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Production of Single Cell Protein by *Aspergillus niger* Using a Mixture of Coffee Husk and Molasses as Substrate

S. D. Said[1,*], M. Zaki[1], Nasrullah[1], Mukhlishien[1], Maulida[2], M. N. Harahap[2], Mentarina Rizki[2]

[1] Chemical Engineering Department, Engineering Faculty, University Syiah Kuala, Banda Aceh, 23111, Indonesia
[2] Undergraduate Chemical Engineering, Engineering Faculty, University Syiah Kuala, Banda Aceh, 23111, Indonesia

[*] *Corresponding author: : syahiddindahlan@usk.ac.id*

Abstract: Gayo is one of the districts in the province of Aceh, one of the largest Arabica coffee producers in Indonesia. So far, coffee husk produced from coffee fruit processing have not been used optimally, causing environmental problems. Efforts to utilize coffee husk as a substrate to produce single cell protein (SCP) can be considered, besides overcoming environmental problems, it will also produce alternative proteins to substitute traditional proteins. A study has been carried out to evaluate the potential of Gayo Arabica coffee husk as a substrate to produce SCP using solid state fermentation with *Aspergillus niger* as a bioconversion agent. The results showed that the C/N ratio and water content of the substrate were variables that greatly influenced protein production. The highest protein production was produced by substrate with a C/N ratio of 10 and a water content of 50% (w/w) with a fermentation time of 4 days. Increasing the level of molasses in the substrate mixture linearly increases protein production.

Keywords: single cell protein, solid state fermentation, *Aspergillus niger*, coffee husk, molasses

1. Introduction

Indonesia is one of the largest coffee producing countries in the world, based on statistical data for Indonesian coffee 2021, Indonesian coffee production continues to increase from year to year. This is illustrated by the increase in coffee production from 2019 to 2021 of 4.43%. Coffee production for 2021 is 786.19 thousand tonnes [1]. The processing of coffee cherries into coffee beans always produces coffee pulp waste of about 40% from fresh fruit or about 26-30% coffee husk from dry coffee cherries [2]. According to Preedy, 2015, for each total weight of coffee produced, depending on the method of the coffee making process, around 30% to 50% coffee skin is produced [3]. if we use the 40% figure, the coffee waste produced in Indonesia per year is approximately 314.5 thousand tonnes. If this large amount can be converted into quality animal feed, it will increase the added value of coffee husk waste, provide benefits for farmers, open up job opportunities and overcome environmental pollution problems.

Coffee husk waste has been used as animal feed, but its application is still very limited, due to the content of anti-physiological and anti-nutritional compounds such as caffeine, tannins and polyphenols [4]. Coffee husk contains the following compounds (dry basis): carbohydrates (58-85%), protein (8-11%), fat (0.5-3%), caffeine (1.3%), tannins (4.5-5.4%), pectin (12.4-13%), cellulose (19-26%) and lignin (20%) [5]. Several studies have been carried out to improve the quality of coffee husks as animal feed. Brand et al., 2000, have conducted a study on the detoxification of coffee husk by solid state fermentation using the *Aspergillus niger* fungus as a bioconversion agent. The results show that *A. niger* can degrade caffeine (92%) and tannins (65%) [4]. According to Wogderess, 2016, the content of tannins and alkaloids compounds can affect livestock health, but the fermentation process using the biological agent *A. niger* can reduce tannin and caffeine levels to a lower level, namely 10% dietary level [5]. In addition to the detoxification process, it is necessary to increase the nutrient content in coffee husk to achieve quality standards for various types of feed. One of the important nutrients is protein. The low protein content in coffee husk for certain feeds can be increased by a fermentation process to produce single cell protein

(SCP). Because it is rich in organic compounds, coffee husk can be used as a substrate for SCP production [6].

SCP is a type of protein that comes from microorganisms, usually single-celled organisms such as fungi. SCP can be used as a source of animal feed. Utilization of SCP as a protein source can be a solution to overcome protein shortages for animal feed in Indonesia, especially in areas where traditional protein sources (fish meal and soybeans) are scarce or unsustainable. SCP has received attention as a potential alternative protein source because its high protein content can be produced using a variety of organic waste materials, such as agricultural residue products, household organic waste, or food processing industry waste [7].

Because each type of organic waste, growth media composition, microbial type, and fermentation conditions will affect the production of SCP, research is needed to obtain optimal conditions for maximum SCP content in fermented substrates. In this work, we studied effect of fermentation time, substrate moisture content, C/N ratio and influence of molasses on SCP production on fermented substrates.

2. Materials and Methods

Coffee husk pretreatment

Coffee husk waste in transparent plastic bags was obtained from farmers who produce Arabica coffee beans in the Gayo highlands, Aceh Province, Indonesia. Before being used as a medium, the coffee husk was washed with tap water, then dried in an oven dryer at 80°C for 24 hours, then ground using a blender and sieved to obtain a size <= 50 mesh. Furthermore, pretreatment was carried out with a low concentration of sodium hydroxide solution (2%) in an autoclave at a temperature of 121°C for 60 minutes to reduce the lignin content. After that, it was washed with water until the pH was neutral, and then dried.

Aspergillus niger inoculum preparation

The fungus used was *Aspergillus niger* obtained from the Microbiology Laboratory, College of Life Sciences and Technology, ITB. The fungus was sub-cultured on slanted agar containing PDA (potato dextrose agar). The subcultures were stored at 4°C. The inoculum for solid state fermentation was prepared from the incubation process of aerial spores of *Aspergillus niger* in a 500 ml Erlenmeyer flask containing 200 ml of liquid medium containing 40 g/L molasses and 7 g/L ammonium sulphate. Incubation took place at 30°C and with a shaking speed of 100 rpm for 3 days. Aerial spores were obtained from agar plates that had been overgrown with *Aspergillus niger* for 7 days. Spore collection was carried out by adding 1 ml of sterile water to the plate, and then scraping with a spatula, then the spore suspension was poured into the liquid medium.

Solid State Fermentation on Mixture of Substrate

10 grams of the mixture of pretreated coffee husks (50%) and molasses (50%) was put into an Erlenmeyer flask (volume 500 ml). The weight of coffee husk and molasses was adjusted according to a predetermined ratio. Then the urea solution was added to the flask in a certain volume according to the desired C/N ratio. Furthermore, the mixture of these substrates was sterilized at 121°C for 15 minutes and then cooled. The mixture was then added with 10 ml of inoculum and next, a certain volume of sterile water was added to achieve the desired initial water content (% w/w) in the substrate mixture. Then continued with solid state fermentation at 30°C with various times, namely: 1, 2, 4, 6, 8, and 10 days. The fermented substrate was analysed for crude protein and water content.

Crude Protein Analysis

SCP production from the fermentation process was estimated by measuring the crude protein content in the fermented product. Samples (fermented products) were analysed using the Kjeldahl method [8]. A sample of 0.51 grams was put into a 100 ml Kjeldahl flask. Then 2 grams of selenium and 25 ml of concentrated H_2S_O4 were added. The Kjeldahl flask containing the mixture was heated in an aluminium block heater until it boils. After cooling, the solution in the Kjeldahl flask was diluted with distilled water until the volume reached 100 ml. 5 ml was taken and put into a distillation flask, then 5 ml of 30% NaOH solution and 3 drops of phenolphthalein indicator were added, and continued with the distillation process.

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The resulting distillate was collected in a volumetric flask containing a solution of 10 ml of a mixture of 2% boric acid and indicator, then titrated with 0.01 N HCL solution.

Formula for calculating crude protein content:

$$\text{crude protein } (\%\ w/w) = \frac{(V_1 - V_2)\ x\ N\ x\ 0{,}014\ x\ fc\ x\ fd}{w}\ x100\%$$

Where :
V_1 : The volume of HCL used for the sample (ml)
V_2 : The volume of HCL used for the blank test (ml)
N : HCl normality (N)
fc : conversion Factor (6.25)
fd : dilution factor
w : sample weight (gram)

3. Result and Discussion

Effect of Fermentation Time on Protein Content

Aerobic solid state fermentation process of mixed substrate (coffee husk pretreatment, molasses and urea) by *A. niger* produced SCP. SCP content in fermented mixed substrate (fermentation product) is measured as crude protein. Figure 1 shows that SCP content increased with increasing fermentation time from 1 day to 4 days. Maximum protein content is achieved after 4 days of fermentation. Kanimozhi and Nagalakshmi, 2014 reported that 4 days of fermentation time was needed by *A. niger* to achieve maximum protein production with wheat bran as a substrate [9]. A similar profile of SCP production was shown by *A. niger* FTCC 5003 which used palm kernel meal as a substrate, but required 5 days of fermentation time to achieve maximum protein production [10]. A shorter fermentation time of 2 days to achieve maximum protein was demonstrated by *A. niger* in the fermentation process using Ginko biloba leaves at various moisture content (40% -70%) and temperature 28°C. The data and reviews above provide information that the different strains of *A.niger*, the type of substrate and the fermentation conditions affect the fermentation time to achieve maximum production.

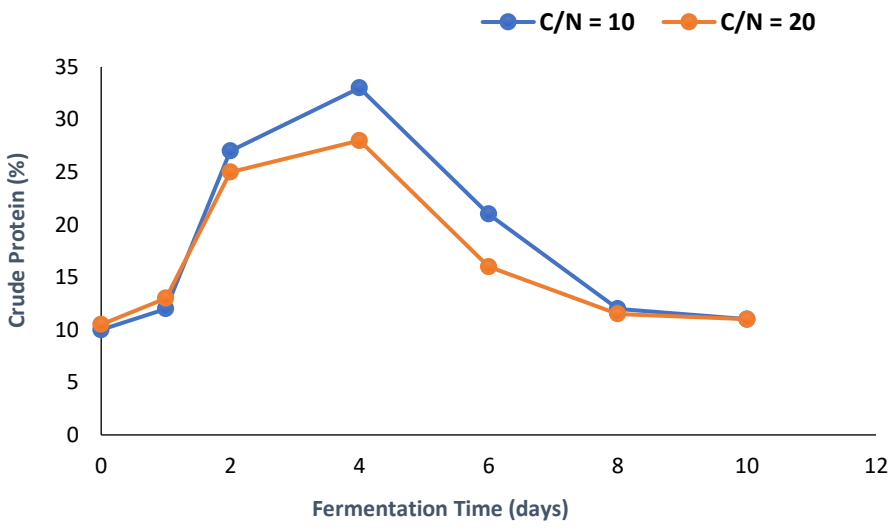

Figure 1. The Profile of crude protein of fermented product during solid state fermentation process by *Aspergillus niger*. Temperature of 30°C; C/N ratio of 10; Water content of 50%.

Effect of C/N Ratio on Protein Production

The carbon element contained in the substrate acts as an energy source, while nitrogen acts as a protein-forming element. In solid state fermentation, the optimum C/N ratio for the growth of the fungus is highly dependent on the type of fungal species involved and the available substrate. To maximize the biomass production of fungi in the fermentation process, setting the C/N ratio is an important parameter in preparing the substrate composition such as using different sources of carbon and nitrogen as well as other additional nutrients.

Figure 2 explains the effect of the initial C/N ratio of the substrate and the fermentation time on the crude protein (SCP) production in the fermented substrate. Increasing the C/N ratio of the substrate, from 10 to 40, tends to reduce SCP production in fermented substrates. The maximum crude protein content of 33% (w/w) was achieved on a substrate with a C/N ratio of 10 and a fermentation time of 4 days. A substrate with a C/N ratio of 10 may have a relatively balanced supply of nitrogen to carbon. Therefore, this substrate results in higher cell biomass. Whereas substrates with C/N ratios greater than 10 contain excess carbon relative to nitrogen, this may limit the availability of sufficient nitrogen for the fungi, and reduce protein synthesis and fungal cell growth.

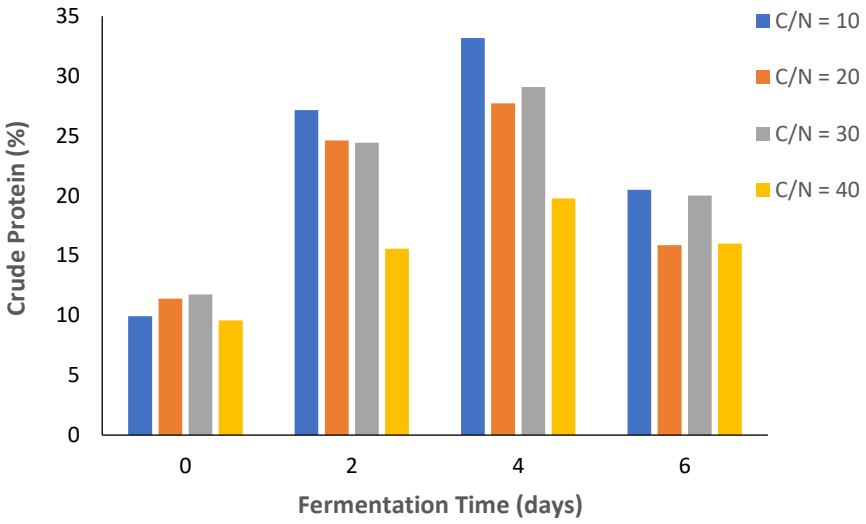

Figure 2. Effect of initial C/N ratio of the substrate on the production of crude protein. At fermentation condition: Temperature of 30°C, Water content of 50%

As a comparison, Oliveira et al., 2017 reported that optimum solid state fermentation conditions by *Aspergillus ibericus* MUM 03.49 using substrate of olive pomace with wheat bran were achieved with a C/N ratio of 25.2 [11]. Anupama and Ravindra, 2001 studied the influence of C/N ratio on the protein yield of solid state fermentation using rice bran as carbon source and sodium nitrate as nitrogen source by *A.niger* at temperature of 30°C, they found that a C/N ratio of 1.387 gave the higher biomass yield of 40.91 mg/g of rice bran [12].

Effect of Water Content on Protein Production

In solid state fermentation the appropriate water content in the substrate to support fungal growth varies greatly, depending on the state of the substrate and the species of fungus. Excess water content on the surface of the substrate can inhibit or reduce the rate of oxygen supply from the air to the surface of the fungal cells, but limited water content causes a reduction in the transfer of essential nutrients from the substrate into the cells. Both excess water and water deficiency will negatively affect the growth of fungi, thus it is necessary to determine the optimal water content.

In this study, the initial water content in the substrate was varied as follows: 50, 60, 70 and 80 % (w/w). From Figure 3 it can be seen that there is a difference in crude protein production, the higher the water content the lower the crude protein production. This shows that the water content has a very significant effect on the production of crude protein. A decrease in crude protein production was observed with an increase in water content from 50 to 80%. The highest crude protein (33.5%) was produced by a

substrate with a water content of 50%. As a comparison, Chi et al, 2015 reported that the optimum water content in solid state fermentation of rapeseed cake with *Aspergillus niger* is in the range of 50 - 60% [13]. Carmo de Oliveira et al., 2019 reported that solid state fermentation using a mixture of chicken feather flour and 15% wheat bran at an initial moisture content of 50% and a temperature of 30oC resulted in higher enzyme production [14].

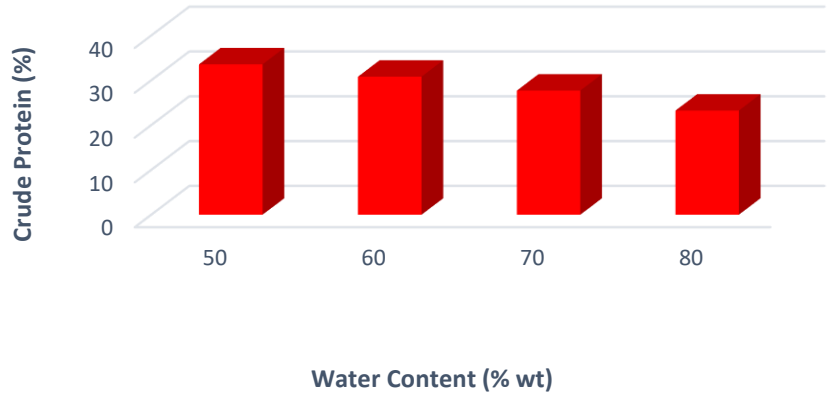

Figure 3. Effect of water content on crude protein production on fermented substrates. Process conditions: Temperature of 30°C and C/N ratio of 10.

Effect of molasses on Protein Production

Molasses is often used as a substrate in solid-state fermentation because it contains the rich nutrients needed for the growth of microorganisms. Carbohydrates are one of the important nutrients as an energy source, consisting of glucose, fructose and sucrose. Apart from that, molasses contains minerals such as calcium, iron, magnesium, and potassium. Molasses also contains B vitamins, vitamin A and vitamin K.

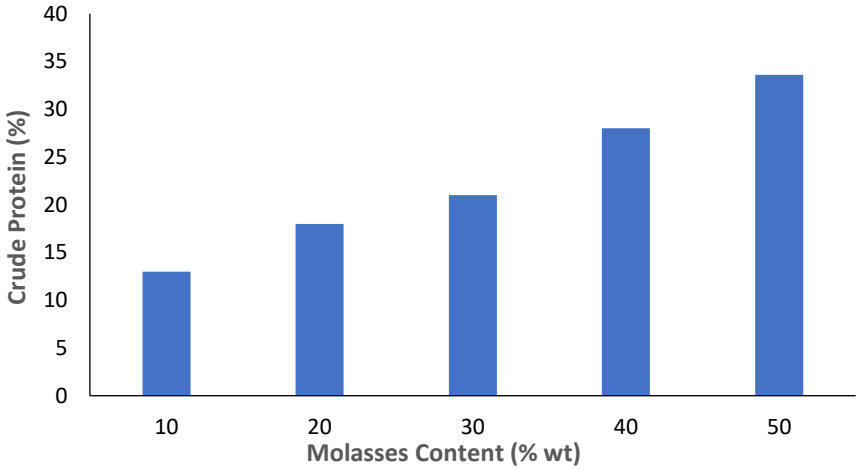

Figure 4. Effect of molasses content in the mixture of substrate on SCP production. Process conditions: Temperature of 30°C, C/N ratio of 10, Water Content of 50%

With its rich nutrition, we used molasses as an additional substrate to supplement the nutritional deficiencies in coffee husks to maximize the growth of *A. niger*. In this study, we evaluated the effect of the molasses content in the substrate mix on protein production. Figure 4 shows a linear

increase in protein production with increasing levels of molasses in the substrate mixture. The substrate with a 10% molasses content after the fermentation process contained 13% crude protein, and an increase in the molasses content to 50% increased the crude protein content of the fermented substrate to 33.6%.

4. Conclusion

A mixture of pretreated coffee husk, molasses and urea is a suitable substrate for the growth of *Aspergillus niger* and production of single cell protein (SCP) in solid state fermentation process at 30ºC. Addition of Molasses to pretreated coffee husks increases SCP production. The C/N ratio and the water content of the substrate are important nutritional balance parameters. Substrate with a C/N ratio of 10 and a moisture content of 50% gave the highest SCP production of 33.6% with a fermentation time of 4 days.

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