Malaysian Cocoa Journal
Volume 13(2)/2021

ISOLATION OF LACTIC ACID BACTERIA FROM COCOA FERMENTATION AS THE POTENTIAL STARTER CULTURES FOR ENHANCING FLAVOUR QUALITY

Khairul B. S.[1] and Husin S.[1]

[1]*Division of Downstream Technology, Cocoa Research & Development Centre Bagan Datuk, Malaysian Cocoa Board, P.O. Box 30, Sg. Dulang Road,36307 Sg. Sumun, Perak, Malaysia.*
Corresponding author: kaybee@koko.gov.my

Malaysian Cocoa J. (2021) 13(2): 149-156

ABSTRACT - *Spontaneous cocoa fermentations involves natural microbes which are inconsistent and contribute to flavour quality variability. A well-performed fermentation using the shallow box has enhanced the flavour quality of Malaysian dried cocoa beans with lactic acid bacteria as dominating microbes. However, the study on the diversity of the lactic acid bacteria species during spontaneous cocoa fermentation using the shallow box is limited. Therefore this study was to evaluate the lactic acid bacteria species involve during the cocoa fermentation using the shallow box and also to isolate dominant species as the potential starter cultures. Cocoa fermentation was conducted using the shallow box for 120 hours and samples were taken every 24 hours. Physicochemical changes such as temperature, total soluble solids and acidity were evaluated. Sensory profiles were also compared to lactic acid bacteria diversity. The dominant lactic acid bacteria was further isolated and identified by characterization of colony morphology and confirmed by molecular method. The study indicated that lactic acid bacteria has been dominated at 48 hours of fermentation with a value of 5.2 X 10^7 CFU/ml and corresponded to the score of sensory properties. A total of six lactic acid bacteria species were isolated and revealed that Lactobacillus fermentum and Lactobacillus rhamnosus is the potential lactic acid bacteria cocoa starter culture.*

Keywords: Cocoa fermentation, shallow box, flavour, lactic acid bacteria, starter cultures.

INTRODUCTION

Cocoa is one of the agricultural commodities in the world and being traded as the fourth in Malaysia (InvestorGuide, 2017; Abdel Hameed and Arshad, 2014). The pod contain about 20 - 50 of seeds which surrounded by a mucilaginous pulp and embedded to a placenta (Zhang and Motilal, 2016). The cocoa seeds have to be processed through fermentation, drying and subsequently followed by roasting, before a unique chocolate flavour is fully developed (Aprotosoaie *et al*., 2016). Among the process, fermentation is a crucial process because it involves enzymatic reaction on the carbohydrate, protein, fat and polyphenols. As a result, the fermentation process produces the precursors of cocoa-specific flavour such as reducing sugars, peptides, amino acids (Voigt and Lieberei, 2014, Romero-Cortes *et al*., 2013) as well as key volatile fractions such as alcohols, esters and fatty acids (Aprotosoaie *et al*., 2016).

Fermentation is begins spontaneously as soon as the cocoa seeds expose to the environment, allows the growth of microorganisms which exist either from a soil, air, dust, banana and plantain leaves as well as gunny used to cover fermenting mass, the utensils and equipment used, the fruit fly, workers hands or husk (De Vuyst and Weckx, 2016; Teng-Sing *et al*., 2016; Hamdouche *et al*., 2015). At the beginning of exposure, the pulp has a relatively low pH (3.0 - 4.0) as well as low oxygen tension where mainly due to the content of 0.5 - 2% citric acid and thickness of the pulp. This condition as well as high sugar content, including pectin and saccharides, provides an excellent medium for a yeast population to flourish on the pulp for about

first 24 to 48 hours of fermentation (De Vuyst and Weckx, 2016; Papalexandratou *et al.*, 2013). The changes of the cocoa pulp physicochemical during the fermentation progress make the new pulp environment more convenient for lactic acid bacteria to grow and become as coexistence with the yeast during the process in between 24 to 72 hours of fermentation (De Vuyst and Weckx, 2016; Ho *et al*., 2015; Crafack *et al.*, 2013). The existence of the lactic acid bacteria continues to change the composition of the cocoa pulp physicochemical and as consequent influences the microbial succession and in which favour the acetic acid bacteria (AAB) to growth (De Vuyst and Weckx, 2016; Ho *et al*., 2014; Papalexandratou *et al*., 2013). Although the succession of the microbial during fermentation is well documented, only yeast diversity during shallow box fermentation was intensively studied. The diversity of the lactic acid bacteria during shallow box fermentation is still limited, thus this project was carried out to study the diversity of lactic acid bacteria during shallow box fermentation and isolate dominant species which could be used as the potential starter cultures.

MATERIALS AND METHODS

Cocoa Fermentation and Drying

Ripe cocoa pods of mixed clones were harvested from the farm of Cocoa Research and Development Centre (CRDC), Bagan Datuk, Perak, Malaysia. A cocoa fermentation was carried out according to the recommendation for five days using the shallow box measuring 30 x 30 x 30 cm^3. The pods were split open by machete within 24 hours harvested. A total of 25 kg of healthy cocoa seeds was placed in each the boxes and covered with a clean gunny sack. The cocoa beans were mixed once at 72 hours by transferring the fermented beans mass from one box to another. Sampling were carried out at predetermined duration 0, 24, 48, 72, 96 and 120 hours of fermentation by scooping out the wet fermented beans diagonally from the top, middle and the bottom layer of mass according to Kelvin *et al*., (2013).

A total of 485 g each of the samples was sun-dried by spreading out uniformly over the platform with dimension of 120 x 60 x 3 cm^3. The platform was placed under the transparent roof to avoid rain. Mixing of the cocoa beans was performed at every three hours to ensure the beans uniformly dried. At night, the platforms were covered with gunny to avoid dewdrops. The practices were repeated until the moisture content reduced to about 7.5%.

Physicochemical analysis

The temperature of each fermentation batches was measured by inserting thermometer and data recorded for every 24 hours before sampling as described by Hernández-Hernández *et al*., (2016). In order to measure total soluble solids (pulp) and acidity (pulp and cotyledons), the pulp layer was peeled from the cotyledons of cocoa beans with a scalpel. Subsequently, the total soluble solids of the cocoa pulp was measured as °Brix using a hand held refractometer (Atago PAL-1, Japan) as described by Paddaa *et al*., (2011). Whereas, the pH of the cocoa pulp and cotyledons was measured using a pH meter (Hanna Thermoscientific, USA) according to Ganeswari *et al*., (2015). All the reading was taken in triplicate and the mean value was reported.

Sensory Analysis

Sensory analysis was carried out in at Cocoa Innovative and Technology Centre (CITC), Nilai by seven MCB trained panelist. A quantitative descriptive analysis with a scale of "0" to "10" was used. Whereby, “0” indicated the absence or minimum while “10” indicated the maximum intensity. The cocoa liquors which were prepared from each of dried samples and placed in a small glass covered with aluminium foil before randomly labeled with selected 3-digit numerical code. In addition, the commercial Ghanaian cocoa liquor prepared by the Barry callebaut, Malaysia was used as reference sample.

Isolation of Lactobacillus

A total of 5 g each of the samples was placed into a sterile falcon tube containing with 15 ml of 0.1% peptone water as soon as sampling has been done. Next, the samples were homogenized vigorously for 3 minutes by using a vortex. Prior to serial dilution, the mixtures were filtered through sterile

gauze to remove the pulp from the microbiological suspension. Then, 100 µl of the filtered mixture was serially diluted at 10-fold dilution for seven serial with 0.1% peptone water. Twenty (20) µl of each the dilutions were spread onto De Man, Rogosa and Sharpe (MRS) media containing cycloheximide (0.05 g\L) and incubated at 32^{0}C. After 72 hours of incubation, the colony-forming unit (CFU) was counted for each of the dilutions. The colonies were subculture by re-streaking onto new MRS media containing cycloheximide and incubated according to the previous condition until a pure single colony obtained. Afterwards, the pure single colonies were observed for colony shape, elevation, margin and opacity (Figure 1.0). The colony morphology is a method to identify the differences characteristics of colony growth on an agar plate and use as a routine procedure, especially in clinical laboratories (Sousa *et al*., 2013). The opacity of colony means either the colony was visually inspected as transparent (clear), opaque (not transparent or clear), translucent (almost clear, but distorted vision–like looking through frosted glass) or iridescent where the colours changing in reflected light (Reynolds, 2018). The differentiated colonies were selected for further identification by molecular analysis.

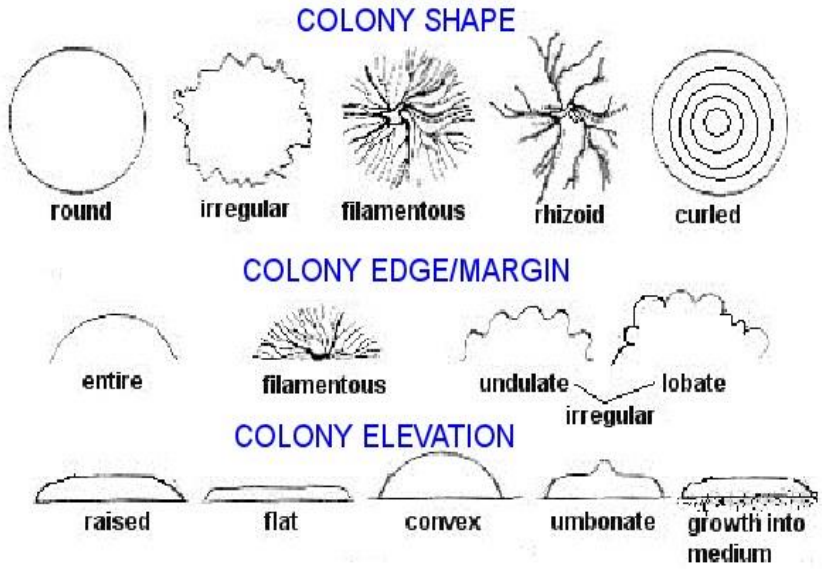

Figure1.0: Characteristics of colony morphology. (Source: Reynolds, 2018).

Identification of Lactobacillus

Identification of Lactobacillus was performed by using general primers LbLMA1-rev (5′-CTC AAA ACT TTC AAA CAA AGT-3′) and R16-1 (5′-CTT GTA CAC ACC GCC CGT CA-3′) for lactobacilli based on method described by Dubernet *et al*., (2002). The pure single colony was subcultured in 1.5 mL MRS broth media and the DNA extracted using DNA extraction kit (NucleoSpin) following the instruction given by the manufacturer. The PCR reaction mixtures were prepared in 50 µl volume containing with 100 ng of DNA per reaction. The amplification was carried out in a thermal cycler (BioTeke) with PCR conditions were 95°C (initial denaturation) for 5 min, followed by 30 cycles of 95°C (30 sec), 55°C (30 sec), and 72°C (30 sec), and final elongation at 72°C for 7 minutes.

The PCR products were electrophoresis on 1.0% (wt/v) agarose gel in 1X TAE buffer at constant voltage 70 V. The size and concentration of amplified products was estimated using 100 bp molecular markers (KAPPA). The gel was stained

with ethidium bromide (5 µg mL–1) for photo documention using Syngene Bio Imaging. After that, the amplified products were purified and submitted to 1st BASE (Malaysia) for reverse and forward sequencing. The sequences obtained were checked for 'noise' before aligned together and compared for species similarity using BLAST database (National Centre for Biotechnology Institute).

Statistic analysis

Statistical analyses were carried out using Minitab version 16.1.0. Comparison of treatment means and significant differences ($P < 0.05$) between treatments means separated were assessed using Tukey Method and reported as means ± standard error of the mean.

RESULT AND DISCUSSION

Table 1.0 shows the physicochemical changes during cocoa fermentation using shallow box and indicated that the onset of the fermentation. The temperature of fermenting mass was significantly increased from 30.7 ± 0.6 to 46.3 ± 0.6 after 96 hours of fermentation and decreased. The pH of cocoa pulp was initially decreased from 4.32 ± 0.02 to 3.89 ± 0.02 after 24 hours of fermentation, and then gradually increased to become less acidic towards the end of cocoa fermentation with a final pH 5.44 ± 0.04. In contrast, initially the pH of cotyledons was 6.56 ± 0.02 and after 72 hours decreased to 4.86 ± 0.01. The pH was subsequently increased until at the end of cocoa fermentation the final pH reached at pH 5.20 ± 0.01. The total soluble solids of the cocoa pulp were decreased from 18.80 ± 0.89 to 12.67 ± 1.41 at the final of fermentation.

Table 1.0. The physicochemical changes during cocoa fermentation using shallow box.

Fermentation Duration (Hour)	Temperature (^{o}C)	pH		TSS (^{O}Brix)
		Pulp	Cotyledons	
0	30.7 ± 0.6^{d}	4.32 ± 0.02^{c}	6.56 ± 0.02^{a}	18.80 ± 0.89^{a}
24	35.7 ± 1.2^{c}	3.89 ± 0.02^{b}	5.85 ± 0.01^{b}	15.93 ± 1.10^{ab}
48	42.3 ± 0.6^{b}	4.30 ± 0.03^{c}	5.12 ± 0.01^{d}	12.23 ± 2.4^{b}
72	45.0 ± 0.0^{a}	4.26 ± 0.04^{c}	4.86 ± 0.01^{f}	13.37 ± 1.69^{b}
96	46.3 ± 0.6^{a}	4.47 ± 0.01^{b}	5.05 ± 0.00^{e}	13.03 ± 1.82^{b}
120	44.3 ± 1.5^{ab}	5.44 ± 0.04^{a}	5.20 ± 0.01^{c}	12.67 ± 1.41^{b}

Means (n=3) followed by different alphabet within the same column were significantly different at Tukey 95% simultaneous confidence intervals.

The increasing in temperature during fermentation is reported as a result of the biological activities of microbial. The yeasts cause a typical alcoholic fermentation occurs by metabolizing pulp sugars and citric acid to ethanol, carbon dioxide, glycerol, acetic acid, succinic acid, and heat. The resulting heat is enough to cause the increasing of mass temperature from an ambient temperature between 25 - 30 °C to 35 - 40 °C within 48 hours (De Vuyst and Weckx, 2016; Ho *et al*., 2014; Papalexandratou *et al*., 2013). The temperature further increased as the lactic acid bacteria use citric acid as a co-substrate and convert it into lactic acid during heterolactate fermentation. Additionally, the exothermic oxidation of ethanol to acetic acids by acetic acid bacteria and better aeration condition liberates more heat, hence rising of mass temperature up to 45 - 50 °C (De Vuyst and Weckx, 2016; Moens *et al*., 2014). The rising of temperature beyond 45 °C is unfavourable not only to the acetic acid bacteria hence resulting in a decline of all microorganisms except for spore-forming aerophilic bacteria types or bacilli. Starting from that moment onward, the bacilli comprise over 80% of microflora and dominate the mass environment. At this phase of fermentation, the pulp layer is totally depleted hence the fermenting mass becomes more aerobic and

together with water formation as well as good aeration promotes the temperature decrease. Decreased of temperature is suggested as an indicator for fermentation to be ended Hernández-Hernández *et al*., (2016).

Conversion of citric acid into lactic acid resultant slightly increases in pH of the pulp as well as changing the composition of the fermenting mass. In addition, the decreasing trend of pH in cocoa pulp may due to the increase of metabolites such as ethanol, glycerol, lactic acid, acetic acid and succinic acid which are derived by utilization of glucose, fructose and citric acid in the pulp by microbes during the fermentation. While, the dripping of the liquefy pulp or the diffusion of the metabolites into the nib which known as nib acidification may be the reason for the pH in the cocoa pulp to increase (De Vuyst and Weckx, 2016).

The scores for the sensory properties of all the liquors prepared from the samples of dried cocoa beans samples are presented in Table 2.0. The result demonstrated that the cocoa liquor prepared from the dried cocoa beans which fermented for 48 hours has the highest intensity of cocoa flavour. The score was lower compared to the commercial Ghanaian cocoa liquor. On the other hand, the scores for bitterness and astringent were slightly higher compared to the commercial Ghanaian cocoa liquor but within reasonable intensity after the cocoa beans have been fermented for 24 hours. While the acidity or sourness of the cocoa beans samples was slightly better than the commercial Ghanaian cocoa liquor.

Table 2.0: Sensory properties for all the liquors from the dried samples of cocoa beans.

Fermentation Duration (Hours)	Score of Flavour Properties			
	Cocoa (7.0)*	Bitter (2.5)*	Astringent (3.0)*	Acid/sour (1.5)*
0	1.8 ± 0.9^{c}	6.4 ± 1.3^{a}	6.1 ± 0.9^{a}	0.9 ± 0.9^{c}
24	3.7 ± 0.9^{b}	4.7 ± 1.1^{b}	4.6 ± 1.0^{b}	1.5 ± 0.8^{bc}
48	5.7 ± 0.7^{a}	4.2 ± 0.8^{b}	4.2 ± 0.8^{b}	2.4 ± 0.5^{ab}
72	4.4 ± 0.4^{b}	4.3 ± 0.9^{b}	4.1 ± 0.9^{b}	2.7 ± 1.3^{ab}
96	3.8 ± 0.8^{b}	4.1 ± 0.6^{b}	4.4 ± 0.8^{b}	2.4 ± 0.9^{ab}
120	4.4 ± 0.5^{b}	4.1 ± 0.5^{b}	4.1 ± 0.7^{b}	3.0 ± 1.0^{a}

Means (n=7) followed by different alphabet within the same column were significantly different at Tukey 95% simultaneous confidence intervals. (Number)* denote as predetermined score by the MCB trained panelist for the commercial Ghanaian cocoa liquor. Definition of scores was given by MCB: 0: Not presence, 1: Very slightly presence, 3: Moderate, 4: Reasonable, 5: Present, 6: Strongly present, 7: Very strongly present and 9: Extremely present.

Table 3.0 shows the total colony count of lactic acid bacteria on the MRS agar during the fermentation process. The lactic acid bacteria population was started to grow after 24 hours of fermentation. The population reached the highest 5.2×10^{7} CFU/mL of total colony count at 48 hours of fermentation. Afterwards, the population dropped until at the end of the fermentation with the final 1.2×10^{4} CFU/mL of total colony count. Among the lactic acid bacteria population, six different single colonies were isolated base on the characteristics of colony morphology (Table 4.0). In this study, four of the isolated colonies were classified as irregular, while the remaining two are circular. Isolation of the bacteria colonies which is based on colony morphology before the identification of lactic acid bacteria species through the polymerase chain reaction (PCR) analysis reduces hassle and cost saving.

Identification of lactic acid bacteria species through the PCR analysis was using a set of primers LbLMA1-rev (5′-CTC AAA ACT TTC AAA CAA AGT-3′) and R16-1 (5′-CTT GTA CAC ACC GCC CGT CA-3′). The LbLMA1-rev is a 21-mer primer developed by Dubernet *et al*., (2002) based on 16S/23S ribosomal RNA intergenic spacer region which starting at position 70 of Lactobacillus acidophilus (U32971). Whereas, the R16-1 is universal primer corresponding to the flanking terminal sequence of the 16S rRNA gene and conserved among various bacteria, including lactobacilli. Amplification with LbLMA1-rev and R16-1 has generated five PCR products with a size of approximately 250 bp and missing of PCR product for LAB2. Sequencing analysis revealed that the isolated lactic acid bacteria species were *Lactobacillus fermentum* (LAB1), *Lactobacillus plantarum* (LAB4), *Lactobacillus delbrueckii subsp. Bulgaricus* (LAB6), *Lactobacillus rhamnosus* (LAB3) and *Lactobacillus acidophilus* (LAB5). Table 5.0 indicates the appearance of the lactic acid bacteria species at different duration during fermentation using shallow box. The study also revealed that the *Lb. fermentum* and *Lb. rhamnosus* have been dominated at the 24 hours of fermentation.

Table 3.0. Total colony count of the lactic acid bacteria on the MRS agar.

Fermentation Duration (Hours)	**0**	**24**	**48**	**72**	**96**	**120**
Total colony (CFU/mL)	0	4.59×10^7	5.2×10^7	2.0×10^6	6.0×10^4	1.2×10^4

Table 4.0. Morphological characteristics of the isolated lactic acid bacteria on the MRS agar.

Isolate	**Morphology**			
	Opacity	**Shape**	**Elevation**	**Margin**
LAB1	Translucent	Circular	Convex	Entire
LAB2	Transparent	Irregular	Raised	Lobate
LAB3	Transparent	Irregular	Flat	Lobate
LAB4	Opaque	Circular	Raised	Entire
LAB5	Transparent	Irregular	Convex	Undulate
LAB6	Transparent	Circular	Raised	Entire

Table 5.0: Appearance of the lactic acid bacteria species during fermentation using shallow box.

Species	**Isolate**	**24**	**48**	**72**	**96**	**120**
Lactobacillus fermentum	**LAB1**	/	/	/	/	/
Lactobacillus rhamnosus	**LAB3**	/	/			
Lactobacillus plantarum	**LAB4**	/	/	/		
Lactobacillus acidophilus	**LAB5**			/		
Lactobacillus delbrueckii subsp. bulgaricus	**LAB6**	/	/	/	/	/

CONCLUSION

As a conclusion, this study has revealed that at least five species of lactic acid bacteria involve in the

diversity of lactic acid bacteria during cocoa fermentation using shallow box. Based on the physicochemical changes, sensory profiles and the total colonies count during fermentation suggested that *Lactobacillus fermentum* and *Lactobacillus rhamnosus* could be used as the potential starter cultures.

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