4. DISCUSSIONS

4.1. The Processing of Natto Making

The processing of natto making includes soaking, steaming, inoculating, fermenting, and aging or ripening (Weng and Fung, 1996). The soaking process before inoculation was important to increase the moisture content of the beans, make the beans edible, to facilitate microbial activity during the fermentation, and to decrease the bitter compound (Pisol *et al*., 2015). The soaked beans were steamed before inoculating by cultures. Based on Shockey and Shockey (2019), there are two ways to cook the beans for natto, which are steaming without pressure and steaming with pressure. Soybeans natto usually need 35-45 minutes at 10-15 pounds of pressure in the steaming process but, for the cowpea need longer time until 60 minutes (1 hour). In this research used steaming with pressure, the beans have a softer texture because at 100°C was the beginning of gelatinization of the starch granules and they need longer to the breakdown of the middle lamella (the outermost layer of cell wall). In this process also to inactivate anti-nutritionals factor such as trypsin inhibitor (TI), hemaglutinin, and cyanogenic glycoside (Sefa-Dedeh *et al*., 1979).

The steamed beans were divided into 16 parts in the stainless steel tray to treat by the different condition of fermentation (time and ratio of co-cultures), this step also used for cooling down the temperature of beans. For the inoculating process based on Jhan *et al.* (2015) that used the ratio 1/10 (beans/cultures) and the same concentration of *Bacillus subtilis* and *Lactobacillus delbrueckii* as the co-cultures. In this research, the concentration of co-cultures were checked by OD_{660} until 1.5 and were standardized using McFarland 5. The spectrophotometer was measured the turbidity directly for estimating the microbial concentrations. While the McFarland standards used to approximate the concentration of cells in a suspension in the CFU/mL (Sutton, 2011). *Kawashima-ya* Natto Starter Spores is pure culture of *Bacillus subtilis* var. natto with $1x10^{10}$ CFU/g. Milner and Makise (2002) has explained the natto bacilli was commonly used concentration of 10^{10} cells/g of dried bacilli powder.

Natto formation usually occurs at 14-18 hours of fermentation at 35-40°C (Wei *et al*. 2001). In this research, cowpea was incubated at 37°C, which is an optimal temperature of *Bacillus subtilis* and *Lactobacilli*. Fermentation time 24, 48, 72, and 96 hours was chosen based on Jhan *et al*. (2015). After the fermentation process, natto was chilled to lower than 10°C for ripening because that process can inhibit fermentation and minimizes ammonia production (Kada *et al.*, 2008). Then, the sample of cowpea natto was taken to measure the pH using pH spear and the brightness (L value) using chromameter. Cowpea natto was extracted using ethanol 50% for 45 min. Ethanol extracts showed has greater antioxidant activity than water extracts (Jhan *et al*., 2015). The extract of natto was used for further analysis such as antioxidant activity (DPPH scavenging activity) and total phenolic content. The sample of cowpea natto also used for sensory analysis by fifteen untrained panellists to evaluate the drawing and smell.

4.2. pH of Cowpea Natto

Natto is one of the Japanese products which made from the alkaline fermentation process that carried out by potential probiotic *Bacillus subtilis* or another *Bacillus* species. Power of Hydrogen (pH) is one of the most important parameters affecting food fermentation. It closely related to the structural changes in phytochemicals during fermentation and microbial growth (Hur *et al*., 2014). Table 2. had shown that there was a relationship between pH and fermentation time and the ratio of co-cultures. The pH value of steamed cowpea (before fermentation process) was 6.72 ± 0.11. At the fermentation time 24 hours to 72 hours of the pH was increased (7.35 ± 0.08 to 9.08 ± 0.07) but, after 96 h the pH was decreased (to 8.63 ± 0.12). Longer time of fermentation (up to 72 hours) led to an increase in the pH of the product. Compare with commercial natto "Goku Kotsubu Okame" that used for sensory test also has pH value 8.12 ± 0.02.

Depends on the final product, natto was categorized as alkaline fermentation product (Parkouda *et al.*, 2009). The main activity of this fermentation is the hydrolysis of proteins to amino acids and peptides and the oxidation of the amino acids leads to the results in the accumulation of ammonia which increases the pH of final products to 8 to 9. The high pH of product inhibits the growth of spoilage microorganisms and the spread of pathogens (Wang and Fung, 1996; Leejeerajumnean *et al*., 2000).

Based on analysis of Two-way ANOVA, there was an interaction between fermentation time and ratio of co-culture on the pH value of cowpea natto. However, at the longest fermentation time (96 hours), the pH was decreased because the *B.subtilis* has reached the optimum time of growth, so it cannot hydrolyze the protein to release ammonia and raise the pH value higher. According to Jhan *et al.*, (2015), the growth of *B. subtilis* in the fermented red beans was decreased after 70 hours of incubation time during the fermentation process. Kim *et al*. (2018) also reported that the highest level of *Bacillus* growth was obtained at pH 9 and the lag phase of *Bacillus* at pH 6-9 at 35°C.

Ammonia was present as volatile NH_3 for the product have an unpleasantly strong ammonia odor. During the hydrolysis of protein, there was a production of ammonia from the utilization of amino acids by the bacteria that produces ammonium with OH-. The pH of product increased and predicted that ammonia is released from the degradation of six amino acids: glutamate, alanine, glutamine, aspartate, asparagines, and arginine (Kada *et al*., 2014).

$$\mathrm{RCH(NH_3^+)COO^-} + \mathrm{nO_2} \rightarrow \mathrm{nCO_2} + \mathrm{nH_2O} + NH_4^+ + \mathrm{OH^-}$$

(Allagheny *et al*, 1996).

The results shown in Figure 4. indicated that for each fermentation time, the higher ratio of lactic acid bacteria leading to the decrease of pH, it because cowpea was acidified by lactic acid bacteria produced by *Lactobacillus delbrueckii* KSM 10. Zhang *et al.* (2014) shown that lactic acid is the main acid component produces by LAB that could reduce pH in fermentation media. pH value of combination cultures *B. subtilis* natto and lactobacilli decreased during the culture period (Hosoi *et al*., 2000).

Meanwhile, at 96 hours of fermentation, natto with the highest ratio of lactic acid bacteria (25% *B. subtilis natto* and 75% LAB) has higher pH than natto with lower ratio of lactic acid bacteria. It because the fermentation conditions (alkaline, pH >8.00) did not allow the lactic acid bacteria to grow well, so it cannot survive and present acidic taste in natto during that fermentation time. The optimum growth pH of *Lactobacillus delbrueckii* subsp. *bulgaricus* was 5.80-6.00 (Jhan *et al*., 2015).

4.3. Color (L Value) of Steamed Beans and Cowpea Natto

Natto products with good quality should be covered with white mucus substances, have characteristic flavor, and light yellow color (Hu *et al*., 2010). In this research, the color changing of cowpea natto was shown as L value (lightness) based on Wei *et al*. (2001). Cowpea was soaked for 16 hours, the color (L value) was 56.82 ± 1.46 and it was decreased after the steaming process with the value of 46.94 ± 1.56. Based on the Wei *et al*. (2001), the steaming process longer than 25 min made the beans darker gradually.

The color changes during steaming were caused by non-enzymatic browning of the Maillard reaction (Taghinezhad, 2015). Maillard reaction is a chemical reaction occurs between reducing sugar and amino acids while foods are processed or cooked at high temperature, that reaction generates different flavors and brown color (Tamanna and Mahmood, 2015). Cowpea contains reducing sugars (glucose and fructose) and high protein contents that led to the Maillard reaction which is the formation of cross-links in the cowpea protein and it caused the decrease in L value (Mwangwela *et al*., 2007)

Based on Table 3. it showed the highest L value of the cowpea natto presented at 48 hours with the value to 51.30 ± 1.64. There was not significantly different of L value between ratio co-cultures L1 and L2 in cowpea natto. At 72 hours to 96 hours of fermentation, the L value was decreased gradually due to the white-colored mucus substances has absorbed the color of cowpea. The seed coat pigments of cowpea contain tannins. Halloin (1982) has explained the tannins are oxidized and polymerize into dark color due to the atmospheric oxygen during fermentation or ripening process.

4.4. Antioxidant Activity of Cowpea Natto

The antioxidant is molecules which can inhibit the oxidation of other molecules. Antioxidant activity is the total capacity of antioxidant for eliminating free radicals in food and cell (Hur *et al*., 2014). In DPPH assay, the antioxidant activity was able to reduce the stable radical DPPH from purple to the yellow colored *diphenyl-picryhydrazine* based on the reduction of DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction (Wonghirundecha *et al*., 2014).

Traditional Asian fermented soy foods such as tempeh, natto, and miso have been known to exhibit stronger antioxidant activity than unfermented steamed soybeans (Jhan *et al*., 2015). Based on Table 4. had shown that the antioxidant activity tends to increase up to 72 hours fermentation time. A significant increase that made the highest value of antioxidant activity was at 72 hours of fermentation when the cultures reached the optimum time of growth with the same ratio of *B.subtilis* and lactic acid bacteria (L2) with the value 47.97 ± 2.49 %.

Based on analysis of Two-way ANOVA, there was an interaction between fermentation time and ratio of co-culture on the antioxidant activity of cowpea natto. The antioxidant was increased during the fermentation process because the particular compounds were released after bacterial hydrolysis. Based on Hu *et al.* (2010) inoculation with *B. subtilis* increases proteolysis. Proteins are hydrolyzed to amino acids and peptides with free radical scavenging activity by proteases produced by *B.subtilis* during fermentation and it was indicated that a high amount of peptides could donate hydrogen atoms which can react with free radicals and convert them into more stable compounds (Hur *et al*., 2014). Protein hydrolysates are important sources of antioxidant and radical-scavenging sequences have been identified from hydrolysis of various proteins (Zhang *et al*., 2014).

In previous study, red beans fermented with *B. subtilis* and *L.bulgaricus* exhibited antioxidant activities, including DPPH radical-scavenging activity (Jhan *et al*., 2015). The presence of lactic acid bacteria affects the increase of antioxidant activity because lactic acid bacteria also hydrolyzed the protein and release peptides (Hur *et al*., 2014). The greatly increase of free amino nitrogen of fermented soybeans by LAB mixed with *B.subtilis* natto showed that the enzyme of LAB and *B.subtilis* natto could work well to hydrolyze the soybean protein (Zhang *et al*., 2014). Moreover, *B.subtilis* natto produces catalase and subtilisin may improve the growth and viability of lactobacilli through their activities in the intestine and it change the protein and peptide content of medium during incubation time as the enhancement effect (Hosoi *et al*., 2000).

The lowest antioxidant activity showed at 96 hours of fermentation with value 35.21 ± 3.36 %. This related to the pH on the previous explanation that the pH was decreased at 96 hours of fermentation because it cannot hydrolyze the protein and raise the pH higher. According to Hur *et al.*, (2014), the increase of antioxidant activity influenced by various factors includes the microorganism species, temperature, pH, solvent water, kind of the food, fermentation time, and aerobic condition.

4.5. Total Phenolic Content of Cowpea Natto

Fermentation has a good influence on the total phenolic content and antioxidant activity of legumes, and it depends on the species of microorganism. Phenolic compounds are antioxidant which contain aromatic rings with one or more hydroxyl and can be divided into two major groups, flavonoids and phenolic acids. They act as antioxidants not only because of their ability to donate hydrogen or electron but also their stable intermediate radical that can prevent the oxidation of food ingredients such as fatty acids and oils (Hur *et al*., 2014).

Based on Table 5. and Figure 6. the total phenolic content of cowpea natto increased gradually until 72 hours of fermentation and decreased at 96 hours. Then, cowpea natto with the same ratio of co-cultures (L2) at 72 hours fermentation time showed the highest level of total phenolic with value 95.67 ± 2.46 µg GAE/ g. It showed the same result with the highest value of antioxidant activity that occurred at 72 hours of fermentation with the same ratio of co-cultures (L2) with value 47.97 ± 2.49 %.

Based on Table 6. also showed that the antioxidant activity has strong correlation (significant at the level 0.01) with the total phenolic content. According to Jayathilake *et al*. (2018) has reported a high positive correlation of antioxidant and total phenol indicating that the overall antioxidant activity of food legumes are dominated by phenolic compounds. Fermented soybeans by *B.subtilis*, Cheonggukjang from Korea also reported a similar result that the total phenol content was increased during fermentation (Cho *et al.*, 2011). While, the effect of lactic acid bacteria on antioxidant activity could be explained by the breakdown of plant cell walls that lead to the liberation of simple phenolic compounds after acid and enzymatic hydroylisis of polymerized phenolic compounds during fermentation process (Hur *et al*., 2014).

After 72 hours of fermentation, the phenolic compounds was decreased caused by the oxidation of tannins (see 4.3). Tannins are considered as high molecular weight phenolics due to large number of phenolic rings in their structures (Siddhuraju and Becker, 2006). Most phenolic compounds are usually found in conjugated forms through the hydroxyl group with sugars (primary glucose) and glycosides in plants (Juan and Chou, 2010). During fermentation, several enzymes are produced such as α-amylase, β-glucosidase, xylanase that can directly in substrate with a consequent release of phenol by the gradual increase for 72 hours of fermentation. The decrease of total phenolic contents after that period probably resulted from the degredation (Oliviera *et al*., 2012).

Hur *et al*. (2014) also explained the fermentation by different microorganisms can modify bioactive compounds in plant-based food. This might be due to the difference in pH of different fermentation when the optimum pH was reached can influence the degradation of the cell walls and enzymes. Moreover, the presence of α-amylase and β-glucosidase inhibitors in cowpea can be beneficial to human health because they can reduce the rate of glucose release during digestion (Jayathilake *et al*., 2018).

4.6. Sensory Analysis

Natto was not widely consumed as compared to other fermented soybean products, even though it is well known in Japan due to its characteristic of the ammonia odor and sticky appearance (Zhang *et al*., 2014). Cowpea natto was compared with the commercial natto from Japan "Goku Kotsubu Okame" by evaluating the drawing item and smell based on the table of sensory evaluation standard (Table 1.). Drawing item includes the sticky appearance, mucus, and viscosity, which is evaluated by stir it by chopsticks. According to Wei *et al.* (2001), In Japan, when natto is served, it stirred vigorously with a pair of chopsticks to generate the stickiness to form a mass of beans. The viscous and sticky texture when natto was stirred depends on the γ-polyglutamic acid (γ-PGA) that forms on the surface of beans and produced by *B.subtilis* var. natto (Kada *et al*., 2008).

Smell properties include the ammoniacal smell, based on the previous explanation, that comes from the hydrolysis of protein to peptides and amino acids. However, color and taste were not evaluated because of there was a very different color of commercial natto (from soybeans) and cowpea natto and natto was not common for Indonesian people. In this sensory test was used natto with 24 hours fermentation time because the longer fermentation time, the ammonia odor became stronger and not acceptable for the panellists. Ma *et al.* (2015) reported that with an increase in the inoculation time, the surface of natto becomes drier, has decreased drawing, stronger ammonia odor, and grows bitter that makes it not acceptable for consumer.

Based on Table 7. the commercial natto has the highest score is 44.80 out of 50 with drawing and smell score are 28.13 ± 2.70 and 16.67 ± 3.74, respectively. The highest score was followed by natto using 75% starter and 25% lactic acid bacteria (L1) with score 32.80 out of 50 with drawing and smell score are 21.80 ± 6.06 and 11.00 ± 3.68, respectively. Based on the table of sensory evaluation standard (Table 1.), commercial natto and L1 natto fell in the same category in which the drawing item scores 21~30 meaning that natto has long drawing, lots mucus, and good viscosity. While for smell with score 11~20 means that natto has a special and strong natto perfume and no ammoniacal smell. The perception of panellist can influence a significantly different score of commercial natto with cowpea natto because the color was very different between soybeans (commercial natto) and cowpea. Meanwhile, there was an addition of rice flour in the ingredients of commercial natto, which can affect the viscosity and smell. Zhang *et al.,* (2014) indicated that the combination of *B.subtilis* natto and lactic acid bacteria in fermentation could improve the odor and sticky appearance of the fermented soybean by maintaining it at an appropriate pH value and providing acidic taste. Additionally, natto was not acceptable to most consumers because of its ammonia odor. So, besides being consumed in sticky form, some functional food companies or biotechnical try to dry it into powder form or make capsulated form sold as a functional food (Weng and Chen, 2010).