III. MATERIALS AND METHODS

3.1 Site of Research

The research was conducted at Balai Riset Budidaya Ikan Hias (BRBIH), Depok from March to September 2011.

3.2 Condition of Fermentation

PKM supply has been ordered from PT. Perkebunan Nusantara VII, Bandar Lampung. The fermentation was made by mixing 42 kg of PKM with 84 L of water (1:2) and put it in the big drum (height 81 cm; diameter 48 cm). The fermentation height in drum is 80 cm. The sample was taken from the depth of 0-10 cm using pipe at some point. At 12 h intervals during 7 days take the sample for analysis of pH, total acid, temperature, and microbial analysis. Analysis of D-mannose was conducted at 0, 36, 48, 72, 96, and 120 hours. Analysis of concentration of volatile organic acid was conducted at 0, 36, 72 and 168 hour. Analysis of fiber was conducted at 0, 36, and 168 hours. Analysis of protein content was conducted at the beginning and at the end of fermentation (Figure 10).

3.3 Microbial analysis

For all determination, 10 grams of fermented sample dispersed into 90 ml of NaCl 0.9% sterile, and then submitted to a mechanical stirrer for 5 minutes to extract cells from the substrate. This corresponds to 10⁻¹ dilution. The extraction step followed by serial dilution, ranging from 10⁻² to 10⁻⁸. From each dilution, 0.1 mL was surface plated for determination of microbial count in each sample of respective agar media, followed by incubation at 30 °C. Total aerobic bacteria were determined on Trypticase Soy Agar (TSA) (Fluka) incubated at 30 °C for 48 hours. Total anaerobic bacteria were determined on Trypticase Soy Agar (TSA) (Fluka) incubated at 30 °C for 48 hours in anaerobic condition. Enumeration of lactic acid bacteria was carried out using De Man, Rogosa and Sharpe (MRS) (Sigma) medium after incubation at 30 °C for 4 days. Enumeration of clostridial bacteria was carried out using Reinforced Clostridial Agar (RCA) (Pronadisa) after incubation at 30 °C for 48 hours in anaerobic condition. Typical
representative colonies were picked up from the higher dilution plate, purified and identified using gram stain and microscopic observation. Yeast and moulds were enumerated on plates of Sabouraud Glucose Agar (SGA) (Fluka) at 30°C for 4 days. The acceptable range of colonies for counting is 30-300 colonies on the plate (Josephson et al. 2000).

3.4 Analysis of pH and temperature

The pH of PKM fermentation analyzed using microcomputer ion meter (Consort). The temperature of PKM fermentation was analyzed using digital thermometer.

3.5 Analysis of total acid

Total acid value deduced by titrating 5 mL of aliquot of sample with 0.1 M NaOH, using 1% phenolphthalein as an indicator. The volume of titrant that causes a permanent colour change of the sample is recorded and used to calculate the total acid value.

3.6 Analysis of fiber

The fiber of fermented PKM conducted by sending the sample to Laboratory of Indonesian Research Institute for Animal Production Bogor, using Van Soeth technique. The percentage of hemicellulose was calculated as the difference between NDF (Neutral Detergent Fiber) and ADF (Acid Detergent Fiber), and percentage of cellulose as the difference between ADF and lignin.

3.7 Analysis of protein content

Mix well 42 kg of PKM + 84 L of water, put in drum during 7 days take the sample for analysis

- pH and temperature
- Protein content
- Concentration of D-mannose
- Total acid
- Volatile organic acid
- Fiber

Microbial analysis

10 g of sample dispersed into 90 ml NaCl 0.9%
(this corresponds to $10^{-1}$)

Extract cell and followed by serial dilution and plating on media agar

- Sabouraud Glucose Agar (SGA)
- De Man, Rogosa and Sharpe (MRS) Agar
- Trypticase Soy Agar (TSA)
- Trypticase Soy Agar (TSA)
- Reinforced Clostridial Agar (RCA)

Incubation in aerobic condition

Incubation in anaerobic condition

Counting and isolation

Figure 10. Schema of the research
3.8 D-mannose Detection Assay

D-mannose concentration of fermented PKM was determined using a four enzyme coupled assay based on the Megazyme International kit (Megazyme International Ireland Ltd.). Mannose was phosphorylated to mannose-6-phosphate by hexokinase (HK) which was subsequently converted to fructose-6-phosphate through the action of phosphomannose isomerase (PMI). Fructose-6-phosphate was then isomerized to glucose-6-phosphate by phosphoglucoisomerase (PGI) and finally, oxidized to gluconate-6-phosphate by glucose-6-phosphate dehydrogenase (G6P-DH). The G6PDH NADP$^+$ cofactor is concurrently reduced to NADPH. Each reaction of enzyme was monitored at 340 nm.

Before analysis, the pH of liquid sample (0.1 ml) was adjusted to 7.6 using 1 M NaOH and then the solution was incubated at room temperature for 30 minutes. Concentration of D-mannose was calculated as follows:

$$c = \frac{V \times MW \times \Delta A}{e \times d \times v}$$

where:
- $c$ = concentration of D-mannose (g/L)
- $V$ = final volume (mL)
- MW = molecular weight of D glucose or D-mannose (180.16 g/mol)
- $e$ = extinction coefficient of NADPH at 340 nm (6300 l/mol/cm)
- $d$ = light path (1 cm)
- $v$ = sample volume
- $\Delta A$ = the absorbance difference between added reagent

(Source: Megazyme International 2005)

3.9 Volatile organic acid analysis

Analysis of volatile organic acid conducted by sending the sample to Laboratory of Indonesian Research Institute for Animal Production Bogor, using Gas Chromatography techniques.