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Effect of Fortification and Fermentation on the Nutritional Value of Sorghum (*Sorghum bicolor* (L.) Moench) Flour

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18

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Abstract

This study focuses on the effect of fortification with soybean flour and fermentation with *Rhizopus oligosporus* on the nutrition of sorghum flour. The result shows that pre-treatment by using dilute alkali solution for 10 h could reduce condensed tannins up to 86.92 %. After 60 h of fermentation, the amount of condensed tannins decreased gradually to 0.24 %. The optimum of the fortification and fermentation experiment characterized by 0.29 % of condensed tannins and 9.09 % of crude protein. These values were obtained by using 7.5 % (w/w) of soybean flour fortification and 2.5 % (w/w) of *R. oligosporus*. The highest swelling power was obtained 8.15 g/g at 95 °C and the water solubility index reaches 26.15 % after fortification and fermentation for 60 h which is caused by changes in the structure of sorghum flour. The proximate analysis shows the results of 79.5 % carbohydrate, 3.1 % fat, 4.8 % moisture content, and 1.6 % ash content. Fermentation using *R. oligosporus* and fortification with soybean flour is feasible to reduce condensed tannins and increase crude protein with higher functional and good rheological properties of sorghum flour. These results could be a good prospect for substituting wheat flour as an alternative food in the future.

Keywords: Sorghum flour, *Rhizopus oligosporus*, Condensed tannins, Fortification

2

Introduction

Sorghum (*Sorghum bicolor* (L.) moench) is one of the cereal plants that has greatly tolerant of marginal land conditions [1] and it can be planted as a companion for plants that require much water to grow such as corn and cane [2]. Having 32 species, sorghum grow in tropical and sub-tropical land areas especially in the Australia-Asia and Southeast Pacific [3,4]. In most countries across the world, sorghum grain was used for livestock feed and also used as a raw material for bioethanol production [5]. Sorghum grain contains high starch within range 64.3 to 73.8 %, crude protein 8.19 to 14.02 %, and bioactive compounds such as tannins 6.77 to 10.66 %. Tannins are polyphenolic compounds that have antioxidant activity and usually categorized into hydrolyzable tannins and condensed tannins [5,6].

The condensed tannins of sorghum grains have advantages and disadvantages in its use for food products. Low concentration of condensed tannins <0.3 % has some health benefits such as antioxidant function and radical scavenging, anticancer, anti-inflammatory, immunomodulatory, cardioprotective, and antithrombotic effect [7]. Nevertheless, high condensed tannins content could play as an anti-nutrient and reduce protein digestibility [8]. Condensed tannins also cause bitter flavour and astringent tastes which cause damage to food products [9]. Therefore, tannins of raw sorghum are less acceptable in food commercial market. Processing sorghum grain to be good flour is very important to reduce condensed tannins compounds to be edible for humans. Based on international standard the maximum tannins concentration permitted for consumption is <0.3 % [10].

As reported in the existing literature, condensed tannins can be reduced by several methods including physical, chemical, biological, and other methods. Soaking using dilute alkali can reduce the condensed tannins of sorghum seed up to 78 % [11]. In addition, the fermentation process with some strain can reduce tannins levels on faba beans up to 68 % [12]. Meanwhile, other studies mention that

fermentation using local yeast bread has reduced condensed tannins levels up to 0.31 % [13]. *Rhizopus* is one of the molds that is often used in the solid-state fermentation process. *Rhizopus* can produce the enzyme tannase which has the potential to hydrolyze condensed tannins in some grains [14]. However, some studies reported that fermentation process could reduce crude protein in food products. *Lactobacillus plantarum* reported reduce crude protein of sorghum up to 11.11 % [15] and using *R. oligosporus* reduce crude protein up to 73.83 % on soybean grains [16]. Currently, there are several methods to increase crude protein and nutrition value of food product, one of which is fortification process. Having high protein more than 30 %, soybean can be used as a fortification in other food materials [17].

The objectives of this study are to identify the effect of fortification with soybean flour and fermentation by using *R. oligosporus* on condensed tannins, crude protein, physicochemical and proximate composition. The results of fortification and fermentation and would provide more information about the possibility of substituting wheat flour as an alternative food product.

Materials and methods

Materials

Sorghum grains and soybean flour were obtained from traditional markets in Semarang (Indonesia). Whereas the *R. oligosporus* was obtained from instant dry yeast (Aneka Fermentasi Industri, Indonesia) with activity $<1.0 \times 10^2$ CFU/gram [18]. Chemical reagents sodium hydroxide, folin-denis' reagent, sodium carbonate, potassium sulphate, cupric selenite, sodium hydroxide, boric acid, and hydrochloric acid were purchased from Merck (Darmstadt, Germany).

Methods

Soaking

The sorghum grains were cleaned from dust and other extraneous material prior to use. Soaked in dilute alkali solution (sodium hydroxide 0.1, 0.2 and 0.3 %) for 10 h. Soaked sorghum grains, then dried in an oven (Memmert 55, Schwabach, Germany) at 55 °C for 2 h. Dried grain was milled and sieved with a 100-mesh sieve to obtain fine sorghum flour and stored in sealed containers for further processing.

Fermentation

For fermentation process the method was refers to Erkan [19]. Sorghum flour was sterilized by autoclave at 121 °C for 15 min and fermented anaerobic at 30 °C for 60 h at sterilized sealed container containing 2.5, 5 and 7.5 % (w/w) of *R. oligosporus*. pH of the substrate was adjusted using vinegar (3.7 - 5.5) for the optimum growth of *R. oligosporus* during the fermentation process. The fermentation was sampled every 6 h during fermentation period of 60 h. Samples were taken and dried in an oven at 55 °C to a constant weight. Dried samples were milled and sieved with a 100-mesh sieve, then stored in the refrigerator at 5 °C for further analysis.

Fortification and fermentation

At this stage, the conventional fermentation with fortified and fermentation on condensed tannin and crude protein were compared. The sorghum flour substrate was fortified with soybean flour to determine the optimum concentration of fortification. Sterilized sorghum flour was mixed using overhead stirrer (Ika Eurostar, USA) at 150 rpm for 30 min with 2.5, 5 and 7.5 % (w/w) of soybean flour. Fortified flour has adjusted the pH value (3.5 - 5.5) and sterilized by autoclave at 121 °C for 15 min. The fermentation process is carried out anaerobic using the optimum concentration of *R. oligosporus* at an earlier stage with a temperature 30 °C for 60 h. Furthermore, optimum samples in variation soybean fortification were analyzed by condensed tannins, crude protein, swelling power, water solubility index, and proximate analysis.

Analytical procedures

Condensed tannins analysis

The Cunniff method was followed to evaluate the concentration of tannins in flour sample [20]. 0.5 g of flour sample dissolved with 10 mL distilled water. Subsequently, 1 mL of sample was put into a 10 mL measuring flask containing 7.5 mL of distilled water, 0.5 mL of folin-denis' reagent, and 1 mL of saturated sodium carbonate solution then incubated at 30 °C for 15 min. The absorption was read using UV-VIS spectrophotometry (GENESYS™ 20 Thermo Fisher Scientific, Karlsruhe, Germany) at the

maximum wavelength of 754 nm. The data are calculated by using standard curves that have been pre-determined.

Crude protein analysis

Samples were analyzed every 6 h using kjeldahl method [21]. 0.51 g of sample digested with 17 mL of concentrated sulfuric acid and catalyst (0.3 g of potassium sulphate and 0.2 g of cuprous selenite in kjeldahl block digestion. This method uses 40 % of sodium hydroxide and 4 % boric acid. The titration was carried out with 0.1 N hydrochloric acid and the Tashiro's Indicator (0.375 g of methyl red and 0.25 g of methylene blue in 300 mL 95 % ethanol) was used to identify the endpoint of the titration.

Swelling power and water solubility index

The fortification and fermentation samples were analyzed with an interval of 6 h using swelling power (SP) and Water Solubility Index (WSI) analysis according to the method performed by [22]. 0.1 g of sample was weighed directly in a cap test tube with 10 mL distilled water. The tube was mixed frequently and incubated at 60, 85 and 95 °C in a water bath for 30 min. The tube had been cooled up to room temperature and centrifugated at 2000x g (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min. The sediment in the tube was weighed and the supernatant was dried in an oven at 100 °C until constant weight. The wet and dry paste weights were recorded after separation for swelling determination. Meanwhile, the wet and dry supernatant weights were also recorded for solubility determination:

$$SP \text{ (g/g)} = \frac{\text{weight of wet paste}}{\text{weight of dried paste}} \quad (1)$$

$$WSI \text{ (%) } = \frac{\text{weight of dried precipitate}}{\text{weight of supernatant}} \times 100 \% \quad (2)$$

Proximate analysis

The proximate analysis to determine the carbohydrate (AOAC) number 955.3, moisture (AOAC) number 972.20, fat (AOAC) number 920.158, and the ash content (AOAC) number 942.05.

Statistical analysis

To understand the difference between condensed tannins and crude protein contents, the study employs analysis of variance (ANNOVA) by using SPSS software.

Results and discussion

Effect of soaking sorghum grain

The result presents condensed tannins level was significantly decreased ($p < 0.05$) when the sorghum grains were soaked in plain water or sodium hydroxide for 10 h. Figure 1 shows at soaked with 0.3 % sodium hydroxide solution condensed tannins concentration decreased sharply from 9.56 to 1.25 %. The decrease in the condensed tannins concentration also occurred in soaked with plain water from 9.56 to 6.72 %. This may due to condensed tannins can be degraded into smaller molecules and dissolved in the dilute alkali or distilled water. A previous study also mentioned the condensed tannins content in mung bean seeds was successfully removed up to 35 % by soaking in plain water [23]. An 82.8% reduction of condensed tannins content was observed after soaking in sodium hydroxide 0.2 M [11]. The reduction of condensed tannins content when grains were soaked in distilled water or alkali hydroxide due to the active group in tannins is a phenolic hydroxyl group. Phenol is easily dissolved in distilled water and dilute alkali [24].

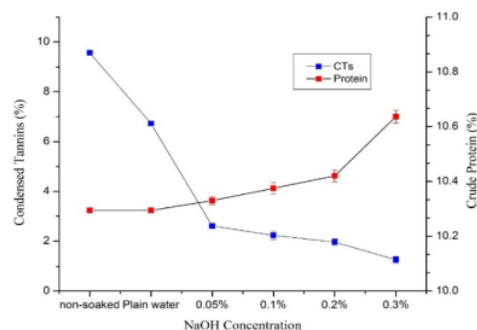


Figure 1 Condensed tannins and crude protein content on soaked sorghum grain.

The crude protein concentration in raw sorghum is 10.29 %. **Figure 1** shows that soaking sorghum grain at 0.05, 0.1, 0.2 and 0.3 % NaOH for 10 h, crude protein concentration slightly increased. The highest increased crude protein concentration from 10.29 % occurs when soaked with 0.3 % sodium hydroxide. As intended, the increase of crude protein concentration may be due to soaked in dilute alkali can cause dry loss meter. Thereby, increasing the concentration of crude protein in sorghum grain. However, the moisture content was not analyzed after the soaking process. This finding is in good agreement with previous research by Ali [11] that soaking sorghum grains into 0.05 to 0.2 % NaOH can increase of crude protein 10.44 to 10.43 %.

Effect of fermentation on condensed tannins content fermentation process

The variation in the concentration of *R. oligosporus* in the fermentation process on condensed tannins concentration did not show a significant difference ($p > 0.05$). Analysis of descriptive statistics, the average condensed tannins content in the mold concentration of 2.5 % has the greatest decrease (0.841) with a standard deviation of 0.413. **Figure 2** shows that there was a gradual decrease in condensed tannins content caused by all molds concentrations in the condensed tannins parameters analyzed. As expected, at first 18 h fermentation there has not been a significant decrease of condensed tannins level in all variation concentration of molds. This is most likely due to *R. oligosporus* is still in a short lag phase. Nevertheless, when the study analyses the results of 42 h it can be found that all *R. oligosporus* concentrations contribute to a decrease in condensed tannins levels to (0.33 - 0.48 %) and the condensed tannins concentration tends to be constant beyond 60 h fermentation. This is due to the decimation of condensed tannins as a substrate and *R. oligosporus* has entered the death phase. As intended, general microorganism growth curves involving short-lag, exponential, stationary, and death phase. The decrease of condensed tannins is caused by *R. oligosporus* which hydrolyses tannins by producing extracellular enzymes in the form of tannases enzyme. Moreover, they also stated that this enzyme has the ability to break down tannins into simple compounds and release free phenolic compounds [25]. This finding is in good agreement with a previous report that *R. oligosporus* capable of degrading hydrolysis of condensed tannins up to 68 % in faba beans fermentation for 6 days [12]. Osman [13] also reported a decrease of 35 % condensed tannins in fermentation varieties sorghum grain using local bread yeast for 24 h.

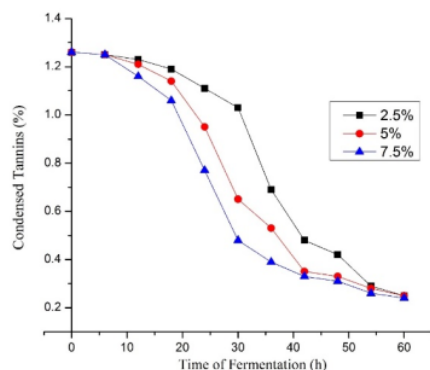


Figure 2 Effect of concentration *R. oligosporus* on condensed tannins content.

Effect of crude protein content on fermentation process

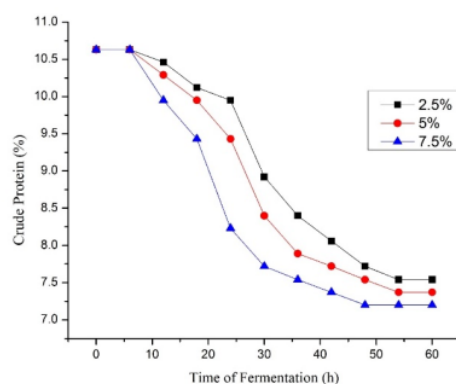


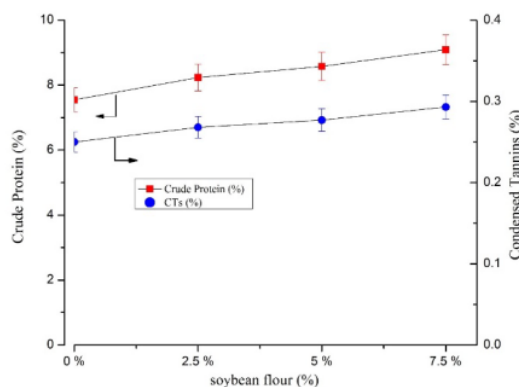
Figure 3 Effect of concentration *R. oligosporus* on crude protein content.

10 The variation in the concentration of *R. oligosporus* on crude protein also showed insignificant results ($p > 0.05$). The degradation of crude protein in sorghum flour during fermentation is shown in **Figure 3**. The results also show that the contents of the crude protein decreased gradually during fermentation by all variation concentration of *R. oligosporus*. At the first 18 h of the experiment, the study found that there was no significant decrease in crude protein content in all variation concentrations of *R. oligosporus*. However, the decreased in crude protein continued until 60 h. The 2.5 % *R. oligosporus* had the lowest decrease in crude protein content by 28.64 %. On the other hand, variation with 7.5 % *R. oligosporus* resulted a decrease in crude protein content up to 33.87 %. *R. oligosporus* grows rapidly up in first 42 h fermentation, then continued with maturing process. Mature and immature ratio of *R. oligosporus* would affect enzyme activity on crude protein hydrolysis. Some authors also report fermentation using *R. oligosporus* that can reduce crude protein. *R. oligosporus* can reduce crude protein in faba beans [16]. Moreover, another study shows that *R. oligosporus* can reduce crude protein in faba beans [12]. In crude protein hydrolysis, amino acid and peptides is assimilated into mild biomass production and then it is oxidized [26]. This may occur depending on the

characteristics of the crude protein source as an energy source for assimilation. The amount and type of substrate as a fermentation medium can cause the spread of mycelium product, condensed tannins, and crude proteins hydrolyzed by enzymes [16].

Effect fortified with soybean flour and fermentation process

At this stage, sorghum flour that has been soaked with 0.3 % NaOH will be fortified by adding soybean flour 2.5, 5 and 7.5 % (w/w) and fermentation performed using the greatest average mold variation in the condensed tannins reduction (2.5 % (w/w) of *R. oligosporus*) and fermentation for 60 h. **Figure 4** shows that the crude protein increases up to 20.69 % in fortification and fermentation flour compared to normal fermentation. The highest increase of crude proteins up to 9.09 % occurred when fortified with 7.5 % soybean flour. This is due to soybean flour has a fairly high crude protein value. In addition, fortification of sorghum flour with soybean flour can help increase the nutritional value [27]. According to Adelakun *et al.* [17], the addition of soybean flour can enrich the types of amino acids such as histidine, methionine, and lysine. **Figure 4** shows condensed tannins content in the fortification process has increased up to 14.9 % from the usual fermentation process. The highest increase in condensed tannins content occurred in the addition of 7.5 % soybean flour which increased to 0.28 %. This relates to the condensed tannins content in soybean flour reaching the amount of 0.063 g in every 100 g of soybeans [26]. Moreover, tannins content in fortified and fermentation flour is still below the maximum limit for consumption of 0.3 % which is permitted by CODEX.



8

Figure 4 Effect of fortification and fermentation on condensed tannins and crude protein content.

Swelling power

The swelling power is the measure of the ability of starch to imbibe water, swell, and reflect of flour to the extent of associative forces within the granules [28]. The SP was analyzed at 6 h intervals during the fortification and fermentation process. **Table 1** shows the highest SP occurs during fermentation for 36 h which is equal to $10.88 \text{ g/g} \pm 0.00$ at 95°C and decreases gradually until the end of the fermentation process, which indicates the higher associative forces. SP of fortified and fermented flour for obtained $8.15 \text{ g/g} \pm 0.14$ at 95°C after 60 h fermentation. Gujska [29] contended that this relates to the strength and character of the micellar network within granule. Moreover, they also stated that this becomes a major factor in the swelling behavior of starch. Therefore, starch is closely related to the structure of micellar and it is resistant to swelling. The relative increase of SP in the fermentation process shows a direct effect on the damage of the starch structure. In addition, the swelling power properties depend on the amylose to amylopectin ratio of starch and the amylopectin structure [30]. This is in line with the previous study that SP increases at the fermentation process 72 h using *R. oligosporus* in soybean flour [31] and corn starch with spontaneous fermentation [32].

Table 1 Effect of fortification and fermentation on SP and WSI.

Fermentation time (h)	SP (g/g)			WSI (%)		
	65 °C	85 °C	95 °C	65 °C	85 °C	95 °C
0	5.70 ± 0.11	6.27 ± 0.09	7.94 ± 0.26	15.76 ± 0.00	20.93 ± 0.03	22.54 ± 0.05
6	6.18 ± 0.49	6.35 ± 0.18	8.13 ± 0.01	15.88 ± 0.01	20.97 ± 0.05	22.78 ± 0.27
12	6.44 ± 0.15	6.51 ± 0.42	8.52 ± 0.01	16.65 ± 0.00	21.19 ± 0.25	22.83 ± 0.09
18	6.63 ± 0.28	6.87 ± 0.00	8.71 ± 0.28	17.18 ± 0.01	21.61 ± 0.23	23.02 ± 0.12
24	6.72 ± 0.07	7.00 ± 0.02	8.95 ± 0.56	20.03 ± 0.13	21.92 ± 0.01	23.48 ± 0.09
30	7.32 ± 0.14	7.85 ± 0.04	10.08 ± 0.19	20.44 ± 0.00	22.28 ± 0.04	24.83 ± 0.07
36	7.40 ± 0.21	8.83 ± 0.63	10.88 ± 0.00	20.96 ± 0.17	22.89 ± 0.03	25.43 ± 0.31
42	7.00 ± 0.01	8.21 ± 0.00	9.17 ± 0.07	21.19 ± 0.04	23.76 ± 0.08	25.70 ± 0.01
48	6.77 ± 0.00	7.74 ± 0.14	9.00 ± 0.01	21.19 ± 0.00	24.10 ± 0.11	25.87 ± 0.04
54	6.70 ± 0.02	7.11 ± 0.02	8.21 ± 0.01	21.20 ± 0.01	24.11 ± 0.02	26.03 ± 0.03
60	6.48 ± 0.24	6.46 ± 0.14	8.15 ± 0.14	21.20 ± 0.01	24.15 ± 0.07	26.15 ± 0.19

Legend: Values are means of 2 replicates standard deviation.

SP: Swelling Power; WSI: Water Solubility Index.

Water solubility index

The water solubility index is one of the reliable criteria to evaluate the behavior of flour in aqueous solution. This parameter measures the ability of flour to form a suspension or dissolve in solution in a water mixture. Moreover, the WSI linked to the presence of dissolved molecules and is an indicator of the degradation of starch. The lower of WSI indicate the minor degradation of the starch and lead to few dissolved molecules in the flour [33]. WSI in different temperatures 65, 85 and 95 °C were analyzed. As the increased temperature, the starch granules vibrated more intensely, breaking intermolecular bonds and allowing more water molecules to participate in hydrogen bonding sites [34]. Table 1 shows that the WSI unfermented flour ranged from 15.76 % ± 0.00 to 22.54 % ± 0.05 at all temperature variations. After the fortification and fermentation process, the WSI increased significantly during fermentation. At the end of fermentation time (60 h) the WSI increased to 21.20 % ± 0.01 to 26.15 % ± 0.19 at all temperatures. This may occur due to the degradation of starch during the fermentation process of sorghum flour by *R. oligosporus*. The result of this process makes the flour ready for digestion. Ojokoh [35] reported that increased WSI after fermentation was related to molecular degradation with the intensity of extrusion condition and a large amount of soluble material is released at high screw speed, high extrusion temperature, and low feed moisture content. This relates to study by Simwaka [36] who reported that WSI increased with the fermentation process in pumpkin, sorghum, millet, and amaranth seed flours.

Proximate analysis of fortified and fermentation flour

Table 2 Properties of fortified and fermented flour.

	Untreated sorghum flour	Fortified and fermented sorghum flour	Wheat flour	Parameter of sorghum flour [10]
Carbohydrate	76.12 %	79.50 %	77.30 %	72 %
Moisture	4.90 %	4.80 %	13.40 %	<15.5 %
Ash	1.80 %	1.60 %	0.70 %	<0.70 %
Fat	3.54 %	3.10 %	1.27 %	1.87 %

Optimum sample with 2.5 % of *R. oligosporus* and fortification 7.5 % of soybean flour was proximate analyzed. Table 2 shows that the carbohydrate content of fortified and fermentation flour was increased by 4.3 % from raw sorghum flour. This relates to the fortification process with soybean flour which has a carbohydrates concentration of 29 %. However, the fermentation process using *R.*

oligosporus actually reduces carbohydrate. Decreased carbohydrate levels after the fermentation process using *R. oligosporus* might be caused by non-partial removal of starch constituents during the solid fermentation process [37]. It has been reported that during the fermentation process of Barley in Solid State Fermentation by *R. oligosporus* can reduce carbohydrate content up to 1.5 % [38]. Carbohydrate concentrations of fortification and fermentation sorghum flour have exceeded carbohydrate concentrations of wheat flour. Therefore, good enough to substitute the other commercial flour.

Moisture content is an important parameter and is considered as a determination of shelf stability. Flour with a high moisture content causes a shorter shelf life compared to drier products. Table 2 shows that the moisture content of fortified and fermented flour has lower content than untreated sorghum flour. This is due to the soaking process using dilute alkali can cause dry loss meter. Furthermore, the moisture content of flour could be affected by temperature, drying method, and drying time. This amount meets the quality standards of the terms of sorghum flour <15 % by [10] and lower than wheat flour as a comparison for flour products. Moisture content decreased during the fermentation process also reported by Ojokoh [39] in pearl millet and acha flour blends.

Ash indicator is one of parameters to indicate the presence of inorganic compounds as minerals in flour. The ash content of untreated sorghum flour was 1.8 %. However, after the fortified and fermentation process, the ash content decreased to 1.60 %. This decrease may occur due to the soaking process with dilute alkali and the leaching of inorganic compounds during the fermentation process. Jaukovic [40] reported that the ash content of maize malt reduced gradually during soaked in NaOH. However, the amount of ash content is considered high as the results are above the quality requirements of sorghum flour parameter, which is between 1.87 % and wheat flour ash content as a comparison. The high ash content of sorghum flour is may cause by the sorghum skin being carried away during the grinding process.

Fat is one of the important nutritional components in food ingredients besides fat also provides a lubrication effect in the compressed polymer. Table 2 shows that fortified and fermented process, the fat content decrease from 3.54 to 3.10 %. This reduction during fermentation related to the fact that the biochemical and physiological changes that occur during fermentation require the lipids contained in the sample for *R. oligosporus* energy sources [41]. This found is in line with the study conducted by [42] that the fermentation process using *R. oligosporus* in solid state fermentation of *Zea mays* can reduce total fat levels by 29.50 %. The fat content of fortification and fermentation flour has met the sorghum flour parameter standard and above the fat content of wheat flour.

Conclusions

After the soaking process using a dilute alkaline, condensed tannins can be degraded into smaller molecules and dissolved in the solution. The results showed that soaking using 0.3 % NaOH had better degradation of condensed tannins in sorghum seeds up to 86 %. The fermentation process using *R. oligosporus* can reduce condensed tannins of sorghum flour to 0.25 %. The reduce of condensed tannins could be caused by *R. oligosporus* produces the enzyme tannase which hydrolyzes condensed tannins into substrates for metabolism during the fermentation process. In addition, *R. oligosporus* was also found to assimilate crude protein in sorghum flour as an energy source during the fermentation process. Fortification using soybean can increase the concentration of crude protein compared to the normal fermentation process. Changes in nutritional value, swelling power, and water solubility index may due to the activity of *R. oligosporus* which changes the structure of sorghum flour. Fortification and fermentation of sorghum flour have great prospects as a food product with good nutritional value. Thus, it is necessary to investigate the processing of sorghum flour using several types of microorganisms to produce good food products.

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