APPENDIX A

Media, Buffer and Experimental Diets

1. Media

The media were autoclaved at 121°C (15 psi) for 15 min.

1.1 Nutrient Broth (NB)

This medium was used for growth and maintenance of bacteria. It contains:

Bacto peptone	3.0	g
Meat extract	5.0	g
Distilled water	1,000.0	ml

The nutrient agar (NA) was prepared by add 15 g of agar to NB.

1.2 Modified Manitol Salt Agar (MYP)

This medium was used for total plate count technique. This ingredient include:

Bacto peptone	5.0	g
Meat extract	5.0	g
D-manitol	5.0	g
NaCl	5.0	g
Phenol red	0.025	g
Agar powder	10.0	g
Distilled water	900.0	ml

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2. Buffer solution

2.1 0.2 M Citrate phosphate buffer (pH 3.0, 4.0, 5.0, 6.0, 6.8, 7.0 and 8.0)

Dissolved X g of citric acid and Y g of di-sodium hydrogen phosphate (Na_2HPO_4) in 950 ml of distilled water. Stir the mixture constantly and adjust to the require pH with 1.0N HCl or 1.0 N NaOH. Make up to 1 liter in volumetric flask

pH		3.0	4.0	5.0	6.0	6.8	7.0	8.0
Citric acid	**(X)**	16.6956	12.9131	10.1918	7.7437	14.1960	3.7090	0.5779
Na_2HPO_4	**(Y)**	5.8346	10.9451	14.6219	17.9295	13.7990	23.3808	27.6112

2.2 0.1 M Phosphate buffer pH 6.0

Dissolved 12.2539 g of sodium di-hydrogen orthophosphate (NaH_2PO_4) and 1.8878 g of di-sodium hydrogen phosphate (Na_2HPO_4) in 950 ml of distilled water. Stir the mixture constantly with a magnetic stirrer and adjust to the require pH with 1.0N HCl or 1.0 N NaOH. Make up to 1 liter in volumetric flask.

2.3 0.2 M Phosphate buffer pH 6.8

Dissolved 13.7990 g of sodium di-hydrogen orthophosphate (NaH_2PO_4) and 14.1960 g of di-sodium hydrogen phosphate (Na_2HPO_4) in 950 ml of distilled water. Stir the mixture constantly with a magnetic stirrer and adjusted to the require pH with 1.0N HCl or 1.0 N NaOH. Made up to 1 liter in volumetric flask.

2.4 0.2 M Sodium citrate HCl buffer pH 3.0

Dissolved 8.4032 g of citric acid and 4.800 g of NaOH in 950 ml of distilled water. Stir mixture constantly and add 4.967 ml of conc. HCl. Adjust to the require pH with 1.0N HCl or 1.0 N NaOH. Make up to 1 liter in volumetric flask.

2.5 0.2 M Tris Maleate NaOH buffer pH 6.8

The buffer was prepared by dissolved 11.8605 g of Tris maleate and 1.8000 g of NaOH in 950 ml of distilled water. Stir the mixture constantly with a magnetic stirrer and adjust to the require pH with 1.0N HCl or 1.0 N NaOH. Make up to 1 liter in volumetric flask.

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APPENDIX B

ANALYTICAL METHODS

1. Determination of protein and soluble peptide

The protein and soluble peptide content was measured by the method of Lowry *et al*. (1951) with bovine serum albumin and tyrosine as standard, respectively.

Reagents

A: 2% sodium carbonate in 0.1N NaOH

B: 0.5% $CuSO_4 .5H_2O$ in 1% sodium citrate

C: 1 N Folin-Ciocalteu's phenol reagent

(2N Folin Phenol was diluted with distilled water to the final concentration in 1N, the solution should be freshly prepared before use.)

D: 1 ml Reagent B + 50 ml Reagent A (or similar ratio)

Make up immediately before use.

Procedure

1. Place 0.1 ml of proper dilution of culture broth (for protein determination) or clear supernatant of reaction mixture (for soluble peptide determination)
2. Add 1 ml of Reagent D into the tube and vortex immediately. Incubate at room temperature for 10 min
3. After the 10 min incubation, add 0.1 ml of Reagent C to sample and vortex immediately. Incubate 30 min at room temperature.
4. Absorbance (OD) of samples was measured at 750 nm.

Concentrations of the samples were compared to the standard curve for determination of values. Distilled water was used instead of sample as a blank.

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Preparation of standard curve of tyrosine

Standards of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mM were prepared from tyrosine. The reactions were carried out with the same procedure as described previously. Absorbance was plotted against various concentrations of standards.

Preparation of standard curve of protein

Standards of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mg/ml were prepared from bovine serum albumin. The reactions were carried out with the same procedure as described previously. Absorbances were plotted against concentrations of standards.

2. Activity assay of other enzymes

2.1 Amylase activity

Amylase activity was determined according to the modified method of Rick and Stegbauer (1974). The amylase activity of the enzyme was determined using soluble starch (Sigma) as a substrate.

Substrate

1% soluble starch (Sigma) in 0.2 M citrate phosphate buffer, pH 6.8.

Procedure

1. Add 1.0 ml of substrate solution to a test tube.
2. Incubate the tube in water bath at 39.5 °C
3. Add 0.5 ml of enzyme, mix well and incubate at 39.5 °C for 10 min.
4. Add 3.0 ml of DNS, mix. Transfer to a rack on the table.
5. Boil exactly 5.0 min in a boiling water bath containing sufficient water. All enzyme blank, glucose standard and the specto zero should be boiled together. After boiling, transfer immediately to a cold water bath.
6. Add 10 ml of distilled water and mix.

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7. Measure the color formed against the spectro zero at 540 nm (OD_{540}). Subtract the color formed in the sample with that of the enzyme blank tube.

8. The spectro zero and enzyme blank, add buffer or enzyme to the tube after the addition of dinitrosalicylic acid (DNS) solution.

One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 μmole of glucose per min at 39.5 °C under the reaction conditions.

2.2 β-Glucanase activity

β-Glucanase activity was determined according to the modified method of Mandels *et al*. (1976). The β-Glucanase activity of the enzyme was determined using β-Glucan from barley (Sigma) as a substrate.

Substrate

1% β-Glucan from barley (Sigma) in 0.2 M citrate phosphate buffer, pH 6.8.

Procedure

1. Add 1.0 ml of substrate solution to a test tube.
2. Incubate the tube in water bath at 39.5 °C
3. Add 0.5 ml of enzyme, mix well and incubate at 39.5 °C for 30 min.
4. Add 3.0 ml of DNS, mix. Transfer to a rack on the table.
5. Boil exactly 5.0 min in a virtuously boiling water bath containing sufficient water. All enzyme blank, glucose standard and the specto zero should be boiled together. After boiling, transfer immediately to a cold water bath.
6. Add 10 ml of distilled water and mix.
7. Measure the color formed against the spectro zero at 540 nm (OD_{540}). Subtract the color formed in the sample with that of the enzyme blank tube.
8. The spectro zero and enzyme blank, add buffer or enzyme to the tube after the addition of dinitrosalicylic acid (DNS) solution.

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One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 μmole of glucose per min at 39.5 °C under the reaction conditions.

2.3 Cellulase

Cellulase activity was determined according to the modified method of Mandels *et al*. (1976). The cellulase activity of the enzyme was determined using Carboxymethyl Cellulose (CMC) as a substrate.

Substrate

2% CMC (BDH) in 0.2 M citrate phosphate buffer, pH 6.8.

Procedure

1. Add 1.0 ml of substrate solution to a test tube.
2. Incubate in water bath at 39.5 °C
3. Add 0.5 ml of enzyme, mix well and incubate at 39.5 °C for 30 min.
4. Add 3.0 ml of DNS, mix. Transfer to a rack on the table.
5. Boil exactly 5.0 min in a virtuously boiling water bath containing sufficient water. All enzyme blank, glucose standard and the specto zero should be boiled together. After boiling, transfer immediately to a cold water bath.
6. Add 10 ml of distilled water and mix.
7. After at least 20 min for pulp settling, measure the color formed against the spectro zero at 540 nm (OD_{540}). Subtract the color formed in the sample with that of the enzyme blank tube.
8. The spectro zero and enzyme blank, add buffer or enzyme to the tube after the addition of dinitrosalicylic acid (DNS) solution.

One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 μmole of glucose per min at 39.5 °C under the reaction conditions.

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2.4 Pentosanase activity

Pentosanase activity was determined according to the modified method of Baily *et al*. (1994). The pentosanase activity of the enzyme was determined using oat spelt xylan (Sigma) as a substrate.

Substrate

1% oat spelt xylan (Sigma) in 0.2 citrate phosphate buffer, pH 6.8.

Procedure

1. Add 1.8 ml of substrate solution to a test tube.
2. Incubate the tube in water bath at 39.5 °C
3. Add 200 µl of enzyme, mix well and incubate at 39.5 °C for 5 min.
4. Add 3.0 ml of DNS, mix. Transfer to a rack on the table.
5. Boil exactly 15 min in a virtuously boiling water bath containing sufficient water. All enzyme blank, xylose standard and the specto zero should be boiled together. After boiling, transfer immediately to a cold water bath.
6. Centrifuge the tube at 2,000 rpm for 10 min.
7. Measure the color formed against the spectro zero at 540 nm (OD_{540}). Subtract the color formed in the sample with that of the enzyme blank tube.
8. The spectro zero and enzyme blank, add buffer or enzyme to the tube after the addition of dinitrosalicylic acid (DNS) solution.

One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 µmole of xylose per min at 39.5 °C under the reaction conditions.

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2.5 Phytase

Phytase activity was determined according to the modified method of Engelen *et al*., (1994). The phytase activity of the enzyme was determined using sodium phytate from rice (Sigma) as a substrate.

Orthophosphate reacts with molybdate in an acid medium to produce a mixed phosphate/molybdate complex. In the presence of vanadium, yellow molybdovanado-phosphoric acid forms. The intensity of the yellow color is proportional to the phosphate concentration. Test results are measured at 415 nm. No interference by phytate occour with this method, which uses molybdovanadate as coloring reagent.

Reagents

(i) Substrate solution

Dissolve 8.40 g sodium phytate ($C_6H_6Na_{12}O_{24}P_6.10H_2O$) from rice (Cat no. P-3168, sigma) in 900 ml buffer solution, adjust to pH 3.0 and 6.8 with 0.1 M Sodium-Citrate-HCl Buffer (pH 3.0) and Tris-Maleate-NaOH Buffer (pH 6.8). Prepare this solution fresh daily.

(ii) Nitric acid slolution

While stirring, slowly add 70 ml nitric acid (65%) to 130 ml water

(iii) Ammonium heptamolybdate stock solution

Dissolve 100 g Ammonium heptamolybdate ($H_{24}Mo_7N_6O_{24}.4H_2O$) (Merck) in 900 ml water, add 10 ml ammonia (25%), and dilute to 1 L with water. this solution may be kept at room temperature shield from light for 1 month.

(iv) Ammonium vanadate stock solution

Dissolve 2.35 g Ammonium vanadate (NH_4VO_3) (Merck) in 400 ml water at 60°C. While stirring, slowly add 20 ml Nitric acid solution, cool to room temperature, and dilute to 1 L with water. Prepare this solution fresh daily.

(v) Color stop mix.

Mix 250 ml Ammonium heptamolybdate stock solution and 250 ml Ammonium vanadate stock solution. While stirring slowly add 165 ml nitric acid (65%), cool to room temperature, and dilute to 1 L with water. Prepare this solution fresh daily.

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Procedure

1. Place the tube containing enzyme dilution 0.5 ml in the water bath, equilibrate each tube for 5 min.

2. Add 1 ml substrate solution at 37.0±0.1°C and mix.

3. At 60 min, terminate the incubation by adding 1.00 ml color-stop mix and mix.

4. Centrifuge all tubes at 2,000 rpm for 5 min.

5. Measure the absorbance at 415 nm with the spectrophotometer after zeroing the instrument with the spectro zero

6. Calculate the corrected absorbance difference by subtracting absorbance blank from that of the corresponding sample standard solution.

7. The spectro zero and enzyme blank, add buffer or enzyme to the tube after the addition of color-stop mix.

Enzyme activity is expressed in activity units (FTU); 1 FTU is the amount of enzyme the liberates 1 μmol inorganic orthophosphate/min under test condition (pH 6.8, 39.5°C, and substrate concentration, sodium phytate at 0.0051 mol/l)

3. *In vitro* digestibility

The *in vitro* incubation conditions simulating the digestion processes in the stomach and small intestine with pepsin followed by pancreatin.

Reagents

1. 0.1 M HCl
2. 0.1 M Phosphate buffer pH 6.0
3. 0.2 M Phosphate buffer pH 6.8
4. 0.6 M NaOH
5. 1.0 M HCl
6. 1.0 M NaOH
7. 20% sulphoaslicylic acid
8. Choramphenicol solution (0.5 g/100 ml ethanol)

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9. Pancreatin solution (50 mg / ml)

10. Pepsin solution (10 mg / ml)

Procedure

Step 1.

1. Weigh 1 g of finely ground diet supplemented with enzymes (ground to pass a screen with 1 mm) to an accuracy of ± 0.1 mg in 125 ml erlenmeyer flask.

2. Sample and blank were tested in triplicate.

3. Add 25 ml of 0.1 M phosphate buffer pH 6.0 to each flask and mix carefully.

4. Add 10 ml of 0.2 M HCl and adjust pH to 2.0 with a 1M HCl (or 1M NaoH solution).

5. Then, add 1 ml of freshly prepared pepsin solution, containing 10 mg porcine pepsin.

6. In order to prevent bacterial growth, add 0.5 ml of choramphenicol solution (0.5 g/100 ml ethanol).

7. Then close the flask with cotton knob and incubate in incubator shaker at 39.5 °C for 6 h.

Step 2.

1. Add 10-ml of 0.2 M phosphate buffer pH 6.8 and 5 ml of a 0.6 M NaOH solution to the mixture, respectively.

2. Adjust pH to 6.8 with 1 M HCl or 1 M NaOH.

3. Mix 1 ml of freshly prepared pancreatin solution containing 50 mg porcine pancreatin.

4. After closing with a cotton knop, place the flasks in incubator shaker at 39.5°C for 18 h.

5. Add 5-ml of 20% sulphosalicylic acid and incubate at room temperature for 30 min.

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6. Collect undigested residues in filtration unit by using dried and pre-weighed filter paper (diameter 12.5 cm).

7. Transfer all material to the filter paper and dry the undigested residue at 80 °C for 12 h. Calculate the *in vitro* digestibility of dry matter from the difference between dry matter in the sample and the undigested residue after correction for dry matter in the blank.

8. Keep the undigested materials together with filter paper at frozen until analysis; the analysis were include crude protein, crude fibre, ash and ether extract. Calculate the in vitro digestibility of these parameters from the difference between the sample and the undigested residue after correction for the blank.

4. Proximate analysis (AOAC., 1995)

4.1 Dry matter

Moisture is evaporated from the sample by oven drying. Dry matter is determined gravimetrically as the residue remaining after drying

Procedure:

1. Dry weighing bottles at 100 °C for 15 hr in an Air Oven and cool in a desiccator.

2. Weigh pre-dried bottles. (W_1)

3. Add 3 g of diet (weighed to the nearest 0.01 g) (W_S) and distribute uniformly.

4. Dry samples to a constant weight at 100°C for 12 h.

5. Remove bottle with diet after drying and place in a desiccator to cool.

6. Weigh the samples after cooling. (W_2)

7. Calculation

Dry matter % (w/w) was calculated as follows:

$$\% \text{ Dry matter} = \frac{W_2 - W_1}{W_S} \times 100$$

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4.2 Crude protein

Crude protein was assayed by Kjeldahl method, the official method. In this method, the proteins and other organic substances are digested with concentrated sulfuric acid in the presence of selenium reagent mixture as catalyst. The present nitrogen is converted into ammonium sulfate ($(NH_3)_2SO_4$). Concentrated NaOH is added to release NH_3 that is distilled, collected in boric acid solution, and quantitated by a titration method.

Reagents:

Sulfuric acid, selenium reagent mixture, sodium hydroxide solution, tashiro indicator, 40% boric acid solution, and hydrochloric acid standard solution, 0.1000 N

Procedure:

1. Transfer sample (1 g) into a Kjeldahl tube.
2. Add selenium reagent mixture (0.5 g) and mix thoroughly.
3. Add H_2SO_4 (20 ml) rinse anything in neck of flask down into bulb.
4. Digestion: Place Kjeldahl tubes in block digestion unit. At the end of digestion, digest should be clear and free of undigested material. Cool the digest to room temperature, add 30 ml H_2O to each tube and mix.
5. Distillation: Place to Kjeldahl tube to distillation unit. Add boric acid (25 ml) solution with indicator to erlenmeyer flask (250 ml) and place on receiving platform, with tube from condenser extending below surface of boric acid solution. At the end of distillation, distillate should be light green solution.
6. Titration: Titrate boric acid receiving solution with standard 0.1000 N HCl solution to first trace of pink. Lighted stir may aid visualization of end point.
7. Calculation

Total nitrogen (%(v/v) or %(w/v)) was calculated as follows:

$$\text{Total Nitrogen, \%} = \frac{1.40 \times (\text{ml HCl, sample} - \text{ml HCl, blank}) \times \text{normality HCl}}{\text{g sample}}$$

To calculate percent "protein" on a total nitrogen basis, multiply percent nitrogen by factor 6.25.

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4.3 Crude fiber

Crude fiber is loss on ignition of dried residue remaining after digestion of sample with 3.125% H_2SO_4 and 3.125% NaOH solution under specific conditions. Method is applicable to grains, meals, flours, feeds and fiber-bearing materials from which fat can be extracted to leave workable residue.

Reagents:

3.125% H_2SO_4 and 3.125% sodium hydroxide solution

Procedure:

1. Transfer sample (W_S) to 600 ml beaker and add 200 ml of 3.125% H_2SO_4.
2. Place beaker on digestion apparatus with preadjusted hot plate and boil exactly for 10 min.
3. Remove beaker and filter through buchner funnel with filter paper (whatman no. 41) covered with diatomaceous earth.
4. Rinse beaker with 50-70 ml of boiling distilled water and wash through buchner.
5. Repeat with three 50 ml portions of water and suck dry.
6. Return all diatomaceous earth and residue to beaker.
7. Add 200 ml of 3.125% NaOH and boil exactly 10 min.
8. Remove beaker and filter as above, transfer diatomaceous earth and residue to porcelain crucible.
9. Dry the residue to a constant weight at 100°C for 12 h, place in a desiccator to cool (15-30 min) and weigh the samples after cooling. (W1)
10. Ignite the residue at 550°C for 6 h, place in a desiccator to cool (15-30 min) and reweigh the samples after cooling. (W2)
11. Calculation

% Crude fiber content was calculated as follows:

$$\% \text{ Crude fiber content} = \frac{W_1 - W_2}{W_S} \times 100$$

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4.4 Ash

Ash was assay by oxidizing all organic matter in a weighed sample of the material by incineration and determining the weight of the ash remaining.

Procedure:

1. Ignite porcelain crucibles in a muffle furnace at around 450-550°C for overnight, cool in a desiccator and weigh after reaching room temperature (W_1).
2. Add 3 g of diets to the pre-ignited crucible (weighed to the nearest 0.01 g) (W_S).
3. Place crucibles with diets on the heater to remove smoke.
4. After removing smoke, place crucibles with diets in the cooled muffle furnace.
5. Ignite for 12 -18 h at about 450-550°C overnight.
6. Turn off the muffle furnace and open after temperature has reached about 250°C
7. Using tongs transfer the crucibles to the desiccator, cool in a desiccator and weigh after reaching room temperature (W_2).
8. Calculation

% Ash content was calculated as follows:

$$\% \text{ Ash content} = \frac{W_2 - W_1}{W_S} \times 100$$

4.5 Ether extract

Fat is extracted from a dry sample with petroleum ether using a special Soxhlet apparatus. The ether extract is collected in a flask. The percentage of crude fat is determined by weight difference.

Procedure

1. Dry round bottom flask with 2-3 pieces of boiling chip at 100°C for 15 h in an air oven and cool in a desiccator.
2. Weigh pre-dried flask. (W_1)

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3. Weigh approximately 3.0 g of ground feed (to the nearest 0.01 g) on sugar filter paper and transfer to an extraction thimble (W_S).

4. Place the thimble inside percolator of the Soxhlet apparatus. Assemble Soxhlet apparatus and extract the sample with petroleum ether for 15 h at a condensation rate of at least 5-6 drops per second.

5. Remove the thimble from the percolator and place it in a beaker and let it dry in the hood for 30 min.

6. Stand the flask in the hood for overnight, dry at 100°C for 60 min, to constant weight. Excessive drying may oxidize fat and give erroneous results

7. Remove to desiccator, cool and weigh accurately (W_2).

8. Calculation

% Ether extract content was calculated as follows:

$$\% \text{ Ether extract content} = \frac{W_2 - W_1}{W_S} \times 100$$

4.6 Nitrogen free extract

% Nitrogen free extract (%w/w) was calculated as follows:

$$\% \text{ Nitrogen free extract} = \% \text{ Dry matter} - (\% \text{ Crude protein} + \% \text{ Crude fiber} + \% \text{ Ether extract} + \% \text{ Ash})$$

5. Blood Urea Nitrogen

Principle

In the Fearon procedure for serum urea quantitation, urea condenses with diacetyl to form diazine. Since diacetyl is unstable, diacetyl monoxime is substituted and generates the required diacetyl in the same reaction mixture. Also, thiosemicarbazide and ferric ions are added to enhance and stabalize the product

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Reagents

Urea nitrogen reagent

1. Add 44 ml of concentrated sulfuric acid and 66 ml of 85% orthophosphoric acid to about 100 ml of distilled water contained in a 1 l volumetric flask.

2. Let the solution cool at room temperature. Then add the following, dissolving each successively: (*a*) 50 mg of thiosemicabazide, (*b*) 2.0 g of cadmium sulfate hexahydrate, and (*c*) 10 ml of a urea solution (26 mg/l of water).

3. Mix and dilute to 1 l with distilled water.

4. Transfer to an amber-colored bottle.

5. This reagent is stable for at least 6 months if refrigerated.

6. The presence of small amounts of urea in the reagent improved the linearity of the standard curve.

Diacetylmonoxime (2.0 g per 1,000 ml distilled water)

1. Add 2 g of diacetylmonoxime to about 900 ml of distilled water in a 1 volumetric flask

2. Mix and dissolve and then dilute to volume.

3. Transfer to an amber-colored bottle.

4. This reagent is stable for at least 6 months if refrigerated.

Urea nitrogen standard (50 mg urea nitrogen per 100 ml)

1. Dissolve 107.25 g of urea in about 50 ml of distilled water in a 100 ml volumetric flask

2. Transfer to an amber-colored bottle.

3. This standard is stable for at least 6 months if refrigerated.

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Procedure

1. Add 5.0 ml of urea nitrogen reagent to each tube.

2. Transfer 50 μl of proper dilution of serum.

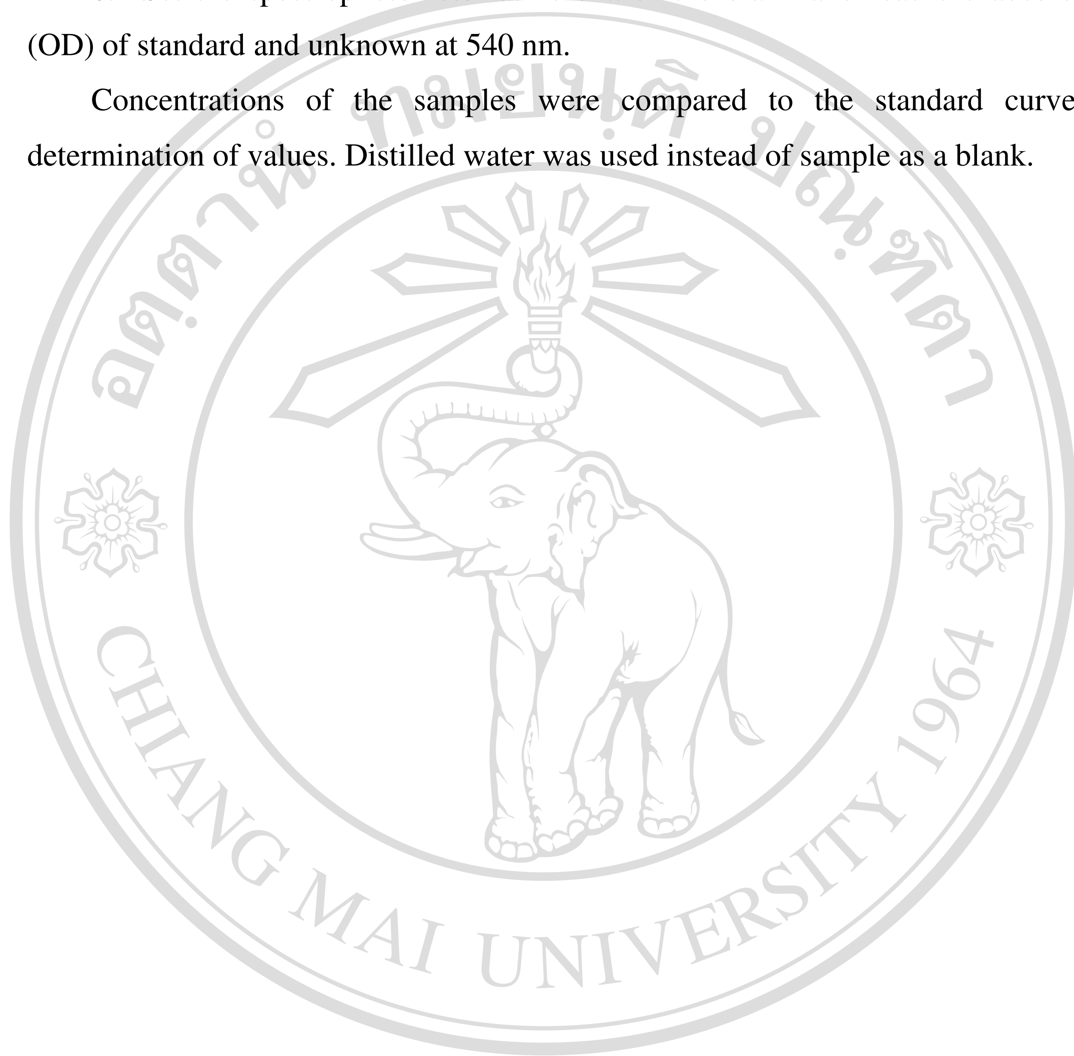
3. Add 0.5 ml of the diacetylmonoxime reagent to the tube and vortex immediately.

4. Place all tubes into boiling bath.

5. Remove all tubes 12 min later, immerse them in cool tap water for 5 min, and vortex immediately.

6. Set the spectrophotometer at zero with the blank and read the absorbance (OD) of standard and unknown at 540 nm.

Concentrations of the samples were compared to the standard curve for determination of values. Distilled water was used instead of sample as a blank.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APPENDIX C

STATISTICAL ANALYSIS

Table 1 ANOVA: Effect of soybean meal concentration on growth and protease production of *Bacillus* sp. FAS001

	Source	df	SS	MS	F	Sig.
Growth	Treatment	8	136.331	17.041	46703.042	0.000
	Error	9	0.003	0.000		
	Total	17	136.335			
Protease	Treatment	8	1558.239	194.780	172.723	0.000
Production	Error	9	10.149	1.128		
	Total	17	1568.388			

Table 2 ANOVA: Effect of initial pH on growth and protease production of *Bacillus* sp. FAS001

	Source	df	SS	MS	F	Sig.
Growth	Treatment	4	97.162	24.291	13835.756	0.000
	Error	5	0.009	0.002		
	Total	9	97.171			
Protease	Treatment	4	6658.065	1664.516	1088.996	0.000
Production	Error	5	7.642	1.528		
	Total	9	6665.708			

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Table 3 ANOVA: Effect of cultivation temperature on growth and protease production of *Bacillus* sp. FAS001

	Source	df	SS	MS	F	Sig.
Growth	Treatment	4	0.772	0.193	244.089	0.000
	Error	9	0.007	0.001		
	Total	13	0.779			
Protease	Treatment	4	2462.831	615.708	528.531	0.000
Production	Error	9	10.484	1.165		
	Total	13	2473.315			

Table 4 ANOVA: Effect of agitation rate and ratio of media to air content on growth and protease production of *Bacillus* sp. FAS001

	Source	df	SS	MS	F	Sig.
Growth	Treatment	8	15.931	1.991	78.487	0.000
	Error	9	0.228	0.025		
	Total	17	16.159			
Protease	Treatment	8	11653.196	1456.649	1136.860	0.000
Production	Error	18	23.063	1.281		
	Total	26	11676.259			

Table 5 ANOVA: Effect of media additive on growth and protease production of *Bacillus* sp. FAS001

	Source	df	SS	MS	F	Sig.
Growth	Treatment	8	0.018	0.002	2.987	0.062
	Error	9	0.007	0.001		
	Total	17	0.025			
Protease	Treatment	8	12160.798	1520.100	163.253	0.000
Production	Error	18	167.604	9.311		
	Total	26	12328.402			

Table 6 ANOVA: Effect of inorganic salt on growth and protease production of *Bacillus* sp. FAS001

	Source	df	SS	MS	F	Sig.
Growth	Treatment	6	133.807	22.301	46886.224	0.000
	Error	7	0.003	0.000		
	Total	13	133.810			
Protease	Treatment	6	35062.657	5843.776	354.699	0.000
Production	Error	14	230.655	16.475		
	Total	20	35293.312			

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Table 7 ANOVA: Effect of inoculum size on growth and protease production of *Bacillus* sp. FAS001

	Source	df	SS	MS	F	Sig.
Growth	Treatment	3	4.351	1.450	2998.279	0.000
	Error	4	0.002	0.000		
	Total	7	4.353			
Protease Production	Treatment	3	506.527	168.842	29.083	0.000
	Error	8	46.445	5.806		
	Total	11	552.972			

Table 8 ANOVA: Effect of cultivation time on growth and protease production of *Bacillus* sp. FAS001

	Source	df	SS	MS	F	Sig.
Growth	Treatment	20	46.836	2.342	1736.890	0.000
	Error	21	0.028	0.001		
	Total	41	46.865			
Protease Production	Treatment	17	45741.298	2690.665	1840.333	0.000
	Error	36	52.634	1.462		
	Total	53	45793.932			

Table 9 ANOVA: The activities of protease powder with different carriers

Source	df	SS	MS	F	Sig.
Treatment	4	789579	197395	311.556	0.000
Error	10	6335.76	633.576		
Total	14	795915			

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Table 10 ANOVA: Effect of enzyme supplement on *in vitro* digestibility of farm piglet diet.

	Source	df	SS	MS	F	Sig.
DM	Treatment	4	328.981	82.245	874.605	0.000
	Error	55	5.172	0.094		
	Total	59	334.153			
CP	Treatment	4	58.919	14.730	401.305	0.000
	Error	10	0.367	0.037		
	Total	14	59.286			
CF	Treatment	4	18.607	4.652	39.030	0.000
	Error	10	1.192	0.119		
	Total	14	19.799			
ASH	Treatment	4	13.492	3.373	37.009	0.000
	Error	10	0.911	0.091		
	Total	14	14.403			
EE	Treatment	4	19.630	4.907	57.019	0.000
	Error	10	0.861	0.086		
	Total	14	20.490			

Table 11 ANOVA: Effect of enzyme supplement on *in vitro* digestibility of growing pig diet.

	Source	df	SS	MS	F	Sig.
DM	Treatment	4	143.349	35.837	367.633	0.000
	Error	55	5.361	0.097		
	Total	59	148.710			
CP	Treatment	4	36.451	9.113	214.458	0.000
	Error	10	0.425	0.042		
	Total	14	36.876			
CF	Treatment	4	16.190	4.047	33.719	0.000
	Error	10	1.200	0.120		
	Total	14	17.390			
ASH	Treatment	4	15.471	3.868	42.951	0.000
	Error	10	0.900	0.090		
	Total	14	16.371			
EE	Treatment	4	13.398	3.349	45.302	0.000
	Error	10	0.739	0.074		
	Total	14	14.137			

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Table 12 ANOVA: Effect of enzyme supplement on *in vitro* digestibility of finishing pig diet.

	Source	df	SS	MS	F	Sig.
DM	Treatment	4	143.349	35.837	367.633	0.000
	Error	55	5.361	0.097		
	Total	59	148.710			
CP	Treatment	4	36.451	9.113	214.458	0.000
	Error	10	0.425	0.042		
	Total	14	36.876			
CF	Treatment	4	16.190	4.047	33.719	0.000
	Error	10	1.200	0.120		
	Total	14	17.390			
ASH	Treatment	4	15.471	3.868	42.951	0.000
	Error	10	0.900	0.090		
	Total	14	16.371			
EE	Treatment	4	13.398	3.349	45.302	0.000
	Error	10	0.739	0.074		
	Total	14	14.137			

Table 13 ANOVA: Effect of enzyme supplement on *in vitro* digestibility of pregnant pig diet.

	Source	df	SS	MS	F	Sig.
DM	Treatment	4	726.599	181.650	2327.631	0.000
	Error	55	4.292	0.078		
	Total	59	730.892			
CP	Treatment	4	21.961	5.490	48.078	0.000
	Error	10	1.142	0.114		
	Total	14	23.102			
CF	Treatment	4	8.992	2.248	16.277	0.000
	Error	10	1.381	0.138		
	Total	14	10.373			
ASH	Treatment	4	10.051	2.513	30.828	0.000
	Error	10	0.815	0.082		
	Total	14	10.866			
EE	Treatment	4	11.937	2.984	74.230	0.000
	Error	10	0.402	0.040		
	Total	14	12.339			

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Table 14 ANOVA: Initial weight of piglets.

Source	df	SS	MS	F	Sig.
Corrected Model	9	12.635	1.404	57.341	0.000
Intercept	1	1941.880	1941.880	79314.364	0.000
TREAT	4	0.105	0.026	1.067	0.379
WEANING	5	12.531	2.506	102.360	0.000
Error	70	1.714	0.024		
Total	80	2017.350			
Corrected Total	79	14.349			

Table 15 ANOVA: Effect of enzyme supplement on final weight of piglets.

Source	df	SS	MS	F	Sig.
Corrected Model	10	86.255	8.626	103.385	0.000
Intercept	1	14.121	14.121	169.249	0.000
TREAT	4	25.271	6.318	75.725	0.000
WEANING	5	1.372	0.274	3.288	0.010
INIT_WT	1	2.525	2.525	30.266	0.000
Error	69	5.757	0.083		
Total	80	33580.940			
Corrected Total	79	92.012			

Table 16 ANOVA: Effect of enzyme supplement on average total feed intake.

Source	df	SS	MS	F	Sig.
Corrected Model	10	32.217	3.222	7.734	0.000
Intercept	1	31.666	31.666	76.020	0.000
TREAT	4	2.791	0.698	1.675	0.166
WEANING	5	3.745	0.749	1.798	0.125
INIT_WT	1	0.129	0.129	0.310	0.579
Error	69	28.742	0.417		
Total	80	42114.580			
Corrected Total	79	60.959			

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Table 17 ANOVA: Effect of enzyme supplement on average daily feed intake at first experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	23224.808	2322.481	101.566	0.000
Intercept	1	1341.109	1341.109	58.649	0.000
TREAT	4	301.776	75.444	3.299	0.016
WEANING	5	1861.121	372.224	16.278	0.000
INIT_WT	1	170.472	170.472	7.455	0.008
Error	69	1577.807	22.867		
Total	80	2911905.884			
Corrected Total	79	24802.614			

Table 18 ANOVA: Effect of enzyme supplement on average daily feed intake at second experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	19796.396	1979.640	65.513	0.000
Intercept	1	4607.524	4607.524	152.479	0.000
TREAT	4	309.410	77.352	2.560	0.046
WEANING	5	692.811	138.562	4.585	0.001
INIT_WT	1	864.818	864.818	28.620	0.000
Error	69	2085.009	30.218		
Total	80	11108186.245			
Corrected Total	79	21881.404			

Table 19 ANOVA: Effect of enzyme supplement on average daily feed intake at third experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	12029.844	1202.984	27.873	0.000
Intercept	1	14489.600	14489.600	335.725	0.000
TREAT	4	582.816	145.704	3.376	0.014
WEANING	5	422.319	84.464	1.957	0.096
INIT_WT	1	316.366	316.366	7.330	0.009
Error	69	2977.982	43.159		
Total	80	22431519.321			
Corrected Total	79	15007.826			

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Table 20 ANOVA: Effect of enzyme supplement on average daily feed intake at fourth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	11683.822	1168.382	19.550	0.000
Intercept	1	20564.757	20564.757	344.095	0.000
TREAT	4	719.349	179.837	3.009	0.024
WEANING	5	264.334	52.867	0.885	0.496
INIT_WT	1	446.965	446.965	7.479	0.008
Error	69	4123.769	59.765		
Total	80	31815793.908			
Corrected Total	79	15807.591			

Table 21 ANOVA: Effect of enzyme supplement on average daily feed intake at fifth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	9162.733	916.273	35.107	0.000
Intercept	1	33890.936	33890.936	1298.529	0.000
TREAT	4	658.793	164.698	6.310	0.000
WEANING	5	1224.452	244.890	9.383	0.000
INIT_WT	1	32.634	32.634	1.250	0.267
Error	69	1800.864	26.099		
Total	80	43451454.413			
Corrected Total	79	10963.597			

Table 22 ANOVA: Effect of enzyme supplement on average daily feed intake at sixth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	11277.303	1127.730	23.162	0.000
Intercept	1	40254.353	40254.353	826.768	0.000
TREAT	4	619.503	154.876	3.181	0.019
WEANING	5	1438.555	287.711	5.909	0.000
INIT_WT	1	202.738	202.738	4.164	0.045
Error	69	3359.529	48.689		
Total	80	54309564.900			
Corrected Total	79	14636.832			

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Table 23 ANOVA: Effect of enzyme supplement on average daily feed intake for whole experimental period.

Source	df	SS	MS	F	Sig.
Corrected Model	10	26043.852	2604.385	4.974	0.000
Intercept	1	7668.891	7668.891	14.646	0.000
TREAT	4	500.778	125.195	0.239	0.915
WEANING	5	2827.231	565.446	1.080	0.379
INIT_WT	1	2728.580	2728.580	5.211	0.026
Error	69	36130.001	523.623		
Total	80	23585597.022			
Corrected Total	79	62173.853			

Table 24 ANOVA: Effect of enzyme supplement on average daily gain at first experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	6090.022	609.002	16.256	0.000
Intercept	1	110.728	110.728	2.956	0.090
TREAT	4	1306.896	326.724	8.721	0.000
WEANING	5	168.167	33.633	0.898	0.488
INIT_WT	1	214.595	214.595	5.728	0.019
Error	69	2585.035	37.464		
Total	80	749800.057			
Corrected Total	79	8675.057			

Table 25 ANOVA: Effect of enzyme supplement on average daily gain at second experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	12925.320	1292.532	16.283	0.000
Intercept	1	2352.730	2352.730	29.640	0.000
TREAT	4	5224.137	1306.034	16.453	0.000
WEANING	5	456.962	91.392	1.151	0.342
INIT_WT	1	205.826	205.826	2.593	0.112
Error	69	5477.081	79.378		
Total	80	4646420.895			
Corrected Total	79	18402.401			

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Table 26 ANOVA: Effect of enzyme supplement on average daily gain at third experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	38969.289	3896.929	36.310	0.000
Intercept	1	6840.600	6840.600	63.738	0.000
TREAT	4	28156.706	7039.177	65.588	0.000
WEANING	5	459.927	91.985	0.857	0.514
INIT_WT	1	168.864	168.864	1.573	0.214
Error	69	7405.317	107.323		
Total	80	10791443.608			
Corrected Total	79	46374.607			

Table 27 ANOVA: Effect of enzyme supplement on average daily gain at fourth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	46764.548	4676.455	53.421	0.000
Intercept	1	15658.910	15658.910	178.879	0.000
TREAT	4	32820.464	8205.116	93.731	0.000
WEANING	5	1179.157	235.831	2.694	0.028
INIT_WT	1	84.945	84.945	0.970	0.328
Error	69	6040.211	87.539		
Total	80	21261000.292			
Corrected Total	79	52804.759			

Table 28 ANOVA: Effect of enzyme supplement on average daily gain at fifth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	19727.511	1972.751	18.172	0.000
Intercept	1	14639.177	14639.177	134.852	0.000
TREAT	4	15234.173	3808.543	35.083	0.000
WEANING	5	595.299	119.060	1.097	0.370
INIT_WT	1	128.730	128.730	1.186	0.280
Error	69	7490.443	108.557		
Total	80	20597912.031			
Corrected Total	79	27217.953			

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Table 29 ANOVA: Effect of enzyme supplement on average daily gain at sixth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	50362.458	5036.246	44.373	0.000
Intercept	1	13361.163	13361.163	117.723	0.000
TREAT	4	45127.038	11281.760	99.402	0.000
WEANING	5	148.313	29.663	0.261	0.933
INIT_WT	1	94.894	94.894	0.836	0.364
Error	69	7831.277	113.497		
Total	80	18514432.098			
Corrected Total	79	58193.735			

Table 30 ANOVA: Effect of enzyme supplement on average daily gain for whole experimental period.

Source	df	SS	MS	F	Sig.
Corrected Model	10	36409.866	3640.987	10.016	0.000
Intercept	1	4837.555	4837.555	13.308	0.001
TREAT	4	23521.724	5880.431	16.177	0.000
WEANING	5	1606.687	321.337	0.884	0.497
INIT_WT	1	675.826	675.826	1.859	0.177
Error	69	25082.126	363.509		
Total	80	10744472.613			
Corrected Total	79	61491.993			

Table 31 ANOVA: Effect of enzyme supplement on feed conversion ratio at first experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	0.461	0.046	3.479	0.001
Intercept	1	0.401	0.401	30.254	0.000
TREAT	4	0.427	0.107	8.041	0.000
WEANING	5	0.044	0.009	0.658	0.656
INIT_WT	1	0.013	0.013	1.004	0.320
Error	69	0.915	0.013		
Total	80	317.508			

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Corrected Total	79	1.377			

Table 32 ANOVA: Effect of enzyme supplement on feed conversion ratio at second experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	0.291	0.029	6.147	0.000
Intercept	1	0.139	0.139	29.506	0.000
TREAT	4	0.279	0.070	14.777	0.000
WEANING	5	0.006	0.001	0.272	0.927
INIT_WT	1	0.001	0.001	0.207	0.650
Error	69	0.326	0.005		
Total	80	193.254			
Corrected Total	79	0.617			

Table 33 ANOVA: Effect of enzyme supplement on feed conversion ratio at third experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	0.521	0.052	25.487	0.000
Intercept	1	0.149	0.149	73.138	0.000
TREAT	4	0.488	0.122	59.730	0.000
WEANING	5	0.001	0.000	0.142	0.982
INIT_WT	1	0.000	0.000	0.036	0.850
Error	69	0.141	0.002		
Total	80	168.839			
Corrected Total	79	0.662			

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Table 34 ANOVA: Effect of enzyme supplement on feed conversion ratio at fourth experimental week.

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Source	df	SS	MS	F	Sig.
Corrected Model	10	0.227	0.023	31.012	0.000
Intercept	1	0.092	0.092	125.291	0.000
TREAT	4	0.215	0.054	73.341	0.000
WEANING	5	0.004	0.001	0.974	0.440
INIT_WT	1	0.000	0.000	0.413	0.523
Error	69	0.050	0.001		
Total	80	120.734			

Corrected Total	79	0.277			

Table 35 ANOVA: Effect of enzyme supplement on feed conversion ratio at fifth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	0.169	0.017	16.546	0.000
Intercept	1	0.164	0.164	160.506	0.000
TREAT	4	0.147	0.037	36.066	0.000
WEANING	5	0.011	0.002	2.215	0.063
INIT_WT	1	0.001	0.001	0.626	0.431
Error	69	0.070	0.001		
Total	80	169.622			
Corrected Total	79	0.240			

Table 36 ANOVA: Effect of enzyme supplement on feed conversion ratio at sixth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	10	0.650	0.065	30.943	0.000
Intercept	1	0.208	0.208	98.761	0.000
TREAT	4	0.617	0.154	73.338	0.000
WEANING	5	0.008	0.002	0.759	0.582
INIT_WT	1	0.000	0.000	0.024	0.877
Error	69	0.145	0.002		
Total	80	237.602			
Corrected Total	79	0.795			

Table 37 ANOVA: Effect of enzyme supplement on feed conversion ratio gain for whole experimental period.

Source	df	SS	MS	F	Sig.
Corrected Model	10	0.493	0.049	7.948	0.000
Intercept	1	0.214	0.214	34.484	0.000
TREAT	4	0.426	0.107	17.201	0.000
WEANING	5	0.022	0.004	0.705	0.622
INIT_WT	1	0.005	0.005	0.793	0.376
Error	69	0.428	0.006		

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Total	80	182.243
Corrected Total	79	0.920

Table 38 ANOVA: Blood urea nitrogen levels at initial experimental week.

Source	df	SS	MS	F	Sig.
Treatment	4	105.241	26.31	3.058	0.017
Error	315	2710.178	8.604		
Total	319	2815.418			

Table 39 ANOVA: Effect of enzyme supplement on blood urea nitrogen levels at second experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	5	5600.571	1120.114	154.703	0.000
Intercept	1	2180.711	2180.711	301.186	0.000
TREAT	4	5531.204	1382.801	190.984	0.000
BUN_W0	1	30.087	30.087	4.155	0.042
Error	314	2273.487	7.240		
Total	320	367114.600			
Corrected Total	319	7874.057			

Table 40 ANOVA: Effect of enzyme supplement on blood urea nitrogen levels at fourth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	5	10109.590	2021.918	277.392	0.000
Intercept	1	591.627	591.627	81.167	0.000
TREAT	4	9839.200	2459.800	337.466	0.000
BUN_W0	1	19.751	19.751	2.710	0.101
Error	314	2288.758	7.289		
Total	320	187195.667			
Corrected Total	319	12398.349			

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Table 41 ANOVA: Effect of enzyme supplement on blood urea nitrogen levels at sixth experimental week.

Source	df	SS	MS	F	Sig.
Corrected Model	5	7760.451	1552.090	206.018	0.000
Intercept	1	1670.598	1670.598	221.748	0.000
TREAT	4	7615.894	1903.974	252.725	0.000
BUN_W0	1	6.546	6.546	0.869	0.352
Error	314	2365.605	7.534		
Total	320	320515.195			
Corrected Total	319	10126.056			

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