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Effect of fermenting cassava with *Lactobacillus plantarum, Saccharomyces cereviseae*, and *Rhizopus oryzae* on the chemical composition of their flour

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Abstract

Cassava production has increased consistenly in Indonesia. Cassava contains toxic substances, including of cyanide acid. Another, it has low content in protein, minerals, and vitamins. Therefore, a special proccessing is important prior to consumption. In this study, modified cassava production without any additional nutrients at different microorganisms and fermentation times, was investigated. Proximate and minerals composition of fermenting flour were also presented. It was found that at the microbial to cassava mass ratio of 1% for fermentation time of 120 h, *L. plantarum, S. cereviseae,* and *R. oryzae* on cassava can increase the levels of protein from 1.92% to 8.58%, 2.29%, and 4.72%, respectively. In addition, it can reduce starch content after fermentation in a number of 55.40%, 71.03%, and 48.20%, respectively. Moreover, the levels of cyanide acid was decreased from 17.5 mg/kg to 1.8, 3.28, and 3.17 mg/kg, respectively.

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Introduction

The total population in Indonesia was last recorded at 245.9 million people in 2012 from 93.1 million in 1960, changing 164 percent during the last 50 years. It represents about 3.51 percent of the world´s total population (Statistic Indonesia, 2012). On the other hand, cassava *(Manihot esculenta)* is a staple food of Indonesian people in addition to rice, sago and corn. This plant is growing well under critical conditions of climate and soil, and flexibel harvesting date, allowing farmers to keep the roots in the ground until needed. Therefore, its production has increased consistently over the last five years.

Cassava is often considered as a low quality raw material, such as low in protein, minerals and vitamins contents (Onwueme, 1978; Aletor, 1993). The low price of cassava is also affected by the properties of fresh cassava are easily damaged due to the presence of tannic acid, a substance that can cause colors in processed cassava products (Hahn, 1992). Another, cassava contains cyanide acid (HCN) that causes toxicity with symptoms of headache, nause, dizziness, diarhoea, and vomiting, sometimes leading to death (Nhassico, 2008). Moreover, high cyanide acid is thought to be the major contributing cause of konzo, an irreversible paralysis of the legs in women of child-bearing age and children (Bradbury, 2006). The presence of these compounds is the major limiting factor to direct utilisation, therefore a great processing is neccessary prior to consumption.

As a commodity, cassava can be processed to produce dried cassava chip, tapioca, ethanol, liquid sugar, sorbitol, monosodium glutamate, and modified cassava flour (mocaf). Several reseachers have focused on the fermentation of cassava with additional nutrients for enhancing detoxification and improving the quality of cassava (Kimaryo and Massawe, 2000; Muzanila *et al*., 2000; Oboh *et al*., 2002; Oboh and Akindah, 2003, 2005). However, the variability of microorganisms (bacteria, yeasts and filamentous fungi) and fermentation time still remains unknown. Therefore, the objective of this work was to produce mocaf without any additional nutrients at a suitable microorganism in a reasonably short time. The proximate composition and mineral content of the mocaf were also discussed systematically.

Materials and Methods

Materials

White cassava tubers were purchased from local markets in Surabaya (Indonesia) with a diameter and length of 2-3 cm and 50-80 cm, respectively. Cassava

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tubers were peeled and cut into dice size with a thickness of about 0,3 cm. Then, they were washed by distilled water and stored at -20°C before use. *Lactobacillus plantarum* (lactic acid bacteria) was obtained from Microbiology laboratory of Airlangga University (Surabaya, Indonesia). *Saccharomyces cereviseae* (baker's yeast) was obtained from Sangra Ratu Boga Company (Jakarta, Indonesia). *Rhizopus oryzae* (tempeh fungus) was purchased from Aneka Fermentation Industry Company (Bandung, Indonesia). Various minerals, such as iron, zinc, calcium, manganese, magnesium, potassium, sodium, copper, and phosphorus were obtained from Sigma Chemicals Company (St. Louis, MO). All solvents and reagents were obtained from commercial sources and of either high-performance liquid chromatography grade or analytical grade.

Cassava fermentation

Cassava fermentation process was carried out by submerged fermentation method. Briefly, a 100 g washed cassava was transferred into a flask. Then, 150 mL of distilled water containing of 1 g microorganism (*L. plantarum, S. cereviseae*, or *R. oryzae*) was added to the flask. The top surface of the flask was covered by cotton (anaerobic condition). The mixture was fermented at a room temperature of 30°C for different times. Afterwards, solid and liquid phases were separated immediately by vacuum filtration. The solid phase on the filter paper was dried by sun drying, then followed by under vacuum until its moisture content of 12-14% and designated as dried fermented cassava. The dried fermented cassava was milled and ground to obtain fermented cassava flour. In order to adjust the standard size of commercial flour, it was sieved by 80 mesh. Finally, the fine of fermented cassava flour and filtrate were collected for further analyses.

Proximate composition

Ash, fiber, protein, and nitrogen free extract contents of dried fresh and fermented cassavas were determined by AOAC (2003). Lipid content of dried fresh and fermented cassavas was analyzed as described by previous work (Gunawan *et al*., 2013). Fixed carbon was measured by ASTM D 3172 (1987). Moisture and mineral contents of dried fresh and fermented cassavas were analyzed using a Halogen moisture analyzer and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-EOS), respectively.

pH analysis

The pH of filtrate obtained from fermentation was determined by pH meter.

Lactic acid analysis

The lactic acid content of filtrate obtained from fermentation was determined by total titrable acidity (GEA Niro, 2006). Briefly, a 25 mL of sample was transferred into a flask. A few drops of phenolphthalein as indicator was added to the flask. Then, the sample was titrated against 0.2 N NaOH. The lactic acid content of the filtrate was calculated as:

$$\text{Lactic acid content (mg/mL)} = N \times V_1 \times 0.090 / V_2 \quad (1)$$

Where N, V_1, and V_2 were the NaOH Normality, NaOH volume, and sample volume. The value of 0.090 was the milli equivalent of lactic acid.

Growth rate

A sample (1 mL) was dissolved in 10 mL of sterile distilled water in a test tube and stirred vigorously. The sample was diluted by the dillution factor of 100. A diluted sample was dropped into the hemacytometer then covered with a deck glass. It was put under microscope, followed by counting the number of cells contained in boxes A, B, C, D, and E. These steps were repeated for three times.

Analysis of cyanide acid content

The cyanide acid content of washed fresh and fermented cassavas was determined by SNI standard (2011). Briefly, a 20 g of fermented cassava was transferred into a Kjeldahl digestion flask. Then, 200 mL of distilled water was added to the digestion flask. The flask was swirled to mix the contents thoroughly for 2 h, then placed on heater to recover cyanide acid as distillate. The distillate was collected in a conical flask containing 20 mL of 2.5% NaOH solution. Next, 8 mL of NH_4OH solution and 5 mL of 5% KI solution as indicator were added to the conical flask. The resulting mixture was titrated against 0.02 N $AgNO_3$ until there was a turbidity. A blank was also run through all steps above. The cyanide acid content was calculated from the amount of $AgNO_3$ used for titration.

$$\text{Cyanide acid content (mg/kg)} = (V_1 - V_2) \times N \times 27 / (V_3 \times W) \quad (2)$$

where V_1, V_2, V_3, N, and W were the blank titration reading, sample titration reading, distillate volume, $AgNO_3$ normality, and sample weight, respectively. The value of 27 was the molecular weight of cyanide acid.

Analysis of starch content

Acid method (AOAC, 2003) was used to determine starch content of washed and fermented cassava. Briefly, a 2 g of crushed sample was put into a flask containing 50 mL of distilled water. The mixture was stirred for 1 h and filtered using a Whatman 42 filter paper. The solid phase on the filter paper was washed with distilled water until reached 250 mL of filtrate. Furthermore, the solid residue on the filter paper was washed with 10 mL diethyl ether, and allowed the ether to evaporate. Afterwards, it was washed again with 150 mL of 10% ethanol for further release of soluble carbohydrates. The filtrate containing soluble carbohydrates was discarded. The solid residue was transferred into a flask containing 200 mL of distilled water. Then, 20 mL of 25% HCl was added to the flask and heated at 100°C for 2.5 h. After cooled, the mixture was neutralized with 45% NaOH solution. The mixture was filtered by vacuum filtration. The resulting solid phase on the filter paper was washed with distillate water until reached 500 mL of filtrate. Furthermore, sugar content of the filtrate was analyzed for according to the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The percentage of starch was determined by multiplying glucose content by factor number of 0.9.

Results and Discussion

Fresh cassava tubers

Cassava is not a native plant of Indonesia, but has become very popular in Indonesia. This plant came into Indonesia in 1852. Recently, it is one of the agricultural commodity in Indonesia, which has been processed into a various finished or semi-finished products with extra value. Cassava is normally processed before consumption as a means of detoxification, preservation and modification. The proximate composition of dried fresh cassava was 1.93 ± 0.04% protein, 0.66 ± 0.01% lipids, 4.24 ± 0.05% fibre, 0.69 ± 0.03 ash and 92.48 ± 1.14% nitrogen free extract. The carbon to nitrogen ratio (C/N ratio) of dried fresh cassava and cyanide acid content of washed fresh cassava were 28 ± 1.23% and 17.5 ± 1.26%, respectively.

It was found that limitation of cassava uses as a food is caused by its high cyanide acid content of 17.5 mg/kg in washed fresh cassava, compared with WHO safe level of 10 mg/kg (FAO, 1991) and its low protein content of 1.93% in dried fresh cassava. Padmaja *et al*. (2009) reported that HCN content in the fresh cassava is 55.1-190.2 mg/kg. The observed of cyanide content for washed fresh cassava was lower than that observed by Padmaja *et al.* (1993). Moreover, Ceballos *et al.* (2006) reported that large difference in protein content (0.95%- 6.42%) were observed in several cassava varieties, gathered for a period of 10 years. The proteins content was estimated on the basis of total nitrogen content under the assumption all proteins contain approximately 16% nitrogen. The nitrogen content was then multiplied by a general factor (6.25) to arrive at the protein content. The variability of cyanide acid and protein contents was attributed to cassava cultivar, harvesting age, and diversity of agronomic factors.

Lipids and fibre contents of dried fresh cassava were 0.66% and 4.24%, respectively. Lipids may contains acyglycerols, free fatty acids, gums, plant pigments, wax esters, and aldehydes. Fibre consists of polysaccharides such as cellulose, hemicellulose, lignin, pectin, and other components associated with the fibrous carbohydrates. The quantity of inorganic materials or minerals, known as ash (0.69%) in the fresh cassava was also measured. These results agree with previous work that fiber and ash contents of cassava ranged 0.77-4.62% and 0.7-2.2%, respectively (Apea-Bah, 2011). Another, Boonnop *et al.* (2009) reported that lipids content in the fresh cassava is 2.3-2.7%. The lipids content of dried fresh cassava in this study was lower than that reported by Boonnop *et al.* (2009). It could be due to cassava cultivar, harvesting age, and diversity of agronomic factors.

Moreover, the nitrogen-free extract consists of non fibrous carbohydrates, such as sugars, and starches. In this study, it was observed that starches content is about 89.4%. This result was higher than that reported by Apea-Bah (2011). Starch content of cassava was between 53.6-75.5 % with the lowest value at 9 months and the highest at 10 months. This may be due to high content of impurities. It was also found that dried fresh cassava contains calcium (38.55 ppm) and iron (2.95 ppm). No other study was found regarding the mineral composition of dried fresh cassava.

Carbon to nitrogen ratio is very important for microbial activities within fermentation process. Microorganisms use the carbon from organic matters (carbohydrates) as a source for energy, and the microorganisms require nitrogen from protein and other nutrients for reproduction. It was found that carbon to nitrogen ratio of dried fresh cassava used in this study is 28.41. On the other hand, Stewart (2006) reported that normal microbial activity requires a carbon-to-nitrogen ratio of about 20 up to 30. Therefore, production of modified cassava flour without any additional nutrients was employed in this work.

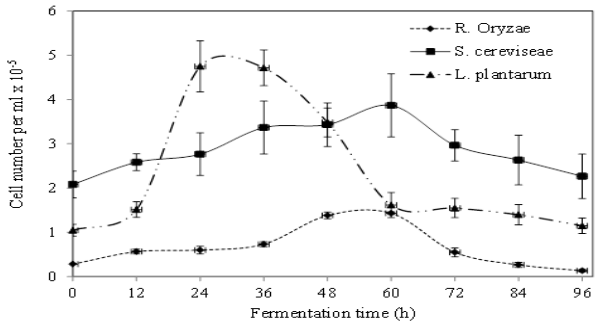

Figure 1. The growth pattern of *L. plantarum, S. cereviseae,* and *R. oryzae* on cassava fermentation

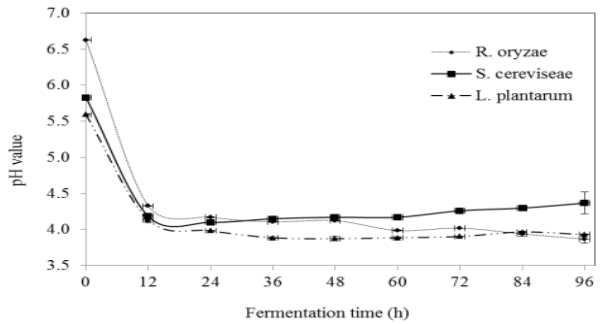

Figure 2. The pH profile of *L. plantarum, S. cereviseae,* and *R. oryzae* cultivations on cassava fermentation.

Microbial growth

Submerged fermentation without any additional nutrients was applied in this study. Microbial growth is defined as microbial population which is increasing of the quantity cellular and structure of organisms. The growth pattern of *L. plantarum, S. cereviseae,* and *R. oryzae* on cassava fermentation is shown in Figure 1. Four phases were detected, such as adaptation phase (lag phase), growth phase (exponential phase), static phase (stationary phase), and mortality phase (death phase).

The growth rate of *L. plantarum* was significantly faster than those of *S. cereviseae,* and *R. oryzae.* It was found that *L. plantarum* growth has entered to stationary phase at fermentation time of 24 h. Moreover, the period of stationary phase both of *R. oryzae* and *S. cereviseae* growth were achieved at about 48 h and 60 h, respectively. No other study was found regarding the microbial growth of *L. plantarum, S. cereviseae,* and *R. oryzae* on cassava submerged fermentation without any additional nutrients.

However, these results were comparable with previous works on cassava fermentation with additional nutrients. Lei *et al*. (1999) reported that log phase (exponential phase) of *L. plantarum* occurs about 20 h at fermention temperature of 30ºC. Wang *et al*. (2004), Pramanik and Roa (2005), Asli (2010), and Polyorach *et al.* (2013) reported that optimal cultivation time of *S. cerevisiae* is approximately 60 to 80 h by highest biomass. On the other hand, above 36 h, *R. oryzae* has entered to stationary phase (Taskin *et al*., 2013).

pH value

Graham *et al.* (1976) stated that pH is one of the most important factors for maximizing growth, which was also found to be true for fermentation temperature. When cultivating *L. plantarum, S. cereviseae,* and *R. oryzae* on cassava fermentation without pH control, the pH profile of cultivations were decreased with time as a result of more latic acid production and accumulation. It was observed that pH condition of *L. plantarum, S. cereviseae,* and *R. oryzae* decreases from 5.6 to 3.9, from 5.8 to 4.4, and from 6.6-3.9, respectively, within fermentation temperature studied at 30ºC (Figure 2).

These results agree with previous works that the final pH for *R. oryzae* cultivation was within the range of 2.0–4.5 (Phrueksawan *et al*., 2012). The optimum pH condition of *L. plantarum* and *R. oryzae* was 3.5-4.5 (Lei *et al.*, 1999) and 4.5-6.0 (Phrueksawan *et al*., 2012), respectively. Moreover, the optimum fermentation temperature of *L. plantarum* and *R. oryzae* was 30ºC and 30-35ºC, respectively. Another, the optimum pH levels for *S. cerevisae* cultivation were from 3.5 to 6.0 and temperature levels were from 20 to 40 °C (Wang *et al*., 2004; Pramanik and Roa, 2005; Asli, 2010; Manikandan and Viruthagri, 2010; Polyorach *et al*., 2013).

To verify the above results, the lactic acid content of the filtrate obtained from fermentation was measured (Data not shown). *L. plantarum, S. cereviseae,* and *R. oryzae* cultivations produced high amounts of lactic acid, leading to a rapid drop in pH, the environment then became selective against less acid-tolerant microorganisms. This indicates that conversion from glucose to lactic acid is the critical step in fermentation on cassava. Phrueksawan *et al.* (2012) reported that *R. oryzae* can convert the sole cassava pulp into lactic acid at the titer of 206.20 mg per g initial dry pulp. *R. oryzae* is capable of utilizing starchy materials and pentose sugars present in the agricultural commodities to produce the optically pure L-lactic acid because *R. oryzae* possesses only L-lactate dehydrogenase while bacteria can produce both D- and L-lactate dehydrogenases.

Cyanide acid content

The laminarin and lotaustralin cassava's cyanogenic compounds are changed to hydrocyanic acid by the action of the laminarase enzyme when roots are crushed or sliced. The effect of fermentation time and different microorganisms on cyanide acid

Table 1. The effect of fermentation time on cyanide acid content[a]

Fermentation	Cyanide acid content, mg/kg		
time, h	*L. plantarum*	*S. cereviseae*	*R. oryzae*
24	7.50±0.12	7.60±0.02	8.10±0.01
48	3.60±0.16	4.05±0.01	4.35±0.03
72	3.40±0.06	3.85±0.01	3.78±0.01
96	3.00±0.02	3.30±0.04	3.27±0.04
120	1.80±0.03	3.28±0.01	3.17±0.04

[a] Obtained from three independent experiments

Table 2. The effect of fermentation time on protein content[a]

Fermentation	Protein content, wt.%		
time, h	*L. plantarum*	*S. cereviseae*	*R. oryzae*
24	2.94±0.29	1.93±0.11	1.95±0.01
48	4.07±0.10	2.01±0.14	2.05±0.04
72	5.56±0.58	2.12±0.12	2.33±0.04
96	6.84±0.29	2.14±0.13	3.50±0.01
120	8.58±0.31	2.29±0.24	4.72±0.01

[a] Obtained from three independent experiments

content is shown in Table 1. Cyanide acid content (lower than 10 mg/kg, WHO safe level) was obtained in all microorganisms studied for fermentation time of 24 h. The longer of fermentation time, the lower cyanide acid content is obtained. The lowest level of HCN in fermented cassava using *L. plantarum, S. cereviseae,* and *R. oryzae* was 1.80, 3.28, and 3.17 mg/kg, respectively, for fermentation time of 120 h.

It was found that this method can reduce significantly the levels of cyanide acid due to immersion (submerged), fermentation temperature, and microbial activities. This is because HCN is easily soluble in water and has a boiling point of 29ºC. Besides, it also means immersion can dissolve compounds linamarin and lotaustralin. Cumbana (2007) reported that immersion of linamarin could inhibit its degradation into cyanide acid. Another, the microorganisms can break down toxins into organic acids and inactivate laminarase enzyme. In this fermentation, microorganisms convert glucose to organic acids that caused pH decreased into ± 4.2. In another hand, laminarase enzyme activity was optimum at pH of 6.0 (Askurrahman, 2010). The lower pH by the fermentation can reduce laminarase enzyme activity to degrade linamarine to cyanide acid. Hence, fermentation contributes to decrease cyanide acid content.

In the fermentation of cassava using *L. plantarum* as starter, the cyanide acid was reduced from 176.3 to 8.2 mg/kg after 120 h (Kimaryo and Massawe, 2000). Boonnop *et al*. (2009) reported that *S. cereviseae* fermented cassava products have very low cyanide acid content of 0.5 mg/kg after 132 h. The cyanide acid contents of product obtained in this study were higher than that reported by Boonnop *et al*. (2009). This could be due to fermentation time and microbial to cassava mass ratio were lower than those reported by Boonnop *et al*. (2009). Moreover, no other study was found regarding the effect of fermenting cassava with *R. oryzae* on the cyanide content.

Protein content

The effect of fermentation times and different microorganisms on protein content is listed in Table 2. It can be seen that protein content increases significantly during the fermentation using *L. plantarum, S. cereviseae,* and *R. oryzae.* Hu *et al.* (2012) reported that fermentation allowed microorganism to convert substrate containing carbon and nitrogen to the protein. Since cassava flour contains carbon and nitrogen, its crude protein was increased after fermentation. Another, Gelinas *et al*. (2007) mentioned that yeast fermentation of starch-based materials enrich the protein contents until 8% from the initial protein (yeast protein).

The longer the fermentation time, the higher protein content was obtained. In this study, the highest protein content in fermented cassava using *L. plantarum, S. cereviseae,* and *R. oryzae* was 8.58, 2.29, and 4.72%, respectively, for fermentation time of 120 h. The increase in protein content is due to the ability of *L. plantarum, S. cereviseae,* and *R. oryzae* to secrete some extracellular enzymes (proteins) into the cassava during fermentation. The fermentation of cassava with lactic acid bacteria *(L. plantarum)* produced proteinase enzyme. Obo and Akindahunsi (2003) reported that the high protein content could be attributed to the ability of the *S. cerevisiae* to secrete some extracellular enzymes such as amylases, linamarase and cellulase into the cassava mash during their metabolic activities, which would lead to yeast growth. Another, Gosh and Ray (2011) mentioned that *R. oryza*e could secrete cellulase, hemicellulase, pectinase, tannase, phytase, amylase, lipase, and protease enzymes.

Boonnop *et al*. (2009) and Polyorach *et al.* (2013) demonstrated that fermentation of cassava chip with Baker's yeast *(S. cerevisae)* could increase crude protein from 2% to 32.4% and 47.5% after 132 h, respectively. Another, the fermented cassava with *S. cerevisiae* enhanced the protein level from 4.4% to

Table 3.The effect of fermentation time on starch content[a]

Fermentation time, h	Starch content, wt.%		
	L. plantarum	*S. cereviseae*	*R. oryzae*
24	69.40±0.30	79.41±0.13	65.50±0.11
48	58.80±0.19	72.92±0.18	50.69±0.16
72	57.70±0.48	72.74±0.58	49.10±0.39
96	56.00±0.60	71.69±0.40	48.80±0.58
120	55.40±0.49	71.03±0.33	48.20±0.76

[a] Obtained from three independent experiments.

10.9% (Oboh & Kindahunsi, 2005). However, the protein contents of product (Table 2) were lower than that reported by Boonnop *et al*. (2009) and Polyorach *et al.* (2013). This is because fermentation time and microbial to cassava mass ratio were lower than those reported by Boonnop *et al*. (2009) and Polyorach *et al. (*2013). Moreover, a recent report has shown that cassava fermentation with *L. plantarum* and *A. niger* increases the level of protein in cassava from 2.37% to 7.68% and 7.60%, respectively. No other study was found regarding the effect of fermenting cassava with *R. oryzae* on the protein content.

Starch content

The effect of fermentation time and different microorganism cultivations on starch content is shown in Table 3. It can be seen that there is a tendency of decrease in starch content in fermented cassava during the fermentation process. The lowest starch content was obtained after fermenting cassava for 120 h using *L. plantarum* (55.40%), *S. cereviseae* (71.03%), and *R. oryzae* (48.20%).

The decrease in starch could be attributed to the possible transformation of starch into glucose as carbon sources for synthesis of protein or fat (Lehninger *et al*., 1982). Furthermore, glucose was converted into the end products, volatile fatty acids (such as lactic, acetic, butyric and propionic acids) (Phrueksawan *et al.*, 2012), and neutral compounds (such as ethanol) (Jennings, 1995).

Sobowale *et al*. (2009) reported that fermentation of cassava chip with *L. plantarum* could decrease starch content from 76.86% to 70.72% after 96 h. When cultivating *R. oryzae* on cassava fermentation, the starch content were decreased from 390 to 40 mg per g of initial dry cassava pulp (Phrueksawan *et al.*, 2012). No other study was found regarding the effect of fermenting cassava with *S. cereviseae* on the starch content.

From all these results, the fermentation of cassava chip with *L. plantarum* could gave a higher protein content and a lower cyanide acid content than those obtained with *S. cereviseae* and *R. oryzae*. It can be also seen that the physical and chemical characteristics of flour produced were similar to that of wheat flour. In wheat flour, the moisture content was less than 14.5%, the starch content was 60-70%, the protein content was higher than 7%, the ash content was less than 0.6% and the cyanide content was undetectable (SNI Standards, 2011). Moreover, it was found that the highest protein content and the lowest cyanide acid content were obtained by fermenting cassava with *L. Plantarum* at microbial to cassava mass ratio of 1% for fermentation time of 120 h.

Conclusion

A strategy for utilization of cassava in production of mocaf was demonstrated. Mocaf flour can be produced by fermentation use *L. plantarum, S. cereviseae,* and *R. oryzae* that are cheap and non pathogenic to increase the levels of protein and decrease the levels of cyanide acid in the mocaf flour. It was found that *L. plantarum* was more efficient than *S. cereviseae,* and *R. oryzae*. This work has also shown that lactid acid is produced as by-product during the fermentation.

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