Identification of lactic acid bacteria and acetic acid bacteria of lampung cocoa beans fermentation

Fahrurrozi1, Stefanie2, A P Purwanto1, D Y Sofia2, S Ratnakomala1

1Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Jl. Raya Bogor KM. 46, Cibinong 16911, West Java, Indonesia

2Department of Biotechnology, Faculty of Life Sciences, Surya University, Great Western Resort Km 2.7 Lt.1 Jalan MH. ThamrinSerpong, Panunggangan Utara, Pinang Kota Tangerang.

E-mail:fahrurrozi@lipi.go.id

Abstract. The quality of chocolate influenced by cocoa varieties, growth conditions, and fermentation. As a part of post-harvesting treatments, cocoa beans fermentation affects biochemical transformations resulting in the aroma, color, and taste of chocolate. Lactic acid bacteria (LAB) and acetic acid bacteria (AAB) along with yeasts are involved during during cocoa beans fermentation. In this study, the diversity of LAB and AAB of cocoa beans fermentation from Lampung will be identified. Fortykgs of cocoa beans undergo spontaneous box fermentation for five days, with 24 hours intervals of sampling. DeManRogosa Sharpe (MRS) Agar and Glucose Yeast Peptone (GYP) Agarwere used for LAB and AAB enumeration, respectively. Morphology of each colony were identified and molecular identification by analyzing 16S rRNA gene. As results, the growth of LAB reach its highest level on the zero and fifth day of fermentation to $10^6$ CFU/mL and the growth of AAB reach $10^6$ CFU/mL as well. Lactobacillus plantarum, L. fermentum and L. pentosus are the main species of lactic acid bacteria, and Acetobacterpasteurianus and A. tropicalis as the main species of acetic acid bacteria. These results describe more about the diversity of Indonesian cocoa that could be applied for starter culture development.

1. Introduction

Indonesia is the third biggest cocoa producer in the world. In 2018, the production of cocoa bean in Indonesia reached to 593.833 tons/year, with several major provinces as producers such as Central Sulawesi, South Sulawesi, Southeast Sulawesi, West Sumatra, West Sulawesi and Lampung [13]. In 2018, the production of cocoa beans from Lampung reached to 5.9% of total cocoa production in Indonesia [13]. Cocoa is one of the main plantation commodities in Lampung, in addition to coconut, oil palm, rubber, cassava and coffee [7].

Chocolate has a different taste because it is influenced by the genotype of cocoa beans, growth conditions and post-harvest processes[1]. One of the critical steps in the post-harvest handling is fermentation. Fermentation affects the biochemical changes in cocoa beans to form the aroma, color and taste of chocolate. Without this process, cocoa beans feel bitter and will not produce chocolate flavor characteristics during further processed [15]. The bitter taste in chocolate caused by polyhydroxyphenols such as catechins, flavan-3-ol, anthocyanin and proanthocyanidin[14]. The
fermentation process is causing the death of bean, initiation of taste formation, discoloration and reduction of bitter taste [1]. Fermentation can also activate the hydrolytic enzyme of seeds that catalyzes biotransformation reactions in beans that express the potential flavor of cocoa beans [18]. Microbes in the fermentation process play an important role in producing acids and alcohol, which will penetrate the placenta and cause cell death. After cell death, biochemical reactions will occur and form precursors to the aroma of chocolate [14].

The main group of microbial populations in cocoa fermentation consists of yeast, lactic acid bacteria and acetic acid bacteria [15]. In the early days of fermentation, yeast will use carbohydrates from the pulp to produce ethanol. Hydrolyzing of carbohydrates by yeast during the first two days of fermentation cause an environmental suitable for lactic acid bacteria. During the fermentation process, the mixing of cocoa beans mass causes environmental conditions to be aerobic [15]. This condition is suitable for the growth of acetic acid bacteria. Acetic acid bacteria metabolize ethanol produced by yeast into acetic acid through an exothermic process [11].

Variations of microbial biodiversity can affect the taste and quality of cocoa [20]. Microbial diversity is different among various regions and countries. Saccharomyces cerevisiae and Pichia kluyveri were found in cocoa fermentation from Ivory Coast and Ghana, while in Ecuadorian cocoa, species P. manshurica identified [8,9,11,12]. In addition, in cocoa fermentation from Malaysia, Ghana and Ecuador, there were also identified species of Lactobacillus fermentum, L. plantarum, Acetobacter pasteurianus and Acetobacter tropicalis [4,10,11,12]. Some species of Bacillus spp. also found near the end of fermentation [11]. Lampung is one of the highest cocoa producing regions in Indonesia and base of several chocolate producers company. To our knowledge, no studies have been identified the microbial diversity of Lampung cocoa beans fermentation.

2. Methods

2.1. Cocoa Fermentation

Forty kg of cocoa beans undergo spontaneous box fermentation in Pringsewu Regency, Lampung. The box made of wooden and was 55 x 55 x 55 cm in size. The box was covered with a banana leaf on the inside. Spontaneous fermentation occurred for five days with 24 hours intervals of sampling, 35 grams each. Fermentation process repeated twice.

2.2. Microbial analysis

Ten grams of cocoa beans diluted in 90 mL 0.85% sodium chloride solution. 1 mL of this solution added to 9 mL of 0.85% sodium chloride solution as 10⁻² dilution. The dilution continuous until 10⁻⁶ dilution. 100 µL of the 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilution would be inoculated to DeManRogosa Sharpe Agar (Merck) for growth of lactic acid bacteria, containing 0.5% of CaCO₃, Glucose Yeast Peptone Agar for growth of acetic acid bacteria, containing 0.3% of NaCl. Plates were incubated at 30°C for 5 days. Colonies were counted by each morphological type. Three representatives of each type of colony was streaked to new medium. Purified colonies then characterized by gram and catalase test. Gram test was done with 3% of potassium chloride solution. Catalase test was done by 3% of H₂O₂ solution.

2.3. Molecular identification

The pure cultures then identified with analysis of 16S rRNA gene with direct colony PCR method. The DNA were amplified with 27F (5’-AGA GTT TGA TCM TGG CTC AG-3’) and 1492R primer (5’- TAC GGY TAC CTT GTT ACG ACT T-3’). 25 µL TE 1X buffer was added to the culture, then incubated with dry bath at a temperature of 95°C for 2 minutes. Then the microtube was centrifuged at 3000 rpm for 60 seconds. 1 µL of the supernatant was taken to be used as DNA template. The bacterial colonies were amplified by PCR kit MyTaqTM HS Red Mix (Bioline, London, United Kingdom). PCR was carried out in 30 cycles with initial denaturation at 96°C for 90 seconds, denaturation at 96°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 90 seconds. The cycle is accompanied by a final extension at 72°C for 5 minutes. DNA sequencing was done by 1st base (Axil Scientific, Singapore). The sequence is then aligned with the ClustalW method.
with MEGA 7 software. The aligned sequence was identified by the BLAST algorithm [2] and database from EzBioCloud[21]. The phylogenetic tree construction was carried out using MEGA 7 software, using the neighbor-joining algorithm and Kimura-2 parameter, with 1000 replication bootstrap analysis.

2.4. Determination of fermentation index
The quality of fermentation was determined by fermentation index. A quarter grams of mashed fermented cocoa beans was diluted in 25 mL of 70% methanol and concentrated hydrochloric acid with the ratio of 97:3. Sample then macerated for 22 hours in 4°C. The fermentation index determined by the ratio of 460 nm and 530 nm absorbance. The cocoa bean considered as sufficiently fermented if the index value is more than one [25].

3. Results and Discussion
3.1. Population of Lactic Acid Bacteria

![Figure 1. Population of lactic acid bacteria during cocoa beans fermentation](image)

The population of lactic acid bacteria varies from day to day. The growth reach its highest level on the zero and fifth day of fermentation to $10^6$ CFU/mL. The growth decrease on the first and second days of fermentation to $10^5$ CFU/mL. On the third day, the population of lactic acid bacteria decreased to $10^4$ CFU/mL and there was no growth in the fourth day. The population increased on the fifth day of fermentation reaching $10^6$ CFU/mL (Figure 1).

The quality of cocoa affect by cocoa varieties, growth condition, fermentation method, and microbial diversity of cocoa bean fermentation. In cocoa bean fermentation, ethanol and acids produced by microbes will penetrate testa and caused bean death, which leads to biochemical reaction that form precursor of chocolate flavour [14]. The diversity of microbes affects cocoa flavour and quality [20]. By knowing the microbial diversity of cocoa bean fermentation, the cocoa quality could be estimated, thus produce consistent quality of cocoa and could be applied for cocoa fermentation starter culture development [3,9,11,12].

The growth of lactic acid bacteria is optimal on the first to the second day because lactic acid bacteria utilize glucose contained in the early days of fermentation to produce lactic acid, acetic acid and ethanol [14]. The decline in the population of lactic acid bacteria on the third and fourth days...
probably due to a temperature increase of up to 45°C (data not shown). On the fifth day of fermentation, lactic acid bacteria that grow may be a species that is more tolerant of ethanol and acid so that it can grow again [10].

3.2. Population of Acetic Acid Bacteria

The population of acetic acid bacteria on zero day of fermentation reached $10^5$ CFU / mL, then increased to $10^6$ CFU / mL on the first day of fermentation. The population of acetic acid bacteria decreased on the second and third days to $10^4$ CFU/mL, then again increased to $10^6$ CFU/mL at the end of fermentation (Figure 2).

The growth of acetic acid bacteria reach its highest level at the end of fermentation. This may be due to high level of oxygen at the end of fermentation because of bean turning during fermentation that favoured the growth of acetic acid bacteria [14].

3.3. Diversity of Lactic Acid Bacteria

After morphological analysis, there are 62 isolates of putative isolates of lactic acid bacteria. Representation of 16 lactic acid bacteria was taken randomly for molecular identification. Based on BLAST analysis of the 16S rRNA gene sequence, the three types of lactic acid bacteria found were Lactobacillus plantarum, Lactobacillus pentosus and Lactobacillus fermentum. L. plantarum is the most abundant species (10 isolates), followed by L. pentosus (5 isolates) and L. fermentum (1 isolates). The distribution of lactic acid bacteria species found during cocoa beans fermentation shown in Table 1. The species L. plantarum can be found in the early stages of fermentation final stage of fermentation. However, species of L. plantarum can also grow less and only on the second and fifth days. The L. pentosus species was found in first, second and fifth day of fermentation.

![Figure 2. Population of acetic acid bacteria during fermentation](image)

Table 1. Species and the number of lactic acid bacteria found in cocoabean fermentation from Lampung.

| Species         | Number of Species per Day of Fermentation |
|-----------------|------------------------------------------|
| L. plantarum    | 10                                       |
| L. pentosus     | 5                                        |
| L. fermentum    | 1                                        |


The biodiversity of lactic acid bacteria of Lampung cocoa during spontaneous fermentation were identified. There are three species of lactic acid bacteria with *L. plantarum* as the dominating species. *L. plantarum* species can be found in fermented cocoa from several regions and its growth can be identified almost throughout the fermentation period [4,10]. However, species of *L. plantarum* can also grow less and only on the second and fifth days, as identified in Ecuadorian cocoa fermentation [12].

Species of *L. plantarum* and *L. fermentum* were also found in cocoa fermentation from Ivory Coast, Ghana, Ecuador and Malaysia [4,10,12,16]. *L. pentosus* is also found in cocoa fermentation from Australia [6]. In cocoa fermentation from the Dominican Republic, *L. pentosus* was identified in the first day of fermentation until the fourth day [5]. Species *L. fermentum* was found on the final stage of fermentation. This result is different to the species *L. fermentum* in Ecuadorian cocoa fermentation which was identified throughout the fermentation period [12]. *L. fermentum* is a species that ethanol, heat and high acid tolerant and this may explain its appearance through the end of fermentation [10]. However, because molecular identification is only carried out on 16 isolates, the distribution and number of species can be increased if identification is carried out on all isolates. Species *L. plantarum* known as homofermentative species and produce high amounts of lactic acid. *L. fermentum* is a heterofermentative species and convert citric acid to lactic acid and acetic acid [23].

### 3.4. Diversity of Acetic Acid Bacteria

After morphological analysis, the number of putative isolates of acetic acid bacteria were 20 isolates. 11 representative isolates of acetic acid bacteria were taken randomly for molecular identification. Based on the results of the BLAST analysis of 24 sequences of 16S rRNA genes, there were two species of acetic acid bacteria identified, *Acetobacter pasteurianus* and *Acetobacter ghanensis*. Ten of eleven isolates were identified as *A. pasteurianus* and 1 isolates identified as *A. ghanensis*. The distribution of the found species of acetic acid bacteria per type of fermentation is described in Table 2. *A. pasteurianus* can be found on the fourth and fifth day of fermentation. *A. ghanensis* was found on the second day of fermentation.

| Species       | 0  | 1  | 2  | 3  | 4  | 5  |
|---------------|----|----|----|----|----|----|
| *A. pasteurianus* |   |    |    |    | 1  | 9  |
| *A. ghanensis*   | -  |    |    |    |    |    |

Abbreviations: A: *Acetobacter*; 0-5: fermentation on day zero to five.

The biodiversity of acetic acid bacteria of Lampung cocoa during spontaneous fermentation were identified. There are two species of acetic acid bacteria, with *A. pasteurianus* as the dominating species. *A. pasteurianus* is a species found in many other regions of cocoa fermentation, such as in Ghana, Malaysia, Brazil, Ecuador and in East Java [3,4,10,11,12,19,22]. Meanwhile, species of *A. ghanensis* have been found in cocoa fermentation from Ecuador and Brazil [12,22]. In other areas of...
cocoa fermentation, *A. pasteurianus* species can be identified throughout the fermentation period, such as Malaysian cocoa fermentation, and can be identified only on the second and third days, as in Ghana's cocoa fermentation [10,11], whereas in Ecuadorian cocoa fermentation, *A. ghanensis* species can be identified on the first to third fermentation [12].

Acetic acid bacteria generally grow on the first day until the fourth day of the fermentation period because of the aeration and utilization of ethanol, citric acid and carbohydrates to produce acetic acid. In this study, *Acetobacter* species were found from the second day to the fifth day of fermentation. In the early stages until the first day of fermentation, no acetic acid bacteria were found based on the results of gram determination, catalase test, clear zone formation and morphological characteristics. This might be because acetic acid bacteria are more difficult to grow in the early stages of fermentation because of the lack of oxygen [20]. Acetic acid bacteria is also more heat and ethanol resistant, thus they can be identified in later stages of fermentation [4]. In cocoa bean fermentation, *A. pasteurianus* and *A. ghanensis* has the ability to oxidize ethanol, mannitol and lactic acid. Ethanol was oxidized into acetic acid, mannitol was oxidized into fructose and lactic acid was oxidized into acetoin [24].

The fermentation was sufficiently done as the fermentation index value is 1.55. The intensity of brown color determined at 460 nm and the intensity of purple color determined at 530 nm. It suggests that the ratio of brown color of the fermented cocoa beans is higher than the purple ones, which indicates a fair fermentation process [25].

3.5. Phylogenetic analysis of lactic acid bacteria and acetic acid bacteria

Sequencing results of lactic acid bacteria were aligned with the ClustalW method with MEGA 7 software. The sequences were then identified with the BLAST algorithm and database on EzBioCloud. The results of the phylogenetic tree construction are shown in Figure 3. A total of 16 bacterial isolates were identified as *Lactobacillus* genus, with a percentage of similarity not less than 99%. A total of 10 isolates were affiliated with species *L. plantarum*, 5 isolates were affiliated with *L. pentosus* and 1 isolates were affiliated with species *L. fermentum, L. delbrueckii* and *Escherichia coli* species are used as outgroup species.
Figure 3. Phylogenetic tree shows the affiliation of lactic acid bacteria species. Phylogenetic trees are constructed using the neighbor-joining method and Kimura-2 parameter. Species of *Lactobacillus delbrueckii* and *Escherichia coli* were used as outgroup.

Sequencing results of acetic acid bacteria were aligned with the ClustalW method with MEGA 7 software. The sequences were then identified with the BLAST algorithm and database on EzBioCloud. The results of the phylogenetic tree construction are shown in Figure 4. A total of 11 bacterial isolates were identified as *Acetobacter* genus acetic acid bacteria, with a percentage of similarity not less than 99%. A total of 10 isolates were affiliated with species *A. pasteurianus* and 1 isolate was affiliated with species *A. ghanensis*. *A. acetii* and *Escherichia coli* species are used as outgroup species.
Figure 4. Phylogenetic tree shows the affiliation of acetic acid bacteria species. Phylogenetic trees are constructed using the neighbor-joining method and Kimura-2 parameter. *Escherichia coli* used as outgroup.

The dendogram showed the affiliation between species of genus *Lactobacillus* and *Acetobacter*. In figure 5, there were two clusters consist of *L. plantarum* that related to *L. pentosus* and cluster of *L. fermentum*. Figure 6 shows the affiliation of *A. pasteurianus* and *A. ghanensis*.

4. Conclusion
Diversity of microbes in Lampung cocoa fermentation consist of three species of lactic acid bacteria; *L. plantarum*, *L. pentosus*, and *L. fermentum*. Identified species of acetic acid bacteria are *A. pasteurianus* and *A. ghanensis*. These species are commonly found in fermented cocoa from other countries. Although it shows the name of the same species, the distribution of these species is different from the fermentation of cocoa from other regions. Species of *L. plantarum* and *A. pasteurianus* are suggested as parts of cocoa bean starter culture.

5. Acknowledgements
We would like to thank Indonesia Institute of Sciences (LIPI) who provided research and working facility.

6. References
[1] Afoakwa EO 2016 Chocolate Science and Technology, Second Edition. Chichester: John Wiley & Sons.
[2] Altschul SF, Madden TL., Schäffer A A., Zhang J., Zhang, Z., Miller W., & Lipman D J. 1997 *Nucleic Acids Research* 3389-3402.
[3] Ardhana, M M & Fleet G H 2003 *International Journal of Food Microbiology* 86 87-99
[4] CamuN., Winter T D., Verbrugghe K., Cleenwerck I., Vandamme P., Takrama JS, Vuyst, L D
2007 *Applied and Environmental Microbiology* 1809-1824

[5] Gálvez, S L, Loiseau G, Paredes J L, Barel M & Guiraud JP 2007 *International Journal of Food Microbiology* 124-130

[6] Ho V T, Zhao J & Fleet G 2015 *International Journal of Food Microbiology* 205 54-67.

[7] Indonesia B 2013 Kelayakan Usaha Komoditi Unggulan UMKM. Retrieved from Info UMKM BankIndonesia: https://www.bi.go.id/id/umkm/kelayakan/komoditi/Default.aspx?Prov=Lampung

[8] Jespersen L, Nielsen D S, Hønholt S & Jakobsen M 2005 *Federation of European Microbiological Societies Yeast Research* 441-453

[9] Koffi O, Samagaci L, Goualie B & Niamke S 2017 *European Scientific Journal* 496-516.

[10] Meersman E, Steensels J, Mathawan M, Wittoecx PJ, Struyf V S & Bernaert H 2013 *Plos One* 8 1-10

[11] Nielsen D, Teniola O, Ban-Koffi L, Owusu M, Andersson T & Holzapfel W 2007 *International Journal of Food Microbiology* 114 168-186

[12] Papalexandratou Z, Falony G, Romanens E, Jimenez J C, Amores F, Daniel HM & Vuyst L D 2011 *Applied and Environmental Microbiology* 7698-7714

[13] Pertanian K2018Statistik Perkebunan Indonesia 2017-2019. Jakarta: Kementrian Pertanian.

[14] Schwan R F & Wheals A E 2004 *Critical Reviews in Food Science and Nutrition* 44 205-221

[15] Thompson S S, Miller K B & Lopez A S 2007 Cocoa and Coffee. In M. P. Doyle, & L. R. Beuchat, *Food Microbiology: Fundamentals and Frontiers*, Third Edition (pp. 837-850). Washington D. C.: ASM Press.

[16] Visintin S, Alessandria V, Valente A, Dolci P & Cocolin L 2016 *International Journal Food Microbiology* 216 69-78

[17] Vos PD, Garrity G M, Jones D, Krieg N R, Ludwig W, Rainey F A, Whitman W B 2009 *Bergey's Manual of Systematic Bacteriology*, Volumer Three. New York: Springer.

[18] Vuyst L D & Weckx S 2016 *Journal of Applied Microbiology* 5-17.

[19] Moreira IM, Miguel M G, Duarte W F, RibeiroDias D & Schwan R F 2013 *Food Research International* 9-17

[20] Schwan R F, Pereira G V & Fleet G H 2015 Microbial Activities during Cocoa Fermentation. In R. F. Schwan, & G. H. Fleet, *Cocoa and Coffer Fermentations* (pp. 129-192). Boca Raton: CRC Press.

[21] Yoon SH, Ha SM., Kwon S, Lim J, Kim Y, Seo H & Chun J. 2017 *International Journal of Systematic and Evolutionary Microbiology* 1613-1617

[22] Papalexandratou Z, Vrancken G, Bruyne K D, Vandamme P & Vuyst L D 2011 *Food Microbiology* 28 1326-1338

[23] Pereira G V, Magalhães K T, Almeida E G, Coelho I d & Schwan R F 2013 *International Journal of Food Microbiology* 161 121-133

[24] Moens F, Lefeber T & Vuyst L D 2014 *Applied and Environmental Microbiology* 1848-1857

[25] Jati M 2008 Physico-chemical changes during cocoa fermentation and key enzymes involved *Review Penelitian Kopi dan Kakao* 47-64