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Selection Of Lactic Acid Bacteria as Starter Culture for Cocoa Fermentation (Theobroma Cacao L.)

Hanni Tsaaqifah [1], Fahrurrozi [2], Anja Meryandini [3,4*]

[1] Biotechnologi Study Program, Graduate School, IPB University, Kampus Dramaga Bogor 16680, Indonesia.
[2] Research Center for Marine and Land Bioindustry, Research Organization for Earth Science and Maritime. BRIN. Jl. Raya Senggigi, Kodek Bay, Malaka, Pemenang, Nort Lombok, West Nusa Tenggara, 83352, Indonesia
[3] Biology Department -Faculty of Mathematics and Natural Sciences- IPB-University, Kampus Dramaga Bogor 16680, Indonesia.
[4] Biotech Center, LPPM-IPB University, Kampus Dramaga Bogor 16680, Indonesia.

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Corresponding Author:
Anja Meryandini
ameryandini@apps.ipb.ac.id

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Abstract: This research aims to select the potential lactic acid bacteria (LAB) for starter culture for cocoa beans fermentation. The bio-control activity against mycotoxigenic fungi were also evaluated. The indigenous LAB was analized from Sukabumi cocoa beans fermentation by spontaneous method for four days (96 hours). The preliminary screening of LAB were observed by clear zone forming, morphology characterization and catalase activity. The LAB were screened for their ability to resist acidity, ethanol, and heat, and their ability to produce high acid and antifungal. The antifungal capability was antagonistic assays to *Aspergillus ochraceus* IPBCC 88.033 on Potato Dextrose Agar (PDA) medium. The result showed that the selected acid bacteria isolate, H 2.34, were antifungal-producing strains, and resist acidity, ethanol and heat, which was suitable property for starter culture and biopreservation agent. The study demonstrated the selection of local Indonesian LAB from Sukabumi fermented cocoa bean to observe the potential of isolate as a starter culture and biopreservation agent.

Keywords: Antifungal; Cocoa bean fermentation; Lactic acid bacteria; Starter culture

Introduction

Cocoa is one of the primary commodities in the Indonesian plantation sector. In the international market, Indonesian cocoa beans are often priced low because their postharvest processing needs to be done appropriately, particularly at the farm level (Kresnowati & Febriami, 2015). On the farm level, cocoa bean fermentations were spontaneous and uncontrollable, resulting in end-products of variable quality. Poor postharvest management may decrease the quality of cocoa beans due to the emergence of contaminating mold. Mold such as Aspergillus, Penicillium, Fusarium, and Mucor, which contaminated the cocoa beans, could potentially lead to produce mycotoxins in cocoa beans (Sulistyo et al., 2014). The presence of toxins in cocoa beans is very unfavorable, because in addition to reducing the quality of cocoa beans, it will also damage the health of the organisms that eat them.

Fermentation is an essential step in the cocoa beans postharvest process. The process is important in removing the pulp, stopping the germination, forming precursor aroma and flavor, and inhibiting contamination of pathogenic microorganisms such as mold (Ganeswari et al., 2015; Moreira et al., 2013; Pereira et al., 2012). The role of various consortiums of microorganisms in cocoa fermentation has been discussed by Moreira et al. (2013), which stated that microbial activity in cocoa pulp involves the succession of microorganisms, especially yeasts and bacteria.

Adding a starter culture from microbial species will control the fermentation process of cocoa, thus providing chocolate with a reliable flavor. The Lactic Acid Bacteria (LAB) play a prominent role in cocoa beans fermentation, which can strongly affect the quality and flavor of the final product. The role of LAB in its fermentation is to hydrolyze sugar and citric acid into organic compounds forming the taste precursors of

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cocoa beans, such as lactic acid, acetic acid, glycerol, and mannitol (Salazar et al., 2022). Aunsbjerg et al. (2015) also reported the ability of LAB to organic acids, fatty acids, hydrogen peroxide, proteinaceous compounds that could inhibit mold growth.

A starter culture is a certain number of microorganism cells added to a substance to produce fermentation products. A good starter culture is a culture that is isolated directly from fermentation by a natural process (Nanasombat et al., 2012). Adding a starter culture will control the cocoa fermentation process and shorten fermentation time (Lefeber et al., 2012; Kresnowati et al., 2013). As a starter culture, LAB should be able to survive changes in fermentation conditions. Adding LAB as a starter culture is essential to the improved postharvest process, producing consistent and reliable cocoa bean quality.

The cocoa fermentation process was spontaneous and uncontrollable, causing the finished product quality of cocoa beans to be unstable. The fermentation of cocoa beans in Indonesia is still under uncontrolled conditions that cause the fermentation process to be inconsistent and result in unreliable end product quality. Therefore, research on the potential development of starter cultures needs to contribute to increasing the quality of cocoa beans. This underlies this research in the search for potential LAB isolates as a starter culture and biopreservation agent candidate for cocoa fermentation. State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Method

Cocoa bean fermentation

The cocoa pods were cut to obtain the beans and surrounding pulp. The bean mass was uniformly mixed and deliberately brought into contact with the pods to provide a source of natural microbial inoculum. About 50 kg of beans were placed into a wooden box fermenter underneath the sack to trap the heat generated during fermentation and incubated for 96 hours, which developed spontaneously due to the growth of the indigenous microflora. The fermenting beans were mixed every 24h during fermentation. The fermentations were repeated twice.

Isolation of Lactic Acid Bacteria

LAB was isolated according to Kouame et al. (2015) method. The samples of beans were taken daily (0, 24, 48, and 72 h) for analyses. Cocoa beans (25 g) were aseptically mixed with 50 mL of de Man Rogosa Sharpe (MRS) broth and vigorously shaken and massaged for 1 min to the homogenate. One milliliter of samples was serially diluted in 0.85% NaCl (w/v) solution. Serial dilutions were made for each sample, and then 1 mL of the appropriate dilution (10-1 until 10-4) was spread-inoculated in duplicate on MRS agar plates containing cycloheximide 100 mg/L. Inoculated agar plates were incubated at 37 °C for 48 hours. The colonies with distinct morphologies (color, shape, and texture) were recorded, isolated, and purified by streaking on the MRS agar. Further, preliminary screening of strains for clear zone forming by MRS agar supplemented with calcium carbonate 1% (w/v), cell morphology, and catalase activity.

Selection of LAB for physiological adaptation to fermentation condition (acid, ethanol, and heat tolerance

The tolerance of LAB isolates performed physiological adaptation in the fermentation conditions to grow in acid, ethanol, and heat. All tubes were inoculated with 1% (v/v) of each isolate with optical density (OD660 nm) value 0.6-0.8, and incubated at 37 oC for 4 h. Total viable each LAB strain counted of each tube was analyzed by spread plating the serially diluted samples on the MRS agar (pH 5.7). For pH tolerance, the medium MRS broth was adjusted to pH 4.0, 4.5, and 5.7 with 5 M hydrochloric acids (HCl). Tolerance of each LAB strain was expressed by decreasing the number of LAB cells (CFU/mL).

Ethanol and heat tolerance was done by growing isolates on MRS agar supplemented with 5%, 10%, and 15% ethanol (v/v) and incubated at 30, 37, 40 and 45 °C for 48 hours. One loop of each 24-hour LAB strain was inoculated on the MRS agar containing different ethanol concentrations and incubated at the above temperature. The grown LAB strains were selected. Each test was repeated three times (triplo).

Screening LAB for total acidity level

Total acidity was measured by using NaOH 0.1 N titration method. LAB culture was incubated for 48 hours on an MRS broth and taken 1.5 mL was centrifuged at 3,000×g at 4°C for 20 minutes. Titration was done by dropping NaOH 0.1 M solution. A total of 1 mL supernatant was diluted with 9 mL distilled water and 2-3 drops of phenolphthalein (PP) indicator.

Screening for antifungal activity of LAB isolates using dual culture method

As described by Shi et al. (2014), the antifungal activity of LAB isolates was determined by the dual-culture plate method with slight modification. The microorganism used as an indicator for antifungal activity was *Aspergillus ochraceus* IPBCC 88.033. This fungal was grown on Potato Dextrose Agar (PDA) plates at 30 °C for 5 days, and then transferred 3.0 mm diameter agar with mycelia from the plate in the center of another PDA agar plate. The selected LAB isolates were inoculated two days ahead and 15 mm apart from the center of the plate. The plates were incubated at 30 °C for

7 days. The diameters of *A. ochraceus* were measured. Antifungal activities were expressed as the inhibition rate as shown at formula 1.

$$H: \frac{(rc - r)}{rc} \times 100\% \quad (1)$$

Formula 1: (H: inhibition index, *rc*: fungal radius without bacteria, *r*: fungal radius with the tested bacteria 15 mm apart from it).

Detection of antifungal activity of cell-free culture supernatant

The fungal inoculation in this procedure was similar to the dual culture method. Agar well diffusion assays performed antifungal assays of cell-free culture supernatant. Before the assays, the selected LAB was twice pre-cultured in MRS broth, at 37 °C for 24 hours. The cell-free supernatant was obtained by centrifuging a bacterial culture at 3,000×g for 30 min at 4 °C. The wells of size 6 mm were made using cork borer in PDA medium, and then different volumes of (50 µL, 150 µL, and 200 µL) of cell-free supernatant were pipetted into the wells separately and 15 mm apart from the center of the PDA plate after four days later.

The antifungal activities were also studied with the concentrated bacterial cell-free supernatants. One liter of bacterial cell-free supernatant adjusted to pH 6.5 using phosphate buffer 0.05 M was used for condensed by vacuum evaporator at 60 °C, 76 cm/Hg, for 45 minutes. Inhibitory activity was determined by the agar-diffusion method using Whatmann No. 1 filter paper. A petri dish was inoculated with 3 mm mycelia of fungal and then concentrated cell-free supernatants were applied to these filter disks. The volume spotted was with different concentrations, such as 50% (10 µL pH 6.5 buffer: 10 µL concentrate of cell-free supernatants) and 100% (20 µL of cell-free supernatants) on the PDA medium, 15 mm apart from the center of the PDA plate containing mycelia. Antifungal activities were expressed as the inhibition rate.

Molecular identification of 16S rRNA genes of selected LAB isolates

The selected LAB's total genomic DNA was extracted using Presto Mini gDNA Bacteria Kit protocol kit (Geneaid GBB 100). The obtained genome DNA was amplified by polymerase chain reaction (PCR) using primers 63 forward 5'- CAG GCC TAA CAC ATG CAA GTC -3 'and 1387 reverse 5'- GGG CGG WGT GTA CAA GGC-3'. Amplification reactions were performed in a total volume of 50 µL. PCR conditions consist of pre-denaturation process (94 °C 4 min), denaturation (94 °C 30 sec), annealing (55 °C 30 sec), extension (72 °C 1 min), and final extension (72 °C 7 min). First Base Laboratories, Malaysia, performed sequencing of purified PCR products. Nucleotide sequences were analyzed and identified using the GeneBank data library and BLAST program.

Result and Discussion

Isolation and identification of LAB from cocoa bean fermentation

The observations at the isolation showed three types of colonies with different morphology (shape, texture, and colony color). First, isolates with spherical colonies, smooth texture, and milky white color. In the second isolate, the colony was round, and the texture was smooth with a cream color. Third, isolates with irregular shapes and cream-colored. A total of 218 isolates were suspected to be LAB, obtained at the isolation and purified by the quadrant scratch method. The 218 isolates were tested to be LAB on solid MRS media containing 1% $CaCO_3$ (w/v), and it 164 isolates formed a clear zone indicating acid production. The clear zone formed around the colony showed that the selected isolates produce acidic compounds which react with $CaCO_3$ and form Ca-Lactate. The absence of catalase was the following selection method, 82 isolates had no catalase, characterized by the lack of gas bubbles after the deposition of H_2O_2 solution. A total of 82 selected LAB isolates were used for the following selection.

Physiological adaptation test of LAB

The temperature, pH, and ethanol levels change during fermentation process (Moreira et al., 2013). LAB, as a culture starter for cocoa fermentation, should be able to survive under fermentation conditions. Our observation showed that all LAB isolates were tolerant when grown at pH 5.7 and slightly decreased cell number at pH 4.5. A total of 55 isolates were found to have better survival under pH 4.0 conditions with a decrease in the number of cells by 1 Log CFU/mL, while the other 27 isolates decreased the number of cells by 2 Log CFU/mL (data not shown). Illeghems et al., (2015) reported that LAB can regulate intracellular pH homeostasis to survive at low pH. Isolate with a decrease in cell count 1 Log CFU/mL at pH 4.0 was selected to be tested on tolerance to ethanol and temperature.

The observation of LAB resistance to temperature and ethanol levels showed that all LAB were able to survive well at 5% ethanol content at 40 °C. Tolerance testing on temperature and ethanol combinations showed that each isolate of LAB had a different survival. The results showed that of the 55 LAB isolates tested, 32 isolates could survive at 10% temperature ethanol at 30 °C, and only 26 isolates could survive at ethanol levels up to 15% at 30 °C and 5% at 40 °C. This test also shows that 3 isolates can survive in ethanol conditions 15% temperature 37 °C. The ability of LAB isolates to survive

under temperature and ethanol conditions is important because, during the fermentation of cocoa beans, there is a change in temperature and ethanol content. Twenty-six isolates able to withstand ethanol conditions of up to 15% of the temperature of 30 ºC were selected to be tested at a later step. Apriyanto, et al. (2019) reported the highest levels of ethanol during cocoa bean fermentation of 5.23%, while Pereira et al. (2012) informed that ethanol content during fermentation was recorded at 8%. After 2 days of fermentation, the temperature changed from 28 ºC - 35 ºC to 40 ºC - 50 ºC (Mahazar et al., 2015).

Total Acidified Acid

The total acid test was pursued to determine the ability of isolates to produce acid. The measured value in total titrated acids is the total of dissociated and non-dissolved. Isolate LAB is superior if it can produce higher acid during fermentation using MRSB medium and under the same fermentation conditions Abedi, et al. (2020) informed that LAB would use sugars in the fermentation liquid to produce lactic acid-induced organic acids.

The total acid measurement results showed that 5 isolates yielded a high total acid concentration of 12.16 m/ mL (Table 1), namely H 0.17, H 2.15, H 2.22, H 2.34, and H 3.1. LAB's ability to produce organic acids is closely related to its role in the fermentation of cocoa beans. These organic acids will form the precursor aroma and flavor of cocoa beans.

Tabel 1. Total acidity level of selected Lactic Acid Bacteria

Isolate Code	Total acidity level (mg/mL)
H 0.3	10,36 ± 0.64
H 0.13	10.36 ± 0.64
H 0.17	12.16 ± 0.64
H 0.24	10.81 ± 1.27
H 0.26	10.36 ± 0.64
H 0.28	9.45 ± 0.64
H 1.22	11.26 ± 0.64
H 2.3	11.26 ± 0.64
H 2.8	10.81 ± 0.64
H 2.14	11.26 ± 0.64
H 2.15	12.16 ± 0.64
H 2.22	12.16 ± 0.64
H 2.26	11.26 ± 0.00
H 2.31	11.26 ± 0.64
H 2.34	12.16 ± 0.64
H 3.1	12.16 ± 0.64
H 3.3	11.26 ± 0.64
H 3.6	11.26 ± 0.64
H 4.6	11.26 ± 0.64
H 4.11	10.81 ± 0.01
H 4.26	10.36 ± 0.64
H 4.36	11.71 ± 0.01
H 4.44	11.26 ± 0.64
H 4.47	10.81 ± 001
H 4.48	9.91 ± 0.01
H 4.51	10.81 ± 0.01

Antagonistic Activity of LAB Isolates Against Aspergillus ochraceus

In this study, some isolates were able to inhibit the growth of molds. 26 LAB isolates were screened for their antagonistic activity *against Aspergillus ochraceus*. The results showed that 26 LAB isolates were positively antagonistic to *A. ochraceus* characterized by the dual culture method. The highest inhibitory zone was owned by isolate H 2.34 with 29% inhibition value (H) followed by H 2.15 with H value 24% (Figure 2, Figure 3). Two isolates with the highest inhibitory zone values were selected for subsequent tests as a starter culture and biopreservation agent candidate. The ability to inhibition of isolates of LAB isolates against *A. ochraceus* varies because each isolate has a different mechanism for inhibiting the growth of *A. ochraceus*.

Figure 1 Control on antagonistic activity of selected LAB against *A. ochraceus* on PDA media incubated at 30ºC for 7 days

Figure 2. Isolate H2.34 on antagonistic activity of selected LAB against *A. ochraceus* on PDA media incubated at 30ºC for 7 days

Figure 3. Isolate H2.15 on antagonistic activity of selected LAB against *A. ochraceus* on PDA media incubated at 30ºC for 7 days

LAB metabolites play a key role in the antifungal activity. Muhialdin et al. (2020) and Yepez et al. (2017) informed the antichaporous inhibition mechanism that occurred due to LAB-produced organic compounds and competition for nutrition. LAB was known to produce antifungal compounds in the form of lactic acid, acetic acid, hydrogen peroxide, formic acid, propionic acid, diacetyl, and specific proteins (Shi & Maryam, 2022). The compounds produced by each isolate vary, so each isolate's ability to inhibit *A. ochraceus* growth also varies. Fossi et al. (2016) reported that a *Lactobacillus plantarum* inhibition index against *A. ochraceus* of 46.5%. Another study of the antagonistic activity of *L. plantarum* was performed by Matei et al. (2014), which exhibits a 25-30% inhibition index against *Penicillium digitatum*. Ryu et al. (2014), identified three active antifungal cmpounds : 5-oxododecanoic acid, 3-OH-decanoid acid, and 3-OH-5-dodecenoic acid from *Lactiplantibacillus plantarum* HD_1 showed inhibitory effect on arange of molds and yeasts. Delavenne et al. (2013) informed that acetic acid is believed to have a synergistic effect with lactic acid in preventing fungal growth.

Antagonistic Activity of LAB Isolate-Free Supernatant Against Aspergillus ochraceus

The presence of antagonistic activity was characterized by forming the inhibit zone index around the well on the PDA media after dropping with a neutral pH supernatant (pH 6.5) and supernatant. A supernatant isolate of LAB H 2.15 and H 2.34 indicates the presence of inhibitory activity against *A. ochraceus* at acidic pH but has not demonstrated inhibitory activity in neutralized supernatant. The absence of inhibitory in the neutralized supernatant indicates that inhibition of mold growth occurring in the acid pH supernatant was due to the presence of organic acid produced by the LAB.

Tabel 2. Antagonist activity of cell-free supernatan of concentrated LAB against *A. ochraceus*

Isolate	Concentration of cell-free supernatan	Inhibition Zone	H (%)
H 2.15	50		
	90	1.30 ± 0.10	8.7
	100	2.83 ± 0.06	18.9
H 2.34	50		
	90	1.43 ± 0.06	9.6
	100	2.93 ± 0.12	19.6

In this study, the antagonism activity of LAB isolate H 2.15 and H 2.34 to *A. ochraceus* by LAB supernatant result of concentration (10X concentrated) was indicated by the inhibition zone around the paper disc (Table II). Table II shows that the highest inhibitory zone index was obtained by isolating LAB H 2.34 with an inhibitory index of 19.6% at 100% cell-free supernatant concentration. These results indicate that isolates H 2.34 and H 2.15 can produce antifungal compounds other than organic acids.

Shi & Maryam, (2022) informed that proteinaceous compounds produced by LAB consist of ribosomal peptides such as bacteriocins, non-ribosomal peptides, and peptides derived has antifungal activity. Antifungal peptides with molecular weight 664 Da – 2024 Da were isolated from *Lp. plantarum* TE10 (Muhialdin et al., 2020) Another publication of antifungal in the form of the protein was done by Rather et al. (2013) informed that *L. plantarum* YML007 has the anti-infectivity activity of a protein with a molecular weight of 1256-617 Da, in which it was damaged if treated with proteinase K and trypsin. Information on concentration from LAB isolate supernatant by vacuum evaporator method was reported by Turnip et al. (2018). The concentration results of LAB isolate *Pediococcus pentosaceus* E2211 showed antimicrobial activity against *Aeromonas hydrophila* with an inhibitory of 4.6 mm with a 100 µL supernatant.

Identification molecular of potential LAB

Based on the analysis of 16S rRNA gene isolate H 2.34 with BLAST-N program, obtained the result that LAB H 2.34 has similarity level of 99% with *Lactobacillus plantarum*. The 99% similarity level obtained is the total value of the total base of the isolates present in GenBank divided by the total number of bases equal to H 2.34.

Conclusion

Selected LAB isolates *Lactobacillus plantarum* H 2.34 has the potential as a starter culture for cocoa fermentation that the character to resist acidity, heat, and ethanol and the ability to produce high acid and antifungal. The isolate also has an antifungal compound capable of inhibiting the mold of *Aspergillus ochraceus*.

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