APPENDICES

Appendix A

Media, Buffer and Experimental Diets

A-1. Media

Composition in each medium was dissolved in distilled water and made the volume up to 1000 ml. The media were autoclaved at 121 C, 15 psi, for 15 min.

A-1.1 Potato dextrose agar (PDA)

This medium was used for maintenance and subculture of fungi. The medium contains

Potato	200 g
Dextrose	20 g
Agar	10 g
Distilled water	1000 ml

For liquid medium, all of the componants except agar were dissolved in distilled water.

A-2. Buffer solution

A-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

A-2.2. 0.1 N glycine-HCl buffer pH 3.2

Dissolved glycine 0.7507 g in 45 ml of distilled water. Stir the solution constantly and adjust to the require pH with 1.0N HCl. Make up to 50 ml in volumetric flask.

A-2-3. 0.1 M phosphate buffer (KH_2PO_4-NaOH) pH 7.0

Dissolved KH_2PO_4 1.3799 g in 45 ml of distilled water. Stir the solution constantly and adjust to the require pH with 1.0 N NaOH. Make up to 50 ml in volumetric flask.

A-2.4. 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 didodium 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 1.0N NaOH. Make up to 1 liter in volumetric flask.

A-2.5. 0.2 M Phospater 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 (NaH_2PO_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 1.0N NaOH. Made up to 1 liter in volumetric flask.

Appendix B
Analytical methods

B-1. Cytotoxicity test

Cytotoxicity of crude β-glucanase powder was determined by the 3-(4,5-dimethylthiazoyl-2-yl) 2,5diphenyltetrazolium bromium (MTT) ASSAY according to the method of Plumb *et al*., (1989).

Sample preparation

Fourteen samples of crude wet-weight were presented to us. All the samples were frozen dried. One gram of each sample was lyzed in 0.1N NaOH for an hour and then were filtrated through 0.2 um filters to be made up at the top concentration of 1 mg/ml. This was the highest concentration we could make.

Cell culture test

The target cells were L929, a mouse lung and BHK(21)C13, a baby hamster kidney standard; well characterized cell lines used for cytotoxicity tests. The L929 and BHK(21)C13 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and Glasgow Modified Eagle's Medium (GMEM), respectively. The other cell line that was used is HepG2, a human hepatocyte cell line. The Hepg2 cells were grown in Minimum Essential Medium (MEM). All cell lines were incubated at 37^{o}C in a fully humidified, 5% CO_2 : air atmosphere.

MTT cytotoxicity test

Metabolism-competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue); this color change is measured spectrophotometrically with a plate reader. It is assumed cells that are metabolically deficient will not survive, thus the MTT assay is also an indirect measurement of cell viability. L929 and BHK (21)C13 were seeded in a 96-well plate with 1000, 1000 and 2000 cells/well, respectively, and incubated for 48 hours. The samples were serial diluted in growth medium to give a concentration of 1, 0.1, 0.01 and 0.001 mg/ml. The samples in each concentration were added to the cells and incubated for 24 hours. The test samples were removed

from the cell cultures and the cells were reincubated for a further 24 hours in fresh medium and then tested with MTT assay. A result was derived from the %survival of the cells compared to controls.

B-2. Aflatoxin test

To test the aflatoxin in the enzyme extract and in the feed trated with the enzyme of the selected strains, the ELISA test kit was used due to the method of the Aflatoxin (Total) Quantitative Test Kit Ser: 15056 Veratox®. The sample extract of selected strains was cultured in wheat bran-soybean medium (WS). The cells and enzyme extract was harvested and preserved by freeze drying before testing.

Sample extraction

Sample were used for quantification aflatoxin. The samples were ground pass 2 mm. size of the sieve. Fifty g of ground sample was diluted in 100 ml of 70% (v/v) methanol through and blended with high-speed blender for 2 min. This sas subsequently filtered by using filter paper by Whatman No.1. The sample solution extract was kept at 4°C.

Quantification aflatoxin in samples by ELISA test kit

1. Add samples solution 100 µl/well and standard solution (0, 2, 5, 10, and 25 ppb) 100 µl/well in the strips (coated with antibody).

2. Add OTA conjugated enzyme 100 µl/well and incubated at room temperature for 5 min.

3. Wash with deionized water 150 µl/well for 5 times. Added substrate solution 100 µl/well and incubated at room temperature in the dark room for 10 min.

4. Add stopping solution 100 µl/well. Read at 650 nm by ELISA reader.

5. Absorbances were plotted against various standard concentrations as the standard curve

B-3. Ochratoxin test

The ochratoxin in the enzyme extract and in the feed treated with the enzyme of selected strains were tested by using the ELISA test kit according to the method of the Ochratoxin Quantitative test Kit Serial: 2516 Veratox®. The cell extracts of

selected strains was cultured in wheat bran-soybean medium (WS). The cell and enzyme extract were harvested and preserved by freezed drying before testing.

Sample extraction

Sample were used for quantification ochratoxin A (OTA). The samples were ground pass 2 mm. size of the sieve. 50 g of ground sample was diluted in 70% (v/v) methanol 100 ml and blended with high-speed blender for 2 minutes. Filtered by Whatman No.1, the sample solution extract was kept at 4^{o}C.

Quantification OTA in samples by ELISA test kit

1. Add samples solution 100 µl/well and standard solution (0, 2, 5, 10, and 25 ppb) 100 µl/well in the trips (coated with antibody)

2. Shake the well for 20 second and incubate at room temperature for 5 minutes.

3. Wash with deionized water 150 µl/well for 5 times. Added substrate solution 100 µl/well and incubated at room temperature in the dark room for 10 min.

4. Add stopping solution 100 µl/well. Read at 650 nm by ELISA reader.

5. Absorbances were plotted against various standard concentrations as the standard curve

B-4. Activities profiles

B-4.1. Amylase activity assay (modified method of Rick and Steuberg, 1974)

Substrate

1% (w/v) soluble starch in 0.2 citrate phosphate buffer, pH 3.0 and 7.0

Procedure

1. Add 1.0 ml of substrate solution to a test tube.
2. Incubate the tube in water bath at 40°C for 5 min.
3. Add 0.5 ml of enzyme, mix well and incubate at 40°C for 10 min.
4. Add 3.0 ml of DNS, mix, and transfer to 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 spectro zero

should be boiled together. After boiling, transfer the tube 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}). Substract the color formed in the sample with that of the enzyme blank tube.
8. For 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 40°C under the reaction conditions.

B-4.2. Cellulase activity assay

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% (w/v) CMC (BDH) in 0.2 M citrate phosphate buffer, pH 3.0 and 7.0.

Procedure

1. Add 1.0 ml of substrate solution to a test tube
2. Incubate the tube in water bath at 40°C for 5 min.
3. Add 0.5 ml of enzyme, mix well and incubate at 40°C for 30 min.
4. Add 3.0 ml of DNS, mix, and transfer to 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 spectro zero should be boiled together. After boiling, transfer the tube 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}). Substract the color formed in the sample with that of the enzyme blank tube.

8. For 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 40°C under the reaction conditions.

B-4.3. 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 3.0 and 7.0

Procedure

1. Add 1.8 ml of substrate solution to a test tube
2. Incubate the tube in water bath at 40°C
3. Add 200 µl of enzyme, mix well and incubate at 40°C for 5 min
4. Add 3.0 ml of DNS, mix, and transfer to rack on the table.
5. Boil exactly 15.0 min in a virtuously boiling water bath containing sufficient water. All enzyme blank, xylose standard and the spectro zero should be boiled together. After boiling, transfer the tube 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}). Substract the color formed in the sample with that of the enzyme blank tube.
8. For 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 40°C under the reaction conditions.

B-**4.4. 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 molybdovanadophophoric 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 agent.

Reagent

Substrate solution

Dissolved 8.4 g of sodium phytate from rice in 950 ml buffer solution, adjust to pH 3.0 and 6.8 with 0.1 M Sodium-citrate-HCl Buffer (pH 3.0) and Tris-Malate-NaOH Buffer (pH 6.8). Prepare freshly

Nitric acid solution

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

Ammonium heptamlybdate stock solution

Dissolve 100 g of ammonium heptamolybdate($H_{24}Mo_7N_6O_{24}.4H_20$) in 900 ml water, add 25% (v/v) ammonia (10 ml), and dilute to 1 L with water. This solution can be kept at room temperature shield from light for 1 month

Ammonium vanadate stock solution

Dissolved 2.35 g of ammonium vanadate (NH_4VO_3) in 400 ml water at 60 C. While stirring, slowly add 20 ml of nitric acid solution, cool to room temperature, and dilute to 1 L with water. This solution may be kept at room temperature shield from light for 1 month.

Color stop mix solution

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

B-4.5. Protease activity assay

Proteolytic activity was determined at the physiological conditions in the animal's digestive tract according to the modified method of An *et al*. (1994). The proteolytic activity of the enzyme was determined using soy protein isolate (SPI) as a substrate.

Substrate

Soy protein isolate (SPI) in citrate phosphate buffer, pH 3.0 and 7.0

Procedure

1. Add 200 µl of substrate solution, 325 µl of citrate phosphate buffer, 625 µl of distilled water and 100 µl of enzyme solution to a test tube and mix well.
2. Incubate the tube in water bath at 40 °C for 30 min.
3. Add 200 µl of 50% trichloroacetic acid (TCA), mix. Transfer to a rack on the table
4. Centrifuge the tube at 2,500 rpm for 10 min.
5. Measure the color formed against the spectro zero at 750 nm (OD_{750}). Substract the color formed in the sample with that of the enzyme blank tube.
6. For the spectro zero and enzyme blank, add buffer of enzyme to the tube after the addition of TCA.

Protease activity was determined as released tyrosine from the supernatants according to a modified Lowry et al. method (1951). One unit of enzyme activity was defined as the amount of the enzyme resulting in the release of 1 ng of tyrosine per min at 40°C under the reaction conditions.

B-5. Proximate analysis

B-5.1. Dry matter

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

Procedure

1. Dry weighing bottles at 100°C for 15 h in a hot air oven and cool in 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 (15-30 min).
6. Weigh the samples after cooling. (W_2)
7. Calculation

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

$$\% \text{ Dry matter} = \frac{W2 - W1 \times 100}{W_S}$$

B-5.2. Crude protein

Crude protein was assayed by Kjeldahl's 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 nitrogen present is converted into ammonium sulfate ($(NH_4)_2SO_4$). Concentrated NaOH is added to release NH_3 that is distilled, collected in H_3BO_3 solution, and quantitated by a titration method.

Reagent:

Sulfuric acid, selenium reagent mixture, sodium hydroxide solution, tashiro indicator, 40% (w/v) boric acid solution, and hydrochloric acid standard solution, 0.1000 N

Procedure

1. Transfer sample 1.0 g (W_S) into a Kjeldahl tube.
2. Add selenium reagent mixture (0.5g) and mix throughly.
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 swirl to mix.

5. Distilled: Place Kjeldahl tube to distillation unit. Add H_3BO_3 (25 ml) solution with tashiro indicator to Erlenmeyer flask (250 ml) and place on receiving platform, with tube from condenser extending below surface of H_3BO_3 solution. At the end of distillation, distillate should be light green or blue solution.
6. Titration: Titrate H_3BO_3 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 :

$$\%\text{Total nitrogen} = \frac{1.401 \times (\text{ml HCl, sample} - \text{ml HCl, blank}) \times \text{normality HCl}}{\text{g sample}}$$

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

B-5.3. Crude fiber

Crude fiber is loss on ignition of dried residue remaining after digestion of sample with 3.125% (w/v) H_2SO_4 and 3.125% (w/v) 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.

Reagent:

3.125% (w/v) H_2SO_4 and 3.125% (w/v) NaOH solution

Procedure

1. Transfer sample 1 g (W_S) to 600 ml beaker and add 200 ml of 3.125% (w/v) 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 bunchner.
5. Repeat with three 50 ml portions of water and suck dry.

6. Return all diatomacous earth and residue to beaker.
7. Add 200 ml of 3.125% (w/v) NaOH and boil exactly for 10 min.
8. Remove beaker and filter as above, transfer diatomaceous earth and residue to porcelain crecible.
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. (W_1)
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.(W_2)
11. Calculation

%Crude fibre content was calculated as follows:

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

B-5.4. 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 procelain crucibles in a muffle furnace a around 450-550°C overnight, cool in a desiccator and weith after reaching room temparature (W_1).
2. Add 3 g of diet to the pre-ignited crucible (weithed 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, overhight.
6. Turn off muffle furnace and open after temparature has reached about 250°C.
7. Using the tongs to transfer the crucibles to the desiccator, cool in a desiccator and weigh after reaching room temparature (W_2).
8. Calculation

%Ash content was calculated as follows:

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

B-5.5. Ether extract

Fat is extracted from a dry sample with petroleum ether using a special Soxhlet apparatus set up. The ether extract is collected in a flask. The percentage of 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 a hot air oven and cool in a desiccator
2. Weigh pre-dried flask. (W_1)
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 a least 5-6 drops per sec.
5. Remove the thimble from 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:

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

B-5.6. Nitrogen free extract

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

%Nitrogen free extract = %Dry matter-(%Crude protein + %Crude fibre + %Ether extract + % Ash)

B-6. Glucan analysis

The assay is specific for mixed linkage [(1-3)(1-4)-β-D-glucan. Kits suitable for performing 100 assays are available from Megazyme. Samples are suspended and hydrated in a buffer solution of pH 6.5 and then incubated with purified lichenase enzyme and filtered. An aliquot of the filtrate is then hydrolysed to completion with purified β-glucosidase. The D-glucose produced is assayed using a glucose oxidase/peroxidase reagent.

Method:

1. Mill barley, oats or fibre sample (approximately 50 g) to pass a 0.5 mm screen

2. Add flour sample (80-120 mg; weighed accurately) to a glass centrifuge tube (16 x 120 mm; 17 mL capacity). Tap the tube to ensure that all sample falls to the bottom of the tube.

3. Wet the sample with 0.2 mL of aqueous ethanol (50 % v/v) to aid dispersion. Add sodium phosphate buffer (4.0 mL, 20 mM,pH 6.5) and stir the contents on a vortex mixer.

4. On mixing, immediately place the tube in a boiling water bath and incubate for 60 sec. Vigorously stir the mixture on a vortex mixer, incubate at 100°C for a further 2 min, and stir again.

5. Incubate the tube plus contents at 50°C and allow to equilibrate for 5 min.

6. Add lichenase (0.2 mL, 10 U) and stir the tube contents. Seal the tube with parafilm and incubate for 1 h at 50°C, with regular vigorous stirring (i.e. 3-4 times) on a vortex mixer. In fact, continuous stirring using a device such as the Megazyme Multistir Incubation Bath (cat. no. G-IBMKIII) is recommended.

7. Add sodium acetate buffer (5.0 mL, 200 mM, pH 4.0) and vigorously mix the tube contents on a vortex mixer.

8. Allow the tube to equilibrate to room temperature (5 min), and centrifuge (1,000 *g*, 10 min). Carefully and accurately dispense aliquots (0.1 mL) into the bottom of three test tubes (12 mL capacity) using a Gilson Pipetman® or a Rainin EDP-2® motorised dispenser.

9. Add β-glucosidase (0.1 mL, 0.2 U) in 50 mM sodium acetate buffer (pH 4.0) to two of these tubes (the reaction). To the third (the reaction blank), add 50 mM acetate buffer (0.1 mL, pH 4.0). Incubate all tubes at 50°C for 10 min.

10. Add GOPOD Reagent (3.0 mL) to each tube, and incubate at 50°C for a further 20 min.

11. Remove the tubes from the water bath and measure the absorbance (510 nm) within 1 h.

B-7. *In vitro* digestibility

Reagents

0.1 M HCl

0.1 M Phosphate buffer, pH 6.0

0.2 M Phosphate buffer, pH 6.8

0.6 M NaOH

1 M HCl

1 M NaOH

20% (w/v) sulphosalicylic acid

Dissolve 50 g of sulphosalicylic acid in 240 ml of distilled water, make up the volume to 250 ml in volumetric flask.

Choramphenical solution (0.5 g/100 ml ethanol)

Dissolve 0.5 g of Choramphonicol in 80 ml of ethanol, make up the volume to 100 ml in a volumetric flask.

Pancreatin solution (50 mg/ml)

Dissolve 2.5 g of porcine pancreatin in 45 ml of distilled water, make up the volume to 50 ml in a volumetric flask.

Pensin solution (10 mg/ml)

Dissolve 0.5 g of porcine pepsin in 45 ml of distilled water, make up the volume to 50 ml in a volumetric flask.

Procedure

Step 1.

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

2. Sample and blank should be 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 tp pH 2.0 with a 1M HCl (or 1 N NaOH solution).

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

6. In order to prevent bacterial growth, especially during the second incubation step, add 0.5 ml of choramphenicol solution

7. Then close the flask with cotton knob and incubate in incubator shaker at 40 °C for 6 h.

Step. 2

1. Add 10 ml of a 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 N 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 40 °C for incubating overnight (18h)

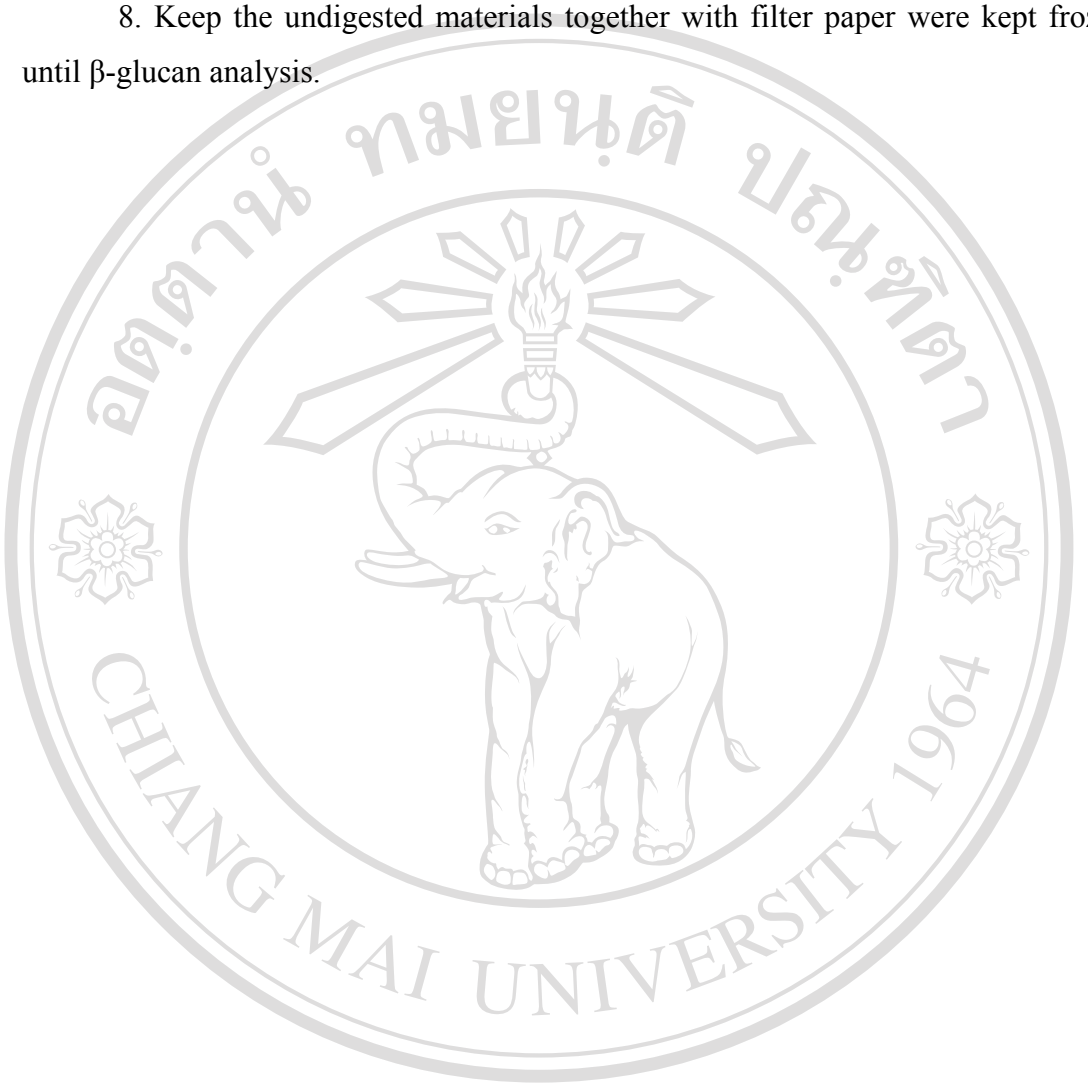
5. Add 5 ml of 20% sulphosalicylic acid, incubate at room temperature for 30 min.

6. Collect the 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 105 °C for overnight. 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 were kept frozen until β-glucan analysis.



APPENDIX C

STATISTICAL ANALYSIS

Table 1 ANOVA: β-glucanase activity screening (Experiment 4.1.1)

1.1 Activity at pH 3.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13297413.624	10	1329741.362	614.432	.000
Within Groups	71417.889	33	2164.178		
Total	13368831.513	43			

1.2 Activity at pH 7.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4060540.429	10	406054.043	106.426	.000
Within Groups	125906.582	33	3815.351		
Total	4186447.012	43			

Table 2 ANOVA: Cytotoxicity test (Experiment 4.1.2.1)

2.1 0.1% mg/ml BHK

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	800.227	5	160.045	8.857	.010
Within Groups	108.420	6	18.070		
Total	908.647	11			

2.1 0.01 mg/ml BHK

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	318.830	5	63.766	14.928	.002
Within Groups	25.630	6	4.272		
Total	344.460	11			

2.3 0.1 mg/ml Hep G2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	860.814	5	172.163	21.066	.001
Within Groups	49.035	6	8.172		
Total	909.849	11			

2.4 0.01 mg/ml Hep G2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	578.667	5	115.733	40.847	.000
Within Groups	17.000	6	2.833		
Total	595.667	11			

Table 3 ANOVA: Aflatoxin test (Experiment 4.1.2.2)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.531	5	.906	.930	.522
Within Groups	5.846	6	.974		
Total	10.377	11			

Table 4 ANOVA: Ochratoxin test (Experiment 4.1.2.3)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	306.740	5	61.348	10.144	.007
Within Groups	36.286	6	6.048		
Total	343.026	11			

Table 5 ANOVA: Resistance to pH and proteolytic enzyme (Experiment 4.1.3.1)

5.1 Incubate at low pH with pepsin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	42.945	2	21.473	1.539	.266
Within Groups	125.600	9	13.956		
Total	168.545	11			

5.2 Incubate at high pH with pepsin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	103.249	2	51.625	.707	.519
Within Groups	657.212	9	73.024		
Total	760.461	11			

5.3 Incubate at low pH with pancreatin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.823	2	1.912	.119	.889
Within Groups	144.575	9	16.064		
Total	148.399	11			

5.4 Incubate at high pH with pancreatin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	91.784	2	45.892	.152	.861
Within Groups	2722.613	9	302.513		
Total	2814.397	11			

Table 6 ANOVA: Resistant to heat (Experiment 4.1.3.2)

6.1 Incubate 2 min, activity at pH 3.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1985.839	2	992.920	33.293	.000
Within Groups	268.413	9	29.824		
Total	2254.252	11			

6.2 Incubate 2 min, activity at pH 7.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	355.665	2	177.833	8.728	.008
Within Groups	183.368	9	20.374		
Total	539.033	11			

6.3 Incubate 5 min, activity at pH 3.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2676.993	2	1338.497	57.917	.000
Within Groups	207.997	9	23.111		
Total	2884.990	11			

6.4 Incubate 5 min, activity at pH 7.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	657.173	2	328.587	11.139	.004
Within Groups	265.481	9	29.498		
Total	922.654	11			

Table 7 ANOVA: Effect of carbon source on β-glucanase production (Experiment 4.2.1)

7.1 Activity at pH 3.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5376622519.778	4	1344155629.944	58.791	.000
Within Groups	342946656.000	15	22863110.400		
Total	5719569175.778	19			

7.2 Activity at pH 7.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	463514416.533	4	115878604.133	28.280	.000
Within Groups	61462279.111	15	4097485.274		
Total	524976695.645	19			

Table 8 ANOVA: Effect of Carbon sources to Nitrogen source ratio (Experiment 4.2.2)

8.1 Activity at pH 3.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6863086124.941	5	1372617224.988	39.276	.000
Within Groups	629069705.289	18	34948316.961		
Total	7492155830.230	23			

8.2 Activity at pH 7.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	692135779.800	5	138427155.960	9.788	.000
Within Groups	254576870.378	18	14143159.465		
Total	946712650.178	23			

Table 9 ANOVA: Effect of media additives (Experiment 4.2.3)

9.1 Activity at pH 3.0

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	5593781600.000	3	1864593866.667	16.019	.000
Within Groups	1396824000.000	12	116402000.000		
Total	6990605600.000	15			

9.2 Activity at pH 7.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	639122000.000	3	213040666.667	17.821	.000
Within Groups	143457600.000	12	11954800.000		
Total	782579600.000	15			

Table 10 ANOVA: Effects of initial moisture content of medium (Experiment 4.2.4)

10.1 Activity at pH 3.0

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	903360244588.317	5	180672048917.663	78.602	.000
Within Groups	41374385311.742	18	2298576961.763		
Total	944734629900.059	23			

10.2 Activity at pH 7.0

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	73739633360.946	5	14747926672.189	53.814	.000
Within Groups	4932925808.279	18	274051433.793		
Total	78672559169.225	23			

Table 11 ANOVA: Effect of inoculum size (Experiment 4.2.5)

11.1 Activity at pH 3.0

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	3329569100.000	3	1109856366.667	7.519	.004
Within Groups	1771391600.000	12	147615966.667		
Total	5100960700.000	15			

11.2 Activity at pH 7.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	731566000.000	3	243855333.333	7.739	.004
Within Groups	378100800.000	12	31508400.000		
Total	1109666800.000	15			

Table 12 ANOVA: Preservation of crude β-glucanase as dry powder (Experiment 4.3)

12.1 Activity at pH 3.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	43.555	2	21.778	1.951	.287
Within Groups	33.482	3	11.161		
Total	77.037	5			

12.2 Activity at pH 7.0

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	534.380	2	267.190	29.416	.011
Within Groups	27.249	3	9.083		
Total	561.630	5			

Table 13 ANOVA: Effect of enzyme supplementation on β-glucan digestibility of raw material (Experiment 4.6)

13.1 Barley

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6157.253	4	1539.313	259.963	.000
Within Groups	59.213	10	5.921		
Total	6216.466	14			

13.2 Oat

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6029.024	4	1507.256	33.652	.000
Within Groups	447.894	10	44.789		
Total	6476.918	14			

13.3 Wheat bran

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.063	4	2.016	10.869	.001
Within Groups	1.854	10	.185		
Total	9.917	14			

Table 14 ANOVA: Effect of enzyme supplementation on production performance for whole experimental period (Experiment 4.8)

14.1 ADFI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	2	.000	.071	.931
Within Groups	.087	18	.005		
Total	.088	20			

14.2 ADG

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	2	.001	.517	.605
Within Groups	.026	18	.001		
Total	.027	20			

14.3 FCR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.116	2	.058	4.076	.035
Within Groups	.256	18	.014		
Total	.372	20			

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