MATERIALS AND METHODS

Place and Time

This experiment was carried out at the Laboratory of Veterinary Public Health (MKAV, Kepayan) Department of Veterinary Service and Animal Industry, Sabah Malaysia from Mei until June 2012.

Material

The materials used in this experiment were rumen liquor of cattle which was obtained from the abattoir (SMTC, Sabah), ice thermos for keeping the rumen liquor, cheesecloth, refrigerator, and centrifuge machine. The material used for determination enzyme activities was centrifuge, thermometer, water shaker, water bath, pH meter, incubator, spectrometer, crude filtrate rumen enzyme, Gas Chromatography, Biochemical analyser and chemical reagent for measuring enzymes activities.

Methods

Sample preparation of rumen liquor

Rumen liquor were collected from slaughtered cattle from the abattoir Sabah Meat Technology Centre (SMTC), Sabah Malaysia and transported to the laboratory used flasks in ice container at 4°C. Samples were filtered in cold condition using four layer of cheesecloth to separate the undigested matter. The filtrate was centrifuged at 10,000 rpm (4°C) for 10 minutes to separate supernatant from microbial cells (Lee et al. 2002). The supernatant was filtered used Wathman filter paper (No. 4) under suction to remove any micro particles. Supernatant was then taken as a source of crude enzyme and stored at 4°C until use.
Enzyme isolation

Supernatant containing enzymes was then reacted with ammonium sulphate (60% w/v) and stirred by magnetic stirrer for 1 hour and kept one night at 4°C. Supernatants were then centrifuged again at 10 000 rpm (4°C) for 15 minutes. The filtrate was taken and added with phosphate buffer pH 7 at the ratio of 10:1 (100 ml supernatant of rumen liquor is dissolved with 10 ml of phosphate buffer pH 7). The precipitates (enzyme source) in phosphate buffer are dialyzed before keeping in freezer for enzyme assay.

Enzymatic Activity Assay

Mannanase activity assayed using 0.5% (w/v) locust bean gum (LBG) in 0 mM sodium citrate buffer, pH 4.0 as substrate (Ghose 1987). The enzyme preparation (200 μL) is added into 1800 μL of substrate. The reaction mixtures are incubated at 40 °C in a water bath for 5 min and 30 min for mannanase. The quantity of reducing sugar released is measure using dinitrosalicylic acid (DNS) method (Miller 1959). One unit of enzyme activity is defined as the amount of enzyme producing 1 mmol of mannose per sec under the assay condition.

Experimental and data analyses

The data obtained from the variable was statistically analyzed according to descriptive analyses.
**In Vitro Experimental Method**

This experiment was conducted to measure the enzyme capabilities to hydrolyze carbohydrates by rumen fluid through *in vitro* test. This experiment was carried out using a method according to Boisen and Egum (1991) and Aslamyah (2006). A total of 25g of feed ingredients were weighed and placed in a plastic container with a lid. The feed was added with rumen filtrate enzyme and stirred evenly. Enzyme doses used were 0, 0.5, 1.0, 1.5, and 2.0% (v/w). Solution volume for each treatment with the addition of enzyme equated with distilled water prior to incubation for 24 h at room temperature.

**Determination of total soluble sugar.**

Measurement of feed carbohydrate hydrolysis by the enzyme is performed by measuring total soluble sugars by the method of Dinitrosalicylic Acid Reagent Methods after incubation. A total of 1g sample rations that have been incubated with rumen fluid enzymes were weighed and put into test tubes. Add 5 ml of distilled water and then vortex for about 1 minute. Centrifuged the mixed at 3000 rpm for 15 min, supernatant is used to measure levels of total soluble sugar rations of feed.

Determination of reducing sugar using Dinitrosalicylic Acid Reagent is based method tests for the presence of free carbonyl group, the so-called reducing sugars. The amount of carbohydrate present is determined by comparison with a calibration curve using a spectrophotometer.

**In vitro digestibility test**

The *in vitro* procedure was modified from that reported by Tilley and Terry (1963). Rumen liquor was taken from slaughtered cattle which were obtained from abattoir at Sabah Meat Technology Centre (SMTC), Sabah, Malaysia. The fluid was strained through four layers of cheese cloth.
McDougal buffer was prepared, then it was added mixed with rumen liquor at the ratio of (4 buffers: 1 rumen liquor). This mixture was saturated with CO$_2$ and warmed at 39°C in a water bath.

This experiment was conducted according to Complete Randomized Design (CRD) with 5 treatments and each treatment with 3 replicate. The exogenous commercial enzymes contain of mannanase (91 927.0 U/g), cellulases (526.0 unit/g), xylanases (3 869.0 U/g), alpha amylase (24 354.0 U/g), protease (345.4 U/g) in a powder form respectively and rumen liquor that used in this experiment consist of mannanase activities (0.0128 IU/L). Meanwhile the commercial yeasts and local yeasts content \textit{Saccharomyces cerevisae} about $4 \times 10^{10} \text{cfu g}^{-1}$, $1.5 \times 10^{10} \text{cfu g}^{-1}$ respectively.

About 500 mg experimental sample Basal ration (BS), BS + 1.5% filtrate enzymes (15ml/kg), BS+0.02% commercial enzyme (25mg/kg), BS+ 1% Local yeasts (10g/kg) , BS+ 0.5% commercial yeasts (5g/kg), then was mixed with McDougall buffer in a ratio 1:4. After gasifying with CO$_2$, tubes were incubated at 39°C. After 48 h the fermentation, 6 ml of HCl solution (20 %) and 5 ml pepsin solution were added and the incubated for 48 h simulating post-ruminal degradation.

Samples were filtered using a dry weighed Whatman filter paper (No. 41) and the residue was washed with boiled water; the filter paper and residue was dried at 100°C overnight and weighed after cooling in desiccators. The content of the crucibles were then incinerated at 475-500°C until a constant weight is achieved. A blank correction without matter was included.

This was calculated by the following

Equation:
\[
\% \text{IVDMD} = \frac{\text{DM samples} - (\text{DM residue-DM blank})}{\text{DM samples}} \times 100\%
\]

The organic matter digestibility (OMD) was calculated by the formula
\[
\% \text{IVOMD} = \frac{\text{OM samples}-(\text{Om residue- OM blank})}{\text{OM samples}} \times 100\%
\]
In Vivo Experimental

Material and Methods

Location and Date
This experiment was conducted at the experimental facilities at the Pusat Pembiakan Kambing, Bongawan, Sabah Malaysia. The chemical feed was analysed at the Food Safety unit of the Veterinary Public Health Laboratory, Kepayan, Sabah, Malaysia and Laboratory of Food Technology, University Malaysia Sabah. This experiment was conducted from June until August 2012.

Animal and diets

The exogenous commercial enzymes contain of mannanase (91 927 U/g), cellulases (526.0 unit/g), xylanases (3 869.0 U/g), alpha amylase (24 354 U/g), protease (34.4 U/g) in a powder form respectively and rumen liquor that used in this experiment consist of mannanase (0.0128 IU/L). Meanwhile the commercial yeasts and local yeasts content Sacchromyeces cerevisae about 4 x 10^{10} cfu g^{-1}, 1.5 x 10^{10} cfu g^{-1} respectively.

Twenty male growing crossbred goats (Feral x Local) averaging 15±2.5kg (initial mean BW±SD). The animals were allocated to 5 dietary treatments in which each treatment contain 4 replicate according to Randomized Complete Block Design (RCBD). The block was made according to their respective body weight. Dietary treatments are formulated to meet NRC nutrient requirements (NRC, 2007) which iso-protein (14%) and iso-energy (ME 2550 Kcal/kg) (Table 1).

The treatments are as follows:

- C = Basal ration B (Control)
- EZ1 = B + 1.5% Filtrate Enzyme (15ml/kg)
- EZ2 = B + 0.02% Commercial Enzymes (25mg/kg)
- YC1 = B + 5 g/h/d Local Yeasts (10g/kg)
- YC2 = B + 2.5 g/h/d Commercial Yeasts (5g/kg)
The experiment was conducted for thirty five (35) days consisted of a 14 day preliminary period, in which animals were adapted to be experimental diet and 21 day trial period.

Table 1 Ingredients and chemical compositions of the experimental diets

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Proportion (%)</th>
</tr>
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<tbody>
<tr>
<td>Palm cake kernel, PKC</td>
<td>50</td>
</tr>
<tr>
<td>Corn meal, CM</td>
<td>10</td>
</tr>
<tr>
<td>Soybean meal, SBM</td>
<td>5</td>
</tr>
<tr>
<td>Rice bran, RB</td>
<td>20</td>
</tr>
<tr>
<td>Pollard wheat</td>
<td>10</td>
</tr>
<tr>
<td>Salt</td>
<td>1</td>
</tr>
<tr>
<td>Mineral mix(^1)</td>
<td>1</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>1</td>
</tr>
<tr>
<td>Molasses</td>
<td>2</td>
</tr>
</tbody>
</table>

Minerals and vitamins (each kg contains): Vitamin A: 10,000,000 IU; Vitamin E: 70,000 IU; Vitamin D: 1,600,000 IU; Fe: 50 g; Zn: 40 g; Mn: 40 g; Co: 0.1 g; Cu: 10 g; Se: 0.1 g; I: 0.5

The basal ration composed of 40% concentrate feed mixture: 60% roughage (signal grass). The concentrate diets were fed in limited amounts (2 % of BW) once daily at 0800 h to minimize refusals and differences in intake among the dietary treatments. The refusals of signal grass were collected daily. Fresh water and mineral blocks were available *ad libitum* throughout the experiment. All goats were given injection for internal worms (Ivermectin) and vitamin A, D₃, and E prior to commencing this experiment. Goats were monitored for any health problems which were treated accordingly. Records were kept of all the health problems. Body weights were measured at the beginning and at the end of experiment.

Data Collection and Laboratory Analysis

Feed offered and feed refused was recorded daily. Feeds and faecal samples were collected from the total collection of each individual goat on each treatment for the last 7 days at morning and afternoon feeding. Combined
samples were dried at 60ºC and ground (1-mm screen) and then analysed for dry matter (DM), organic material (OM), crude protein (CP) content (AOAC 1997) and crude fibre (Van Soest et al. 1994).

Rumen fluid was collected using stomach tube connected with a vacuum pump at 0 and 4h post feeding. The pH and temperature of the rumen fluid were immediately measured by means of a portable pH and temperature meter. Rumen fluid samples were then filtered through two layers of cheese cloth and divided into two portions.

The first portion of rumen fluid was used for analysis of volatile fatty acids (VFA) and Ammonia (NH₃-N). 1M H₂SO₄ solution (5 mL) was added to 45 mL of rumen fluid. The mixture was centrifuged at 10,000 rpm for 15 minutes and the supernatant was stored at -20°C prior to VFA analysis by Gas Chromatography (GC). Ammonia concentration was determined by phenol-hypochlorite method of Broderick and Kang (1980). The second portion was used for a total direct count of bacteria and protozoa with a haemocytometer by the methods of Galyean (1989).

A blood sample (about 10 ml) was drawn from the jugular vein at the same time as rumen fluid sampling (at 0 and 4 h post-feeding) and centrifuged at 5,000 rpm for 10 minutes. The supernatants were stored at -20°C until analysis of blood urea nitrogen (BUN) using Biochemical analyser (Microlab 300).

**Experimental Design and Data analysis**

All the data were analysed as a Randomized Complete Block Design (RCBD) using one way analyses of variance (ANOVA). Treatment means were statistically compared using Duncan’s Multiple Range Test to identify differences between means. Analysis of covariance (ANCOVA) was used to test for differences in ADG weight change between the supplemented diets groups. In each ANCOVA, the initial body weight of the animals was used as the covariate to control for pre-existing differences. A linear regression was used to correlate individual ADG rates within each supplemented diets group. Homogeneity of regression assumptions were tested and met in each analysis. Significance was set at a p value of 0.05 for differences in means and correlation coefficients.