ANNEXURE – I
TOTAL PENTOSAN ESTIMATION

Principle
Pentosan in the sample is converted to furfural by boiling with HCl, which is measured with HCl, which is measured colorimetrically using orcinol-iron reagent.

Reagents
1. 12% HCl (w/w) 101.6 ml HCl (sp. gr. 1.18) made up to 1 L with distilled water
2. Stock iron solution 0.990 g Ferric ammonium sulphate in 1 L concentration HCl
3. Oncinol-iron reagent 333 ml stock iron solution
   467 ml concentration HCl
   200 ml distilled water
   2.0 g Orcinol (phenol)
4. Standard xylose solution 0.5 g D-Xylose + 1.0 g sodium benzoate in DW – made up to 500 ml in volumetric flask

Procedure
- To 400 mg sample, 100 ml 12% HCl and broken porous was added and heated so that the rate of distillation was 30 ml per 10 min.
- Run 12% HCl was run from the funnel every 10 min to make up the original volume and 360 to 400 ml was distilled.
- The condenser was rinsed down with 12% HCl.
- The distillate was cooled and made up to 500 ml with 12% HCl.
- A duplicate determination in the same manner using 10 ml standard xylose solution in place of the flour was performed.

Furfural determination

Pipette out 1.0 ml and 2.0 ml aliquot portions of the distillate was pipetted out into two test tubes.
Standard curve: 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 ml of the standard xylose distillate was pipette out into 5 test tubes (0.0 to 50 μg xylose).
- Each aliquot was adjusted to 3.0 ml with 12% HCl to which 9.0 ml Orcino – iron reagent was added.
- The tubes were closed with small glass bulbs and placed in boiling water bath for 30 min and then cooled rapidly under cold running water.
- Optical density of the colour formed was read at 670 nm.
- The amount of xylose in the sample was determined using standard xylose graph.

Total Pentosan in the sample (%) = % xylose X 0.97
ANNEXURE – II

PECTIN ESTIMATION

Principle
Pectin is extracted from plant material and saponified. It is precipitated as calcium pectase by the addition of calcium chloride to an acid solution. After thoroughly washing to eliminate chloride ions, the precipitate is dried and washed.

Reagents

1. 1 N Acetic acid : 57.6 ml glacial acetic acid in 1 L DW
2. 1 N NaOH : 40 g NaOH in 1 L DW
3. 1 N calcium chloride : 55 g calcium chloride in 1 L DW
4. 0.3 N HCl : 9.27 ml HCl in 1 L DW
5. 0.01 N HCl : 0.309 ml HCl in 1 L DW
6. 0.05 N HCl : 1.545 ml HCl in 1 L DW
7. 1% silver nitrate : 1 g AgNO₃ in 100 ml DW

Procedure

- To 50 g sample in 1 L beakers, 300 ml 0.01 N HCl was added and boiled for 30 min.
- The sample was then filtered under suction, washed with hot water and the filtrate was collected.
- To the residue, 100 ml of 0.05 N HCl was added, boiled for 20 min, filtered under suction and the filtrate was collected.
- The residue was boiled for 10 min with 100 ml of 0.3 N HCl, filtered under solution and the filtrate was collected.
- The filtrates were pooled and the volume was made up to 500 ml.
- To 100 to 200 ml aliquot of the filtrate in 1 L beakers, 250 ml water was added. The acid was neutralised with 1 N NaOH using phenolphthalcin indicator. The final solution was allowed to stand overnight.
- 50 ml of 1 N acetic acid was added. After 5 min, 25 ml 1 N CaCl₂ was added slowly and allowed to stand for 1 hour.
- After 1 hour, the mixture was boiled for 1 to 2 min, cooled and filtered through pre-weighed Whatman No.1 filter paper.
- The precipitate was washed with almost boiling water until the filtrate was free from chlorine which was confirmed by testing with AgNO₃.
- Filter paper with residue (calcium pectate) was dried overnight at 100°C, cooled in desicator and weighed.

The calcium pectate content in the sample was calculated as follows:

\[
\% \text{ calcium pectate} = \frac{\text{Wt. Of Ca. Pectate} \times 500 \times 100}{\text{ml of filtrate taken} \times \text{sample wt.}}
\]

\[
\% \text{ Pectin} = \frac{\% \text{ calcium pectate} \times 100}{110}
\]

ANNEXURE – III

CELLULOSE ESTIMATION

Principle
Cellulose undergoes acetolysis with acetic-nitric reagent forming acetylated cellodextrins which get dissolved and hydrolysed to form glucose molecules on treatment with 67% H₂SO₄. This glucose molecule is dehydrated to form hydroxy methyl furfural which forms green coloured product with anthrone.

Reagents
- Acetic – Nitric reagent: 150 ml of 80% glacial acetic acid and 15 ml concentration nitric acid.
- Anthrone reagent: 200 mg anthrone dissolved in 100 ml concentration H₂SO₄. Prepared fresh and chilled for two hours before use.
- 67% H₂SO₄

Procedure
- 3 ml acetic – nitric reagent was added to 0.5 g sample in a test tube and vortex mixed.
- The tube was placed in water bath at 100⁰C for 30 min, then cooled and centrifuged for 15-20 min.
- The supernatant was discarded, the residue was washed with distilled water, centrifuged and again the supernatant was discarded.
- To the residue 10 ml of 67% H₂SO₄ was added and allowed to stand for 1 hour.
- 1 ml of the above solution was diluted to 100 ml with DW. To
- 1 ml of this diluted solution, add 1 ml DW and 10 ml of anthrone reagent was added and mixed well.
- The tube was heated in boiling water bath for 10 min, cooled and the colour was measured at 630 nm.

Standard curve
- To 100 mg cellulose in a test tube, add 10 ml of 67% H₂SO₄ was added and allowed to stand for 1 hour.
- 1 ml of the above solution was diluted to 100 ml.
- 0.2 ml, 0.4 ml, 0.6 ml ....... 2 ml of the diluted solution in different test tubes was taken corresponding to 20 – 200 µg of cellulose and the volume was made up to 2 ml with DW. To this 10 ml anthrone reagent was added, boiled to develop colour as in case of sample.

Calculation
The amount of cellulose in the sample was calculated using the standard graph.

ANNEXURE – IV
TOTAL NON-STARCH POLYSACCHARIDE ESTIMATION

Principle
Starch in the sample is completely removed enzymatically after solubilisation and Non-starch Polysaccharides (NSP) is measured as the total sugars released by acid hydrolysis.

Reagents
1. Pullulanase solution:
   Pullulanase suspension was diluted 1:100 with DW immediately before use.
2. α-amylase solution:
   Approximately 700 mg pancreatin powder and 9 ml DW put into centrifuge tube and vortex mixed. Then mixed for 10 min on magnetic stirrer, centrifuged for 10 min. The supernatant solution was used.
3. Dinitro salicylate solution:
   Dinitro salicylic acid – 20 g + phenol – 4 g + sodium sulphite – 1 g + potassium sodium tartrate – 400 g in 1 L 2% NaOH and made up to 2 L with DW.
4. Acetone
5. Ethanol 85%
6. Dimethyl sulfoxide
7. 0.1 M sodium acetate buffer (pH 5.2)
   10.5 ml of 0.2 M acetic acid (11.46 ml in 100 ml) +
   39.5 ml of 0.2 M sodium acetate (27.2 g of CH₃COONa.3H₂O in 1000 ml). Made up to 100 l with DW.
8. 2 M H₂SO₄ : 106.9 ml concentration H₂SO₄ in 1 L DW
9. 12 M H₂SO₄ : 641.4 ml concentration H₂SO₄ in 1 L DW
10. 50% saturated benzoic acid: 1.7 g benzoic acid in 1 L DW
11. Arabinose
12. Xylose
13. Glucose
14. 3.9 M NaOH : 156 g in 1 L DW

Procedure
- 100-200 mg dry sample (1 g wet sample) was taken in 50 or 60 ml screw cap tube.

- Fat extraction and drying:
  - To the sample 40 ml acetone was added and mixed on magnetic stirrer for 30 min.
  - Supernatant was removed after centrifugation without disturbing the residue.
  - The tube was placed in beaker of 65-70°C water on stirrer / hot plate and mixed for 2-3 min until the residue appeared dry.

- Dispersion and hydrolysis of starch:
- 2 ml Dimethyl sulfoxide was added and mixed for 2 min on magnetic stirrer at room temperature and then placed in a beaker of boiling water for 1 hour.
- After 1 hour the tube was removed and immediately 8 ml of 0.1 M sodium acetate buffer (pH 5.2) pre-equilibrated at 50°C was added and vortex mixed then placed in 42°C water bath for 2-3 min.
- Immediately 0.5 ml α-amylase solution followed by 0.1 ml pullulanase solution was added and vortex mixed.
- The capped tube was incubated at 42°C, mixed intermittently for the first hour and left for 16 hours (overnight).
- The capped tube was incubated at 42°C, mixed intermittently for the first hour and left for 16 hours (overnight).
- 40 ml absolute alcohol was added, mixed well by inversion and left for 1 hour at room temperature.
- The tube was then centrifuged for 10 min and the supernatant was removed without disturbing the residue.
- The residue was washed twice with 50 ml of 85% ethanol, mixed well by inversion, magnetic stirred, centrifuged and the supernatant was removed.
- To the residue, 40 ml acetone was added, stirred for 5 min, centrifuged for 10 min and the supernatant was discarded.
- The tube was then placed in a beaker of 65-70°C water on stirrer / hot plate for 2-3 min until the residue appeared dry.

- **Acid hydrolysis of NSP**
  - To the dried residue, 2 ml of 12 M H₂SO₄ was added and immediately dispersed by vortex mixing.
  - The tube was left at 35°C for 1 hour with occasional mixing.
  - After 1 hour, rapidly 22 ml water was added, mixed and then placed in broiling water for 2 hours with continuous stirring. Then cooled to room temperature.

- **Estimation of NSP**

  **Standard solution**

  Using Arabinose : Xylose : Glucose (3 : 4 : 3 by weight) solutions of 1.0, 2.0, 3.0 and 4.0 mg total sugar / ml in 50% saturated benzoic acid were prepared.

  Immediately before use, the above solution were diluted 1:1 with 2 M H₂SO₄ to provide standards of 0.5, 1.0, 1.5 and 2.0 mg sugar / ml in 1 M H₂SO₄.

  - Into separate tubes, the following solutions were taken : 1 ml blank solution (1 ml 50 % saturated benzoic acid + 1 M H₂SO₄ v/v).
  - 1 ml each of standard solution
  - 1 ml of sample hydrolysate
  - To each of the tubes 0.5 ml of 0.5 mg glucose / ml and 0.5 ml 3.9 M NaOH was added and mixed.
  - 2 ml DNS solution was added to each tube and vortex mixed.
  - All the tubes were placed in boiling water bath for 10 min and then cooled to room temperature.
  - 20 ml deoinised water was added to the above tubes and mixed thoroughly by inversion.
  - Absorbance was measured at 530 nm.

**Calculation**

Per cent of NSP in the sample was calculated as follows:
Total NSP % = \frac{AT \times VT \times 100 \times 0.89}{AS \times WT}

Where,

AT - Absorbance of the text solution

VT - Total volume of the test solution (24 ml)

AS - Absorbance corresponding to 1 mg sugar /ml taken from the line of best fit from the sugar standard.

WT - Sample weight (mg)

0.89- Scale factor to convert the experimentally determined monosaccharides to polysaccharides.

ANNEXURE – V

CELLULASE ACTIVITY ASSAY

Principle

The assay is based on the production of reducing sugars i.e., glucose from 50 mg of Whatman No.1 filter paper which then reacts with dinitro salicylic acid reagent to produce colour. The colour produced is proportional to the amount of reducing sugars released and in turn the cellulase activity in the sample.

Unit definition

One filter paper unit (FSU) is defined as the amount of enzyme required to liberate 1 micro mole of glucose from 50 mg of Whatman No. 1 filter paper per minute at 50°C and pH 4.8.

Reagents:

- Citric acid monohydrate
- Sodium hydroxide
- 3,5-dinitro salicylic acid
- D(+) glucose
- Potassium sodium tartrate tetrahydrate
- Phenol
- Sodium meta sulphite
- Sodium meta tri-sulphite
- Whatman No.1 filter paper

Preparation of reagents

1. **0.05M citrate buffer (pH 4.8)**
   
   210 g of citric acid monohydrate was dissolved in 750 ml DW to which NaOH pellets were added until the pH reached 4.3. The solution was diluted to 1000 ml and was diluted the pH was made up to 4.8. This is 1M stock citrate buffer. The stock solution was diluted 1:20 to prepare 0.05M citrate buffer. pH was adjusted to 4.8 with acetic acid or NaOH.

2. **DNA reagent**
   
   a. 8 g of DNS monohydrate was dissolved in 500 ml of DW.
   b. 24 g NaOH was dissolved in 200 ml DW
   c. 5 g phenol was dissolved in 80 ml Dwd.
   d. 20 ml of DNS solution (a) was added to the phenol solution (c).
   e. 120 ml of NaOH solution (b) was added slowly to the remaining solution (a) and stirred thoroughly.
   f. 200 g of potassium sodium tartarate tetrahydrate was added to solution (e).
   g. 5 g of sodium meta tri-sulphite was added to the solution (d) and dissolved.
   h. Solutions (f) and (g) were mixed and the volume was made up to 1 L with DW was filtered. The resulting solution was filtered through absorbent cotton and 5 g of sodium meta bi-sulphite was added and stored in a dark bottle.
Standard Graph

- 300 mg of D(+) glucose (oven dried at 105°C for 2 hrs) was dissolved in 100 ml of 0.05 M citrate buffer.
- Different dilutions of the standard glucose solution was prepared as follows:

<table>
<thead>
<tr>
<th>Standard glucose solution (ml)</th>
<th>Citrate buffer (ml)</th>
<th>Mg of glucose in the solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.5</td>
<td>0.0</td>
</tr>
<tr>
<td>0.1</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>0.2</td>
<td>1.3</td>
<td>0.6</td>
</tr>
<tr>
<td>0.3</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>0.4</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>0.6</td>
<td>0.9</td>
<td>1.8</td>
</tr>
<tr>
<td>0.7</td>
<td>0.8</td>
<td>2.1</td>
</tr>
<tr>
<td>0.8</td>
<td>0.7</td>
<td>2.4</td>
</tr>
<tr>
<td>0.9</td>
<td>0.6</td>
<td>2.7</td>
</tr>
<tr>
<td>1.0</td>
<td>0.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

- To each of the above tubes 3 ml DNS reagent was added, boiled for 5 min, cooled under cold running water.
- The absorbance was read at 540 nm against reagent blank (tube with 0 mg glucose).

Enzyme assay

- Different dilutions of the enzyme sample (1:20, 1:40, 1:80, etc.) were prepared.
- 1 ml citrate buffer was taken in a series of test tubes. To each tube, 0.5 ml of different enzyme dilutions was added. The tubes were incubated at 50°C for 5 min.
- After 5 min, to each of the above tubes, 50 mg Whatman No. 1 filter paper (1x6 cm) was added and vortex mixed.
- The tubes were incubated in water bath at 50°C for 1 hour.
- After 1 hour the tubes were cooled and 3 ml DNS reagent was added.
- Simultaneously the tubes were kept in boiling water bath for 5 min.
- The tubes were then cooled and add 15 ml DW added.
- Enzyme blank.
  Similar to the above tubes except that 0.5 ml enzyme dilution was added following 3 ml DNS addition.
- Absorbance of the sample tubes against enzyme blank was read at 540 nm.

Calculation
The amount of glucose released was found out using the standard graph. Cellulase activity in the sample was calculated as follows:

\[
FPU/g = \frac{\text{Mg of glucose released} \times \text{Dilution factor} \times 100}{180 \times 60 \times 0.5}
\]

ANNEXURE – VI

XYLANASE ACTIVITY ASSAY

Principle
The assay is based on the production of reducing sugars from oat spelt xylan which reacts with 3.5 – Dinitro salicylic acid (DNS) to produce colour. The intensity of colour produced is proportional to the amount of reducing sugars released which in turn is proportional to the xylanase activity in the sample.

Unit definition
One unit of xylanase is defined as the amount of enzyme required to produce 1 micro mole of xylose per minute at pH 4.8.

Reagents
- Citric acid monohydrate
- Glacial acetic acid
- Oat splet xylan (Sigma)
- E.5 Dinitro salicylic acid
- D(+) xylose
- Potassium sodium tartarate tetrahydrate
- Sodium hydroxide
- Sodium meta b-sulphite
- Sodium meta tri-sulphite
- Phenol

Preparation of reagents
1. **0.5M citrate buffer (pH 4.8)**
   210 g of citric acid monohydrate was dissolved in 750 ml DW to which NaOH pellets were added until the pH reached 4.3. The solution was diluted to 1000 ml and was diluted the pH was made up to 4.8. This is 1M stock citrate buffer. The stock solution was diluted 1:20 to prepare 0.05M citrate buffer. pH was adjusted to 4.8 with acetic acid or NaOH.

2. **Xylan substrate**
   2 g of oat spelt xylan and 1-2 pellets of NaOH was taken in a mortar pestle and ground. To this citrate buffer was added until the volume reached approximately 80 ml. The pH was adjusted to 4.8 with glacial acetic acid. The final volume was made up to 100 ml and kept in refrigerator (2-8°C) until use.
   ‘XYLAN SUBSTRATE SHOULD BE PREPARED FRESH”

3. **DNA reagent**
   a. 8 g of DNS monohydrate was dissolved in 500 ml of DW.
   b. 24 g NaOH was dissolved in 200 ml DW
   c. 5 g phenol was dissolved in 80 ml Dw.
   d. 20 ml of DNS solution (a) was added to the phenol solution (c).
e. 120 ml of NaOH solution (b) was added slowly to the remaining solution (a) and stirred thoroughly.
f. 200 g of potassium sodium tartrate tetrahydrate was added to solution (e).
g. 5 g of sodium meta tri-sulphite was added to the solution (d) and dissolved.
h. Solutions (f) and (g) were mixed and the volume was made up to 1 L with DW was filtered. The resulting solution was filtered through absorbent cotton and 5 g of sodium meta bi-sulphite was added and stored in a dark bottle.

**Standard Graph**
- D(+) Xylose was dried to constant weight at 105°C desiccator.
- 225.2 g of D(+) xylose was dissolved in approximately 80 ml of 0.05M citrate buffer. The volume was made up to 100 ml with the same. This given the stock solution of 15 micromoles xylose/ml.
- Different solutions of the standard xylose solution was prepared in a series a of test tubes as follows.

<table>
<thead>
<tr>
<th>Substrate (2% xylan ml)</th>
<th>Standard Xylose solution (ml)</th>
<th>Citrate buffer (ml)</th>
<th>Micro moles of xylose in the solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8</td>
<td>0.0</td>
<td>0.200</td>
<td>0.0</td>
</tr>
<tr>
<td>1.8</td>
<td>0.066</td>
<td>0.134</td>
<td>1.0</td>
</tr>
<tr>
<td>1.8</td>
<td>0.100</td>
<td>0.100</td>
<td>1.5</td>
</tr>
<tr>
<td>1.8</td>
<td>0.133</td>
<td>0.067</td>
<td>2.0</td>
</tr>
<tr>
<td>1.8</td>
<td>0.167</td>
<td>0.033</td>
<td>2.5</td>
</tr>
<tr>
<td>1.8</td>
<td>0.200</td>
<td>0.00</td>
<td>3.0</td>
</tr>
</tbody>
</table>

- All the tubes were placed in 40±1°C water bath for 5 minutes and then 3 ml DNS reagent was added into each tube and the tubes were placed them in boiling water bath for exactly 5 min. Immediately the tubes were cooled under flowing water. 15 ml DW was added to each tube, mixed well and the Absorbance was read against reagent blank (tube with 0 μ moles xylose) at 540 nm.

**Enzyme assay**
- For each sample to be analysed, 1.8 ml of xylan substrate was added into a row of 5 test tubes, 3 for the test and one for the enzyme blank. All the tubes were incubated for 2-3 minutes at 40±1°C. Enzyme blank was prepared by adding 3 ml DNS reagent to the 4th tube followed by 0.2 ml of the enzyme dilution.
- The reaction was stopped by adding 3 ml DNS reagent to each tube. The tubes were covered and placed in boiling water bath for exactly 5 minutes.
- The tubes were cooled under flowing tap water and then 15 ml DW was added.
- The absorbance was read at 540 nm against enzyme blank.

**Calculation**
Micromoles of xylose released was calculated by comparing the absorbance values of the samples with the standard graph. The xylanase activity in the sample was calculated as follows:
I.U./g = \frac{\text{Micromoles of xylose released} \times \text{Dilution factor}}{\text{Wt. Of the sample taken} \times \text{Incubation time}}

ANNEXURE – VII

PECTINASE ACTIVITY ASSAY

Principle

The assay is based on the release of reducing groups (galacturonic acids) from pectin which is then made to react with iodine. The residual iodine is titrated using solution thiosulphate solution. The amount of residual iodine is inversely proportional to the amount of galacturonic acid released.

Unit definition

One unit of pectinase (polygalacturonase) is defined as the amount of enzyme required to release 1 milli mole per min at 25°C and pH 4.0.

Reagents

- 1 M sodium carbonate – 10.6 g of Na₂CO₃ in 100 ml DW.
- 0.1 M iodide – 1.3 g iodine in 100 ml DW.
- 2 M H₂SO₄ – 10.91 ml concentration H₂SO₄ in 100 ml DW.
- 0.05 N sodium thisulphate – 12.41 g in 1000 ml DW.
- 0.1 N NaOH – 0.2 g NaOH in 500 ml DW.
- 0.5% pectin solution – 0.5 g pectin in 100 ml DW. pH adjusted to 4 using 0.1 N NaOH.
- Enzyme dilution – 250 lmg enzyme in 10 ml Dw.

Standard curve

100 ml of D-galacturonic acid monohydrate was adjusted in 100 ml DW to give 1 mg sugar/ml. Different dilutions from the stock solution was made as follows:

<table>
<thead>
<tr>
<th>Stock sugar solution (ml)</th>
<th>Pectin solution (ml)</th>
<th>Sugar in the mixture (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>5.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.1</td>
<td>4.9</td>
<td>0.1</td>
</tr>
<tr>
<td>0.2</td>
<td>4.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>
To each of the above dilutions, 0.9 ml 1 M Na₂CO₃ and 5 ml 0.1 N iodine solution was added and allowed to stand for 20 min at room temperature. Immediately after 20 min, 2 ml of 2 M H₂SO₄ was added and the residual iodine was titrated using 0.5 N Na₂S₂O₅. The volume of Na₂S₂O₅ run down was noted.

**Assay**

- 1 ml of enzyme dilution was mixed with 0.5% pectin solution and incubated at 40°C.

- At different intervals, 5 ml of the aliquot was taken out to which 0.9 ml 1 M Na₂CO₃ and 5 ml 0.1 N iodine was added.

- The mixture was stirred thoroughly and allowed to stand at room temperature for 20 minutes.

- After 20 minutes, the reaction mixture was acidified with 2 ml of 2 M H₂SO₄ and the residual iodine was titrated with 0.05 N Na₂S₂O₅.

The sugars released was calculated by comparing the volume of Na₂S₂O₅ run down with standard curve.

ANNEXURE - VIII

Phytase Activity Assay

Principle

Phytase acts on phytic acid to release inorganic phosphate. The determination of released inorganic phosphate is based on the colour formed by the reduction of a phosphomolybate complex.

Reagents:
- Sodium citrate
- Citric acid
- Sodium phytate
- Trichloroacetic acid
- Ascorbic acid
- Ammonium molybdate
- Conc. sulphuric acid
- Potassium dihydrogen orthophosphate

Equipments
- Water bath set at 37\(^\circ\) C
- Stop clock
- Grade A glassware
- Range of automatic pipettes
- Spectrophotometer set at 820 nm
- Thermometer
- Vortex mixture
- Phosphate free glassware

Unit definition

One phytase unit (FYT) is the amount of enzyme which liberates under standard conditions, one micromoles of inorganic phosphate from sodium phytate in one minute.

Preparation of reagents
- 0.2M Sodium citrate
  - Weigh 58.8 g of sodium citrate and dissolve in 800 ml of distilled water. Make up the volume with distilled water to 1 litre.
- 0.2 Citric acid
  - Weigh 42 g of citric acid and dissolve in 800 ml of distilled water and make up the volume with distilled water to 1 litre.
- Citrate buffer
  - Adjust the pH of the citrate solution to 5.0 with 0.2M citric acid (the consumption of citric acid should be about 385 ml.)
- Substrate preparation
  - Dissolve 1 g of sodium phytate in about 70 ml of citrate buffer. Adjust the pH to 5.0 with citric acid and adjust the volume to 100 ml with buffer.
- 15% TCA solution
  - To prepare 100 ml of 15% TCA, weigh 15g of TCA in a 150ml beaker. Add 60 ml of distilled water and dissolve. Make the volume to 100 ml in a volumetric flask with distilled water.
- 10% Ascorbic acid
  - Weigh 10 g ascorbic acid and dissolve in 75 ml of distilled water. Make up the volume to 100 ml with distilled water.
- 2.5% Ammonium molybdate
- Dissolve 2.5g in 80ml distilled water and make up to 100 ml with distilled water in a volumetric flask.
- 1M sulphuric acid
- Add 55.6 ml of conc. sulphuric acid to 800 ml distilled water. Allow to cool and make to 1000ml with distilled water.
- Reagent C
- Mix three volumes of 1 M sulphuric acid with 1 volume of 2.5% Ammonium molybdate, then add 1 volume of 10% ascorbic acid solution.

**Standard graph**

4.5M phosphate stock solution: Dissolve and dilute 306.2mg of KH2PO4 (dried in desiccator with silica) to 500 ml with water in a volumetric flask
Make the following dilutions from 4.5M phosphate stock solution in distilled water from the stock solution and use these as standards.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Phosphorus m moles</th>
<th>Phytase activity FYT/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/2</td>
<td>90</td>
<td>0.24</td>
</tr>
<tr>
<td>100/1.5</td>
<td>67.5</td>
<td>0.18</td>
</tr>
<tr>
<td>100/1</td>
<td>45</td>
<td>0.12</td>
</tr>
<tr>
<td>100/0.75</td>
<td>33.75</td>
<td>0.09</td>
</tr>
<tr>
<td>100/0.5</td>
<td>22.5</td>
<td>0.06</td>
</tr>
<tr>
<td>100/0.25</td>
<td>11.25</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Pipette 4 ml of each dilution to two test tubes. Pipette 4 ml of water in one of the tube which serves as a reagent blank. Add 4 ml Reagent C and vortex. Incubate at 50°C for 20 minutes then cool to room temp. immediately. Measure the absorbance at 820nm against reagent blank. Prepare a standard curve by plotting the absorbance against phytase activity (FYT/ml). A new standard graph must be plotted with each series of assays.

**Preparation of dilutions**

Samples are diluted in citrate buffer. Weigh the sample accurately in a volumetric flask, dissolve in the buffer and fill to the mark and stir. Dilute further if necessary.

**Assay Procedure:**

**Hydrolysis**

Pipette out 1ml of sample dilution in two test tubes. Add 2 ml of 15% TCA to one of the tubes (blank) and vortex. Put the tubes without TCA in a water bath at 37°C and let them equilibrate for 5 min. Using a stop clock start the hydrolysis by adding sequentially at proper intervals 1 ml of substrate (equilibrate for about 5 min at 37°C) to each tube and vortex. After exactly 15 min. of incubation stop the reaction by adding 2
ml of TCA to each tube. Vortex and cool to room temperature. Add 1ml of substrate to the blank tubes.

Released orthophosphate determination:

- Pipette 0.4ml of each sample after hydrolysis in test tubes. Add 3.6ml of water to each tube. Add 4 ml of reagent C and vortex. Incubate at 50°C for 20 min. and cool to room temp. Measure the absorbance against that of reagent blank at 820nm.

**Calculation:**

Subtract the blank absorbance from sample absorbance (The difference can be 0.1-1).

\[
\text{Phytase activity FYT/g} = \frac{\text{Conc. of the sample from graph} \times \text{dilution factor} \times 40}{15 \times 1000}
\]

40 = dilution factor used to develop the colour
15 = time taken to release the phosphate
1000 = conversion factor from mmoles to micro moles.
ANNEXURE - IX

Amylase Activity Assay

Principle:
Alpha amylase breaks down the alpha (1,4) glycosidic linkages of buffered starch to yield maltose and smaller dextrins. The breakdown products are reacted with an iodine solution and the colour produced changes from blue to a red brown as starch is broken down. The change in colour is followed spectro-photometrically and the end point (dextrinization time) determined. For accuracy, the dextrinization time should take place between 10 and 20 min.

Unit definition
One alpha amylase unit is the amount of enzyme which breaks down 5.26 mg of starch per hour under the conditions specified in this procedure.

Reagents:  
Sodium acetate anhydrous  
Glacial acetic acid  
Sodium chloride  
Starch lintner  
Calcium chloride dihydrate  
Iodine crystals  
Potassium Iodide anhydrous

Preparation of reagents:
- 1M Sodium acetate
  Weigh 82 g of sodium acetate, add to 800 ml of deionised water and mix. When dissolved make up to 1 litre in a volumetric flask.
- 1M Acetic acid
  Measure 57 ml of acetic acid using a graduated cylinder. Slowly add to 500 ml of deionised water in a 1 litre volumetric flask, bring slowly up to the mark and mix.
- 1M Acetate buffer pH 5
  Measure 80 ml of 1M sodium acetate into a beaker. Using a continuous readout pH meter, slowly adjust the pH to 5.0 with 1M acetic acid.
- Diluent
  Dissolve 0.58 g of sodium chloride and 2.22 g calcium chloride dihydrate in 800 ml of deionised water. Add 2 ml of 1M acetate buffer (8.3) and bring to 1 litre in a volumetric flask with deionised water (The pH of the diluent is 4.89)
- Buffered starch substrate
  Boil 100-120 ml of water. Weigh 1.39 g dry weight of lintner starch in a 25 ml beaker. Add 2-4 ml of water to it and make a slurry. Ensure that it is free from lumps. Add the slurry to boiling water and start the stopwatch immediately. Once it starts boiling
stir it for 10 sec and then continue boiling for 15-20 sec. The alternate boiling and stirring should be carried out for 5 min. At the end of the 5th min, cool the beaker in an ice cold water bath. Add 21 ml of 1M acetate buffer (8.3) to the substrate and make up the volume in a 200 ml volumetric flask with water. Prepare Substrate freshly.

- Stock Iodine solution
  Dissolve 5.5 g of Iodine crystals and 11 g of potassium iodide in 150 ml of deionised water, make up the volume in a 250 ml volumetric flask. Store in a dark stoppered container.
- Working iodine solution
  Dissolve 10 g of potassium iodide in 200 ml of deionised water and add 1 ml of stock iodine solution, make up the volume in a 250 ml volumetric flask with deionised water.

Assay Procedure

Pipette 10 ml of buffered starch substrate into a test tube for each sample being tested. Also keep enzyme dilutions in the test tubes and pre-incubate both the enzyme and the substrate at 40°C for 4 min. Add 5 ml of enzyme dilution to the tubes containing substrate giving a time interval between the samples. The time interval is noted. Prepare a series of 12 test tubes for each test sample, add 5 ml of working iodine solution to each tube. After exactly 10 min. and at the same time intervals take 1 ml from the reaction mixture and add to 5 ml of working iodine solution and vortex. Determine the optical density of the solutions at 620 nm using deionised water to zero the spectrometer. Measure the reaction time from enzyme addition to the time when the sample OD reaches 0.5.

Calculation:

\[
\text{Amylase activity U/g} = \frac{60 \times 10 \times 6.95 \times \text{diln}}{5.26 \times \text{wt} \times 5 \times t} \times \frac{158.5 \times \text{diln}}{t \times \text{wt}}
\]

where 60 = 1 hour in minutes, 10 = ml of starch substrate, 6.95 = mg of starch in 1 ml of solution, 5.26 = mg of starch hydrolysed per hour (conversion to units), wt = weight of test sample (g), 5 = volume of enzyme solution added (ml) and t = time of reaction (min).
Table. 11. Effect of enzyme supplementation (A or B) on hen-day egg production (%) in layers fed varying levels of sunflower meal and deoiled rice bran-based diets

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SFM + DORB</th>
<th>Enzyme</th>
<th>Period</th>
<th>Mean ±SE</th>
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<tr>
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Means within column bearing common superscripts do not differ significantly (P<0.05)
Table 11a. Analysis of variance on hen-day egg production

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NS = Non significant

Table 12. Effect of enzyme supplementation (A or B) on egg weight (g) in layers fed varying levels of sunflower meal and deoiled rice bran based diets

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Means within column bearing common superscripts do not differ significantly (P<0.05)
Table. 12a. Analysis of variance on egg weight

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NS = Non significant (P < 0.05)

Table. 13. Effect of enzyme supplementation (A or B) on feed intake (g/bird/day) in layers fed varying levels of sunflower meal and deoiled rice bran based diets

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<td>1.120.16±1.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.119.57±0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.121.56±0.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.122.12±0.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.121.78±0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.120.82±0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>20+20</td>
<td>A</td>
<td>1.114.75±1.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.114.87±1.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.114.76±1.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.116.92±0.75&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.116.92±0.83&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.116.38±0.91&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.115.62±0.42&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>20+20</td>
<td>B</td>
<td>1.113.89±0.40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.114.19±0.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.113.15±0.42&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.116.03±0.98&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.115.70±0.51&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.115.75±0.51&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.114.79±0.31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
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<td>25+25</td>
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<td>1.127.92±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.125.64±0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.125.32±0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.127.58±0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.127.45±0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.128.22±0.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.127.02±0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>25+25</td>
<td>A</td>
<td>1.120.60±0.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.120.48±0.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.119.76±0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.121.80±0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.122.37±0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.122.66±0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.121.28±0.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>25+25</td>
<td>B</td>
<td>1.120.08±1.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.119.04±1.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.119.41±1.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.120.88±0.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.121.36±0.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.121.91±0.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.120.45±0.41&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means within column bearing common superscripts do not differ significantly (P<0.05)
Table 13a. Analysis of variance on feed intake

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>df</th>
<th>MSS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<tr>
<td>Treatments</td>
<td>11</td>
<td>170.55*</td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>4.14</td>
</tr>
</tbody>
</table>

* - Significant (P< 0.05)

Table 14. Effect of enzyme supplementation (A or B) on feed efficiency per dozen of eggs in layers fed varying levels of sunflower meal and deoiled rice bran based diets

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SFM+DORB %</th>
<th>Enzyme</th>
<th>Period</th>
<th>Mean ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>10+10</td>
<td>0</td>
<td>1.43±0.02 def</td>
<td>1.44±0.03 f</td>
</tr>
<tr>
<td>2</td>
<td>10+10</td>
<td>A</td>
<td>1.38±0.01 ce</td>
<td>1.38±0.02 ef</td>
</tr>
<tr>
<td>3</td>
<td>10+10</td>
<td>B</td>
<td>1.37±0.02 g</td>
<td>1.37±0.02 g</td>
</tr>
<tr>
<td>4</td>
<td>15+15</td>
<td>0</td>
<td>1.50±0.02 e</td>
<td>1.49±0.01 fde</td>
</tr>
<tr>
<td>5</td>
<td>15+15</td>
<td>A</td>
<td>1.45±0.04 defe</td>
<td>1.45±0.02 def</td>
</tr>
<tr>
<td>6</td>
<td>15+15</td>
<td>B</td>
<td>1.42±0.01 def</td>
<td>1.41±0.01 fg</td>
</tr>
<tr>
<td>7</td>
<td>20+20</td>
<td>0</td>
<td>1.58±0.01 b</td>
<td>1.58±0.01 b</td>
</tr>
<tr>
<td>8</td>
<td>20+20</td>
<td>A</td>
<td>1.51±0.02 d</td>
<td>1.51±0.01 cd</td>
</tr>
<tr>
<td>9</td>
<td>20+20</td>
<td>B</td>
<td>1.49±0.03 de</td>
<td>1.49±0.02 de</td>
</tr>
<tr>
<td>10</td>
<td>25+25</td>
<td>0</td>
<td>1.70±0.02 a</td>
<td>1.66±0.02 a</td>
</tr>
<tr>
<td>11</td>
<td>25+25</td>
<td>A</td>
<td>1.69±0.01 b</td>
<td>1.59±0.01 b</td>
</tr>
<tr>
<td>12</td>
<td>25+25</td>
<td>B</td>
<td>1.58±0.02 b</td>
<td>1.56±0.03 bc</td>
</tr>
</tbody>
</table>

Means within column bearing common superscripts do not differ significantly (P<0.05)
**Table 14a. Analysis of variance on feed efficiency per dozen of eggs**

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>df</th>
<th>MSS</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
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<td>0.037*</td>
<td>0.032*</td>
<td>0.034*</td>
<td>0.049*</td>
<td>0.044*</td>
<td>0.037*</td>
<td>0.183*</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>36</td>
<td>0.0019</td>
<td>0.0016</td>
<td>0.0015</td>
<td>0.0018</td>
<td>0.0009</td>
<td>0.0007</td>
<td>0.0031</td>
<td></td>
</tr>
</tbody>
</table>

* - Significant (P< 0.05)

**Table 15. Effect of enzyme supplementation ( A or B) on feed efficiency per kilo of eggs in layers fed varying levels of sunflower meal and deoiled rice bran based diets**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SFM+DORB %</th>
<th>Enzyme</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Mean ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10+10</td>
<td>0</td>
<td>2.08±0.04efgh</td>
<td>2.07±0.05def</td>
<td>2.07±0.01def</td>
<td>2.08±0.01c</td>
<td>2.06±0.02fg</td>
<td>2.08±0.02d</td>
<td>2.07±0.01</td>
</tr>
<tr>
<td>2</td>
<td>10+10</td>
<td>A</td>
<td>1.98±0.03b</td>
<td>1.98±0.04fg</td>
<td>2.01±0.04fg</td>
<td>2.01±0.03e</td>
<td>1.99±0.03gh</td>
<td>2.02±0.02de</td>
<td>2.00±0.01h</td>
</tr>
<tr>
<td>3</td>
<td>10+10</td>
<td>B</td>
<td>2.02±0.03dghi</td>
<td>1.96±0.03g</td>
<td>1.97±0.04g</td>
<td>2.01±0.06c</td>
<td>1.95±0.02h</td>
<td>1.97±0.02e</td>
<td>1.98±0.01h</td>
</tr>
<tr>
<td>4</td>
<td>15+15</td>
<td>0</td>
<td>2.20±0.05abc</td>
<td>2.14±0.01abc</td>
<td>2.12±0.04c</td>
<td>2.18±0.02ad</td>
<td>2.18±0.02cd</td>
<td>2.18±0.03c</td>
<td>2.17±0.01de</td>
</tr>
<tr>
<td>5</td>
<td>15+15</td>
<td>A</td>
<td>2.09±0.07efgh</td>
<td>2.06±0.03efg</td>
<td>2.07±0.04def</td>
<td>2.08±0.03e</td>
<td>2.07±0.01ef</td>
<td>2.08±0.03d</td>
<td>2.07±0.01</td>
</tr>
<tr>
<td>6</td>
<td>15+15</td>
<td>B</td>
<td>2.05±0.02ghi</td>
<td>2.02±0.02fg</td>
<td>2.03±0.03efg</td>
<td>2.06±0.03e</td>
<td>2.02±0.03ghi</td>
<td>2.05±0.03a</td>
<td>2.04±0.01g</td>
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<tr>
<td>7</td>
<td>20+20</td>
<td>0</td>
<td>2.29±0.02bci</td>
<td>2.28±0.03b</td>
<td>2.30±0.02b</td>
<td>2.36±0.04b</td>
<td>2.33±0.02b</td>
<td>2.32±0.01b</td>
<td>2.31±0.01bc</td>
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<tr>
<td>8</td>
<td>20+20</td>
<td>A</td>
<td>2.17±0.03def</td>
<td>2.17±0.03cd</td>
<td>2.18±0.01c</td>
<td>2.34±0.04cd</td>
<td>2.21±0.02c</td>
<td>2.21±0.01c</td>
<td>2.20±0.01d</td>
</tr>
<tr>
<td>9</td>
<td>20+20</td>
<td>B</td>
<td>2.14±0.04ef</td>
<td>2.13±0.03ade</td>
<td>2.12±0.01cabc</td>
<td>2.21±0.02a</td>
<td>2.13±0.03ade</td>
<td>2.16±0.01c</td>
<td>2.15±0.01f</td>
</tr>
<tr>
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<td>25+25</td>
<td>0</td>
<td>2.47±0.03a</td>
<td>2.39±0.03a</td>
<td>2.44±0.04a</td>
<td>2.47±0.02a</td>
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<td>2.46±0.02a</td>
<td>2.45±0.01a</td>
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<td>A</td>
<td>2.32±0.04b</td>
<td>2.28±0.02b</td>
<td>2.31±0.02b</td>
<td>2.35±0.04b</td>
<td>2.34±0.03b</td>
<td>2.35±0.03b</td>
<td>2.32±0.01b</td>
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<tr>
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<td>25+25</td>
<td>B</td>
<td>2.28±0.02cde</td>
<td>2.24±0.05bc</td>
<td>2.26±0.02b</td>
<td>2.32±0.05bc</td>
<td>2.28±0.02b</td>
<td>2.31±0.03b</td>
<td>2.28±0.01c</td>
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</table>

Means within column bearing common superscripts do not differ significantly (P<0.05)
Table 15a. Analysis of variance on feed efficiency per kilo of eggs

<table>
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<tr>
<th>Source of Variance</th>
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<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>Mean</th>
</tr>
</thead>
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<td>0.071*</td>
<td>0.081*</td>
<td>0.093*</td>
<td>0.104*</td>
<td>0.092*</td>
<td>0.423*</td>
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<tr>
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<td>0.005</td>
<td>0.004</td>
<td>0.0032</td>
<td>0.004</td>
<td>0.002</td>
<td>0.002</td>
<td>0.003</td>
</tr>
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</table>

* - Significant (P< 0.05)

Table 16. Effect of enzyme supplementation (A or B) on intestinal viscosity (cP) in layers fed varying levels of sunflower meal and deoiled rice bran based diets

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SFM + DORB %</th>
<th>Enzyme</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<tr>
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<td>A</td>
<td>1.42b</td>
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<td>3</td>
<td>10+10</td>
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<td>4</td>
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<td>0</td>
<td>1.49d</td>
</tr>
<tr>
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<td>A</td>
<td>1.34e</td>
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<td>1.35j</td>
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<td>25+25</td>
<td>B</td>
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</table>

Means with no common superscripts differ significantly (p<0.05)
### Table. 16a. Analysis of variance on intestinal viscosity (cP)

<table>
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<tr>
<th>Source of Variance</th>
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</tr>
</thead>
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</tr>
<tr>
<td>Error</td>
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</tbody>
</table>

* - Significant (P< 0.05)

### Table. 17. Effect of enzyme supplementation (A or B) on faecal moisture (%) in layers fed varying levels of sunflower meal and deoiled rice bran based diets

<table>
<thead>
<tr>
<th>Period</th>
<th>Treatments</th>
<th>SFM+DORB</th>
<th>Enzyme</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Mean ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10+10</td>
<td>0</td>
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<td>74.84±0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.76±1.12&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>76.61±1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.63±0.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.89±0.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.53±1.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.71±0.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>10+10</td>
<td>A</td>
<td></td>
<td>69.85±1.28&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>72.03±1.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.68±1.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.37±1.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.48±0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.44±1.65&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>71.98±0.64&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>10+10</td>
<td>B</td>
<td></td>
<td>72.61±1.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>73.41±0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.38±1.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.72±1.14&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>70.02±1.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75.29±1.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.57±0.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>15+15</td>
<td>0</td>
<td></td>
<td>72.92±0.54&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>70.60±0.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.84±1.25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>72.96±1.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.98±0.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75.68±0.70&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>72.83±0.54&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>15+15</td>
<td>A</td>
<td></td>
<td>70.12±1.18&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>69.33±0.98&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>67.22±1.17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>74.17±0.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68.86±1.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71.56±1.09&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>70.21±0.60&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>15+15</td>
<td>B</td>
<td></td>
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* - Significant (P< 0.05)

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Means within column bearing common superscripts do not differ significantly (P<0.05)
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Fig. 2. Skeletal structure of plant cell wall

Fig. 3. Classification of NSP

- Lignin
- Cellulose
- Hemicellulose
- Betaglucans
- Pectins
- Oligosacch.
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**Profit (Rs.):**

| -     | 91   | 114 |
| -     | 156  | 200 |
| -     | 142  | 224 |
| -     | 149  | 195 |

**Figure 4:** Effect of enzyme supplementation on hen-day egg production (46 to 70 weeks)

- **SFM + DORB**
- **SFM + DORB + ENZ. A**
- **SFM + DORB + ENZ. B**
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Fig. 5. Effect of enzyme supplementation on egg weight (48 to 78 weeks)
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**Fig. 6. Effect of enzyme supplementation on feed intake (46 to 70 weeks)**

- SFM + DORB
- SFM + DORB + ENZ. A
- SFM + DORB + ENZ. B
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<th></th>
<th>Profit (Rs.)</th>
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**Fig. 7.** Effect of enzyme supplementation on feed efficiency per dozen eggs (46 to 70 weeks)
<table>
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<th>Enzyme</th>
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<th>Profit (Rs.)</th>
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<td>2.45</td>
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</tbody>
</table>

**Fig. 3. Effect of enzyme supplementation on feed efficiency per kilo egg (64 to 78 weeks)**

- SFM + DORB
- SFM + DORB + ENZ. A
- SFM + DORB + ENZ. B
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>HEP %</th>
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<th>1.38</th>
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<table>
<thead>
<tr>
<th>Profit (Rs.)</th>
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<th>114</th>
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<td>200</td>
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<tr>
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<td>142</td>
<td>224</td>
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<tr>
<td></td>
<td>149</td>
<td>195</td>
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</table>

**Fig. 9. Effect of enzyme supplementation on intestinal viscosity (46 to 70 weeks)**

- SFM + DORB
- SFM + DORB + ENZ A
- SFM + DORB + ENZ B

Intestinal viscosity (cP)
<table>
<thead>
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<th>Enzyme</th>
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<th>Profit (Rs.)</th>
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<tr>
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<td>224</td>
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<tr>
<td>0</td>
<td>61.79</td>
<td>195</td>
</tr>
</tbody>
</table>

**Fig. 10. Effect of Enzyme supplementation on faecal moisture (% to 70 weeks)**
Plate 1. Effect of enzyme A supplementation on intestinal viscosity

Fluid extracted from the digesta of layers fed enzyme ‘A’ supplemented groups showing variation in colour intensity when compared to enzyme non- supplemented groups ‘O’
Plate 2. Effect of enzyme B supplementation on intestinal viscosity

Fluid extracted from the digesta of layers fed enzyme ‘B’ supplemented groups showing variation in colour intensity when compared to enzyme non-supplemented groups ‘O’