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The Effect Of Cellulase Content On Phytoestrogens Formation (Genistein) Materials On Fermented Soybean Waste *Aspergillus niger*

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Abstract

The activity of the cellulase enzyme and the content of phytoestrogen (genistein) forming is determined during the fermentation of soybean waste with Aspergullus niger. The analysis is performed at fermentation incubation time of 0, 48, 96, 144 and 192 hours and treatment with addition of micro nutrient (V2) and without micro nutrient (V1). Data analysis on cellulase enzyme activity on phytoestrogen forming material is performed using T test. The average of cellulase content of fermented soybean waste increases from 0.032-0.139 IU / ml (V1) and 0.061-0.158 IU / ml (V2) after 48 hours into 0.238 IU / ml (V1) and 0.245 IU / ml (V2) after 192 hours The average of genestein levels in the fermented soybean waste increases as well from 0.101 - 0.573 mg / g (V1) and 0.114 - 0.587 mg / g (V2) after 48 hours and increase into 0.722 mg / g (V1) and 0.699 mg / g (V2) after 192 hours. Statistical analysis indicates that the treatment of V1 and V2 has an effect on the increase of cellulase enzyme content and genistein level (P <0.05). The correlation between enzyme activity of cellulase and genistein on soybean waste fermented with Aspergillus niger is significant (R2 = 0,949). The relationship between the enzyme performance and its formation material is running synergistic, cellulase enzyme activity and genistein level experience significant increase during the fermentation process.

Keywords: Soybean waste, Aspergillus niger, fermentation, cellulase enzyme and genistein

Background

Soybeans are an important source of food, in addition to having many advantages, soybeans can also be used to meet the needs of human food, animal feed, and useful for raw materials of various industries (Kalsum, 2008). Soy is a very important source of protein in life, but it also contains high isoflavones. Isoflavones are a compound of flavonoid groups found mostly in beans, especially in soybean (*Glycine max)* (Chang, 2009). The total content of isoflavone compounds in soybean is 1.2-4.2 mg / g of dry sampling (Yunidarwati, 2015). There remains a lot of isoflavon content in soybean waste as well, soybean waste is a by-product of the final waste of large industry and home industry. Soybeans also contain high isoflavones and proteins that can also be used as a substitute material to increase chicken egg production (Muis *et al.*, 2009). Isoflavone compounds in soybean waste are glycosides (daidzin, genistin and glycine) and aglikon (daizein, genitein and glycetein) (Dhaubhadel, 2011). These compounds have various biological activities such as being able to inhibit the growth of cancer cells, antioxidants, and phytoestrogens and reduce the risk of heart disease (Chae and Ha, 2011).

Phytoestrogens are estrogen-like chemicals in plants that act as precursors to the body's metabolism. These phytoestr-ogens are natural chemicals that can interact with estrogen receptors (genistein) (Teekachunhatean, 2013). Phytoestrogens are natural decomposition found in plants that have much in common with estradiol. Estradiol found in plants is called phytoestrogens (Jefferson *et al.,* 2002).

Isoflavones in the form of aglycons possess better bioactivity than in glycoside forms (Pandit, 2011), whereas isoflavones in soybean have glycoside forms (Teekach-unhatean, 2013). Isoflavones glycosides might undergo conversion into aglycons through the fermentation process (Huynh, 2015). Fermentation is the process of chemical changes in an organic substrate through the activity of enzymes produced by

microorganisms, such as bacteria, mold and others (Suprihati, 2010). One of mold that has been widely used in feed fermentation process is *Aspergillus niger*. This mold is capable of producing cellulase enzyme, this enzyme is capable of degrading cellulose, with its working principle involving three types of enzymes that work synergistically, i.e. *endo-* and *ekso-1.4-* β- *glucanase* and β- *glucosidase.* The β- *glucosidase* enzyme is capable of converting isoflavone glycosides into isoflavone aglikon (Muuray *et al.*, 2003).

According to Putri (2013), the addition of *Aspergillus niger* fermented soybean waste in chicken ration can increase the number of chicken egg follies. It is assumed that there is an increase of phytoestrogens contained therein. The largest phytoestrogen in soybeans is genistein, isoflavones in the form of aglycons after undergoing conversion in the *Aspergillus niger* fermentation process (Jefferson *et al.*, 2002). The objective of this study is to determine the content of cellulase enzyme on the formation of phytoestrogens (genistein) on soybean waste fermented with *Aspergillus niger.*

Materials and Methods

This study was conducted in June 2015. The preparation of *Aspergillus niger* fermented soybean waste was conducted at the Veterinary Public Health Laboratory of the Faculty of Veterinary Medicine, Syiah Kuala University of Banda Aceh. Examination of cellulase and genistein levels was carried out at FMIPA Chemistry Laboratory of Syiah Kuala University of Banda Aceh. This study is an experimental study using a variance analysis design consisting of two treatments (F1 and F2). The experiment of this study was the effect of cellulase content and genistein levels in the fermented soybean waste with additional micro nutrient (F2) and without micro nutrient (F1) on the length of incubation.

The process of making *fermented soybean waste*

Inoculum was developed from *Aspergillus niger* isolate on rice substrate. Mold was isolated on a rice substrate that had been mixed with water (1: 1) and was checked until it was half cooked. Then the culture was incubated at room temperature and covered with a plastic bag (Nurliana *et al.*, 2013).

The *fermented soybean waste* was prepared in the following way: the soybean waste was squeezed with a napkin to decrease the water concentration. Then it divided into two parts: (F1) soybean waste without micro nutrients and (F2) soybean waste with additional micro-nutrients i.e. 2% ammonium nitrate, 15% sucrose, 2.4% potassium hydrogen phosphate, 0.5% magnesium sulfate and 0, 01%. Then added with 8 g of inoculum until they evenly distributed, stored on a plastic tray and cover with plastic. It incubated at room temperature for 0, 48, 96, 144, and 196 hours.

Sample Preparation

The fermented soybean waste was fed into 0.5 g of reaction tubes. In each tube aqua solution was added by 5 ml, then was shaken quickly for 1 hour using *multi tube vortexer*. The sample was then centrifuged at 3000 rpm speed for 10 minutes. Then the supernatant fluid was separated and the cellulase content and phytoestrogens (genistein) level was examined with a spectrophotometer.

Examination of cellulase content

Testing of cellulase enzyme activity using modified DNS method from Wood (1998) in Desi, (2012). To make a solution of DNS (Dinitro salicylic acid) using a formulation in 100ml aqua, 1 g of DNS, 0.2g of phenol, Na_2SO_3 (0.05g), NaOH (1gr) were required.

For the blank preparation, 1.8ml of substrate (CMC 1%) was added with 0.2 ml of acetate buffer pH 5. Then for cellulase activity test, 1.8 ml of 1% CMC substrate was added with 0.2 ml of crude enzyme extract then it was homogenized then incubated at 30° C for 30 min (activity of enzyme was terminated in boiling water for 15 min). This solution was taken by 1 ml then added with 1 ml of DNS, then reheated on boiling water for 15 minutes. Absorbance was measured at wavelength λ = 575 nm

with a spectrophotometer. The cellulase activity was expressed in unit of international unit per milliliter (IU / ml).

Measurement of Phytoestrogens (Genistein) Level

Genistein testing was carried out using the standard genistein (4^1, 5, 7-trihidrosi isoflavone, $C_{15}H_{10}O_5$ with molecular weight 270.24 obtained from japan with 99% purity) the genistein standard was in the form of powder. So before usage, it could be diluted first. As much as 15 mg of standard genistein was added with methanol to reach 100 ml volume, then sonication was performed for 5 minutes.

Genistein testing was carried out using ICH guidelines (International Conference on Harmonization), where samples were added with the standard of examined content. For the blank preparation, 5 ml of the genistein standard was added with 1 ml of $AlCl_3$ (2%). Then for genistein testing, 3 ml of standard genistein was added with 0.6 ml of crude enzyme extract then were homogenized (vortex). 1 ml of this solution was taken and added with 1 ml of $AlCl_3$ (2%) then was homogenized (vortex). Measurement of absorbance of genistein level at wavelength λ 382 nm with spectrophotometer (Isabella, 2008).

Data analysis

Data analysis of cellulase enzyme level on phytoestrogen forming material (genistein) was carried out using ANAVA, Nexted by LSD (Least Significant Difference) test that aims to see the effect between the length of incubation and treatment.

Results and Discussion

Content of cellulase enzyme

Cellulase enzyme is a mixture of several enzymes namely endoglucanase, exoglucanase and β-glucosidase (Ul-Haq et al, 2005). Cellulase enzyme serves as a catalyst for hydrolyzing cellulose present in soybean waste. Cellulase enzyme is capable of converting isoflavone glycoside compound into isoflavone aglikon (Maier et al, 2000). The results indicated that the content of soybean waste fermented with *Aspergillus niger* was affected by nutrients in feed and the length of incubation. The average value of cellulase content in soybean waste fermented with *Aspergill-usniger* with different incubation periods can be seen in Figure 1.

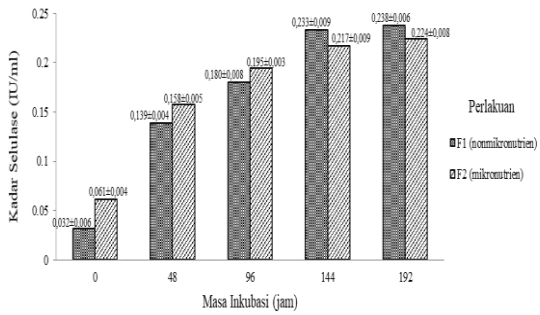

Figure 1. Average (±SD) cellulase enzyme levels in Aspergillus niger fermented soybean waste.

The cellulase content of the fermented soybean waste with nutrient additives was higher than the non-nutrient soybean waste, but the resulting average value was relatively the same and did not show any significant difference. The cellulase content of the fermented soybean waste increases as the incubation period increases. The average cellulase content of fermented soybean waste increased from 0.032 to 0.139 IU / ml (F1) and 0.061 - 0.158 IU / ml (F 2) after 48 hours into 0.238 IU / ml (F1) and 0.245 IU / ml (V2) after 192 hours. Statistical analysis using T-test indicates that treatment of F1 and F 2 has significant effect on cellulase content ($P < 0.05$). When it was observed from the incubation period of 0, 48, 96, 144, and 192 hours it was found that there was a significant difference during the fermentation process. Tests of this cellulase enzyme content aims to prove that *Aspergillus niger is* able to degrade cellulose to produce glucose with the help of the cellulase enzyme complex it produces. Cellulose content ranging from 0 to 48 hours indicated significantly elevated levels of cellulase activity up to 192 hours. In the 0-hour state, it had a value of 0.032 to 0.061 (IU / ml), this is due to the occurrence of

natural fermentation during the immersion process, which allowed the occurrence of contamination of organisms (Purwadaksi, 2007). In 48 to 96 hours state, cellulase produced indicated significant increase. At that range of time, cellulose degradation process reached to maximum performance, this is called exponential phase. In this diffuse, microbes split rapidly and constantly follow the logarithmic curve (Balasaravanan, 2013). However, this cellulase content did not necessarily result in an increase, in an incubation of 144 - 192 hours, the results were static by a difference of ± 0.005 IU / ml. This situation is affected by the growth of microbes and the medium where they grow such as nutrient content and environmental conditions (temperature and humidity). In this phase, the number of cell population grows equal to the number of dead cells (Maryandini, 2009).

This is consistent with the study report of Safriani (2013) that the highest enzyme activity on fermentation is obtained after day 4, but decreasing on day 8. Pasaribu, 2013 states that the effect of enzyme activity on fermentation period at the beginning of fermentation until the increase of fermentation period indicates that the activity of enzyme produced increases until fermentation period of 4 days, but after 4 days, the activity of producing enzyme is low. Spore-forming organisms usually produce enzymes in the post exponential phase (stationary). So it can be assumed that when the enzyme production is high, the mold has been in that phase (Suhartono, 1989).

Levels of phytoestrogens (Genistein)

Fermented soybean waste plays an important role in improving the isoflavone aglikon. One of the major isoflavone aglikon compounds present in soybean dregs is genistein (Yoan, 2006). Genistein can be obtained from isoflavone glycoside transformation by deglicosylation reaction due to cellulase enzyme activity (β-glucosidase). This enzyme attacks glucose that binds to the flavanoid in the position of C3 and C7 (Pandit, 2011). Genistein is an isoflavone used as phytoestrogens. Phytoestrogens are natural decompositions found in plants that have much in common with estradiol (Jefferson et al., 2002). Estradiol in plants is called phytoestrogens. According to Winarsi (2005) isoflavones are naturally synthesized by plants, the biosynthesis takes place gradually starting from sinnamic acid, kumar acid, flavones and isoflavones. Based on the biosynthesis, isoflavones are classified as secondary metabolite compounds (Pawiroharsono, 2001). The average value of phytoestrogens (Genistein) in *Aspergillus niger* fermented soybean waste with different incubation periods can be seen in Figure 2.

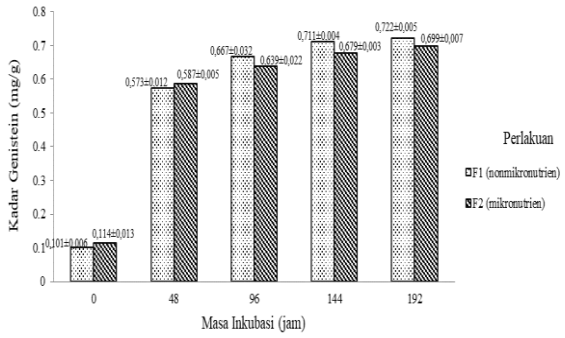

Figure 2. Average (±SD) genistein content in *Aspergillus niger* fermented soybean waste.

The level of genetein of fermented soybean waste given the nutrient is higher than the non-nutrient soybean waste. The level of genestein in the fermented soybean waste increases as the incubation period increases. The average of genestein levels in the fermented soybean waste increased by 0.101 - 0.573 mg / g (F1) and 0.114 - 0.587 mg / g (F2) after 48 hours and after 192 hours of genestein levels to 0.722 mg / g (F1) and 0.699 mg / g (F2). Statistical analysis using T test indicated that treatment of V1 and V2 had an effect on genestein level ($P < 0.05$). *Aspergillus niger* fermented soybeans waste with nutrient additives yielded a relatively the same of average value with the average value of *Aspergillus niger* fermented soybean waste without nutrients additives. When it was observed at the incubation period of 0, 48, 96, 144, and 192 hours it was found that there was significant difference during the fermentation process. Testing of genestein

levels aims to prove that *Aspergillus niger* is able to break glycoside bonds in soybean waste during the fermentation process so as to convert genistin into genistein (Sumarna, 2009). Genistein level from 0 to 48 hours indicated significant increase of up to 192 hours. In the 0-hour state, it had a value of 0.101-0.114 (mg / g), this is due to the natural conversion between geistin (isoflavone glycosides) into genistein (isoflavone aglikon) at the time of immersion with the aid of cellulase enzymes produced by microorganisms. Genistin found in soybean waste can be hydrolyzed by β-glucosidase into genistein (5, 7, 4'-trihydroxy isoflavones) and glucose (Garlok, 2000).

In the 48 to 192 hours state, genistein produced showed significant increase. At the range of time, it was assumed that the performance of the resulting enzyme reached to maximum, the production of β-glucosidase enzyme increases in line with cell growth in the logarithmic phase because it is associated with primary metabolism in the cell. As the statement that (Tagliaferri et al., 2007) the increased levels of genistein by β-glucosidase hydrolysis occurs at days 4.7 and 9, the highest concentration of genistein occurs on day 9. Mold can produce hormones that could stimulate the growth of its host, antibiotic agents and other useful secondary metabolites. Mold is also able to increase its host plant growth hormone (Lingga, 2010). Based on the result of study conducted by Putri et al, 2013, the addition of fermented soybean waste in chicken ration can increase the number of chicken egg follicles, but the addition of non-fermented soybean waste does not affect the increase of the number of follicles. Estrogen hormone in poultry can serve to stimulate the development of secondary sex nature, affect growth and fat deposition, affect the growth and development of follicles and is essential for the synthesis of egg's albumin (Glover and Assinder, 2006). Fani, 2013 states with the addition of 10g of Aspergillus niger fermented soybean waste into 85g of commercial feed given as hen feed aged 15-16 months shows the average value of eggshell thickness of 0.39 ± 0.028 mm more than non-fermented eggshell 0.36 ± 0.024mm.

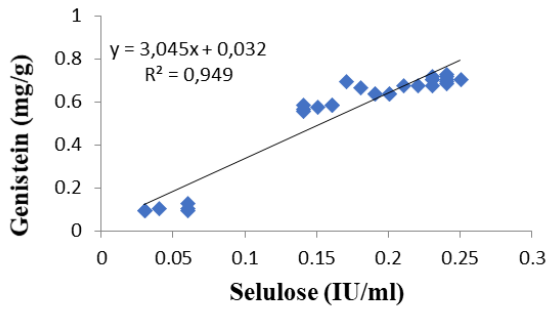

Figure 3. Graph of correlation of cellulase and genistein levels in Aspergillus niger fermented soybean west.

The correlation test showed a positive value in the increase of cellulase enzyme content which then also increased the genistein level R2 = 0.949. The increase cellulase enzyme activity and genistein levels are also affected by the length of the incubation period in the fermentation of soybean pulp. The presence or absence of micro-nutrient does not significantly affect the content of cellulase enzyme and genistein levels, it produces a positive correlation value R2 = 0.949. The development of cells depends on the nutrient content of the fermentation substrate, the addition of micro-nutrients can accelerate the action of enzymes but may affect physical changes in products such as water content on more substrates and faster decay processes. This can be observed starting from 192 hours of fermentation.

The optimum period limit on fermentation is 3-6 days, at which time the microorganisms are in the exponential growth phase, in this phase the microbes divide rapidly and constantly follow the logarithmic curve. In this phase the substrate remains in good condition, with good shape and distinctive odor. Whereas in the fermentation on day 7 onwards, the substrate has begun to break down and been odor, it is feared that the cattle do not like it and does not want to eat it (Chiou and Cheng, 2001). Sinurat (2001) reports that the optimum limit of fermented feeding in poultry rations was 5% for broiler, 15% for laying chicken, chicken and duck.

Conclusion

Based on the research results, it can be concluded that the addition of micronutrient and fermentation time 48 hours up to 144 hours can increase the cellulase enzyme level to the formation of phytoestrogens (genistein) on Aspergillus niger fermented soybean waste.

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