Physicochemical properties and characterization of fermented cassava flour by lactic acid bacteria

N L M Isa¹, F Kormin¹*, A C Iwansyah², D Desnilasari², A Hasan¹

¹Department of Technology and Natural Resources, Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia, Pagoh Educational Hub, 84600 Pagoh, Johor, Malaysia
²Research Centre for Appropriate Technology, Indonesian Institute of Sciences, Jl. KS. Tubun No. 5 Subang West Java, Indonesia, 41213

*Corresponding author: faridahk@uthm.edu.my

Abstract. Cassava tuber was used to produce fermented cassava flour with aid of lactic acid bacteria which is Lactobacillus plantarum, Lactobacillus bulgaricus and Pediococcus pentosaceus. This study aimed to determine effect different starter culture on physicochemical properties and characterization of cassava flour by lactic acid bacteria. Various type of cassava flour (L. plantarum, L. bulgaricus, P. pentosaceus and mixed starter culture) were made as the variable in determining their effect on physicochemical properties and characterization of the fermented cassava flour. The physicochemical properties that conducted on fermented cassava flour was protein content, moisture content and water activity analysis, pH and total titratable acidity analysis. Highest protein content with value (45.49%) was found in fermented cassava flour with L. bulgaricus. It was found that fermentation cassava with microbial starter culture shown the increasing amount of the protein content of the flour. This is due to the action of enzymes produced by the microbial starter culture. The fermented cassava flour also been characterized by using Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD) and Scanning Electron Microscopy (SEM). In conclusion, this study shows that protein content of cassava flour increases due to the fermentation by microbial starter culture.

Keywords. L. plantarum, L. bulgaricus, P. pentosaceus, mocaf, protein

1. Introduction
Cassava flour is a popular plant that widely used as food source for human consumption and animal feed in most developing country. This is because cassava flour is rich of carbohydrates mainly 20 to 25% of starch which provide energy source to human [1]. Cassava flour contains low protein content which is less than 1.5% of fresh weight [2]. This is because the composition of the peel and the parenchyma is different in which the peel tends to have more protein, fiber, sugars and cyanogen compared to the parenchyma [3]. Therefore, fermentation process is one of the methods that can increase protein content. According to Gunawan [4], fermentation process with microbial starter in production cassava flour had ability on increasing the protein content of the cassava flour. Besides, fermentation process also enhances the reduction of cyanogen [5]. This is because fermentation process can eliminate cyanohydric acid in the cassava tuber. Therefore, additional of microbial starter helps in enhancing the fermentation process. The microbial starter or endogenous or exogenous microbial have ability to release linamarase.
enzyme which helps in converting cyanide-containing compound into acetone cyanohydrin and decompose to hydrogen cyanide (HCN). This compound is volatile compound thus it can be releases to the air or dissolve in water to remove the compound [6].

Moreover, lactic acid bacteria are used in this study as microbial starter because its ability to produce enzyme, linamarase which specified to reduce the cyanide compound in cassava flour. Selection of microbial starter is important to reduce the cyanide compound in the cassava flour effectively. It can be single culture or mixed culture. There are three strain of different lactobacilli will be used which are L. plantarum, L. bulgaricus and P. pentosaceus as individual and mixed starter culture to reduce the toxicity and increasing functional aspect of the cassava flour. This study aimed to determine effect different starter culture on physicochemical properties and characterization of fermeted cassava flour by lactic acid bacteria.

2. Materials and Methods

2.1. Materials
Cassava tuber and commercial cassava flour were purchased from local market, Batu Pahat, Malaysia. Starter culture (L. plantarum, L. bulgaricus and P. pentosaceus) were supplied by Research Center for Appropriate Technology, Indonesian Institute of Sciences, Indonesia. Sulfuric acid, potassium sulfate, 50% of sodium hydroxide, 2% of boric acid solution, methylene blue, methyl red, hydrochloric acid, 7.5% Na₂CO₃, methanol, ethanol and ammonium hydroxide were purchased from Sigma-Aldrich, Singapore.

2.2. Production of fermented cassava flour
Cassava tubers were peeled and cut into two parts. Then, the cassava was washed with distilled water. The cassava tubers were grated into smaller size by using grater. The cassava (1 kg) were fermented by soaking in 1 L of water at ratio of (1:1, v/v) mixed with of starter culture containing Lactobacillus plantarum, Lactobacillus bulgaricus and Pediococcus pentosaceus. Fermentation mixture were stirred and covered with plastic film and then were fermented at 25°C for 24-120 hours. The cassava was filtered and pressed, the cassava was transferred to aluminium trays and were dried in oven at 40-50°C for 24 hours to reduce moisture content to 12-14%. The dried fermented cassava was milled to obtain fermented cassava flour.

2.3. Experimental design
This study employed a completely randomized design (CRD) with three replications on fermentation of cassava flour with L. plantarum (LP), L. bulgaricus (LB), P. pentosaceus (PP) and their mixture (MC). Commercial cassava flour were used as control. The parameters tested were: physicochemical properties such as protein content, moisture content, water activity (a_w), pH and total titratable acidity. Besides, the fermented cassava flour was characterized by using fourier transform infrared spectroscopy (FTIR), x-ray diffraction (XRD) and scanning electron microscopy (SEM).

2.4. Procedure Analysis

2.4.1 Protein content
Crude protein content was determined by Kjeldahl method in which involve digestion, neutralization and distillation of samples [7]. Fermented cassava flour (2 g) were placed in digestion tube with 24 mL of concentrated sulfuric acid (H₂SO₄). Then, potassium sulfate catalyst (14 g) were added and digestion. The tubes were cooled, and distilled water (500 mL) were added into tube. The tube was stored at room temperature overnight and then the samples were distilled. Sodium hydroxide (25 mL; 50%) was added to the digested sample solution, then boric acid solution (25 mL; 2%), 4 drops of methylene blue and 4 drop of methyl red were added to receiver conical flask. Distillate was collected in the receiver conical flask, and titrated with hydrochloric acid (0.05 M) until the indicator colour changed.
2.4.2 Moisture content Moisture content of the cassava fermented flour were determined by using moisture analyzer (model A&N Weighing Malaysia MX-50 Moisture Analyzer). Samples (5 g) were placed on the aluminum pan and weighed. The heating temperature was standardized to 200°C. Moisture content of the samples was expressed in percentage (%) and the reading was done in triplicate at room temperature of 25°C.

2.4.3 Water activity \( (a_w) \) Water activity \( (a_w) \) of samples were measured by water activity meter (DECAGON; model Aqua Lab). The samples were prepared in triplicated with cassava fermented flour (1 g) was placed in the sample holder. The sample was placed in the water activity meter and the measurement was recorded.

2.4.4 pH value The value measured using pH meter (Eutech pH 700). pH meter was calibrated using standard buffers at pH 4, pH 7 and pH 10 before used. Samples are put into beaker glass, dilute, then the pH meter electrode is dipped into filtrate obtained from fermentation and left to show a stable number. Samples were measured triplicates.

2.4.5 Total titratable acidity The lactic acid content of cassava flour was determined by total titratable acidity. Samples (10 g) were weighed and dissolved with 250 mL of distilled water. The mixture was shaken and mixed for 30 minutes. Then, the mixture was filtered by using filter paper. 25 mL of the filtrate was transferred into conical flask. A few drops of phenolphthalein were added to the conical flask as indicator. Then, the sample were titrated against 0.2 N NaOH. The lactic acid content of the filtrate will be calculated equation 1:

\[
lactic\ acid\ content = \frac{N \times V_1}{V_2} \times 0.090 \tag{1}
\]

where \( N, V_1, \) and \( V_2 \) were the NaOH normality, NaOH volume, and sample volume, respectively. The value of 0.090 was the milli equivalent of lactic acid.

2.4.6 Fourier transform infrared spectroscopy (FTIR) FTIR spectra of fermented cassava flour illustrated absorption bands with characteristic frequency attributed to different functional groups and all spectra were obtained using Perkin Elmer (model Spectron two UATR, USA) [8]. Samples were prepared using the standard KBr pellet technique, which was used to determine the background of the sample spectra. A few milligrams of fermented cassava flour were mixed with 0.5 g of potassium bromide. The mixture was turned into pellet form by applying pressure. The pellet was placed between interferometer and detector in the sample holder of the spectroscope. The spectra of the fermented cassava flour were recorded in transmission mode from 4000 to 400 cm\(^{-1}\), with spectral resolution of 4 cm\(^{-1}\) at room temperature. Each spectrum was rationed against a fresh background spectrum recorded from the bare crystal. The crystal was cleaned with absolute ethanol to remove any residual to collect background spectrum. Each sample was scanned in triplicates.

2.4.7 X-ray diffraction (XRD) Samples (2.0 g) was weight and transferred to silicon sample holder. The sample was adjusted by using glass slide until fully cover the sample holder. The crystal phase of the fermented cassava was identified using X-ray diffraction (XRD, D2 Phaser Bruker) with Cu Ka (\( \lambda = 1.541\ A \)) radiation in 2\( \theta \) from of 10-80° with scanning step of 0.04°/sec [9].

2.4.8 Scanning electron microscopy (SEM). Scanning Electron Microscope (SEM) is an electron microscopic analysis produced the drawn sample by scanning the sample using a focused beam of electrons. Starch granule characteristics were obtained using Scanning Electron Microscope COXEM (model Em-30AX). Fermented cassava flour sample were sprinkled onto double-sided cellphone tape attached to aluminum stubs. These samples were compress with air to remove uneven surface on the
aluminum stubs. These sample then were coated with gold- palladium at 10 milliamps for 3 minutes (Quorum Sputter Coater, Q300T D) [10].

2.5 Statistical Analysis
The data are presented in mean ± standard deviation (sd). Data were analyzed by analysis of variance (ANOVA), with a confidence interval of 95% and tested for normality of the data first. Duncan's multiple comparison test was used to determine the average difference between each treatment. The data processing program used is Microsoft Excel 2013 and Statistics Software for Windows.

3. Results and Discussion

3.1 Physicochemical properties of fermented cassava flour
The moisture content of fermented cassava flour was range 11.32% to 12.56% (table 1). The commercial cassava flour was had the highest moisture content and water activity (a_w) which is 13.85% and 0.647, respectively (P<0.05). Besides, cassava flour with mixture starter culture shown the lowest value of moisture content which is 11.32%, while cassava flour with L. plantarum (LP) had the lowest water activity (a_w) which at 0.471. This is because fermentation affects the chemical composition of the flour thus reducing the moisture content of the cassava flour [11]. Low water activity can prevent growth of microorganism [12].

However, these values is acceptable because moisture content higher than 12% may enhance microbial growth therefore low level of moisture content of cassava flour are favorable and increase the shelf life of the flour [13]. The low moisture content of fermented cassava indicates as good stable shelf life for packaged and stored stability [14].

| Samples | Moisture (%) | Water activity (a_w) | Total titratable acidity (%) | pH |
|---------|--------------|---------------------|------------------------------|----|
| LP      | 11.75±0.09^cd| 0.467±0.022^de      | 0.063±0.007^ab              | 3.89±0.01^e |
| LB      | 11.61±0.09^cd| 0.512±0.006^c       | 0.048±0.007^bc              | 4.10±0.00^d |
| PP      | 12.56±0.09^b | 0.562±0.001^b       | 0.048±0.012^bc              | 4.10±0.01^e |
| MC      | 11.32±0.10^c | 0.471±0.010^de      | 0.034±0.007^de              | 4.50±0.01^b |
| Control | 13.83±0.03^a | 0.647±0.003^a       | 0.019±0.007^de              | 4.80±0.02^e |

Data were presented mean±standard deviation (SD) (n=3). LP (L. plantarum); LB (L. bulgaricus); PP (P. pentosaceus); MC (mixed 3 starter). a>b>c>d>e. Values followed by different letters in same columns showed significant differences (P<0.05).

Fermented cassava flour with L. plantarum strain (LP) had the highest total titratable acidity (0.063%), while the commercial cassava flour had the lowest total titratable (table 1). This results because homofermentative L. plantarum only produced lactic acid as product resulting higher production of lactic acid.

The sample with L. plantarum had lowest pH value which at 3.89 (table 1). The pH values of LP, LB and PP are statistically lower than MC (P<0.05). This results was agreement with Tefera et al. [15], fermentation of cassava by action of single species of microorganisms shows reduction in pH level.

This is due to production of lactic acid by L. plantarum through degradation of carbohydrates [16]. Therefore, resulting to acidification in which also increase the total titratable acidity [17]. Low pH of cassava flour can prevent multiplication of pathogenic microorganisms [18]. Protein content of different type of fermented cassava flour are shown at figure 1.

Based on Figure 1, fermented cassava flour with L. bulgaricus starter culture had the highest protein content (45.49%), followed by the others (P<0.05). This result is due to the lactobacilli action in which
degradation of cassava tuber during fermentation process. Besides, increasing protein content in cassava flour was due to the activities and number of microorganism present during fermentation. The proteolytic activities of enzymes produced by the lactic acid bacteria [16]. According to Ongol et al. [19], reported fast acidification during fermentation is regarded as a useful property of L. bulgaricus. The increasing protein content in fermented cassava flour may due to the ability microorganism synthesize amino acid [20] [21].

![Figure 1](image)

**Figure 1.** Protein content of different type of fermented cassava flour. Data are presented mean±standard errors (n=3). LP (L. plantarum); LB (L. bulgaricus); PP (P. pentosaceus); MC (mixed 3 starter); control (non-fermented). a>b>c>d. Values followed by different alphabetic in bar shows significant differences (P<0.05).

3.2 Characterization of fermented cassava flour
Characterization of fermented cassava flour was determined by using Fourier Transform Infrared (FTIR), X-Ray Diffraction (XRD) and Scanning Electron Microscopy (SEM).

3.2.1 Functional group Fourier Transform Infrared Spectroscopy (FTIR) is used to detect a range of functional group of fermented cassava flour. The FTIR spectra of starches are presented in figure 2. Spectra of starches bands originate mainly from vibration of amyllose and amylopectin. This is because amyllose and amylopectin are the main components of starches.

The peaks was observed in the regions below 800 cm\(^{-1}\) (figure 2), the fingerprint region was between 800 and 1500 cm\(^{-1}\), the region between 2800 and 3000 cm\(^{-1}\) are C-H stretch region and the region between 3000 and 3600 cm\(^{-1}\) is for O-H stretch region [22]. The presence of absorption band around 1000-1100 cm\(^{-1}\) (figure 2). It is indicated that starch possess C-O functional group [23]. Besides, the peak at 1336 cm\(^{-1}\) was originated from CH\(_2\) bending and C-O-H bending. The band region around 3000-3600 cm\(^{-1}\) are indicated as stretching vibration O-H bond of starch [24]. All samples have starch component in the cassava flour (figure 2). All the O-H related peak was significantly equivalent to moisture content and shows that the sample was strongly reflect the surrounding environment. FTIR also used to study starch in rubbery and glass states. Amorphous and crystalline phase starch were identified by FTIR at band around 1022 cm\(^{-1}\) and 1047 cm\(^{-1}\), respectively [25]. According to Wahyuni *et al.*, [9], cassava flour should have characteristic absorption at band 3000-3600 (O-H stretching) which indicate the carbohydrates, 2933 and 2881 (C-H stretching of CH\(_2\)) to determine fat, 1190-950 (C-O stretching) and 1630 cm\(^{-1}\) in presence of water.
3.2.2 Crystallinity

Crystallinity of the fermented cassava flour were determine using X-ray Diffraction (XRD). The characteristic peaks of fermented cassava XRD pattern as seen in figure 2. The main crystalline peaks in XRD pattern are attributed to the crystalline peaks of starch which consist of two which is amylose and amylopectin.

All fermented cassava flour shows the crystalline peak around 15, 17, 18 and 23. Fermented cassava flour is a type A starch because it showed strong diffraction peaks at around 15, 17, 18 and 13. Type A starch present mainly in cereal starches such as maize starch and wheat starch [26].

Table 2 showed that fermented cassava flour with *L. plantarum* (LP) had the lowest crystallinity index (44.31%) followed by LB and PP. According to Putri *et al.* [27], the fermented cassava flour by higher amylolytic of lactic acid bacteria presented the lowest value of relative crystallinity. *Lactobacillus plantarum* strain is homofermentative which produced high lactic acid bacteria which resulting to lowest crystalline index. Besides, the diffractograms of the fermented cassava flour samples showed narrow peaks which contributes to hydrolysis on amorphous areas during fermentation resulting intact the crystalline areas [28].
Figure 3. XRD patterns of fermented cassava flour with *L. plantarum* (a), *L. bulgaricus* (b) and *P. pentosaceus* (c).

| Samples | Integrated intensity on diffraction angle (2θ) | Crystallinity index (%) |
|---------|-----------------------------------------------|-------------------------|
|         | 15° | 17° | 18° | 23° |                        |
| LP      | 815 | 867 | 990 | 788 | 44.31                  |
| LB      | 781 | 864 | 907 | 865 | 48.81                  |
| PP      | 770 | 885 | 899 | 858 | 48.83                  |

Data were presented mean. LP (*L. plantarum*); LB (*L. bulgaricus*); PP (*P. pentosaceus*). Values followed by different letters in same columns showed significant differences (P<0.05).

3.2.3 Surface morphology Scanning electron microscopy was used to determine the microstructure of cassava flour after fermented with *L. plantarum* and *P. pentosaceus*. The morphologies were shown in figure 4. The microstructure of fermented cassava flour with *L. plantarum* and fermented cassava flour with *P. pentosaceus* are seen having the starch granules with 2000 X magnification. Both micrograph
of starch granules has similar sizes because both samples undergo fermentation process. This is because cell wall of cassava tubers become broken and change the starch granules into small size during fermentation [29].

**Figure 4.** the surface morphology of cassava flour fermented with *L. plantarum* (a) and *P. pentosaceus* (b)

The fermented starch granule showed partly broken granule and has rougher surface compare to the native starch which has smooth surface with some irregular surface [9]. This is due to proteolytic activity which removed the protein matrix that covered the starch resulted in releasing of its granules [30]. Moreover, fermentation process by microbial starter tend to form irregular structure of starch granule and lose smooth structure of the flour [31].

4. Conclusion
In conclusion, fermented cassava with lactic acid bacteria were showed the higher value in physicochemical properties. Fermented cassava with *L. bulgaricus* had shown higher protein content compared with other fermented. Besides, the mixture of starter culture also showed reduction in moisture content cassava flour. Fermented cassava flour with *L. plantarum* recorded higher in total titratable acidity and indicate lower pH of the cassava flour. This is important to prolong the shelf life of the cassava flour. Fermentation of cassava were shown significant increase in protein content of the cassava flour.

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