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Effects of different fermentation approaches on the microbiological and physicochemical changes during cocoa bean fermentation

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Abstract

The influence of different fermentation methods and turning of cocoa beans on the cocoa bean's quality was studied. Both shallow box covered with banana leaves (SBBL) and shallow box without banana leaves (SBWL) were used throughout fermentation (120 hours). The initial microbial load for SBBL and SBWL was 5.35±0.18 and 5.19±0.21 log CFU/g before increased to 6.27±0.08 and 6.17±0.03 log CFU/g, respectively at the end of fermentation (120 hours). The titratable acidity of the cocoa beans increased steadily until 72 hours before decreased slightly to 1.34±0.07 (SBBL) and 0.75±0.15 (SBWL) at the latter stage of fermentation. The cocoa beans fermented under SBBL were less acidic than those found in SBWL. Turned cocoa beans produced better quality of cocoa with less acidic compared to the one without turning. Cocoa beans with periodical turning recorded higher percentage of brown beans for both SBBL (73%) and SBWL (69%); percentage of purple beans decreased to about 7-8% for cocoa fermented in respective methods mentioned above. No slaty beans were recorded throughout the study. This study suggests that the use of shallow box with banana leaves can produce cocoa beans with superior quality.

Introduction

Cocoa beans are obtained from the ripen Theobroma cacao pods which commonly planted in the West Africa, South America and some tropical regions around the world (Ardhana and Fleet, 2003). They are the essential source of cocoa butter and the raw ingredients for the manufacturing of chocolates, cakes, biscuits, ice cream and candy products. The raw cocoa beans usually have an unpleasant flavor that need to undergo subsequent treatments including fermentation, drying and roasting in order to obtain its unique sensory characteristics (Wood and Lass, 2001).

In fact, the freshly harvested cocoa beans tend to undergo spontaneous fermentation once the beans inside the mucilaginous pulp are removed from the pods. The beans are then subjected to various fermentation approaches either from the most common heaps fermentation to the use perforated boxes or tray that left to ferment for 5-7 days (Thompson *et al*., 2001). Literally, the fermentation is initiated by indigenous microorganisms that naturally present at the fermenting sites, the pod surfaces as well as the soil. Indeed, the microbial succession profile throughout spontaneous fermentation has been clearly elucidated (Beckett, 2000). These include the yeasts, acetic acid and lactic acid bacteria, bacilli and filamentous fungi (Garcia-Armisen *et al*., 2010; Lima *et al*., 2011). The microbial fermentation triggers many chemical reactions that promote promising biochemical characteristics of the beans. For instance, the bioconversion of sugar (inside mucilage) into intermediate organic components such as ethanol, lactic acid, acetic acid and other organic acids which will inhibit the continuous germination of the beans (Lopez and Dimick, 1995; Pereira *et al*., 2012b). Several enzymatic reactions also take place contributing to the formation of desirable flavor and color of the beans. The color of the beans will change from purple to brown which usually used as indicator for the maturity stage of the beans. Moreover, this spontaneous biochemical changes inside the bean also reduce the bitterness and astringency of the beans (Lagunes-Galvez *et al*., 2007).

The approaches used in spontaneous cocoa bean fermentation differs among the producing countries as followed the local preferences, for instant the methods being used, fermentation duration, pod or bean selection and postharvest treatments which will

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have significant impact on the quality of end products (Lagunes-Garvez *et al.*, 2007; Camu *et al.*, 2008b; Kostinek *et al.*, 2008). The cocoa planters in Malaysia are adopting boxes, trays or containers for improved fermentation to substitute the conventional banana heaps fermentation. It is understood that the above methods produce better quality of fermented cocoa beans as compared to the latter one as it minimize the contamination due to natural microbiota or handling throughout the process. According to Guehi *et al.* (2010a), the cocoa bean fermentation generally require shorter time at start and peak of cocoa crop but longer towards the end of the crop when the mucilage is less available for fermentation. Indeed, the fermentation is facilitated with good aeration condition which can be done by periodically turning on the seeds. This will improve the aeration and reduces the residual levels of organic products (especially lactate and acetate) in the beans (Thompson *et al.*, 2001; Leal *et al.*, 2008). Nevertheless, there's less information on the use of banana leaves in assisting spontaneous shallow box cocoa bean fermentation. Therefore, the present study aims to determine the influence of banana leaves together with turning on the microbiological and physicochemical changes of the cocoa beans during fermentation.

Materials and Methods

This study was conducted at the Fermentation Laboratory of Cocoa Research and Development Center, Hilir Perak during June – August 2011.

Harvest of cocoa beans

The freshly harvested cocoa beans were obtained from Malaysian Cocoa Board, Hilir Perak Station. The fully ripe cocoa pods (*Theobroma cacao* L.) were harvested and opened using sterilized knife without damaging the beans. The cocoa beans were removed carefully from the placenta prior to fermentation.

Spontaneous cocoa bean fermentation and sample preparation

The harvested and cleaned cocoa beans were subjected to fermentation. Four batches of fresh cocoa beans were used throughout the spontaneous fermentation. The first two batches were used for shallow box fermentation covered with banana leaf (SBBL) and shallow box fermentation without banana leaf (SBWL). The banana leaves used in this study was originated from Musa Paradisia fa. Corniculata or commonly known as pisang tanduk. Another two batches were SBBL and SBWL with turning respectively. An approximately 100 kg of cocoa beans were placed in four different boxes with the measurement of 42 cm (w) x (L) x 32 cm (H) as suggested by Jinap *et al*. (1994).

The beans in the boxes were turned once after 72 h and stopped after 5 days; while the beans without turned was designed as control. Turning is simple which involved the transfer of the beans from a shallow box to another to allow subsequent fermentation. In addition, the old banana leaves in the SBBL were changed by new leaves to help keep the heat within the beans. Besides, it also avoids possible vapor accumulation during fermentation. The base of the shallow box do have small hole to allow the drainage of acidic liquid due to liquefaction of mucilaginous pulp and aeration of the cocoa beans. All the fermentation was carried out simultaneously to minimize the influence of time, weather and cross contamination throughout the study.

Sampling of the cocoa fermentation

An approximately 150 g of beans were taken randomly from each fermentation box at the same depth (25-30 cm below the surface) for every 24 h interval until the end of fermentation (120 h). The sample were stored in sterilized sampling bag and kept in freezer box at 4-5°C prior further analysis.

Microbiological analyses involve in cocoa's pulp fermentation

A total of 20 g sample was mixed homogenously with 180 mL of 0.1% peptone water using aseptic stomacher bag. A 10-fold serial dilution was made in the solution and 0.1mL of aliquot from each dilution was spread over respective agar plates. The total plate count (TPC) was determined using Plate Count Agar (PCA); while the total yeast and mould count were determined using Potato Dextrose Agar (PDA). All the plates were incubated at 37°C for 24 h and 25°C for 48-72 h respectively. The colony counting was done at the plate within the highest dilution factor and the microbiological data were transformed into number of colony forming unit (CFU/g).

Physicochemical analyses of cocoa's pulp fermentation

The determination of pH was simple as an approximately 5 g of ground nibs were homogenized in 45 mL warm distilled water. The mixture was filtered using Whatman Number 4 filter paper and left to cool at 20-25°C. The filtrate was then measured for pH using a pH meter (Metler Toledo MP120). Meanwhile, the titratable acid was determined by titrating a further 10 mL aliquot to an end point pH of 8.1 with 0.1N NaOH solution. The data reported as mole of sodium hydroxide/100 g sample (Nazaruddin *et al*., 2006).

The cut test was performed to evaluate the

fermentation quality of the cocoa bean as followed to Hii *et al*. (2006). A total of 300 pieces of cocoa beans were cut lengthwise through the middle using knife and both halves were examined in full day light according to cross sectional color of the beans. Each defective type of bean was counted separately and the results were expressed as percentage of the beans examined. Visual observations on the quality of bean will be compared to the cut test chart as suggested by ISO 2451 standard (ISO, 1973), the classification on beans quality includes: the fully purple, half purple, fully brown, half brown and slaty.

Statistical analysis

All data obtained from the microbiological and physiochemical analyses were subjected to one-way ANOVA and Duncan's Multiple Range Test using SPSS (version 17) at 0.05 confident levels.

Results and Discussion

Microbiological quality of spontaneous cocoa bean fermentation

The spontaneous cocoa beans fermentation has been studied in different planting locations using different approaches. Studies revealed that the yeasts, lactic acid bacteria and acetic acid bacteria are the main microfloras involve in spontaneous cocoa bean fermentation as each of them are responsible to the synthesize and production of related metabolites such as ethanol, lactate, acetate, heat and also volatile precursors (Lopez and Dimick, 1995; Misnawi and Teguh, 2010). Despite the same fermentation approaches are being applied, spontaneous cocoa bean fermentation generally leads to the variation of end product quality and also the microbial diversity. The investigation on the microbial load during this spontaneous fermentation using respective approaches provides meaningful information on the possible dominant microflora throughout the process. In this study, the initial total plate count (TPC) for both SBBL and SBWL was high as the former one recorded 5.35 log CFU/g while the later one was 5.18 log CFU/g, respectively. The high microbial load might be attributed by the spontaneous fermentation that favors many microfloras to thrive after the onset of fermentation. In fact, this is a common scenario in most fermentation as the growing environment is suitable together for microorganisms to multiply. However, the microbial load was peak at 96 h for both SBBL (6.75 log CFU/g) and SBWL (6.39 log CFU/g), respectively before slightly dropped at 120 h (Table 1). The increase ($p<0.05$) of the microbial load at 96 hrs might due to the proliferation of dominating microfloras especially filamentous fungi, yeasts or those lactic and acetic acid producing bacteria. Moreover, the excellent nutrients contains in the cocoa beans as well as the biochemical changes inside in the beans also play significant role in the above scenario (Camu *et al*., 2007).

Table 1. Microbiological loads of cocoa beans using different fermentation approaches

Parameters	Fermentation (Hours) Microbial load (log CFU/g)					
	0	24	48	72	96	120
Total plate count (TPC)						
SBBL*	5.35± 0.18^{c}	5.17± 0.15^{d}	5.56± 0.20^{c}	5.93± 0.06^{b}	6.75± 0.12^{a}	6.27± 0.08^{a}
SBWL	5.19± 0.21^{d}	5.33± 0.14^{c}	5.59± 0.23^{b}	5.74± 0.07^{b}	6.39± 0.11^{a}	6.17± 0.03^{a}
Total yeast mould count (TYMC)						
SBBL	3.73± 0.13^{c}	3.59± 0.17^{d}	3.77± 0.09^{c}	3.99± 0.22^{b}	4.23± 0.52^{a}	3.92± 0.27^{b}
SBWL	3.07± 0.28^{e}	3.42± 0.29^{d}	3.37± 0.33^{d}	3.65± 0.24^{c}	4.18± 0.61^{a}	3.84± 0.35^{b}

*SBBL – shallow box with banana leaves; SBWL – shallow box without banana leaves

Mean values having a common letter within the same line and column are not significantly different according to Duncan's multiple range test at the 5% level

The total yeast mould count (TYMC) of SBBL was always higher than those recorded in SBWL (Table 1). This phenomenon can be explained by the present of additional mould or filamentous fungi that accidentally introduced to the fermentation via banana leaves. The current result was corresponded to Thompson and Fleet (2003) and Tagro *et al.* (2010) that the change of banana leaves will bring additional fungal spores to the fermentation. Similar to certain fermentation process, the filamentous fungi and mould are playing central part to breakdown the cocoa pulp before transform them into valuable secondary metabolites as required by subsequent microorganism groups. The TMC for both fermentation approaches showed an increasing trend after the onset of fermentation and slightly decreased at the end of fermentation (120 hrs). The slight decrease of microbial load might attribute by the depleting of essential nutrients as most of the sugars originated from the mucilage pulp were converted into alcohol and other metabolites. In fact, the accumulation of excessive ethanol might limit the growth of mould or filamentous fungi. Major yeasts and moulds genera that commonly present in this stage include *Saccharomyces*, *Candida*, *pichia*, *Rhodotorula*, *Saccharmycopsis*, *Hanseniaspora*, *yarrowia* and *Zygosaccharomyces* (Daniel *et al.*, 2009; Lima *et al*., 2011; Tristeza *et al*., 2012). However, the elucidation on TPC and TMC using both fermentation approaches did not change much at the latter stage of fermentation period (96 and 120 hours) as compared to those recorded at the initial fermentation stages.

Physicochemical changes of the cocoa beans during fermentation

Apparently, cocoa fermentation is a complex biochemical reaction which can be divided into anaerobic hydrolytic and subsequent aerobic or oxidative phases. The pH for the cocoa beans that underwent fermentation was ranging from 3.75±0.18 to 7.12±0.19 (Table 2). During the first two days of fermentation, the pH recorded a decreasing trend for both fermentation approaches (SBBL and SBWL) before slight increased at the end of the fermentation. The initial fermentation stage was acidic (low pH) as the cocoa pulp contained certain amount of citric acid that favors the yeast to grow. However as fermentation went on, the dominant yeasts with good pectinolytic activity degraded the pulp and removed the citric acid to allow subsequent bacteria to grow. This explained why there's slight increase of pH from 3.84 ± 0.10 (48 hours) to 4.63 ± 0.20 (72 hours) for SBBL. Once the fermenting environment became more aerobic, the lactic acid bacteria (LAB) would mediate fermentation and converting the intermediate metabolites into lactic acids.

Table 2. Physicochemical changes of cocoa beans under different approaches throughout fermentatio

Parameters	Fermentation (Hours)					
	0	24	48	72	96	120
pH						
SBBL*	4.00± 0.05^{c}	3.75± 0.18^{d}	3.84± 0.10^{c}	3.63± 0.20^{a}	4.15± 0.12^{b}	4.21± 0.08^{a}
SBWL	4.23± 0.11^{d}	3.86± 0.09^{c}	3.97± 0.13^{b}	3.88± 0.22^{b}	5.18± 0.16^{a}	7.12± 0.19^{a}
Titratable acid						
SBBL	1.78± 0.05^{c}	2.54± 0.13^{d}	2.8± 0.09^{c}	2.15± 0.02^{b}	1.80± 0.22^{a}	1.34± 0.07^{b}
SBWL	1.78± 0.12^{e}	2.65± 0.19^{d}	2.69± 0.17^{d}	2.24± 0.11^{c}	1.28± 0.26^{a}	0.75± 0.15^{b}
Temperature (°C)						
SBBL	27	36	43	46	48	45
SBWL	26	35	42	47	44	40

*SBBL – shallow box with banana leaves; SBWL – shallow box without banana leaves

Mean values having a common letter within the same line and column are not significantly different according to Duncan's multiple range test at the 5% level.

At the latter stage of fermentation, acetic acid bacteria (AAB) oxidized the ethanol produced by yeasts and LAB into acetate and further to carbon dioxide and water. Nevertheless, the acetic acid was highly volatile and seldom accumulated under aerobic condition. Therefore, the pH of the cocoa bean in SBWL was higher than those recorded in SBBL at the latter stage of fermentation (Table 2).

Unlike the pH, the titratable acidity is a more appropriate indicator to measure the total acid level in any fermentation process and usually both parameters are negatively correlated. The acidity of cocoa beans fermented in SBBL is significant higher (1.34±0.07) than those found in SBWL. In a microaerophilic condition (using banana leaves), it usually favor the growth of lactic acid bacteria who will utilize the fermenting substrates before convert them into secondary metabolites especially the lactic acid. According to Lagunes-Galvez *et al*. (2007), the synthesis of lactic acid is very common among lactic acid fermentation, especially cocoa fermentation that carried out under anaerobic condition. On contrary, the acidity of the cocoa bean under SBWL were dropped at the end of fermentation (4 and 5th day) because of low accumulation for organic acid (acetic acids) compared to cocoa beans fermented under SBBL.

The temperature was risen steadily from 27 to 45 (SBBL) and 26 to 40 °C (SBWL), respectively after the onset of cocoa fermentation. The steady increase on the temperature might associate to the release of heat from cocoa biomass throughout the process. Initially, the yeasts were the dominant species that utilize the available fermentable substrate (sugar) before convert them into ethanol and further to acetic acid via microbial succession. The conversion of fermentable substrate into desired metabolite by-products is performed exothermically, and hence assists in the increase of temperature (Schwan and Wheals, 2004).

Influence of turning and fermentation approaches on the physical quality of cocoa beans

Literally, the use of swallow box to ferment the cocoa beans is aimed to minimize the cross contamination level compared to those performed under heaps fermentation. However, it must accomplished by frequent turning on beans to ensure fermentation is carried out homogenously. In an original state (without turning the beans), the beans prepared in shallow box covered by banana leaves (2.64±0.20, Table 3) exhibited slightly higher acidity level compared to those uncovered with banana leaves (2.27±0.15). The use of swallow box together with banana leaves indirectly create limited aeration space which favor most lactic and acetic acid bacteria to survive. In such microaerophilic environment, the organic acids (lactate) level will increase significantly (Schwan, 1998). However, the continuous increase of acidity level is not favorable for any cocoa beans fermentation as it will influence the end product quality prior harvesting.

Turning on beans is commonly practiced for medium to large scale of cocoa beans quantity to undergo even or homogenized fermentation throughout the process. According to Thompson *et*

Table 3. Effect of turning on the quality of cocoa beans.

Parameters	With 1 turning		Without turning	
	SBBL	SBWL	SBBL	SBWL
TPC (log CFU/g)	7.83±0.14[a]	6.86±0.21[b]	6.97±0.18[b]	6.35±0.06[c]
TMC (log CFU/g)	4.12±0.22[b]	5.36±0.17[a]	4.57±0.09[c]	4.98±0.13[b]
Acidity	1.96±0.12[a]	1.85±0.27[a]	2.64±0.20[b]	2.27±0.15[c]
Temperature (°C)	48	45	46	42

*SBBL – shallow box with banana leaves; SBWL – shallow box without banana leaves

Mean values having a common letter within the same line and column are not significantly different according to Duncan's multiple range test at the 5% level

al. (2001), the turning on fermenting cocoa beans will improve their aeration, reducing the lactate and acetate levels in those beans. This will definitely improve the bean quality with less acidic. As overall, the acidity level of the turned beans under SBBL (1.96±0.12) and SBWL (1.85±0.27) was lower than those without turning. Turning creates aeration on the beans that favors the yeasts to extend their growth. Hence, more sugar will be utilized and by yeasts prior converting them into ethanol. The accumulation of ethanol indeed limits the other bacteria groups (especially lactic acid bacteria) to grow. Apart from that, the turning on beans also causes the dissociation of mucilage which favors the acetic acid bacteria to oxidize the sugar into ethanol and further into acetate which is highly volatized under aerobic or drying condition. Moreover, turning also increased the total microbial load of SBBL (7.83±0.14 log CFU/g) and SBWL (6.86±0.21 log CFU/g) respectively compared to those without turning. For the cocoa beans fermented using SBBL, their microbial load was higher ($p<0.05$) than those in SBWL because the change of old banana leaves by new leaves might accidentally increase the microbial load especially the filamentous fungi group. Meanwhile, the filamentous fungi or yeast group prefer to grow within limited space in the swallow box under microaerophilic condition also explained why their load (TPC) was higher than those without turning. Generally, the turning elevated the temperature inside the bean mass in the cocoa bean fermentation. The beans in SBBL approach recorded the highest temperature (48°C) as compared to others as this was due to the growth of microorganisms that assisted the conversion of sugar into ethanol followed by related metabolites under exothermic condition. This explains why the temperature increases faster to optimum (48°C) for the turned beans compared to those were not turned. The current result was in line to those reported by Guehi *et al.*, 2010b).

Figure 1 shows the influence of turning on the physical quality of beans resulted from different

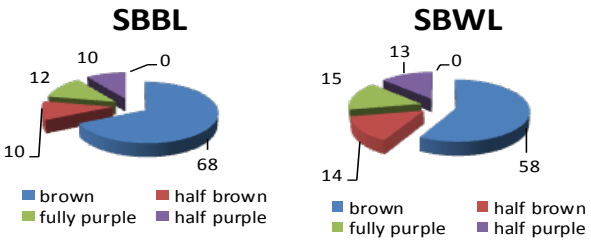

Figure 1a. Physical quality of cocoa beans without turning using different approaches.

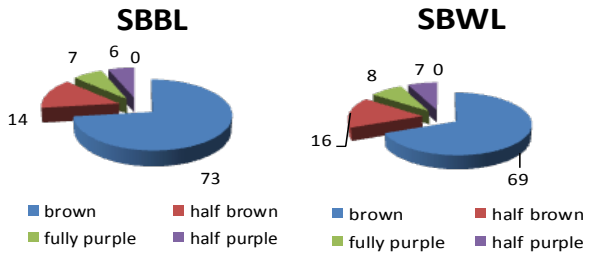

Figure 1b. Effect of turning on physical quality of cocoa beans among different approaches.

fermentation approaches that evaluated using cut test method. All the beans showed no sign of insect damages or fungal infections at the early of the study. In figure 1a, the cocoa beans without turning recorded 68 (SBBL) and 58% (SBWL) of brown bean, which is relatively lower than those found in the turned beans under SBBL (73%) and SBWL (69%, Figure 1b) respectively. Meanwhile, the beans without turning exhibited highest fully (10%) and half purple (10%) beans compared to the turned beans in whatever the studies approaches. On contrary, the percentage for both fully and half purple beans were significantly reduced to 6 (SBBL) and 7% (SBWL) after the cocoa beans were turned during fermentation. The significant decrease in percentage of purple beans using different approaches could be related to the microaeration condition created by turning as well as the use of banana leaves that allow fermentation of sugars into desired metabolites. In fact, the high percentage of purple beans usually acts as indicator of imperfect cocoa bean fermentation which associated with low quality beans. Turning on the cocoa bean was very important as it induced aerobic condition which favors many biochemical reactions to occur (Thompson *et al*., 2001). Turning on cocoa beans will increase the temperature which activated relevant enzymatic reactions which allow the pigment degradation on the beans. This will eventually led to the change of internal bean's color from purple to brown after fermentation done. In this study, no slaty beans were detected throughout fermentation for both methods applied and this showed that cocoa beans were perfectly fermented under applied methods in this study.

Conclusion

Current study demonstrated that the use of different fermentation approaches together with turning on cocoa beans have influences on studied parameters. Among all, fermentation in shallow box with banana leaves (SBBL) resulting better quality of cocoa beans for their microbiological and physicochemical performances. The beans under SBBL exhibited highest quality at 72 hours of fermentation. Turning on the cocoa beans facilitated better fermentation as it improved the total amount of brown beans, while reduced the percentage of purple beans for both approaches. This technique is feasible and can be promoted among local cocoa planters to initiate a homogenous fermentation process for better cocoa beans quality. Further study will be focus on the effect of different fermentation approaches towards the cocoa beans flavor and to standardize the fermentation process using up-scaling approach.

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