The effect of civet coffee isolate and time fermentation on Robusta coffee protein profiles

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Abstract. This research aims to determine the effect of the isolate’s concentration of civet coffee and fermentation time on the protein concentration profile and protein molecular weight profile of Robusta coffee which fermented in vitro, in vivo, and without fermentation. Isolates of civet coffee made from microbial civet feces and made in five variants concentrations (2.91x10^8 CFU/mL; 2.91x10^7CFU/mL; 2.91x10^6CFU/mL; 2.91x10^5 CFU/mL; 2.91x10^4 CFU/mL). In the fermentation process coffee Robusta beans marinated with various concentrations of coarse isolates. The coffee beans are fermented analysed by UV-Vis spectrophotometer and the results obtained in vitro protein concentration of the lowest 50.95 μg/mL (fermentation time of 60 hours and isolates concentration of 2.91 x 10^8 mg/mL), coffee fermentation in vivo (167.44 μg/mL), and coffee without fermentation (217.61 μg/mL). The protein molecular weight profile is determined based on the results of electrophoresis. The higher the concentration of isolates civet coffee in the fermentation process, the more protein bands appear, and vice versa.

1. Introduction

One of Indonesia's leading plantation commodities that has a high export value and to provide substantial foreign exchange for the country is coffee. About 60% of the total national coffee production is exported to major destination countries such as the United States, Germany, and Japan [1].

Currently, Indonesian coffee which is exported is only limited to coffee beans, not a product that is ready for consumption. Therefore it is necessary to research the semi-wet or dry fermentation coffee processing technology which is more easily applied by farmers. One method that needs to be developed is the natural fermentation process of the digestive system of mongoose animals which has been proven to produce coffee beans with better quality and flavour [2].

Civet coffee is a type of coffee that has been processed through a short fermentation in the digestion of civet animals (Paradoxurus Hermaphroditus). The enzymes in the civet digestive tract can produce coffee with distinctive flavour and aroma [3]. Improved quality of civet coffee flavour is caused by low protein content and high-fat content compared to regular coffee [4]. Coffee beans that
are usually processed into civet coffee are Robusta coffee and Arabica coffee, where these beans generally contain minerals, caffeine, trigonelline, fat, chlorogenic, aliphatic acids, oligosaccharides, polysaccharides, amino acids, proteins, and human acids [5].

Bengkulu Province is one of the Robusta coffee-producing provinces in Indonesia, especially in the Rejang Lebong district. Robusta superior grade coffee can produce coffee beans throughout the year, more resistant to pests and leaf rust, and resistant to extreme temperatures. This is the reason why civet coffee producers in Bengkulu prefer to use this type of coffee as a basic ingredient for civet coffee. The optimal temperature for Robusta coffee growth is 21-24 °C.

Civet coffee production on a large scale causes problems for the civet population. Many civets are hunted to be used as civet coffee-producing animals. this directly disrupts the mongoose population. To cope with the growing demand for civet coffee, producers cannot only expect production from mongoose animals. The utilization of mongoose animals as fermentation agents is considered to torture mongoose animals as well as threatening the survival and preservation of mongoose animals. One way to produce civet coffee without disturbing the mongoose animal population is to do fermentation by using coarse isolate of mongoose coffee that comes from civet feces.

2. Methods

2.1. Research time and location

This research was conducted in November 2015 - March 2016 at the IHPT Laboratory, Argo ecotechnology Laboratory, Chemistry Education Laboratory, Biomedicine Laboratory, Faculty of Medicine, Bengkulu University and SBIH Ruyani Laboratory (Learning Nature Harmony From the Facts).

2.2. Determination of Robusta coffee protein concentration profile

Microbes from civet feces mixed with Robusta coffee beans were incubated for 24 hours in a 0.9% Physiological NaCl solution, forming a precipitate. The liquid which is at the top of the sediment is mixed into the liquid Nurien Borth (NB) and incubated for 24 hours, the result of this incubation is called the coarse isolate. The coarse isolates obtained are then made into dilution series variations 10-1–10-6. The smallest dilution series (10-6) is taken and implanted on PCA media. Planting is carried out by the top planting method and incubated for 24 hours. The results obtained were then calculated the number of microbial colonies using "Colony Counter" to determine the amount of microbial concentration for each dilution series.

This fermentation process is done by soaking the Robusta coffee beans and a little flesh with isolates from various concentration variants (2.91x108 CFU / mL; 2.91x107CFU / mL; 2.91x 106CFU / mL, 2.91x105 CFU / mL; 2, 91x104 CFU / mL). Then incubated according to a predetermined time variation (24,36,48, and 60 hours). And stirring is done every 3 hours. After the fermentation process is complete, Robusta coffee beans are washed with 70% alcohol and then rinsed with distilled water.

Protein isolation is done by grinding fermented coffee beans, then homogeneous using Tris-HCL buffer pH 7.4 for 24 hours. Next homogenate is filtered with filter paper and centrifuged. The centrifugation process is carried out in 2 stages, the first is carried out at 4.500 rpm for 15 minutes and the second stage is carried out at 13.500 rpm for 30 minutes to separate the precipitated protein by dissolution, the pellet is taken because it is a precipitation protein, the determination of protein concentration from the pellet will be carried out.

The determination of protein concentration was carried out using a Uv-vis spectrophotometer at a wavelength of 570 nm. The method of determining the concentration of protein used is the Biuret method using a standard protein BSA (Bovin Serum Albumin). BSA solution is made in a biuret solution with various concentrations of 20 μg / mL, 40 μg / mL, 60 μg / mL, 80 μg / mL, 100 μg / mL.
2.3. Determination of Robusta coffee protein molecular weight profile
The electrophoretic gel is made for bottom-PAGE and upper-PAGE with a concentration of 12%. The gel is allowed to stand for several minutes. Chip with some wells that are ready to be filled with protein samples. The protein standards used in this analysis are broad range prestained SDS-PAGE BIO-RAD with the following criteria: myosin (210KDa), β-Galactosidase (125KDa), bovine serum albumin (101KDa), Ovalbumin (56.2 KDa) Carbonic anhydrase (35.8KDa), soybean trypsin inhibitors (29 KDa), lysozyme (21 KDa), aprotinin (6.9 KDa).

Electrophoresis was carried out at a constant voltage of 220 V and stopped when the tracer colour (bromophenol blue) had moved to reach the lower end of the gel or ± 60 minutes. The 1-D PAGE produced was then coloured with Coomassie Bio-safe [6]. Protein bands are observed and photographed directly using a digital camera. The number of components is determined by the number of stains obtained from the separation results. Then the molecular weight is analysed by comparing the protein band in the sample with the standard protein band.

2.4. Data analysis techniques
Data analysis techniques for laboratory experiments were carried out based on the results of calculations of protein concentrations contained in the samples used. The concentration of this protein can be determined by calculating the absorbance value shown by the Uv-Vis spectrophotometer. The protein concentrations obtained were then analysed using Microsoft Excel 2010. In this research, a real difference test was performed using the parametric statistical analysis of variance (ANOVA) test, namely the Randomized Group Design (RCBD) and continued with the CRD (Randomized Complete Design).

3. Result and discussion
3.1. Protein concentration profile of Robusta coffee
Based on the results of this research showed that the Robusta coffee protein concentration decreased to 166.67 μg / mL when compared with Robusta coffee fermented in vitro (fermentation time of 60 hours and microbial concentrations of rough isolates 2.91 x 10^8 CFU / mL). This result is different when compared with in vivo fermented civet coffee, the concentration of pure Robusta coffee protein has decreased to 50.17 μg / mL.

Average in vitro fermentation concentration data for each concentration and time variation can be plotted in Figure 1:
Based on Figure 1, it can be seen that the lowest protein concentration of Robusta coffee beans in vitro is 50.95 μg/mL (fermentation time is 60 hours and the concentration of crude isolates is 2.91 x10^8 CFU/mL). Based on the curve it can be seen that the longer the fermentation time and the greater the concentration of civet coffee isolates used, the concentration of Robusta coffee protein will be lower. This is because the longer the fermentation time, the more content of substances used by bacteria to survive so that the amount of food remaining less and less including protein compounds.

As for the concentration of microbes, the greater the concentration of microbes in coarse isolates, the greater the concentration of protein which decreases, this is due to the large amount of microbial concentration that works to reduce the protein in coarse isolates so that the content of substances used by microbes to survive will be more numerous, so that food substances the remaining will be less so as well as protein compounds [7]. The longer the fermentation time, the longer bacteria will decompose the compounds contained therein such as sugar, protein, cellulose [8].

Several factors that influence microbial growth are the availability of nutrients, water, temperature, pH, oxygen, reduction oxidation potential, the presence of inhibitors, and the presence of other microorganisms [9]. Differences in growth in microbes are caused by physiological diversity and different responses to physical conditions and the environment [10].

Robusta coffee with low protein concentration is indeed the coffee desired in this research because coffee with low protein concentration has a delicious taste and has a bitter taste. Protein generally acts as a form of bitter taste in roasted coffee so that civet coffee is not as bitter as ordinary coffee because of its low protein content. This is consistent with the opinion of Marcone [4] improvement in the quality of civet coffee flavour caused by a low protein content and high fat content compared to ordinary coffee. Low protein content can reduce bitter taste, while high fat content can increase body. In addition to reducing the bitter taste in coffee, low protein content in civet coffee can also support a diet program without protein.

3.2. Molecular weight profile of Robusta coffee

Determination of the molecular weight of Robusta coffee protein in vitro fermentation was carried out using electrophoresis. Electrophoresis is a protein separation technique based on the movement of charged protein molecules in an electric field (isoelectric point). The speed of molecules moving in the electric field depends on the charge, shape and size. Electrophoresis results can be seen in the image below:

Based on figures 2 and 3, the higher the concentration of coarse isolates used in the fermentation process, the colour of the protein bands produced in the electrophoretic gel will be thicker and the number of bands produced will be more than that of samples fermented with isolates that have lower concentrations. This occurred in almost all samples of Robusta coffee protein fermented in vitro,
except for Robusta coffee protein samples with an isolate concentration of $2.91 \times 10^8$ CFU / mL and isolate concentration of $2.91 \times 10^8$ CFU / mL with 48 hours fermentation time (Figure 1). This is thought to occur because the Robusta coffee protein has been completely hydrolysed into amino acids during the fermentation process.

Samples with high isolate concentrations generally form more bands during the electrophoresis process. This shows that the hydrolysis process is going quite well, although not perfectly. This means that most protein molecules are hydrolysed into amino acids, but some are not. Proteins that are hydrolysed into amino acids will later form many bands on the electrophoretic gel according to their respective molecular weights.

Figures 2 and 3, several Robusta coffee protein samples only have one band on the top of the electrophoresis gel. The protein band at the top of the electrophoresis gel is a large molecular weight protein, a protein with a large molecular weight will not be able to go down to the bottom of the well, because the protein down to the bottom of the well is a protein with smaller molecular weight.

The fermentation time did not significantly influence the results of the research. This can be seen where the protein band produced does not decrease based on the length of time of fermentation, but decreases along with the decline in the concentration of microbes used in the fermentation process for each time of fermentation. This might be due to the imperfect fermentation process or it could also be caused by protein denaturation before the fermentation process is carried out.

3.3. The linkages between the results of the analysis of concentration profiles and the molecular weight profile of Robusta coffee proteins

The results obtained from this research indicate that the results of the analysis of protein concentration profiles (Figure 1) are not always directly proportional to the results of the protein molecular analysis weight profiles in Robusta coffee (Figures 2 and 3). This can be seen from protein samples with (Luwak coffee isolate concentration $2.91 \times 10^8$ CFU / mL and isolate concentration $2.91 \times 10^7$ CFU / mL with fermentation time 48 hours) which has a high protein concentration value based on the results of the UV-vis spectrophotometer, but it does not appear on the molecular weight band on the electrophoretic gel. It is suspected, the protein read by the UV-Vis spectrophotometer is the total protein of all types of proteins, including protein fractions (oligoproteins).

The absence of protein bands in electrophoresis process, may be caused by the undetectable amount of protein as a whole, which is thought to be only protein with a molecular weight that is not too small that can form a band on the electrophoresis gel, whereas for proteins with very small molecular weights as in the form of oligoproteins or even in the form of fractions, cannot form a band because it is suspected that when the electrophoresis process takes place this type of protein molecule has come down perfectly to the part that the well, making it difficult to detect even though the process of purification and colouring in the gel has been carried out.

The purity of the analysed protein also seems to be one of the gaps in the results obtained. The coffee protein analysed is thought to be not purely Robusta coffee protein but has been mixed with proteins from proteolytic bacteria and proteins from protease enzymes. When the process of protein hydrolysis takes place, it is suspected that the protease enzyme derived from proteolytic bacteria and proteolytic bacteria itself also hydrolyses along with proteins from coffee beans. As a result, the protein analysed both on the spectrophotometer and with electrophoresis is a mixture of protein from coffee bean protein, protease enzyme protein, and protein from proteolytic bacteria. It is strongly suspected that the band at the top of the electrophoresis gel is a Robusta coffee protein sample band, while the protein band that appears irregularly in the middle and bottom of the electrophoresis gel is thought to be a protein derived from proteolytic bacteria and protease enzymes that have undergone hydrolysis in during the fermentation process.

The thickness of the polish of electrophoresis gel (the blue colour that extends along the electrophoresis well) produced by each protein sample is different, depending on the concentration of protein remaining after the running process is complete. Samples that have a thicker polish colour are thought to have more protein concentrations when compared to samples that have a thinner polish.
colour. This has a connection with the fermentation process, in which a properly hydrolysed protein will go down the gel completely when the running process is carried out, so that the protein concentration in the gel well is reduced or even absent so that when reacted with Coomassie blue, the resulting blue colour is not so thick or even does not appear, whereas in samples with BM it is likely that when the running process is complete there is still a lot of protein concentration in the gel well so that when Coomassie blue is added, the protein will react with Coomassie blue to form a blue colour along the gel electrophoresis well.

4. Conclusion
Fermentation time and concentration of coarse civet coffee isolates are inversely proportional to the Robusta coffee protein concentration. Whereas the concentration of Luwak Robusta coffee isolates used in the fermentation process is directly proportional to the appearance of protein sample bands on the resulting electrophoretic gel. While the fermentation time did not have a significant effect because no significant differences were found between each sample with different time variants.

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References
[1] Rahardjo P 2013 Kopi (Jakarta: Penebar Swadaya)
[2] Fauzi M 2008 Isolasi dan Karakterisasi Bakteri Asam Laktat Biji Kopi Luwak Jurnal Keteknikan Pertanian Tropis dan Biosistem 3(1) 265-273
[3] Panggabean E 2011 Buku Pintar Kopi (Jakarta: Agromedia Pustaka)
[4] Marcone M F 2004 Composition and properties of Indonesia palm civet coffee (Kopi Luwak Arabika) and Ethiopian civet coffee Food Research International 37 901-912
[5] Clarke R J and Macrae R 1987 Coffe Technology vol. 2 (London: Elsevier Applied Science)
[6] Wilson K 1994 Protein and Enzyme Techniques in Practical Biochemistry (Cambridge University Press)
[7] Setyatwan H 2007 Peningkatan Kualitas Nutrisi Duckweed Melalui Fermentasi Menggunakan Trichodermah zianium JIT 7(2) 113-116
[8] Oktadina F D, Bambang D A and Hermanto M B 2013 Pemanfaatan Nanas (Anannas Comosus L.)
[9] Waluyo L 2007 Mikrobiologi Umum (Malang: UMM Press)
[10] Pelezar M J and Chan E C S 2007 Dasar-Dasar Mikrobiologi 1 (Jakarta: UI Press)