 ml of the sample.
5. Mix the sample with agar medium by gently rotating the tube between your palms.
6. Pour the contents of the tubes into the corresponding labelled petri plates and allow to solidify.
7. Incubate the plates in inverted position at room temperature.

**b) Spread Plate Technique**

1. Use the nutrient agar plates as prepared in your earlier experiment.
2. Serially dilute the given culture as given in section b (pour plate technique).
3. Label the nutrient agar plates corresponding to the dilutions.
4. Place 0.1 ml from the each dilution in seperate nutrient agar plates.
5. Sterilize the 'L' rod with alcohol and then by flaming it.
6. Cool the rod and place the rod gently on the surface of the agar. Spread the sample over the surface of the agar medium by rotating the petriplate manually or by placing it on the rotating plate disc.
7. Incubate the plates in inverted position at the room temperature.

Observe the well seperated colonies from these different techniques. Pick up isolated colonies with the sterile inoculation needle and inoculate into the liquid medium. Incubate them as before. After sufficient growth make wet mount of the cultures and observe under microscope. If the cultures are pure (axenic) they may be individually transferred to agar slants and labelled.

**c) Streak Plate Technique**

1. Use the nutrient agar plates prepared as earlier.
2. Sterilize the inoculation needle by flaming it red hot and allow it to cool for 30 seconds.
3. Holding the culture tube in your left hand, take the tube near flame and remove cotton with your right hand and flame the mouth of the tube for a few seconds.
4. Touch the culture with the needle loop and take the culture.
5. Place it on the agar plate and streak it on the plate once.
6. Resterilize the loop and cool it as above and take the culture from one end of the plate to other end and complete the streak as indicated.

**OBSERVATION AND RESULT**

## MAINTENANCE AND PRESERVATION OF PURE CULTURES

The following four methods used for maintenance and preservation of pure cultures.

The methods are:

1. Refrigeration
2. Paraffin Method
3. Cryopreservation
4. **Lyophilisation**

### Refrigeration

Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold-rooms. This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped.

Thus their growth continues slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

### Paraffin Method

This is a simple and most economical method of maintaining pure cultures of bacteria and fungi. In this method, sterile liquid paraffin in poured over the slant (slope) of culture and stored upright at room temperature.

The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium. This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture is preserved for several years.

### Cryopreservation

Cryopreservation (i.e., freezing in liquid nitrogen at-196°C) helps survival of pure cultures for long storage times. In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents such as glycerol, that prevent the formation of ice crystals and promote cell survival.

**Lyophilisation (Freeze-Drying)**

In this method, the culture is rapidly frozen at a very low temperature (-70°C) and then dehydrated by vacuum. Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years.

Lyophilized or freeze-dried pure cultures and then sealed and stored in the dark at 4°C in refrigerators. Freeze- drying method is the most frequently used technique by culture collection centres.

## 2. STAINING TECHNIQUE –GRAMS AND FUNGAL

**Aim**

To study the various staining techniques for identify the morphology of microorganisms.

**Background**

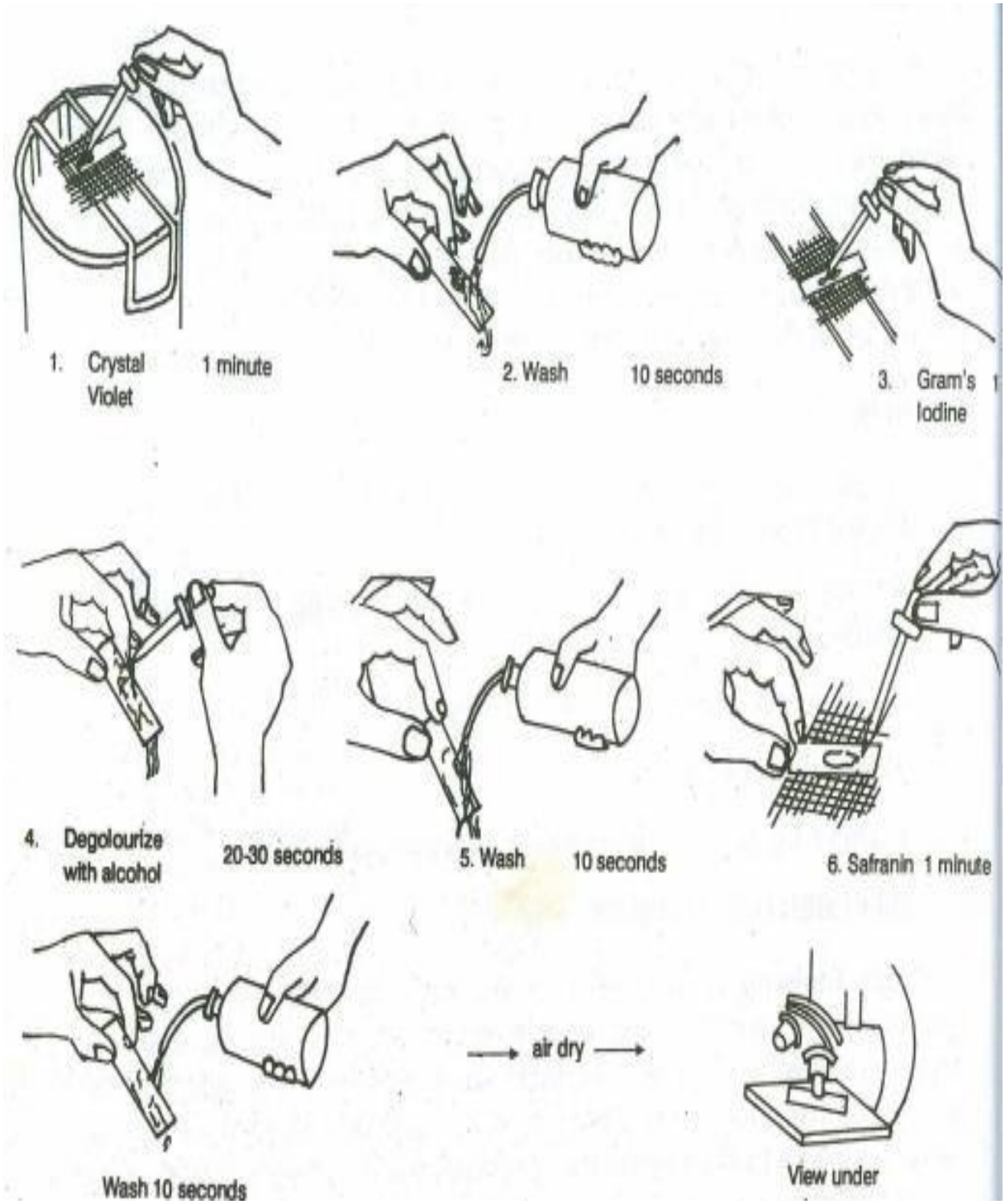
Staining is the method of artificially producing colour in micorogranisms to allow for the visualization under the microscope; Stains are employed not only to make the organisms visible but also understand their structure and chemical nature.

**A. GRAM STAINING**

Gram Staining is employed to visualize and differentiate between organisms. It is useful in presumptive identification of organisms before carrying out several other tests such as biochemical tests. The cultures used for Gram stain reaction should be less than 24 hr. old. Reasons for failure to stain positively are age of culture, low pH of medium or both. True Gram negative organism will not become gram positive although increasing alkalinity make them appear as positive. Increase in acidity may cause Gram positive organism to appear Gram negative. Cocci are generally Gram positive (except *Neisseria*).

**Materials required**

Glass slides, 24 hrs old cultures of *Escherichia coli and Bacillus subtilis*, Crystal violet, Potassium Iodide/Iodine Solution, Acetone-Alcohol and Safranin.




**Gram Staining**

**Procedure**

1. Prepare the smear on the slides with the bacterial cultures as done for the simple positive staining method.
2. Stain it for one minute with crystal violet solution (Gram's stain) washes it in tap water.
3. Apply the Iodine solution (Mordant) for 1 min. Wash in tap water.
4. Decolourize with alcohol by adding drop wise on the tilted slide until all free blue colour has been removed (20-30 sec). Wash it in tap water.
5. Flood the slide with safranin (counter stain) for one minute. Wash it in tap water and air dry.
6. Examine the stained smear under the oil immersion objective to determine which organism is Gram positive (Violet colour) and which is Gram negative (Pink colour).

**B. FUNGAL STAINING**

**Aim**

To identify the fungi by lacto phenol cotton blue staining

**Principle**

The lacto phenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components: phenol, which will kill any live organisms; lactic acid which preserves fungal structures, and cotton blue which stains the chitin in the fungal cell walls.

**Materials Required**

- Slide
- Inoculation loop
- Bunsen burner
- Fungal Culture
- Cover slip
- LPCB stain

**Procedure**

1. Take a clean dry glass slide
2. A drop of LBCB stain was placed in the centre of the slide
3. With the help of inoculation loop a small portion of colony was scratched and placed in the drop of stain
4. Then it was teased into small bits with the help of teasing needle
5. A cover slip was placed on top and gentle pressure was applied for even spreading
6. It was then observed under the microscope.

**OBSERVATION AND RESULT**

## 3. MOTILITY –FLAGELLAR STAINING, HANGING DROP AND SOFT AGAR ANALYSIS.

### A) FLAGELLAR STAINING

**Aim**

To Stain Flagella of cell by using Leifson‟s method

**Background**

- Bacteria have two types of locomotory organs and that are Flagella and pili.
- Here we are studying about flagella staining.
- Flagella is a thin,hair like structure made up protein called as flagellin.
- It size ranges from 20 µ to 200 µ in length.
- Flagella is one of the most important locomotory organ .It is mainly made up of three parts- 1) Basal body 2) Filament 3) Hook.
- Flagella is generally present in rod shape bacteria and very few cocci shape bacteria posses flagella.
- As flagella are very thin and hair like they cannot be easily observed under microscope.
- So a special technique is design to increase thickness of flagella as well as stain it.
- Due to this technique we can observe structure of flagella easily under microscope.

**Requirement**

1. Flagellated cell culture slant.
2. Leifson‟s stain.
3. 1 % Methylene blue.
4. Distilled water.

**Procedure**

1. First of all take two hours old flagellated cell culture slant and add two to three drops of sterile distill water in the slant with the help of sterile pipette.
2. Note that the distill water is added slowly without disturbing the growth of cells.
3. After addition of distill water incubated the slant for 20 minutes.
4. Then take a drop of suspension from the slant and place the drop on a clean slide which is kept in slanting position.

5. The drop should flow slowly from one end of slide to other end to avoid folding of flagella on cell.
6. Allow smear to air dry here we don‟t use heat fixation treatment .
7. After air drying the slide is flooded with Leifson‟s stain till a thin film of shinny surface appear.
8. After this give a gentle stream of water wash treatment to a slide.
9. Now treat the slide with 1 % methylene blue treatment for 1 minute.
10. Give the slide water wash treatment, air dry and observe under oil immersion lens.

**Preparing Agar Slants**

1. Allow the test tubes containing the agar medium (step 8) to cool down to about 45 to 50°C
2. Keep them in a slanting position by resting the plugged end over the glass rod on the table
3. Leave the tubes in this position until the medium has cooled to room temperature and solidified to look opaque.
4. Store these tubes for later use.

**B) HANGING DROP TECHNIQUE**

**Aim**

To observe the motility of bacteria.

**Background**

Heat fixing and staining are rather severe treatment for a bacterial cell and they cause considerable changes in morphology. To avoid these changes, bacteria can be observed in their living state by means of wet mount technique. In this method there is usually little difficulty with highly motile organisms but feebly motile organism may require prolonged observation of individual cells. Bacterial movement must be distinguished from "Brownian movement". Brownian movement is vibratory movement caused by invisible molecules striking the bacteria displacing them for short distance. True motility of the bacteria may lead to the bacteria to move from one place to other relatively longer distance. Therefore it is best to use hanging drop

preparation for this purpose. For observing motility of pathogenic organism it is safer method is to inoculate semisolid medium and observe growth and motility.

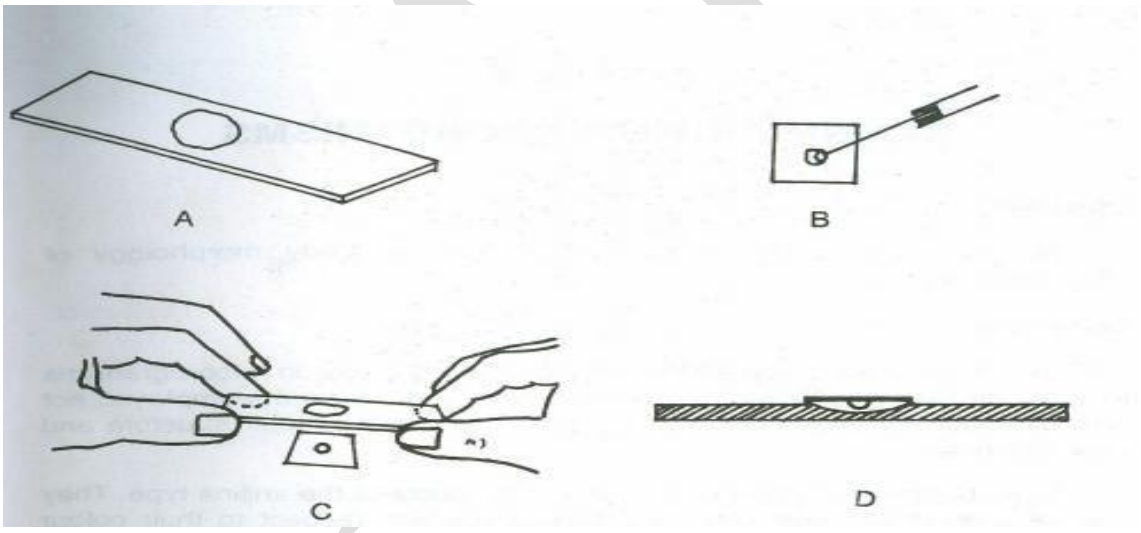
**Materials required**

A broth culture of motile bacteria, Proteus sp., Microscope, Single cavity slides, Coverslips, Vaseline, Soft agar medium.

**Procedure**

**a) Hanging drop method**

1. Place a very small drop of bacterial culture in the centre of a cover slip.
2. Place a small drop of Vaseline at each corner of the depression side of the cavity slide.
3. Invert the slide (cavity side) over the cover slip. The cover slip adhere to the glass slide and when the cover slip is inverted,the hanging drop is suspended in the well.
4. Bring the edge of the hanging drop into the focus with 10X objective betore turning to the high power objective to observe motility.




Preparation of hanging drop

A. Cavity slide (vaselin applied over the edges)
B. Placing loopful organism at the centre of cover glass
C. Cavity slide placed over cover slip
D. Complete hanging drop slide for viewing

**b) Stab culture method**

1. Prepare the semisolid nutrient agar medium (0.7% agar) and distribute them in test tubes and sterilize them.
2. Allow the tubes to cool for 1 hr.
3. Sterilize the inoculation loop by flaming. Then cool it for a while.
4. Take a loopful of culture from the broth culture.
5. Remove the plug from the tube of semisolid medium, flame the mouth and stab medium with inoculation loop containing culture.
6. Flame the neck of the tube. Plug with the cotton.
7. Incubate for 48 h at 37°C in an incubator.
8. Examine growth of the bacteria in the medium in the tube. If the cloudiness appears indicate the motility of the bacteria. Non-motile organism grows only on the line of the stab.

## C) SOFT AGAR ANALYSIS

### Introduction

One of the most generally accepted method by assaying for survival in a measured of the ability of the cells to form culture in isolation. This is usually achieved by simple dilution of a single cell suspension and determination of survival by counting by colonies, that forms some drug may be necessary to be a colony size. Analysis as well this may be done by counting the number of cells per colony only possible in small colonies by measuring absorption of colonies stained with 1% crystal.

### Aim

To quantitative cells and to count the end of the no. of colony forming units in semi solid (0.3%) agar and asses the toxic effect of given drug.

**Materials Required**

All volumes are calculated to 4 plates per point.

**Base Agar**

1.Melt 1% agar in microwave cool to 40°C in a water bath warm 2×RPMI + 20% FCS to 40°C in water bath allow at least 30mins for temperature equilibrate.

2. Mix equal volume of the 2 solution to give 0.5% agar + 1x RPM I +FCS.

3. Add 1.5ml per 35m petridish allow to set the plates can be stored at 4°C for upto 1 week.

**Top Agar**

1. Tripsinization cells and count it is very important to have a positive control line.

2. Your require 5000 cells/plate, therefore you need 20,000/ tube adjust cells count to 2,00,000 cells /ml.

3. Add 0.1ml of suspension to 10ml yellow capped centrifuge tube.

4. Labelled 35mm petridish with base agar appropriately temperature.

5. For plating add 3ml 2×RPMI+10% or 20% FCS and 3ml 0.7% agar to a tube, mix gently and add 1.5 ml to equal replicate plate.

6. Incubate the assay plate at 37°c in humidified incubator for 10-14 days.

7. Stain the plates with 0.5ml of 0.005 % crystal violet for more than 1 hour, count colonies using a dissection microscope.

**OBSERVATION AND RESULT**

## 4. GROWTH CURVE (BACTERIA AND FUNGI) AND BIOMASS ESTIMATION

**Aim and Objectives**

1. To investigate some factors contributing to microbial growth
2. To construct microbial growth curves

**Introduction**

There are many factors controlling microbial growth, including temperature, oxygen, pH, and water availability. All bacteria have a minimum, maximum, and optimum temperature for growth. At low temperatures, enzymatic activity is slowed and growth is minimal. At high temperatures, enzymatic activity is high, and growth is rapid. Some bacteria are strict aerobes, and will not grow well under conditions where $O_2$ concentration is low. Others are facultative anaerobes, which grow best with lots of $O_2$, but can switch to anaerobic respiration if $O_2$ levels are reduced. Some microbes are halophiles (have an absolute requirement for moderate to high concentrations of salt) or halo tolerant (can tolerate some salt in their environment, but do not require it for growth). Microbes that cannot tolerate high salt become dehydrated and cannot grow. In this lab, we will investigate the effects of either temperature or oxygen on the growth of three different species of bacteria.

**Materials:**

- 2 - 200 mL TSB cultures of *E.coli, Staphylococcus aureus*
- Spectrophotometer and cuvettes
- 10 mL tube of TSB to use as blank

Procedure

1. Obtain two 200 mL cultures of bacterium.
2. Decide which factor you want to manipulate (temperature or aeration rate), and how you are going to vary them (i.e. what two stir plate settings or what two temperatures). Label the flasks with the experimental condition.

3. Use sterile TSB to zero the spectrophotometer at 686 nm (reading absorption). Record the absorption of each of your two cultures in Table. Use a separate Pasteur pipette for each culture. Discard the samples in the waste beaker after each measurement.
4. Set up your cultures and note the time in Table. This is time "0".
5. Determine O.D. readings every 20-30 minutes for both of your cultures. Record the data in Table.
6. Use your conversion factor to determine the concentration of cells at each time point for both cultures, and record the results in Table.
7. Construct growth curves of cells/mL vs. time for both of your experimental conditions. Label the phases of growth seen in each curve.
8. Determine the $\log_{10}$ of each cell concentration where the cultures were in exponential phase, and record in Table.
9. Construct growth curves of $\log_{10}$ cells/mL vs. time for both of your experimental. Only include those time points where cultures were in exponential phase (e.g. the plot should be linear).
10. Fit a regression line to each curve, and include the equations on the graph.
11. From the slope (m) of each line, determine g (remember that g = 0.301/m).

a. _____g =_____min.

b. _____g =_____min.

## MEASUREMENTS OF CELL BIOMASS CONCENTRATION

### Introduction

The cell density can be quantified in two basic ways: as grams of dry or wet weight per liter of sample, or as number of viable/dead cells per ml. The cells in a sample can be separated from the broth and weighed while they are wet, or the cells may be thoroughly dried before weighing. The dry weight measurement usually gives a much more consistent result than the wet weight. Alternatively, the number of cells can be counted either by successively diluting the original sample and plating on a Petri dish, with the help of a microscope and a counting chamber, or with an automated cell counter such as a Coulter counter or a cytoflowmeter. The plating method

detects only the viable cells; whereas, the automated cell counters can only detect the total number of cells.

All of the above methods either require the availability of expensive equipment or the substantial investment of time. In reality, the most often used method simply monitors the optical density of the sample. The absorbance of the sample measured in a spectrophotometer is correlated to either the dry weight or the number of cells per volume.

Biomass concentration is one of the most critically needed measurements in fermentation studies. It is also one of the most difficult and unreliable ones. For example, all the above dry/wet weight methods and all the automated counting equipment fail completely if the broth contains other insoluble particulate matter, which is often the case in a practical fermentor. Similarly, the optical density measurement only has limited usefulness if the fermentation broth is not clear. In addition, these methods cannot distinguish the viable cells from the dead ones. On the other hand, the standard plate count can detect viable cells among other particulate matters. However, the method requires elaborate preparations, and it takes 24-48 hours for the cells to be incubated and counted; the cost of Petri dishes and media can also be prohibitive. Consequently, the direct plate count is useless in feedback control of a fermentation process; it is mainly used industrially to countercheck other measurements, especially the optical density.

In this experiment, the cell density of a given sample will be measured by wet weight and dry weight.

**Reagents and Instruments**

- Flask of culture
- Nutrient (YPG) agar
- Sterile water
- Graduated cylinder
- Filtration unit with vacuum pump
- Filter membrane or weighing pan
- Centrifuge

- Oven, 100 ºC
- Balance
- **Procedures**

**Dry/Wet Weight Measurement**

1. Dry in an oven an empty aluminum weighing pan or a sheet of cellulose acetate filter membrane, 47mm in diameter, 0.45µm in pore size. Weigh them and store them in a desiccator lined with Drierite (anhydrous $CaSO_4$).
2. Stir the flask to suspend the culture evenly. Pour out 100 ml of the culture into a graduated cylinder.
3. Separate the cells from the broth either by centrifugation at 10,000 g for 5 minutes or by filtration. In the case of centrifugation, carefully discard the clear broth and scrape the cell paste from the centrifuge tube into a weighing pan. Rinse the centrifuge tube with a few ml of water. Pour the rinse water into the weighing pan, as well. In the case of filtration, the culture is poured into the holding reservoir fitted on the filter membrane. A vacuum is applied to pull the liquid through the membrane. Rinse the reservoir with a few ml of water and scrape any paste adhering to the glassware. The wet weight of the culture is measured immediately after all the water has been pulled through.
4. Dry the cell paste in an oven set at 100ºC. The cells will be charred and the filter membrane will be burned if the temperature of the oven is set too high. Measure the weight of the pan/filter plus the cell paste periodically until there is no further decrease in the dry weight. It will take 6-24 hours to dry the sample completely, depending on the oven temperature and the thickness of the paste. Calculate the difference in the weight, and express the dry weight in g/l.

**OBSERVATION AND RESULT**