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Candida sp. as a starter culture for cocoa (*Theobroma cacao* L.) beans fermentation

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Abstract

Two cocoa bean fermentation methods (spontaneous fermentation and the use of starter culture) for 7 days fermentation were compared in terms of safety and quality fermented beans. *Candida* sp. was used as a starter culture in this study. The safety of the fermented cocoa beans were measured by the growth colonies of pathogenic microorganisms namely *Bacillus cereus, Escherichia coli, Salmonella* sp., *Staphylococcus aureus*, and *Pseudomonas* sp., on *Bacillus cereus* agar, eosin-methylene blue (EMB) agar, xylose lysine deoxycholate (XLD) agar, Baird-Parker agar (BPA), and *Pseudomonas* agar, respectively. *B. cereus, E. coli* and *Salmonella* sp. were early present in both fermentations. *Candida* sp.-fermentation showed detection of *B. cereus* at 5.34 $\log_{10}$ CFU/g and absence after 24 hours of fermentation while in spontaneous-fermentation *B. cereus* was too few to count. Moreover, the $\log_{10}$ *E. coli* number in *Candida* sp.-fermentation and spontaneous-fermentation were reduced from 5.72 to 3.66 and from 7.15 to 4.46 on day 1 to day 3, respectively. There were no presences of pathogenic microorganisms on day 5 and day 7 for both fermentations. In term of quality, proximate analysis of spontaneous-fermentation resulted that the content of moisture, ash, fat, crude protein, crude fibre and carbohydrate was 56.47%, 2.32%, 3.17%, 7.02%, 28.14% and 2.88%, meanwhile for the *Candida* sp.-fermentation was 53.96%, 2.19%, 3.44%, 8.25%, 25.46% and 6.70%, respectively. This study showed that both fermentations are considered to be safe and there is no significant difference in proximate value in fermented cocoa beans from spontaneous-fermentation and *Candida* sp.-fermentation.

Introduction

Cocoa (*Theobroma cacao* L.) was first discovered in South America by Mayas and Aztecs people. The cacao plant was then spread from America to Europe by cortes (Verna, 2013). Cocoa has been used as food, beverage and medicine in early centuries (Dillinger *et al*., 2000). Chocolate today is often served as a hot drink, dessert or snack.

Cocoa fermentation, is a crucial step in the process of technological transformation of cocoa into chocolate (Villeneuve *et al*., 1989; Biehl *et al.*, 1993; Schwan and Wheals, 2004), is essentially led by yeasts and various genera of bacteria, including *Bacillus*, acetic acid bacteria and lactic acid bacteria (Schwan, 1998; Thompson *et al*., 2013). Yeasts have been reported as predominant microorganisms during cocoa beans fermentation. *Saccharomyces cerevisiae*, *Kluyveromyces marxianus, Pichia membranifaciens, Pichia kudriavzevii* and some *Candida* spp. are most often dominant (Ardhana and Fleet, 2003; Jespersen *et al*., 2005). Yeasts degrade mucilaginous pulp surrounding the seed by using pectinolytic enzyme (Schwan *et al*., 1997). This enzymatic activity caused metabolites production and conditions that cause bean death and initiate chocolate flavor precursors from biochemical reactions within the bean (Lima *et al*., 2011; Ho *et al*., 2014). According to Schwan and Wheals (2004), *Candida* was present up to the end of fermentation when the temperature was approximately 50°C. Species of *Candida* were also detected during the early stages of fermentation and gave significant growth at higher temperature of 40°C (Ardhana and Fleet, 2003).

Thus, the used of *Candida* sp. as a starter culture in cocoa fermentation needs to be investigated in order

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Table 1. Changes in the pH of cocoa bean during fermentation

Fermentation Period (Day)	0	1	2	3	4	5	6	7
Spontaneous	3.00± 0.00	3.00± 0.00	4.00± 0.05	4.54± 0.13	4.82± 0.06	4.38± 0.05	7.66± 0.13	7.97±0.01
+*Candida* sp.	3.00± 0.00	4.00± 0.00	4.24± 0.23	4.49± 0.40	4.81± 0.24	4.06± 0.02	7.29± 0.35	7.68±0.18

Values are means (n = 3) ± SD

Table 2. Changes in temperature during fermentation cocoa bean (°C)

Fermentation Period (Day)	0	1	2	3	4	5	6	7
Spontaneous	28.0± 0.00	29.6± 0.58	36.0± 3.0	41.3± 2.5	41.6 ± 2.0	35.0 ± 1.0	33.3 ± 0.5	33.0 ± 0.0
+*Candida* sp.	28.0± 0.00	29.5± 0.71	36.5± 0.7	42.0± 1.4	42.5 ± 2.1	36.5 ± 2.1	33.5 ± 2.1	31.5 ± 2.1

Values are means (n = 3) ± SD. Both fermentations were carried out at non fix temperature.

to produce safe and high quality cocoa products. In this study, *Candida* sp. culture was added to the cocoa beans to observe the safety and quality of fermented beans compare to the spontaneous fermentation. The result of this study might be useful for the cocoa industry in order to produce high quality of cocoa products.

Materials and Methods

Yeast cultures and media

Yeast strains (*Candida* sp.) were provided by Barry Callebaut Services Asia Pacific Sdn. Bhd. Yeast extract (Merck, Darmstadt, Germany) and molasses (Liqueur Agency Sdn. Bhd., Malaysia) were used in fermentation media preparation. Yeast cultures were grown on yeast peptone dextrose (YPD) agar containing 1% yeast extract (Oxoid, Basingstoke, UK), 2% peptone (Oxoid, Hampshire, England), 2% glucose (Merck, Darmstadt, Germany) and 2% agar (Oxoid, Hampshire, England). After 48 hours of incubation, the single colony of yeast strains were inoculated into sterilized 10 mL YPD broth and incubated at 30°C for 24 hours. The inoculums were inoculated (1% v/v) aseptically onto the respective media contained molasses and yeast extract, and incubated in orbital shaker (agitated at 180 rpm) at 30°C.

Cocoa samples and cocoa bean fermentation

The cocoa pods were obtained from cocoa plantation in Jengka, Pahang. The mature pods received were in size range of 7-10 inches. The pods were cut to obtain the white and mucilaginous content (beans). The cocoa beans were scrapped out and select for the fermentation process, where the spoiled and germinated beans were discarded. The cocoa beans were weighed into stainless steel bowl approximately 5 kg for each batch. *Candida* sp. culture was inoculated on cocoa beans. After addition of starter culture, beans were manually mixed before being poured into basket. Both basket of *Candida* sp.- fermentation and spontaneous fermentation basket were covered with banana leaves. *Candida* sp.-fermentation and spontaneous fermentation were lasted for 7 days with turn after every 24 hours. Samples of beans (100 g total, from locations throughout the fermenting mass) were taken daily for microbiological and chemical analyses. Samples for microbiological analysis were used immediately while those for chemical analysis were stored at−20 °C until examined.

Temperature and pH measurement

The temperature was measured using a digital thermometer (model 15 077 8B Fischer Scientific, USA) at random points inside the box. The pHs of the cocoa beans were determined according the method as reported by Nazaruddin *et al*. (2006). Five grams samples of nibs were homogenized for 30 s in 100 ml of hot distilled water and then vacuum filtered through Whatman No. 4 filter paper (GE Healthcare, Buckinghamshire, UK). A 25 ml aliquot was pipetted into a beaker and the pH was measured using a pH meter (MP230, Mettler-Toledo, Switzerland).

Microbiological analysis

Enumeration of bacteria was done according to the method described by Ho *et al*. (2014). Fermented cocoa beans (25 g) were aseptically mixed with 225 ml of 0.1% peptone water in a stomacher bag and manually shaken for 5 min to give a homogenate suspension of the pulp material. One ml of the suspension was serially diluted in 0.1% peptone water until ten-fold dilutions and 0.1 ml samples from each

Table 3. Microbial cell counts during fermentation of cocoa beans (log_{10} CFU/g)

Fermentation Period (Day)	0		1		3		5		7	
	Sponta-neous	+*Candida* sp.	Sponta-neous	+*Candida* sp.	Sponta-neous	+*Candida* sp.	Sponta-neous	+*Candida* sp.	Sponta-neous	+*Candida* sp.
B. cereus	TFTC	5.34	TFTC	0	0	0	0	0	0	0
E. coli	TFTC	TFTC	7.15	5.72	4.46	3.66	0	0	0	0
***Salmonella* sp.**	TFTC	TFTC	0	0	0	0	0	0	0	0
S. aureus	0	0	0	0	0	0	0	0	0	0
***Pseudomonas* sp.**	0	0	0	0	0	0	0	0	0	0

TFTC = Too few to count
0 = No colony growth

Table 4. Changes in proximate composition of cocoa beans during fermentation

Fermentation Period (Day)	0		1		3		5		7	
	Sponta-neous	+*Candida* sp.	Sponta-neous	+*Candida* sp.	Sponta-neous	+*Candida* sp.	Sponta-neous	+*Candida* sp.	Sponta-neous	+*Candida* sp.
Moisture	59.77^{a} ±2.82	59.53^{ab} ±4.59	62.55^{ab} ±2.93	57.61^{ab} ±6.36	58.28^{ab} ±5.04	60.94^{ab} ±1.66	55.97^{ab} ±5.44	52.88^{b} ±3.45	56.47^{ab} ±1.55	53.96^{ab} ±1.22
Ash	1.74^{abc} ±0.28	1.56^{abc} ±0.18	1.96^{abc} ±0.08	2.36^{a} ±1.04	0.96^{c} ±0.37	1.19^{bc} ±0.52	1.20^{bc} ±0.16	1.22^{bc} ±0.22	2.32^{a} ±0.18	2.19^{ab} ±0.10
Fat	0.90^{e} ±0.74	0.61^{e} ±0.18	2.58^{c} ±0.22b	0.32^{e} ±0.27	0.95^{de} ±0.19	1.53^{cde} ±0.93	5.22^{a} ±0.60	2.28^{bcd} ±0.90	3.17^{b} ±0.45	3.44^{b} ±0.49
Crude Protein	0.32^{c} ±0.12	0.33^{c} ±0.13	1.65^{c} ±0.50	1.86^{bc} ±0.45	6.08^{a} ±0.94	7.01^{a} ±0.84	5.39^{ab} ±1.18	6.57^{a} ±0.59	7.02^{a} ±3.09	8.25^{a} ±3.08
Crude Fibre	20.28^{b} ±2.48	22.95^{b} ±4.04	23.94^{b} ±1.38	22.23^{b} ±4.10	25.77^{b} ±5.98	23.98^{b} ±4.65	28.61^{ab} ±4.11	34.93^{a} ±2.77	28.14^{ab} ±1.12	25.46^{b} ±2.30
Carbohydrate	17.00^{a} ±4.06	15.02^{ab} ±8.12	7.33^{abc} ±2.48	15.62^{ab} ±8.03	7.97^{abc} ±4.20	5.35^{bc} ±3.71	3.62^{c} ±4.56	2.13^{c} ±0.50	2.88^{c} ±2.25	6.70^{abc} ±3.08

Values are means (n = 3) ± SD. Values with different superscript are significantly different at $p < 0.05$

of three consecutive dilutions were spread onto plates of different agar media. All inverted plates were incubated at 37°C for 24 hours. After incubation, bacterial colonies were counts and calculated in log_{10} CFU/g.

Proximate analysis

The moisture, crude fat, crude protein and ash were determined following the procedures in AOAC (2005) methods 931.04, 963.15, 970.22 and 972.15, respectively. Carbohydrate was determined using 'by difference' method. All the analyses were performed in triplicate and the mean values reported.

Statistical analysis

Statistical analysis was performed using MINITAB software for analysis of variance (ANOVA). During this analysis, the Turkey's test was used in order to identify the significance of difference (P = 0.05) between those treatments. Then, the results were interpreted as means ± standard deviation (SD) of duplicate analysis.

Results and Discussion

In this study, the fermentation of cocoa beans was carried out using basket fermentation. The beans were covered with banana leaves to conserve the heat generated during fermentation. Beans were turned after every 24 hours to ensure uniform fermentation and increase aeration (Schwan and Wheals, 2004). This study was set up to determine the effect of added culture (*Candida* sp.) to the fermentation of cocoa beans on its safety and quality.

The pH and temperature of fermenting beans were taken every day from Day 0 to Day 7. At harvest, the cocoa bean pulp was acidic (pH 3.0) and increased by the end of fermentation (pH 8.0). An increase in pH towards the end of fermentation was caused by citric acid conversion (Crafack *et al*., 2013). The pH values of fermenting cocoa were shown in Table 1. The temperature of the fermenting beans was increased during the fermentation process from 28°C and reached maximum during Day 4 at 42°C and decreased continually to 33°C at the end of fermentation process (Table 2). The temperature of the fermenting mass increases due to exothermic reactions of acetic acid bacteria (Schwan and

Wheals, 2004), thereby limiting the growth of many microorganisms. Contrary to this, Thompson *et al*. (2001) reported an increased temperature, in combination with increased pH and aeration is associated with growth of aerobic spore-forming *Bacillus* sp.

In microbiology analysis, five major bacteria associates with food safety were investigated and interpreted as $\log_{10}$ CFU/g (Table 3). In this study *E. coli* presence in the fermenting cocoa beans from the Day 0 to Day 3. However, during the Day 0 the amount of colony growth on the agar was TFTC (too few to count) for both spontaneous fermentation and *Candida* sp.-fermentation. It shows an increment after 24 hours of fermentation and decrease during Day 3, 7.15 to 4.46 $\log_{10}$ CFU/g and 5.72 to 3.66 $\log_{10}$ CFU/g for spontaneous and *Candida* sp.-fermentation respectively. It reduced and showing the absence at Day 5 and day 7. Meanwhile, *B. cereus* presents only during Day 0 with amount of 5.34 $\log_{10}$ CFU/g only in *Candida* sp.-fermentation, and TFTC in spontaneous fermentation for Day 0 and Day 1. *Salmonella* sp. gives only TFTC during Day 0 and showed no growth during the fermentation period onwards. *S. aureus* and *Pseudomonas* sp. result no colony growth in both fermentation throughout the process. .

In the proximate analysis, comparison is conducted on the percentage of moisture content, ash content, fat content, crude protein content, crude fibre content , and carbohydrate content (by difference). Moisture content showed significant different ($p<0.05$) between Day 1 spontaneous fermentation (59.77 ± 2.82) and Day 5 *Candida* sp.-fermentation (52.88 ± 3.45). In comparison of ash content, it show no significantly different between spontaneous fermentation (1.74 ± 0.28, 2.32 ± 0.18) and *Candida* sp.-fermentation (1.56 ± 0.18, 2.19 ± 0.10) on Day 0 and Day 7 respectively. This followed as well with crude fibre content, where spontaneous fermentation gives 20.28±2.48 on Day 0 and 28.14±1.12 on Day 7 and *Candida* sp.-fermentation gives 22.95 ± 4.04 (Day 0) and 25.46 ± 2.30 (Day 7). There are no significant different in fat content and crude protein content at the end of fermentation process between spontaneous and *Candida* sp.-fermentation. Both qualities were only show significant different between the comparisons to fermentation period, where fat content is increased from 0.90 ± 0.74 to 3.17 ± 0.454 (spontaneous) and from 0.61 ± 0.18 to 3.44 ± 0.49 (*Candida* sp.) and crude protein content from 0.32 ± 0.12 to 7.02 ± 3.09 (spontaneous) and from 0.33 ± 0.13 to 8.25 ± 3.08 (*Candida* sp.). The details of the results are shown in Table 4. A decrease in protein content of fermented beans was observed from data obtained by Krähmer *et al.* (2015). The content of proteins decreases due to proteolytic digestion of the cocoa storage proteins. This degradation goes along with an increase of the free amino acid content.

Conclusion

The main conclusion to be drawn from the results of this research is addition of *Candida* sp. on cocoa beans fermentation inhibit the growth of pathogenic microorganisms. In term of quality, there was no apparent difference in bean composition between spontaneous and *Candida* sp.-fermentation. *Candida* sp.-fermentation ensured both the quality and safety of the end product. However, further studies would be interesting to determine volatile compounds produced from both fermentations.

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