CHAPTER 3

MATERIAL AND METHODS
MATERIAL AND METHODS

MATERIALS

The details of various raw material and chemicals used for experimental work are described below.

3.1 Raw Material Used

The following raw materials were used during experimental work:

Anionic Surfactant: Linear Alkyl Benzene Sulphonate (LABS, Acid Slurry)
Alpha Olefin Sulphonate (AOS)
Nonionic Surfactant: Alcohol Ethoxilate
Builders: Soda Ash (Commercial Sodium Carbonate)
Sodium Tripolyphosphate (STPP)
Zeolite
Sodium Silicate (Commercial)
Fillers: Sodium Sulphate (Commercial)
Dolamite
Common Salt (NaCl)

All the above materials were procured from local detergent manufacturer M/S Kanpur Detergent Pvt. Ltd., Chaubepur, Kanpur Dehat and local market of Kanpur as commercial samples used for detergent manufacture.
3.2: Enzymes Used:

The following four enzymes were used in the study are:

1) **Protease:** Protease used in detergent formulation is Savinase 8.0T in granular forms. Savinase 8.0T was procured from Novo Nordisk Enzymes Pvt. Ltd., Bangalore. Savinase is a serine-type protease produced by submerged fermentation of a genetically modified *Bacillus* microorganism.

2) **Lipase:** Lipase enzyme was used in detergent formulation is Lipolase 100 L in granular forms. It was procured from Novo Nordisk Enzymes Pvt. Ltd., Bangalore. Lipolase is a lipase from *Thermomyces lanuginosus* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism.

3) **Amylase:** Amylase enzyme was used in detergent formulation is Termamyl in granular forms. Termamyl was procured from Novo Nordisk Enzymes Pvt. Ltd., Bangalore.

4) **Cellulase:** Cellulase enzyme was used in detergent formulation is Cellulzyme 1.0L. It was supplied by Novo Nordisk Enzymes Pvt. Ltd., Bangalore. Cellulzyme is a cellulase complex produced by submerged fermentation of a *Humicola* microorganism.

3.3: Chemicals Used:

The following chemicals were used for various analyses during experimental work are described below.

<table>
<thead>
<tr>
<th>Name of Chemical</th>
<th>Grade</th>
<th>Suppliers / Make</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>LR Grade</td>
<td>Qualigens</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>LR Grade</td>
<td>Ranbaxy</td>
</tr>
<tr>
<td>Chemical</td>
<td>Grade</td>
<td>Supplier</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Benzethonium chloride</td>
<td>AR Grde</td>
<td>Qualigens</td>
</tr>
<tr>
<td>White oil</td>
<td>LR Grde</td>
<td>Ranbaxy</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>GR Grde</td>
<td>Qualikems</td>
</tr>
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<td>Coconut oil</td>
<td>LR Grde</td>
<td>Ranbaxy</td>
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<td>S. D. Fine chemicals</td>
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<td>Carbon Black</td>
<td>AR Grde</td>
<td>Qualikems</td>
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<td>Sodium tungasate</td>
<td>AR Grde</td>
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<td>Sodium molybdate</td>
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<td>Phosphoric acid</td>
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<td>Tyrosin</td>
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<td>Casein</td>
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<td>3, 5 dinitro salicylic acid</td>
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<td>Glucose</td>
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<td>Merck</td>
</tr>
<tr>
<td>α-naphthol</td>
<td>LR Grde</td>
<td>Merck</td>
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</table>
Sulphuric acid AR Grade Qualikems
Starch LR Grade Merck
Cholesterol LR Grade Qualigens
Triethanolamine LR Grade S.D. Fine Chemicals
Linolenic acid LR Grade S.D. Fine Chemicals
Oleic acid Commercial S.D. Fine Chemicals
Squalene Commercial S.D. Fine Chemicals
Palmetic acid Commercial S.D. Fine Chemicals
Steric acid Commercial S.D. Fine Chemicals
Olive oil Commercial Figaro

Cloths: The 100 % cotton cloths used for making swatches were procured from Narsing Giriji Mills, Solapur, India.

Blood: Stable Blood was collected from Pathology.

Lipstick: Red Lipstick (Lakme Pvt. Ltd.) was purchased from local market of Kanpur.

Soup Powder: Corn soup powder and tomato soup powders were purchased from Grocery market.

METHODS

3.4: Methods for Performance Evaluation:

Various performance of experimental samples were evaluated by using the B. I. S. methods as follows:
3.4.1: **Active Matter** (164):

A solution of sample (Anionic detergent) with methylene blue was shaken with chloroform which dissolve the methylene blue salt of the sample. The mixture was titrated with benzethonium chloride (cationic active agent) which after it has combined with all the free anionic detergent, begin to displace methylene blue from the salt. The end point was taken when sufficient methylene blue has been displaced into the aqueous layer to produce phase of equal color intensity. As the reaction is not stoichiometric, it was essential to carry out standardization using a known anionic detergent similar in nature to the unknown.

Anionic active matter as sodium alkyl benzene sulfonate was calculated as follows:

\[
\text{Anionic active matter present by mass} = \frac{342 \times V \times I \times 5}{M}
\]

Where,

- 342 = Molecular mass of sodium alkyl benzene sulfonate taken for calculation
- V = Volume in ml. Of benzethonium chloride solution added
- I = Molarity of benzethinium chloride solution, which has taken 0.04M for tests
- M = Mass in gm of the sample taken which was taken 4.5 gm for tests.

3.4.2: **Active (Reserve) Alkalinity and Total Alkalinity**:

10 gm of detergent sample was mixed in 500 ml distill water. pipet out 50 ml. of the solution in a conical flask, adds 2-3 drops of phenophthalin indicator. This solution is titrated with 0.1N HCl. The end point will be V1 (volume of HCl). Add 2 drops of methyl
orange indicator to the solution and titrated again with 0.1N HCl. The end point will be
V2 (vol. of HCl).

Calculation:

\[
\text{Active Alkalinity} = 0.31 \times \frac{V1}{W}
\]

\[
\text{Total Alkalinity} = 0.31 \times \frac{V2}{W}
\]

Where \( W \) = Weight of sample

3.4.3: Foaming Power (165):

Ability to produce foam. A mass of small gas cells separated by thin film of liquid
and formed by the juxtaposition of bubbles giving a gas dispersed in a liquid is known as
"Foam".

Foaming power was characterized by volume of foam obtained under specific
experimental conditions. The decay of this volume of foam during the five minutes
following its formation was also relevant. This was measured by Ross-Miles method. In
this method, a measured cylinder of 1 lit. capacity and a separating funnel of 500 ml,
capacity was placed about 50 cm from the bottom of the cylinder. Walls of the cylinder
were rinsed with 50-ml sample solution. Then the funnel was filled with rest of the
solution. Now stop clock had opened, when all of the solution had run out of the funnel,
start the stopwatch. Reading of the foam height was taken and other reading were taken at
the interval of 5 minutes up to 30 minutes. Reading was taken by measuring the foam
production at the top of the foam column at the highest average height to which the rim
of the foam has reached. This height is proportional to the volume of air remaining in the foam.

3.4.4: Relative Dispersing Power (166):

A known amount of finally divided carbon black was suspended in different concentrations of the sample solutions for different duration of time. After a definite interval of time say 1 hr, 3 hrs and 24 hrs. A known amount of the solution was taken from the center of the solution and the amount of solid dispersed in the liquid was determined.

25 gm of different sample was dissolve separately under test in sufficient amount of water and volume of each solution was made up to 1 liter. 4 gm of carbon black was taken in 250 ml graduated cylinder, 5 ml white oil and 40 ml 2.5 percent sample solution was added, then volume was made up to 200 ml by adding more water. Similar solution of all the samples in separated cylinder was prepared. Each cylinder was stoppered, held them upright and tilted in clockwise direction to invert with stopper down and restored back in the same way. This procedure was repeated 10 times and then kept the cylinder stationary without disturbing the contents. After a period of one hour, 5 ml of solution from the center of each cylinder was pipet out and transferred each aliquot portion to a previously tared petridish. Solutions in different dished was evaporated on water bath and dried the residue at 105-110°C to constant weight. The procedure was repeated, but pipeting out the percentage of solid dispersed by each sample was calculated by:

\[
C = \frac{W - D}{4000}
\]

Where

C = Percentage of solid dispersed
$W = \text{Weight in mg of the residue, and}$

$D = \text{Weight in mg of the surface active agent present in 5 ml. Of the solution of}$

Surface active agent when dried at 105-110°C to constant weight.

3.4.5: Wetting Power (167):

A skein of grey cotton yarn was submerged in the test solution and time taken for the air in the yarn to be replaced by penetration of the solution was recorded. The end point was observed at the moment when the skein sinks. 500 ml test solution in water was prepared by dissolving 5 g of the surface active agent under test. 250 ml of solution was transferred to a 600 ml beaker and 250 ml water added to it. This solution was transferred to 500 ml graduated cylinder. A hook, anchor and skein was taken to the cylinder containing test solution. The hook was dipped into the solution until the surface was just touched. Then skein was dropped in the solution and simultaneously stopwatch started the sinking time was attained when the skein sinks and the hook just reached the level of the anchor. This time was noted.

3.4.5: Detergency Test (168):


Preparation of Fabric Specimen:

White cotton fabric were put in a 0.8% NaOH solution and boiled for 1 hour then the solution was decanted. Treated fabric was rinsed with distilled water several times until the pH of the rinse was neutral. Treated fabric were then put in 0.13% neutral soap solution, boiled for 30 minutes, and rinsed thoroughly until the fabrics were free of soap. Fabric was air dried overnight at room temperature prior to staining and detergency tests. For the
purpose of removing the finishing agents from the finished cloth prior to soiling the cloths were pre-treated. The fabric was scoured with hot water for over night then fabric was rinsed with normal water. After that the fabric was scoured with an almost neutral soap solution of .30 % concentration at 90°C for 15 min. The fabric was finally rinsed a number of times with soft water until the rinsing was free from soap. The fabric was allowed to dry at room temperature.

**Fabric soiling:**

Various types of soils were prepared and soiled with fabric for evaluating the performance of enzyme based detergent.

**Indian Standard soil** (168): the soil consisted 9.0 gm of coconut oil, 4.5 gm of coconut oil fatty acid, 1.87 gm refined mineral oil, .90 gm of lanolin (anhydrous), 3.5 gm of homogenous stable colloidal suspension of graphite and rest to make 1000 ml of carbon tetra chloride. All the contents of soiling mixture were taken in the trough. Swatches (10 cm × 10 cm) were dipped in this solution one by one and then dried at room temperature. This process was repeated at least for five times. These swatches were taken for washing tests.

**Denatured egg/cotton swatches** (169): Whole egg were homogenized with 0.5% drawing ink and 0.1% ethoxilated nonyl phenol. Cotton swatches were immersed in the soiling mixture and, immediately thereafter squeezed between rubber rollers. After drying overnight at ambient temperature, the egg protein was denatured by soaking the swatches for 10 min in tap water at 60°C, rinsing in running tap water, squeezing between rubber roller and, finally, drying overnight at ambient temperature.

**Native egg/cotton** (169): The procedure is identical to that described for denatured egg/cotton, except that the denaturation procedure is omitted.
Spinach / cotton (169): Cotton swatches were immersed in spinach juice squeezed between rubber roller and dried at room temperature. The impregnation with spinach juice was repeated. In order to denature the protein, the dry swatches were then soaked in tap water at 70°C for 10 min, rinsed in running tap water, squeezed between rubber rollers and finally dried overnight at ambient temperature.

Blood/cotton swatches (169): Fabric was immersed in citrated blood (collected from a Krishna pathology, Kanpur), immediately thereafter squeezed between rubber rollers and dried overnight aged by soaking for 8 min in tap water at 60°C, followed by rinsing, squeezing and drying at ambient temperature overnight.

Blood/ Milk/ carbon black (170): Cotton cloth soiled with a composition containing 30 parts stabilized blood, 30 parts water, 0.4 part carbon black.

Cacao / Milk / Sugar (170): Cotton cloth dipped in a soil solution containing 5 parts cacao, 2.5 parts sugar, 50 parts milk and 50 part water and than air dried.

Olive Oil/Carbon black (170): cotton cloths dipped in a soil solution containing 0.5 part carbon black, 1 part olive oil, 100 part water and ethoxilated nonyl phenol as emulsifier.

Preparation of oily dye stain (171): First, an artificial carbon black colloidal suspension (CABL) was prepared as follows: arabic gum (3.2 g) was dispersed in 15-ml distilled water and heated to dissolve. Carbon black (2.3 g) was combined with 10 ml of 95% ethyl alcohol in a porcelain grinder, 25 ml water was added; the mixture was ground for 30 min. Dissolved gum arabic was added to the mixture, followed by additional grinding for 2 min. the mixture was transferred to a large beaker, including several rinses of the grinder. Total volume of the liquor was brought to 1,500 ml with ethyl alcohol/distilled water (1:1, vol/vol). The diluted solution was the so called CABL.
Second, oily liquor was made from castor oil, liquid paraffin, and lanolin (1:1:1, w/w), with lecithin (0.4 g/ml in 50% ethyl alcohol) as an emulsifier. The weight ratio of lecithin and mixed oil was 2:1.

To prepare the oily dye stain, oily liquor (25 ml) was added gradually into a beaker that contained heated (55°C) CABL (500 ml). The oily-liquor flask was rinsed thoroughly with 25 ml of 50% ethyl alcohol, and all the rinses were poured into the heated beaker. The oily-CABL mixture was heated (55°C) for 30 min with continuous stirring and then cooled to 45°C before filtration through fine cloth to remove the foam in the upper layer. The filtered oily-dye liquor (stain) was used to soil the dried fabrics.

**Lipstick Stain:** The red lipstick (lakme pvt. Ltd.) was uniformly rub in the 5*5 cm² cotton fabric surface.

**Corn Soup:** corn soup powder (noor pvt. Ltd.) was dissolved in water and boiled. After cooling the soup the cloths were dipped in the solution and air-dried.

**Tomato soup:** Soup powder was dissolved in water, boiled and cool the soup and then cotton cloths was dipped in the solution and air-dried.

**Particulate matter/Synthetic Sebum Soil (172):**

Air born particulate combined with synthetic sebum. Collect the vacuum cleaner dirt or street dirt as a natural soil. The soil was removed from the filter cloths of a small vacuum cleaner and the entire contents of the dust bag were put into a large beaker partially filled with ethanol. The mass was alternatively stirred and decanted until the supernatant liquid was clear. This removed all the oil and reduced the organic content. After drying on the steam bath, the contents were sieved through a 200-mesh screen to remove large particles. Now 86 g of air born particulate was mixed with 8 g carbon black,
4 g black iron oxide and 2 g yellow iron oxide. The particulate mixture was then suspended in the emulsified sebum and this mixture was padded on the cloth.

**Synthetic Sebum Composition:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmatic Acid</td>
<td>10%</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>5.0%</td>
</tr>
<tr>
<td>Coconut Oil</td>
<td>15%</td>
</tr>
<tr>
<td>Paraffin</td>
<td>10%</td>
</tr>
<tr>
<td>Spermaceti</td>
<td>15%</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>20%</td>
</tr>
<tr>
<td>Squalene</td>
<td>5.0%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.0%</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>10.0%</td>
</tr>
<tr>
<td>Linolenic Acid</td>
<td>5.0%</td>
</tr>
</tbody>
</table>

Heat 50 gm of synthetic sebum until melted. Add 4 gm of Oleic Acid 8 gm of triethanolamine and 4 gm of particulate mixture. Thoroughly mix. Add an amount of water equal to the volume already measured and stir. Then add 50 more ml of water again and restir. Homogenize this mixture twice with a hand homogeniser. Make up to 1000 ml with water at room temperature and mix again.

**Clay on Cotton Cloth** (173): To prepare clay swatches, local clay is sieved through a #200 sieve. Then, 200 g of clay are mixed with 800 ml of deionized water in a blender at high speed. The dispersion can be applied to the cloth by padding or with a doctor blade.

**Size of Test Fabrics:**

The dimension of all swatches was 10 X 10 cm.

Soiled fabric were aged three days at room temperature (25°C) with a relative humidity of 70%. The whiteness of the fabric was determined by reflectometer with pure white magnisium carbonate as a reference standard. Five reading from each side of the fabric were taken and the average value of ten reading was regarded as the whiteness intensity.
**Washing test:**

The detail of the procedure used for washing is given in Table 1. After washing, the test materials was rinsed in running tap water and ironed. Whiteness was measured with an Elrepho reflection photometer with filter R-46 against an MgO-standard. Detergency was calculated as follows (174):

\[
\text{Detergency (\%)} = \left[ \frac{(R_w - R_s)}{(R_o - R_s)} \right] \times 100
\]

Where \( R_w \), \( R_s \), and \( R_o \) are the reflectance of washed cloth (after washing), of soiled cloth (before washing), and of unsoiled cloth, respectively.

All washing experiments were carried in duplicate in the Terg-O-Tometer tests.

**Table 3.1: Washing Procedure Used**

<table>
<thead>
<tr>
<th>Apparatus</th>
<th>Terg-O-Tometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>100 rpm</td>
</tr>
<tr>
<td>Volume of washing</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Solution, mL</td>
<td>4 g/liter</td>
</tr>
<tr>
<td>Detergent conc., g/liter</td>
<td>3 No. of swatches /L</td>
</tr>
<tr>
<td>Soaking time</td>
<td>15 min.</td>
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<tr>
<td>Washing time, min</td>
<td>15 min.</td>
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<tr>
<td>Rinsing time, min</td>
<td>10 min.</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>40 ppm</td>
</tr>
<tr>
<td>Water hardness, ppm</td>
<td>200 ppm</td>
</tr>
</tbody>
</table>
3.5: Enzyme Assay Method

3.5.1: Assay Method of Alkaline protease:

Anson Method (175): The activity of alkaline protease was estimated by using the modified Anson’s method. This method is based on liberation of tyrosine in a trichloroacetic acid filtrate and was colorimetry estimation using folin reagent.

Reagents

1) Folin-Ciocalteau reagent: this is a solution of sodium tungasate and sodium molybdate in phosphoric and hydrochloric acid. Two-fold dilution of this commercial reagent was made with an equal volume of distilled water.

2) Standard tyrosine solution: 0.1 mg/ml aqueous solution in distilled water.

3) Casein- solution: 0.5% (w/v) casein (Hammersten) solution in 0.2M Glycine- NaOH buffer (pH 10.0) was made by heating at 70°-75°C for 5 minutes.

4) Trichloro-acetic acid solution: 5% (w/v) aqueous solution in distilled water.

5) Sodium- carbonate solution: 0.4 M aqueous solution in distilled water.

6) Buffer Solution : 0.2 M Glycine-NaOH buffer

    Stock solutions:

    A: 0.2 M Glycine solution (15.01 g in 1000 ml.)

    B: 0.2 M NaOH

(pH 10.0) 50 ml of A + 32 ml of B, diluted to a total of 200 ml with distilled water.

(pH 9.0 ) 50ml of A + 8.8 ml of B; diluted to a total of 200 ml with distilled water.
Standard Curve for Tyrosine

A varying concentration (0.01 mg/ml to 0.1 mg/ml) of tyrosine solution were prepared by taking 0.1 to 0.9 ml of standard tyrosine solution (0.1 mg/ml) in different test tubes and the volume was made up to 1 ml. With distilled water, 4 ml of 0.4 M Na₂CO₃ and 1 ml two fold diluted Folin reagent was added in to each test tube and the contents were thoroughly mixed. After standing for 30 min, absorbance was measured at 660 nm and a calibration curve was plotted between absorbance and tyrosine concentration (Fig. 3.1).

Procedure for determination of enzyme Activity

A 2 ml aliquot of casein solution (0.5% w/v) was added to a test tube and equilibrates in a 37°C water bath for 5 minutes. 0.05 mg of an enzyme was added to the above aliquot and was incubated at 37°C for 10 minutes. At the end of the incubation period the reaction is terminated by addition of 4 ml of 5% (w/v) trichloroacetic acid. After standing for 10 minutes the suspension was allowed to pass through filter paper.

A zero time incubation blank (control) was prepared by reversing the sequence of addition enzyme and 5% (w/v) trichloroacetic acid. For the blank solution enzyme was not added to the aliquot.

Again a set of three tubes with 0.5 ml supernatant from blank control and sample were prepared with the addition of 4 ml of 0.4 M Na₂CO₃ and 0.5 ml two-fold diluted Folin reagent. After 30 min the blue color developed was read at 660 nm on UV-visible spectrophotometer (Model UV-1601) against blank. The values for the controls were subtracted from those of the samples.

Calculation of Enzyme activity:
Enzyme Activity = \( \frac{\Delta A \times D}{T \times W} \) units/ml

Where

\( \Delta A \) = Concentration difference of tyrosine corresponding to difference in absorbance of sample and control.

\( D \) = Dilution factor i.e. factor by which enzyme was suitably diluted with buffer.

\( t \) = time-period of incubation (10 min.)

\( W \) = weight of enzyme taken for incubation.

Unit of Enzyme Activity

One unit of enzyme activity is defined as that amount of enzyme which catalyses the release of Folin positive amino acids equivalent to 1 nano-moles of tyrosine in 1 minute under assay conditions. Activity is expressed as units per mg of enzyme.

3.5.2: Assay Method of Lipase activity.

One unit of activity is defined as the amount of enzyme that liberates 1 \( \mu \text{mol} \) equivalent to fatty acid from olive oil in 1 min under the analytical condition. Activity of lipase was expressed as its hydrolytic activity measured by olive oil emulsion method.

Requirement:

1) Phosphate buffer (pH 7.0)

Stock Solution

A) 0.2 M solution of monobasic sodium phosphate (27.8 gm in 1000 ml distill water)

B) 0.2 M solution of dibasic sodium phosphate (53.65 gm of \( \text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O} \) in 1000 ml distill water).
2) Lipase solution: 1mg. Lipase in 10 ml phosphate buffer.

3) Olive oil

4) 0.05 N NaOH Solution

Procedure:

Lipolytic activity of lipase was tested with olive oil emulsion, by the surfactant addition method of Kawase et al (176), with some modification. Lipolase (2.5u/ml) was dissolved in .05 N glycine buffer (pH 9.4). Substrate was prepared by emulsifying 25 ml olive oil with 75 ml polyvinyl alcohol (PVA) (4% PVA in glycine buffer, pH 9.4), henceforth refereed to as olive oil emulsion. PVA served as surfactant to stabilize the olive oil emulsion. In the typical assay of lipolytic activity, glycine buffer (4mL) and olive oil emulsion (5 ml) were combined in a clean 100-ml Erlenmeyer flask. The pH of the mixture was adjusted as necessary by the addition of 10mM NaOH and then preincubated in water bath until the desired temperature was reached. Lipolytic activity was started by addition of the dilute lipase solution (1-ml) into the flask, and incubation in the water bath was continued at the respective optimum temperature with shaking (250 rpm). After 10 minutes, 15 ml of 95% ethyl alcohol was added to reaction mixture to stop the hydrolysis, and the fatty acid content in the mixture was determined by titration to the end point (phenophthalein as indicator) with .05 N NaOH. As a blank, the assay procedure was conducted by adding the enzyme after addition of 95% ethyl alcohol. Activity of lipase (unit/g) was calculated as follows:

Unit/g = (S-B) 50 f / (t) (W)

Unit/ml = (S-B) 50f / (t) (V)
Where, \( S \) = volume (ml) of 0.05 N NaOH to titrate lipase hydrolysates; \( B \) = volume (ml) of 0.05 N NaOH to titrate blank solution; \( f \) = dilution factor for lipase solution; \( 50 \) = \( \mu \)mol of NaOH contained in 1 ml of 0.05 N NaOH solution; \( t \) = reaction time (10 min); \( W \) = weight (g) of the lipase powder in 10 ml assay mixture; and \( V \) = volume (ml) of the lipase solution added to the assay mixture.

### 3.5.3: Assay Method of Amylase:

For determination of amylase activity at 50\(^{\circ}\)C with DNS reagent using 1 % starch in phosphate buffer (pH 7) as substrate. One unit of enzyme activity may be defined as microgram of glucose liberated from hydrolysis of 1 % starch by 1 ml. of enzyme in one minute.

### Analytical Method:

#### 3.5.3.1: Determination of Reducing Sugar using 3-5 dinitrosalicylic acid reagent (177):

**Materials:**

1) Sodium potassium tartrate (dissolve 300 gm of this salt in 500 ml. Distilled water.

2) 3-5 dinitroslaicylic acid (dissolve 10 gm of this reagent in 200 ml of 2 M NaOH)

3) Dinitro Salicylic reagent (prepared this fresh by mixing solution (1) and (2) and making upto 1 liter with distill water)

4) NaOH (2 mol/liter)

5) Stock sugar standard (0.1 % glucose)

**Procedure:**

To 1 ml. of properly diluted sugar solution, 3 ml. of DNS reagent was added and kept for 5 minutes in boiling water bath, cooled and 20 ml. of distilled water was added.
Optical density was then determined at 540 nm against a reagent blank. A calibration curve (Fig.3.2) was prepared with D-glucose solution as standard. The concentration of glucose solution was in the range of 200 µgm to 1000 µgm. The concentration of reducing sugar was determined from the calibration curve (Fig.3.2).

3.5.3.2: Estimation of Starch (178)

Material

1) 95 % alcohol, 50 % alcohol, 10 % alcohol solution.
2) Concentrated HCl
3) 1 N NaOH
4) α-naphthol
5) Concentrated H₂SO₄
6) 1 % starch solution

Procedure:

10 ml of 1 % starch solution plus 1 ml of 1 % enzyme solution was heated to 50°C. To above starch solution 100 ml. of 95 % alcohol was added and centrifuged till the precipitate settled at bottom. Residue was filtered and washed with 50 % alcohol until the filtrate gave no test for sugar (to a few ml of filtrate in a small narrow test tube. 10 % alcoholic solution of α-naphthol was added then 1 ml. conc. H₂SO₄ allowed to flowed slowly down at the side of test tube. If any red ring would appear, it shows presence of sugar.

Residue was transferred to a 500 ml conical flask with about 100 ml of distilled water and 20 ml. conc. HCl and placed a funnel in the neck of the flask to prevent evaporation and kept in a boiling water bath for 2.5 hr. then it was cooled and neutralized
with NaOH. Determination of reducing sugar was carried out with the help of DNS reagent and starch concentration defined in terms of reducing sugar

\[ \% \text{Starch} = \% \text{Reducing Sugar} \times 0.90 \]

### 3.5.4: Assay Method of Cellulase:

One unit of enzyme activity may be defined as microgram of glucose liberated from hydrolysis of 1% CMC by 1 ml. of enzyme in one minute. Cellulase activity was routinely assayed by measuring the rate of formation of reducing sugar from CMC as a substrate, in 0.1 M-glycine/NaOH (pH 9.5) as the reaction buffer (179). The reducing sugars liberated from CMC were quantified as glucose by the 3,5-dinitrosalicylic acid procedure (3.4.3.1).

**Material:**

1) Sodium potassium tartrate (dissolve 300 gm of this salt in 500 ml. Distilled water.
2) 3-5 dinitrosalicylic acid (dissolve 10 gm of this reagent in 200 ml of 2 M NaOH)
3) Dinitro Salicylic reagent (prepared this fresh by mixing solution (1) and (2) and making upto 1 liter with distill water)
4) NaOH (2 mol/liter)
5) 0.1 M glycine/NaOH buffer (pH 9.5)
6) CMC solution (add 10 gm. of CMC to 800 ml boiling distill water with continuous stirring. To this add 100 ml glycine/NaOH buffer. The volume make up to 1 lit with distill water).
7) Enzyme solution (1mg enzyme in 10 ml glycine/NaOH buffer)

**Procedure:**
I ml of 1 % CMC solution and 1 ml enzyme solution was taken in a test tube. This mixture was incubated at 40°C for 30 minutes. 3 ml. of DNS reagent was added in the test tube, and then test tube was kept in boiling water bath for 5 min. and then cools. The absorbance was read at 550 nm to know the amount of sugar from the graph (Fig. 3.2) (1 mg of glucose = .0925 enzyme activity.)
Fig. 3.1: Standard curve for Tyrosine
Fig. 3.2: Calibration Curve for Glucose