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Amino acid analysis of cocoa fermented by High Performeance Liquid Chromatography (HPLC)

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ABSTRACT

The aims of the study was to improve quality of cocoa bans by fermentation of sun dried cocoa beans. The characteric fermented cocoa beans was determined by maesuring amino acids cocoa beans during fermentation. The fermentation process used 3 level treatment i.e. control (without inoculum), mixed culture of microbies added at the begining fermentation. Regulated temperature during fermentation, respectively 35 ° C (24 hours), 45 ° C (24 second clock), 55 ° C (24- hour three) and 35 ° C (last 48 hours) and then fermentation was conducted for 120 hours. The result show all cocoa beans total amino acids hydrophobic increase during fermentation from 7.21 to 7.46 ìg/g for control, 8.19 to 8.26 ìg/g for addition of inoculum at begining of fermentation and from 7.56 to 8.74 ìg/g for addition of inoculum at the begining and midle of fermentation.

Keywords: Amino acids, Bulk cocoa beans, Cacao beans, Fermentation, HPLC analysis, Lindak clone.

INTRODUCTION

Fermentation is at the core of the processing mechanism of cacao beans because it can enhance the flavor of cocoa. The reason for the fermentation of cacao is to enhance biochemical reactions in the seed that led to the formation of aroma precursors, taste and color, reduction of bitter taste and astringent, and improvement of the physical appearance of cocoa. In addition, fermentation facilitates the release of the pulp layer that cling to the seed and hardens the seed coat into shell-like (Misnawi *et al*., 2005; , 2013).

Cocoa fermentation is basically conversion of pulp's sugar and citric acid into organic acids by microorganism (Camu et al., 2008; Ardhana and Fleet, 2003). The organic acids induce enzymatic reaction inside the beans resulted biochemical changes generating several compounds critical for aroma, taste, and color formation (Biehl et.al, 1985; Afoakwa *et.al*, 2014). This process was done by heaping the cocoa beans inside closed container or basket for 5-7 hour with turning once every 2 days. Without fermentation, cocoa beans will taste bitter, astringent, and without distinctive cocoa aroma after processing (Schwan and Wheals, 2004).

Dry cocoa beans lost most of their moisture content and substrate. Around 35% water is needed during fermentation, used for enzymatic reaction inside the beans and microbial growth in pulp (Schwan and Wheals, 2004) as the media for enzyme substrate interaction to mediate hydrolysis and oxidation to generate precursors for cocoa taste, color, and aroma. Substrate is any substances convert by microorganism during fermentation, such as pulp's sugar and citric acid, which metabolized into organic acids. Acids will then diffused into the beans and induce enzymatic reaction for taste, aroma and color (Afoakwa *et al*., 2014). Considering this critical role, rehydration is done for cocoa beans prior to fermentation.

Amino acid has a significant correlation with the formation of flavor in normal fermentation circumstances. The formation of amino acids in the maximum number can be used as a guide for determining the appropriate length of time to obtain fermented cocoa beans that have good flavors. Misnawi *et al*. (2005) argues that the best cocoa aroma is usually produced from cacao beans that have high levels of free amino acids and reducing sugar reached maximum levels. Voigt et al. (1994) found that free amino acids and oligopeptides are essential aroma precursors. The combined activity of enzymes, endoprotease aspartate and carboxy peptidase, on cacao beans protein is required for the formation of certain cocoa aroma precursors.

According to Biehl and Passern (1982); de Brito *et al*. (2000), after fermentation, high-quality cacao beans must have approximately 8-14 mg/g of cocoa beans in the dried substances. Component of chocolate aroma consists of volatile compounds, which are mainly formed by the reaction of amine and carboxyl groups. Precursor flavour of chocolate unique aroma producer are composed of

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hydrophobic amino acids, peptides hidrophilic and reducing sugars (Mulono et al., 2016).

Composition of amino acid is very important, because it can predict the process of synthesizing flavor compounds. There are many studies on the determination of amino acid number in cacao beans (Puziah et al.,1998; de Brito *et al*., 2000), including High Performance Liquid Chromatography (HPLC) method.

HPLC is a chromatography system that its motion phase is passed quickly, facilitated by pressure and pump and the result is detected by an instrument. The main advantages of using HPLC is volatile amino acid derivates are not required and its ability to separate D and L forms of amino acids. Selection of ion chromatography technique is based on the ability to perform simultaneous detection, how easy to be operated, high speed of analysis and accuracy of results and the stability of column separator so that it can be used, and can be used for quantitative analysis and qualitative simultaneously (Ardianingsih, 2009).

In this study, the analysis of component of amino acids that compose the protein of cacao beans is conducted. The protein content of seed is interesting to be investigated further on the components of its constituent amino acids because it is associated with the aroma and flavor formed on chocolate products.

Experiment on non-fermented dry cocoa beans was conducted to measure change amino acid in cocoa beans during fermentation. Submersion cocoa beans unfermented was done to restore the water content such as fresh cocoa beans before fermentation. Research on fermented dry cocoa beans using unferrmented cocoa bean was done successfully in the engineering lab PAU, UGM. The study aimed to obtain optimal fermentation products by determining the concentration and types of free amino acids present in cacao beans using HPLC (High Performeance Liquid Chromatography).

MATERIALS AND METHODS

Preparation of Cocoa beans: Cocoa beans were taken from yellowish-orange ripe pods identified with tapped hollow sound, then dried inside cabinet dryer at 40 °C until moisture content was reduced to 15%.

Fermentation of dried beans was done in glass jar; each contained 100 gram. Regulated temperature during fermentation, respectively 35 ° C (24 h), 45 ° C (24 second clock), 55 ° C (24-hour three) and 35 ° C (last 48 h). The first treatment is the control treatment or without the addition of inoculum, the second treatment (IA) using inoculum of *S. cerevisiae (FNCC 3056), L. lactis (FNC 0086) and A. aceti (FNCC 0016)*, respectively each of 10^8 cfu / g are given simultaneously at the beginning fermentation. The third treatment (IB), administration of gradually yeast inoculum at the start of fermentation, lactic acid bacteria at the start of the second 24 hours and acetic acid bacteria at the start of a 24-hour party with the same microbial population with the second treatment.

Amino Acid Analysis of fermented cocoa beansUsing HPLC: HPLC (Model 1100, Agilent Technologies, Waldrbom Germany) provided with a binary pump with degasser micro vacum degasser, thermostat controlled auto sampler. Column compartment, a G1321 detedctor flourescence, and a G1315A diode array detector was used for amino acid analysis. AccQtag column (3,9x150mm) was used for separation of amino acids. Nollet (1996) methodology was adoped for amino acid analysis of fermented cocoa beans using HPLC. The measurement of amino acid was performed in two phases: liquid hydrolysis and derivatization proceeded by chromatographic analysis. The method of liquid hydrolysis was 5 ml HCL 6 N hydrolyzed at 110 °C for 22 hours in vacuum condition. After hydrolysis, the tube was cooled and the solution was filtered using spartant-HPLC, then diluted with water at 1:20 v/v ratio. Condition of HPLC was measured at 37 °C, the mobile phase contains of 60% acetonitril - accqTag Eluent A, gradient system and flow rate was 1.0 mL per minute. Fluorescence detector had 250 nm excitation and 395 nm emission. Injecting volume was 5 ìL.

Experimental design: A 3 x 3 full factorial experimental design was used for the study. The principal factors investigated were inoculum added (control (TI), inoculum added in the beginning (IA), inoculum added in step (IB) and fermentation time (0, 72, 96 & 120 h).

Statistical analysis: SPSS software IBM version 22 was used to analyze the data. Analysis of Variance (ANOVA)was used to test the level of significance. Least significant difference (LSD) was used to separate and compare the means, and significance was accepted at 5% level ($p < 0.05$).

RESULTS AND DISCUSSIONS

Table 1 reveals that cacao beans fermented for 120 h (control (TI)) showed higher amino acid content, specifically for aspartic acid, glutamic acid, alanine, isoleucine, proline and valine (hydrophobic). Those are significantly different with amino acids produced by cacao beans fermentation at 72, 96 and 120 h. Amino acid deteriment specifically alannine 1.59 ± 0.01 increase to 1.60 ± 0.01, tyrosine 0.89 ± 0.01 decrease to 0.67±0.01, valine 1.08±0.01 increase to 1.19 ± 0.01, phenylalanine 1.53±0.01 decrease to 1.51±0.01, isoleucine 1.03±0.01 decrease to 1.03±0.01 and leucine 1.85 ± 0.01 increase to 1.99 ± 0.01.

Table 2 show that cocoa beans of added inoculum mixed microbies in beginning, fermented 120 h produced higher amino acids, specifically for aspartic acid, glutamic acid, alanine, isoleucine, proline, valine (hydrophobic). Those are significantly different with amino acids produced by cacao beans of fermented 72 h, 96 h, 120 h. Amino acid

Table-1: Changes in the concentration of free amino acids (ig/g) during the fermentation of cocoa beans on control (TI) on the hour to 72, 96 and 120 hours.

	Fermentation time (hours)			
Hidrophobic				
Alanine	1.59b	1.55d	1.65b	1.60c
Tyrosine	0.89b	0.73b	0.65a	0.67a
Valine	1.08b	1.21b	1.12a	1.19d
Phenylalanine	1.53b	1.46c	1.31d	1.51d
Ileucine	1.06c	1.01a	1.01c	1.03c
Leucine	1.85a	1.89a	1.76b	1.99b
Methionin	0.73a	0.73b	0.72b	0.73b
Total	**7.65**	**8.58**	**8.22**	**8.72**
Acidic				
Aspartic acid	3.99a	4.35c	4.43c	3.83c
Glutamic acid	15.95a	15.11a	14.6b	15.14b
Serin	1.41d	1.28c	1.37b	1.46c
Histidin	0.83a	0.82b	0.79c	0.82c
Total	**22.18**	**21.56**	**21.05**	**21.25**

Tablel-2: Changes in the concentration of free amino acids (ug / g) during the fermentation of cocoa beans on the addition of inoculum treatment beginning on the hour to 72, 96 and 120 hours

	Fermentation time (hours)			
	0	**72**	**96**	**120**
Hidrophobic				
Alanin	1.57d	1.52d	1.66a	1.61a
Tyrosin	0.85d	0.77c	0.61a	0.62b
Valin	0.94d	1.07b	0.99b	1.06b
Phenylalanin	1.43c	1.35b	1.19b	1.35d
Ileusin	1.02c	0.96b	0.97b	1.07d
Leusin	1.66d	1.72a	1.60c	1.83a
Methionin	0.72c	0.71a	0.70c	0.72a
Total	**8.19**	**8.10**	**7.72**	**8.26**
Acidic				
Glutamic acid	15.86a	15.05b	15.03c	15.42b
Serin	1.39b	1.25b	1.35c	1.44d
Histidin	0.82a	0.81a	0.78a	0.80a
Total	**21.88**	**21.58**	**21.69**	**21.41**

deteriment specifically alannine 1.57 ± 0.01 increase to 1.61 ± 0.01, tyrosine 0.85±0.01 decrease to 0.62±0.01, valine 0.94±0.01 increase to 1.06 ± 0.01, phenylalanine 1.43±0.01 decrease to 1.35±0.01, isoleucine 1.02±0.01 increase to 1.07±0.01 and leucine 1.66±0.01 increase to 1.83±0.01.

Table 3 show that cocoa beans of graadually added inoculum microbies in fermented 120 h produced higher amino acids, specifically for aspartic acid, glutamic acid, alanine, isoleucine, proline, valine (hydrophobic). Those are significantly different with amino acids produced by cacao beans of fermented 72, 96 and 120 h. Amino acid deteriment specifically alannine 1.37 ± 0.01 increase to 1.65 ± 0.01, tyrosine 0.78±0.01 decrease to 0.63±0.01, valine 0.82±0.01 increase to 0.95 ± 0.01, phenylalanine 1.26±0.01 decrease to 1.19±0.01, isoleucine 0.92±0.01 just same after 120 h and leucine 1.41±0.01 increase to 1.48±0.01.Most types of amino acids in the cacao beans

Jinap et al. (2008) described that amino acid is a precursor of certain aroma that acts as a substrate for a Maillard reaction to form methylpyrazines (Eichner et al., 1994).

The introduction of High Performance Liquid Chromatography or HPLC expose a new dimension in the analysis of proteins, peptides and amino acids, especially in terms of the effectiveness of separation, speed of analysis and sensitivity of detection. This is because by using HPLC, analyte mixtures can be directly separated in the appropriate columns, such as on the method of amino acid analysis (Sumarno *et al.*, 2002). Flavor formation in cocoa related with flavor and aroma of chocolate starts from the quality of raw materials and the processing mechanism. Flavor of cocoa beans will appear after going through the process of fermentation and drying. Fermentation is a very vital stage of processing mechanism to guarantee chocolate products have good taste. Fermentation is also very influential in the development of aroma and flavor as well as in the reduction of astringent and bitter taste. Wrong fermentation practice damage the taste which cannot be repaired through subsequent processing modifications. Study has shown that the unfermented cocoa beans contain no aroma precursors in their seeds (Harrington, 2011).

The results of this study showed that the fermentation could increase the concentration of amino acid, types of amino acids, hydrophobic amino acids and other amino acids as shown in Table 1, 2 and 3. This happened because in after stages (24 hours fermentation), fermentation

Tabel-3: Changes in the concentration of free amino acids (ig/g) during the fermentation of cocoa beans on the gradually added of inoculum on the hour to 72, 96 and 120 hours

	Fermentation time (hours)			
	0	**72**	**96**	**120**
Hidrophobic				
Alanin	1.37a	1.32b	1.75b	1.65c
Tyrosin	0.78a	0.69a	0.611b	0.63c
Valin	0.82a	0.95d	0.87c	0.95c
Phenylalanin	1.26c	1.18c	1.03a	1.19a
Ileusin	0.92d	0.87b	0.82c	0.92b
Leusin	1.41b	1.47a	1.35b	1.48d
Methionin	0.65a	0.64a	0.63d	0.64c
Total	**7.21**	**7.12**	**7.06**	**7.46**
Acidic				
Aspartic acid	3.21	4.59	4.65	3.15
Glutamic acid	15.74c	15.31a	14.89b	15.31a
Serin	1.24c	1.19a	1.30b	1.38a
Histidin	0.79b	0.76b	0.74b	0.77a
Total	**20.98**	**21.85**	**21.58**	**20.61**

occurs in the seed pulp that will produce alcohol and acetic acid. At the moment, the precursors have not been created in the seed. According to Sulistyowati (1988), flavor precursor formation begins after the seed dies caused by the presence of acetic acid, alcohol diffusing into the beans and the heat that arise simultaneously which are the products of microbial activity during the fermentation process. As the seed died, decomposition of cells happened, so that no biological barrier separated the enzyme and its substrate. On the third day ahead, cocoa bean cells began to break and at that time, the formation process of flavor precursors began, because the enzymes in cocoa beans that was initially covered inside the cells begin to come out and mix with the other components. Hydrolytic enzymes in cocoa beans include amylase, lipase, the amino-acid decarboxylase, peroxidase, and poligalakturonase catalyzed the formation of this precursor compound. Hydrophobic amino acids such as alanine, tyrosine, valine, isoleucine, leusine and phenylalanine are special aroma precursors for the formation of cocoa aroma (Voigt, *et al.*, 1993).

According to Crafack *et al.* (2014), the amino acid content will increase after fermentation, but it will not change the composition. The accumulation of hydrophobic amino acids during drying will undergo degradation during roasting (Crafack *et al*., 2014). Result is the formation of volatile compounds such as aldehydes, ketones, pyrazines and furans which affect the formation of the chocolate flavor. Cocoa bean has varied concentration of amino acids. However, generally, unfermented and three days of fermentation seeds produce more aspartic acid, glutamic, leucine, isoleucine, valine, histidine, serine, threonine and lysine and significantly different from 72 h, 96 h and 120 h fermentation. Based on observations on the 72 h of feremention concetration acetic acid control 10%, mixed microbies added 15% and gradually added inoculum 19%. If more concetration acetic acid to decrease pH. Besides pH, temperature also affects the activity of enzymes. In high temperatures, enzyme activity will increase result in complete cell decomposition, so that the produced flavor do not have bitter taste and astringent (Sulistyowati, 1988).

Based on the active side of the peptide bond in the termination process, there are two types of enzyme protease, exopeptidase and endopeptidase. At pH 4.5, exopeptidase becomes active to cut the outer side of the polypeptide chain and forms amino acids. At pH 5.5 endopeptidase enzymes will be active, therefore in that pH range will be found more peptides and less amino acid. Proteolitic activity has significant role in the formation of aroma (Biehl *et al.* 1985).

Voigt et al. (1993); Misnawi *et al*. (2004) and Gu *et al*. (2013) stated that in addition to reducing sugars, peptides and hydrophobic free amino acids such as alanine, tyrosine, valine, isoleucine, leucine and phenylalanine are specific flavor precursors for the formation of cacao aroma. Furthermore, the hydrophobic amino acids are considered as the most potent precursor formation for methylpirazins. The study of Jinap *et al*. (2008 and 2010) and Yusep *et al.* (2002) showed that hydrophobic free amino acids and acid group amino acids directly contribute to the formation of cocoa flavor because they act as substrates for Maillard reaction to form methylpirazins. Methylpirazin is allegedly associated with chocolate flavor intensity, while the hydrophobic amino acids are considered to be the most potent precursor formation for methylpirazins. According to Atmaja (2013), the reformation of cocoa bean proteins into amino acids and peptides by the activation of enzyme protease, aspartic endoprotease and carboxypeptidase produce amino acids and oligopeptides. Aspartic endoprotease in cocoa beans cut the substrate proteins on hydrophobic amino acid residues to produce oligopeptides wchich have hydrophobic amino acids. The greater amino acid was produced from the protein breakdown reaction, the higher protease activity occurred.

CONCLUSION

Hydrophobic amino acids at the end of fermentation increased, hydrophobic acids were detected, namely; alanine, leucine, ileusin, phenilalanin, valine, tyrosine and metheoinin. Hydrophobic amino acids as aroma precursors, namely alanine, leucine, ileusin, valine and phenilalanin. The total hydrophobic amino acid in the treatment of inoculum stages. This shows that the addition of inoculum may gradually degrade proteins into a more hydrophobic amino acids.

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