Effect of Pods Storage and Fermentation Duration on Bioconversion of Proanthocyanidins in Malaysian Cocoa Beans

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***Abstract*—These two factors being studied where pods storage duration were varied as 0, 2, 4 and 6 days. Meanwhile, the fermentation duration of cocoa beans differs from day 0 until day 5. The sample that being ferment were defatted before undergo the extraction process. The extraction process undergo overnight by immersing the sample with the methanol solvent. The total proanthocyanidins content were analyzed by using UV Spectrophotometric and High-Performance Liquid Chromatography (HPLC) analysis. Multiple linear regression method was used in order to relate the relationship between concentrations of proanthocyanidins with the absorbance value taken from UV Spectrophotometric analysis. The regression value of this research was R^2 = 0.9486. The total proanthocyanidins contents in all samples from different pods storage and fermentation duration are varied from the both analyses. ANOVA analysis definitely revealed that fermentation duration has a significant effect to the bioconversion of the proanthocyanidins in Malaysian cocoa beans. Meanwhile, the pods storage might have 2.36E-08 ± 1.2E-05 effect toward bioconversion of proanthocyanidins. Since it < 0.05, it is considered negligible. It can be concluded that pods storage duration does not have significant effect to the proanthocyanidins bioconversion.**

Keywords— Proanthocyanidins, HPLC, Cocoa Beans, Pod Storage

I. INTRODUCTION

A dessert with lots of sugar and fat known as chocolate are made up from cocoa beans. *Theobroma cacao* is the scientific name for the cocoa tree. The cocoa tree usually grown in a sea-level tropical forest with little seasonality, for example, Ivory Coast, Ghana, Malaysia and northwestern South America (Edi et al., 2014). Chocolate gained its popularity as a nutritionally impenetrable product. As the knowledge has been improved day by day, benefits of cocoa bean have been known widely by the people around the world. Demands for the cocoa beans and cocoa product fluctuated based on the statistics given by Malaysian Cocoa Board regarding the export and import of the cocoa beans and cocoa product (Board, 2016). However, the supply is not available ad per demand since the statistics on the production of cocoa beans by region decrease by each year. Other than that, the problem that also being arise is the product from Malaysian cocoa beans have been not being commercialized and well known as the product from other countries and the bioconversion of proanthocyanidins in Malaysian cocoa beans have been giving the significant effect toward the quality of cocoa beans or not. Therefore, this research was done to investigate in more detail about the compound or factor that have in Malaysian coco beans that give more impact to the flavor and quality of the product from Malaysian cocoa beans.

Many studies have shown that cocoa powder has more antioxidants than other product (Afoakwa, Paterson, Fowler, & Ryan, 2008; Krähmer et al., 2015). Polyphenolic compounds also known as flavonoids are important in cocoa beans due to its potential cardiovascular health benefit, antioxidant protections and cholesterol controller in the body. The flavonoids contain a lot of useful component such as anti-inflammatory, anti-carcinogenetic properties and powerful antioxidant which 20 times greater than nutrition C and 50 times higher than vitamin (He, Wallace, Keatley, Failla, & Giusti, 2009; Rahul, Deeba, Vaqar, Mohammad Imran, & Hasan, 2015) .

Phytochemicals’ profile in cocoa beans differs for among cocoa beans and cocoa-containing food and various cultivars. Generally, cocoa is devoured food ingredients. The polyphenols content may be different from various foods such as wine, tea or vegetables. However, the proanthocyanidins is around 58 to 65% of the total polyphenols that can be obtained in the cocoa seeds (Niemenak, Rohsius, Elwers, Omokolo Ndoumou, & Lieberei, 2006). The content and composition of polyphenols differ intensely depending upon a few variables which are genotype, origin, degree of ripeness and processing of the beans (Kothe, Zimmermann, & Galensa, 2013).

Fermentation becomes a crucial process in order to determine the biochemical changes in the type and concentration of final cocoa flavor (Krähmer et al., 2015). The right choices of the fermentation process of cocoa beans depend on the countries of origin due to the improvement of the reasonable flavor as well as the flavor precursor (Ho, Zhao, & Fleet, 2014). Malaysian Cocoa Board has been endorsing five days fermentation period using a shallow box with a single turning on the third day as a standard fermentation preparation for the Malaysian cocoa beans (Khairul Bariah Sulaiman & Yang, 2015). However, the Malaysian Cocoa Board has been recommended the fermentation using a shallow box (Khairul Bariah Sulaiman, 2014). Malaysian cocoa beans are usually known for its low cocoa flavor, high acidity and astringent (Reed & Cocoa, 2010). Therefore, it has been reported that this technique being able to produce Malaysian cocoa beans with low acidity and stronger cocoa flavor (Zaibunnisa Abdul, 2002).

Whereas, the pods storage have the effect to the quality of Malaysian cocoa flavor focusing on the acidity of the cocoa beans had been investigate by (Afoakwa, Quao, Budu, Saalia, & Takrama, 2012) and (Meyer, Biehl, Said, & Samarakoddy, 1989). According to Khairul Bariah Sulaiman, (2016) pods storage process does not important due to Malaysian beans had been stated to be enhanced in flavor quality if the pods are kept up to 10 days preceding to fermentation. However, pods storage process turn out to be compulsory since cocoa cultivation area is conquered by small holdings nowadays. Pods storage have been done not only to

reduce volume as pulp preconditioning but it also can decrease of nib acidification, increase in cocoa flavor (Meyer et al., 1989) and also as an approach to collect their pods until abundant beans is obtained for fermentation (Khairul Bariah Sulaiman, Tajul Aris Yang, & Hamizah, 2016).

Polyphenolic cell which is a kind of parenchyma cells from cotyledons can give abundant of polyphenol from the cocoa seeds (Afoakwa et al., 2008). The contribution of three major molecules which are sugars, proteins and polyphenols shows that various chemical reactions arise inside and outside of the cocoa beans throughout fermentation development (Afoakwa et al., 2008). Chemical and biochemical reaction affected the flavor feature of cocoa beans during fermentation and drying. Polyphenols keep complex biochemical reactions which is important to cocoa flavor and the color of the cocoa throughout the processing. As a consequence of cell destruction throughout the fermentation process, polyphenols radiate from the storage cells, flavonoid glycosides are hydrolyzed and anthocyanidins are changed over to colorless pseudo base. Meanwhile, the catechin experience non-enzymatic oligomerization and proanthocyanidins are moved into more intricate structure (Tanaka, Matsuo, & Kouno, 2009).

Polyphenol in the cocoa beans is reported consist of flavanols, anthocyanins and proanthocyanidins with high level of flavan-3-ols represent as monomers of epicatechin and catechin (Mazor Jolić, Radojčić Redovniković, Marković, Ivanec Šipušić, & Delonga, 2011). These compound give astringent and bitter sensation and contribute essentially to the green and fruity flavor of cocoa liquors. All the bioconversion of the polyphenols with flavonoids, anthocyanins and proanthocyanidins content differ throughout the fermentation process. Nonetheless, these chemical reactions happen for the duration of fermentation cannot be observed by the naked eyes. In spite of the fact that the level of polyphenols generally diminishes where there were stated cases in which their concentration unaltered or even increase (Rusconi & Conti, 2010).

The fresh seed of cocoa beans rapidly changes its color from purple, pink and ivory during fresh into slaty, fully purple, partly purple-brown and fully brown after fermentation and drying process. More than that, these color changes reported to be related with the varieties of cocoa and the way of postharvest handling (Khairul Bariah Sulaiman & Yang, 2015). However, some problem being arise which is the product from the Malaysian cocoa beans have been not being commercialized and well known compare to product from other countries yet the information about the bioconversion of polyphenol compounds related to the quality of Malaysian cocoa beans is still lacking. Therefore, this research is done to investigate in more detail about the compound or factor that have in the Malaysian cocoa beans will give more impact to the flavor and quality of the product from the Malaysian cocoa beans.

The result in this research can be useful to the Malaysian Cocoa Board in the determination the bioactive compound that exists in the Malaysia cocoa beans. This finding of this study can be applied to the Malaysian Cocoa Board to increase the quality and productivity of the cocoa beans as well to develop more products from the cocoa beans. This study will shows the main compound that exist in the cocoa beans as well to detailed out the bioactive compound that being in the specific wavelength and compare with the previous study done by Khairul Bariah and Tajul Aris Yang (Khairul Bariah Sulaiman et al., 2016)

II. METHODOLOGY

A. Materials

The ripe and health cocoa pods of mixed clones were kindly provided from the Cocoa Research and Development Centre, Hilir Perak, Malaysia. Standard of catechin and quercetin were also provided by Malaysian Cocoa Board. Methanol, acetic acid, n-hexane and acetonitrile were taken from the Chemistry Laboratory at the Faculty Chemical Engineering.

B. Sample Preparation

The ripe and health cocoa pods was chosen in order to determine the compound content in the cocoa. Cocoa Research and Development Centre (CRDC) have done the collecting of the cocoa pods which come from mixed clone. The pods storage, fermentation and drying process/method was being done according to the recent studies that have been done by (Khairul Bariah Sulaiman & Yang, 2015). For pods storage duration were being done by kept the cocoa pods in a basket and left for a few days under dry and well circulated air surroundings which specified on the studies done by (Khairul Bariah Sulaiman & Yang, 2015). Meanwhile, fermentation was done in six different days in the shallow box with a specific measurement. The healthy fresh beans were being utilized for this fermentation. Then, the beans were dried on the dried platform by using natural drying method up until not less than 7.5% moisture content (Khairul Bariah Sulaiman & Yang, 2015).

C. Defatted powder

The roasted beans were cut manually in order to remove the shells from the beans to leave just the cocoa nibs (ICCO, 2013). Analytical grinded is utilized to grind the nibs into the fine powder. The fine cocoa powders undergo alkalization usually with n-hexane to develop the color and flavor. According to the (Khairul Bariah Sulaiman & Yang, 2015), the powder was put in the n-hexane and then being shaken hardily where the solution were thrown away. Buchner funnel being utilized to flushed the left over powder with 15 ml n-hexane and kept for further analysis (Khairul Bariah Sulaiman & Yang, 2015).

D. Extraction of Sample

The defatted powder weighed amount of 0.1g and being placed into the 15 ml dark glass bottle in order to extract the compound in the cocoa beans. The powder was suspended with 10ml methanol solvents. The samples were shaking vigorously for five minutes. This process is to enable the dispersion of sample. The samples were undergoing compound extraction process by being placed in the incubator at 4^{o}C overnight before filtered through a Whatman No.1 filter paper. Filtrate was divided for spectrophotometric and High Performance Liquid Chromatography (HPLC).

E. Total proanthocyanidins determination

Total proanthocyanidins were measured by using UV-Vis Spectrophotometer. The method was optimized to measure flavanols and proanthocyanidins using catechin as a standard.

Catechin standards- The catechin stock solution was prepared by dissolving 0.1g catechin in 10 mL methanol and shake vigorously for a few minute to ensure uniform mixing. Next, the stock solution was diluted using methanol to prepare 10, 20, 40, 60, 80, 100 mg/L catechin.

Spectroscopic analysis- All experiment was done by preparing the cuvettes either by adding 1mL of each catechin standard or sample extract was subjected to the visible spectrum reading. The reading was taken using UV-Vis Spectrophotometer. The catechin standards were taken the reading at 640nm wavelength. The experiment was repeated three times and the mean value was taken. Linear Regression method was being used by Least Squares method using Microsoft Office Excel. The average total of proanthocyanidins content of sample was reported in milligram catechin equivalents per gram defatted cocoa powders.

Spectrophotometric analysis

About 1mL filtrate were placed in a prepared cuvette was subjected to visible spectrum reading. The readings were taken by a UV-Vis Spectrophotometer between 300-500 nm wavelengths with the increment of 10 intervals. Results were observed and plotted in the Microsoft Office Excel.

Statistical analysis

Data of total average proanthocyanidins content was analyzed by using multiple linear regression method in Microsoft Office Excel. This method utilized in order to relate the relationship between concentrations of proanthocyanidins with the absorbance value taken from UV Spectrophotometric analysis. Data taken will evaluate the effect of pods storage and fermentation duration on its bioconversion in Malaysian Cocoa Beans.

F. High Performance Liquid Chromatography (HPLC)

The determination of proanthocyanidins compounds was performed on High Performance Liquid Chromatography (HPLC) where the system equipped with a sampler, solvent degasser, quaternary pump, column heater, photodiode array and fluorescence detectors. The column was used as a stationary phase in which a solvent. It is also known as mobile phase where it continuously applied to the column. The sample that comprises proanthocyanidins compounds injected to the column together with a mobile phase at a certain time (Robbins et al., 2009). The detector used in the HPLC is an UV detector which has been set at a wavelength of 280 nm. The methods of normal HPLC analysis taken from (Departament de Medicina i, Universitat Rovira i, Segura-Carretero, Fernández-Arroyo, & Cádiz-Gurrea, 2014) have been adapted to the analysis in determination of proanthocyanidins. The HPLC have been set the parameter to the appropriate condition where flow rate of 1mL/min and the column was saved at a temperature of 30°C with an injection volume of 20µL.

In the Saucier's experiment (Saucier, Mirabel, Daviaud, Longieras, & Glories, 2001), solvent A consist of water with 5% acetic acid become the stationary phase in determination of proanthocyanidins. Meanwhile, the mobile phase is the solvent B where comprises with methanol and 5% of acetic acid. The gradient of the solvent parameter was achieved using a linear gradient which have been set isocratic/isothermal with 50-50% solvent A and B.

III. RESULTS AND DISCUSSION

A. Changes of total proanthocyanidins content

In the spectrophotometric analysis, catechin was used as a standard curve. The standards were measured at 640 nm wavelength. The graph of standard curve was plotted in the Microsoft Office Excel and the R^2 value obtained was 0.9486. The R^2 is a statistical measure that relates how close the data are to the fitted regression line. The higher R^2, the better the linear graph fits to the data. R^2 equivalent to 0.9486 is considered high and accepted to the further calculations.

Source of Variation	SS	Df	MS	F	P-value
Fermentation duration	89.5327	5	17.9065	2.283	0.06735
Pods storage	748.029	7	106.861	13.63	2.36E-08
Replicate	274.316	35	7.8376		
Total	1111.88	47			

The average total proanthocyanidins concentrations of cocoa beans in all storage and fermentation duration were calculated. The total proanthocyanidins content present among the 24 samples varied between 0.164 ±0.00 to 2.0358 ± 0.10 mg/g relies on the pods storage and fermentation duration (*Figure 1)*. The unfermented sample from pods without storage duration is at the second lowest average total proanthocyanidins content (0.250 mg/g). The lowest total proanthocyanidins content is unfermented sample from pods with six days storage. In fact, the total proanthocyanidins content in all samples from pods with six days storage remained the lowest after 24, 48, 72, 96 and 120 hours fermentation durations. However, unfermented sample from pods without storage increase in the average total proanthocyanidins content throughout the fermentation duration.

In comparison with total proanthocyanidins content using Spectrophotometer analysis with the total polyphenols content, Figure 1 shows graph were totally fluctuated upon fermentation hours increases which means that the color observation itself might not be full depend of. According to Bonvehí (2005), polyphenols content gradually decreased upon fermentation duration from 0 to 8 yet the graph show the fluctuated pattern in polyphenol concentration from days 0 to 3.

The total proanthocyanidins content of all samples shows fluctuated pattern except for the samples unfermented pods. The unfermented pods storage sample showed the increment in the total proanthocyanidins content throughout the fermentation duration. However, most of the samples showed total proanthocyanidins content decreased more than 50% and the final concentration in between 0.7 ± 0.00 to 2.00 ± 0.10 mg/g at the end of fermentation duration.

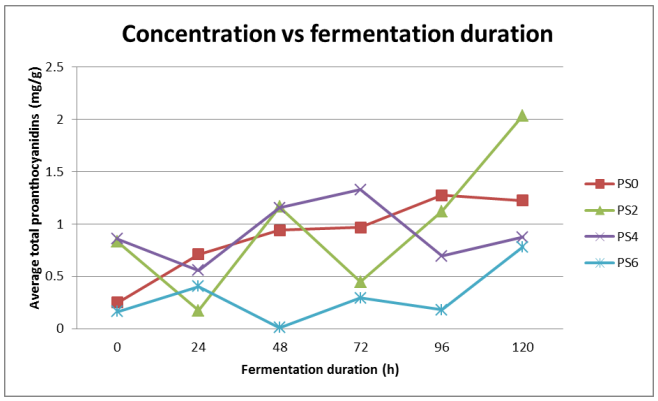

Figure 1: The total proanthocyanidins content (mg/g) in all samples from different pods storage and fermentation duration.

The average total proanthocyanidins content was additional assessed by the ANOVA in order to determine the replication, pods storage and fermentation have unpredictability effect on the bioconversion of total proanthocyanidins during fermentation process. There is a proof for fermentation duration have significant effect to the bioconversion of proanthocyanidins where $\alpha = 0.067$ (Table 1). Yet, there is no significant proof for the pod storage effect. The variance component evaluations showed that the unpredictability attributable to the pods storage, fermentation duration and replication was 8.05, 67.28 and 24.67 % of the total unpredictability respectively.

Table 1: ANOVA to study effect of replication, pods storage and fermentation duration on the bioconversion of total proanthocyanidins based on Spectrophotometric analysis.

Polyphenols have three main groups which are catechin also known as flavonoid, anthocyanins and proanthocyanidins. These compound represent certain percentage in the cocoa beans such as 5% to 10% of anthocyanins, 29% to 38% of flavonoid and 58% to 65% of proanthocyanidins (Aprotosoaie, Luca, & Miron, 2016). In

the recent studies shows that the total proanthocyanidins will be decrease as the fermentation increase (Ioannone et al., 2015). This is because of the polyphenol where it is diminishes essentially due to enzymatic browning and diffusion out of the beans throughout the fermentation and drying process. This can be shown by the decrement of (-) –epicatechin content after fermentation and drying process. Moreover, the level of (-)- epicatechin reduced by approximately 50% in dried and unfermented beans (Tabasco et al., 2011).

B. Phenolic Spectra

The spectra analysis of 4 samples from all the 24 samples extract at the wavelength range between 300 to 500 nm is accessible in the *Figure 2*. In overall, all the extracts comprise high peaks of absorption within in the first range of 300 to 320 nm and 360 to 370 nm. He et al., (2009) have mentioned that the flavonoids known as a pigment that have the ability to absorb light in the visible spectrum in the wavelength range of 400 to 500 nm. Moreover, anthocyanin is one of the flavonoid pigments that absorb light between 490 to 550 nm. It is can be concluded that the second peak range in between 400 to 550 nm in all 24 samples is anthocyanin. Other than that, it is suggested that the first peak might be proanthocyanidins where it has been reported that proanthocyanidins present at 319 nm (Andújar, Recio, Giner, & Ríos, 2012).

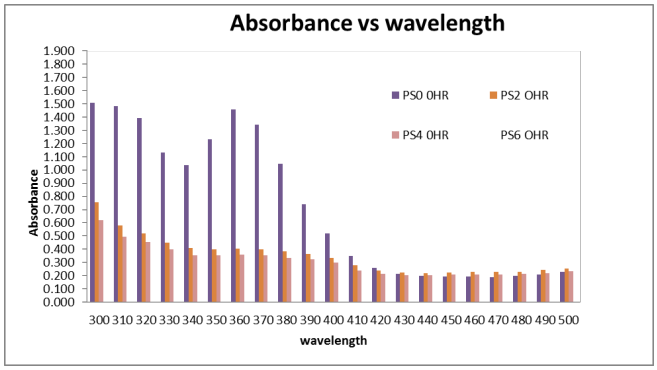

Figure 2: The spectra analysis of 4 from all the 24 samples extract in methanol solution at the wavelength range between 300 to 500nm.

The spectrophotometric analysis should be carried out in the acidic medium. Therefore, extraction using methanol could be the most effective solvent for extracting proanthocyanidins. This is because of the catalytic role of the acid in the reaction of proanthocyanidins and spectrophotometer (Sun B., 1998).

C. High Performance Liquid Chromatography (HPLC)

As previously described by (Departament de Medicina i et al., 2014), the use of HPLC column and UV detection at emission wavelength of 280 nm considered to be one of the most optimum HPLC method for accurately evaluating the amount of proanthocyanidins present in fermented and unfermented cocoa beans. The standard curve has been generated by using external standard quantitation for HPLC method. The external standard (ESTD) quantitation procedure is the basic quantification procedure in which both calibration and unknown samples are analyzed under the same conditions.

In determination of proanthocyanidins in cocoa extract, calibration curve was constructed using provided catechin from the Cocoa Research and Development Centre, Hilir Perak, Malaysia. The area of each proanthocyanidins peak was recorded and the relationship was determined when plotted the area against the concentration. By using the external standard (ESTD) quantification, it only uses one concentration of catechin standard. Therefore, 1000ppm of catechin standard has been selected. The proanthocyanidins content of the cocoa extracts were calculated and plotted in the graph by using Microsoft Office Excel. Individual percent areas for proanthocyanidins compound were also reported to be related to the total proanthocyanidins content of each sample.

HPLC and UV-visible spectrophotometric analyses of proanthocyanidins show a quite similar quantitative data (*Figure 3*). Moreover, HPLC analysis is helpful in isolating compounds while eliminating interferences that might be present in the pH differential method where compounds those produce a brown color depending on the pH of the solution. This variability in the quantification of the compounds is due to differences in other compound's extinction coefficients and absorption in the visible region. The results of the HPLC analysis were consistently higher compared to the result from UV Visible Spectrophotometric analysis throughout this research.

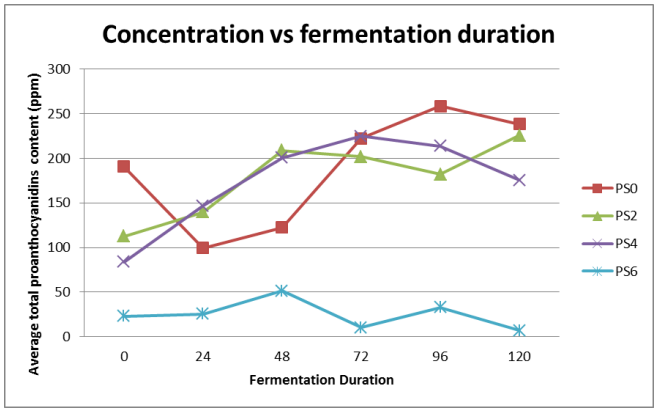

Figure 3: The total proanthocyanidins content (ppm) in all samples from different pods storage and fermentation duration

Source of Variation	SS	Df	MS	F	P-value
Fermentation duration	19677.8	5	3915.6	2.277	0.099579
Pods storage	109219.9	3	36406.6	21.17	1.2E-05
Replicate	25797.2	15	1719.82		
Total	154594.9	23			

Table 2: ANOVA to study effect of replication, pods storage and fermentation duration on the bioconversion of total proanthocyanidins based on HPLC analysis

Compared to the data observed in phenolic spectral, the unfermented pods sample without storage duration become the second lowest content of proanthocyanidins during the 0

hours fermentation duration. However, unfermented pods sample with 96h storage duration have the highest content of proanthocyanidins which approximately 250 ± 0.5 ppm. Other than that, the lowest average total proanthocyanidins content in both analyses is longest fermented sample from pods storage duration (PS6). For PS6, the graph fluctuated as there is an increment in the fermentation duration.

Sample from pods without storage and with two and four days storage showed the increase in total proanthocyanidins content after 24 hours fermentation. Most of the samples showed the total proanthocyanidins content increase as well as increase in fermentation durations. However, based on the observation on the color of the sample (*Figure 4-Figure 7*), it is reported that unfermented pods sample without the storage duration supposed to have the higher total proanthocyanidins. Other than that, it also shows that as the pods storage and fermentation duration increased the color of the sample become less pink and slowly turns to brown color. This showed that the total proanthocyanidins content in the samples decrease as the duration increase.

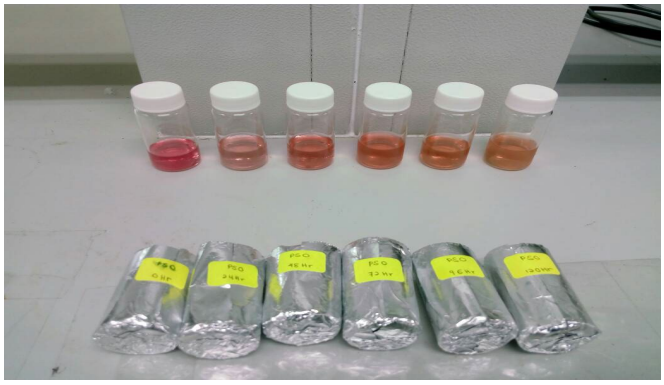

Figure 4: The color observation on the PS0 with the increment of fermentation duration (0, 24, 48, 72, and 96,120 h)

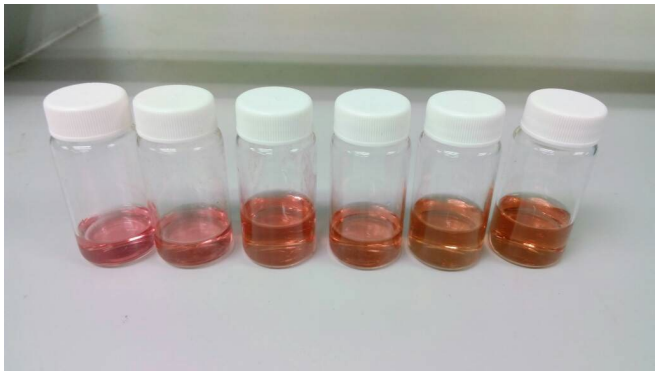

Figure 5: The color observation on the PS2 with the increment of fermentation duration (0, 24, 48, 72, and 96,120 h)

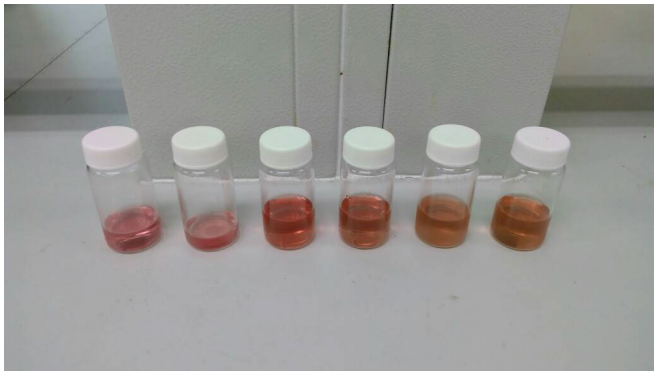

Figure 6: The color observation on the PS4 with the increment of fermentation duration (0, 24, 48, 72, and 96,120 h)

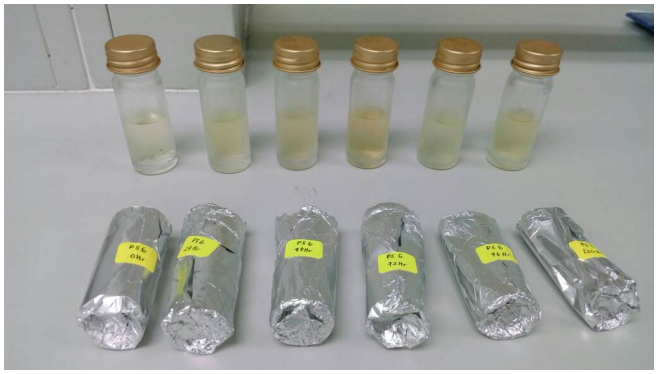

Figure 7: The color observation on the PS6 with the increment of fermentation duration (0, 24, 48, 72, and 96,120 h)

From this figure, higher total proanthocyanidins should be in pink color. The color will turn from pink to the brown color where the proanthocyanidins decrease in the solvent. Therefore, from this color observation, it can be stated that unfermented pods without storage should have higher total proanthocyanidins. Meanwhile, PS6 have less content of total proanthocyanidins. However, PS6 have the appropriate result where it has the least proanthocyanidins content in both analyses. There are some different results in both analyses due to some reasons. In the UV Visible Spectrophotometric analysis, the absorbance for the blank must be 0.000 nm and 0% to avoid any absorbance reading error for all samples. Other than that, cuvette used supposed to be a disposable cuvette. However, we are using a recycle cuvette in this research where there might be a contamination in the cuvette. The cuvette must be clean without having a scratch and must be hold rightly when being put into the spectrophotometer. Therefore, there might have significant effect to the absorbance reading throughout this study. There are differences in the result might be due to the weather condition during the fermentation process. This can be prove where throughout fermentation, microbial progressions can be occur with the changes of environment such as temperature, pH and oxygen availability (Bordiga et al., 2015).

IV. Conclusion

As the conclusion, the total proanthocyanidins contents in all samples from different pods storage and fermentation duration are varied from the both analyses. ANOVA analysis definitely revealed that fermentation duration has a significant effect to the bioconversion of the proanthocyanidins in Malaysian cocoa beans. Meanwhile, the pods storage might have 2.36E-08 ± 1.2E-05 effect toward bioconversion of proanthocyanidins. Since it < 0.05, it is considered negligible. It can be concluded that pods storage duration does not have significant effect to the proanthocyanidins

bioconversion. Other than that, it also revealed that proanthocyanidins higher at the wavelength range from 300 to 320nm and 360 to 370nm. In addition, further study must be carried out in order to determine and characterize more detail about the compound exist in the Malaysian cocoa beans.

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