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Improvement of Fermentation Performance and Cocoa Bean Quality by Addition of Lactic Acid Bacteria Starter

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Fermentation is an essential step in the formation of aroma and flavor of cocoa beans. Uncontrolled fermentation cause the mycotoxin contamination in cocoa, which is produced by *Aspergillus* sp., *Fusarium* sp., *Trichoderma* sp. and *Penicillium* sp. Yeast, lactic acid bacteria (LAB) and acetic acid bacteria are the dominant microorganisms in cocoa beans fermentation. LAB is one of the indigenously present microorganisms in cocoa beans that is relatively easy to obtain. The addition of LAB starter on the cocoa bean fermentation process improved the fermentation performance and the quality of the fermented cocoa bean. The addition of LAB starter to the cocoa bean fermentation increased the sugar consumption rate, and could accelerate the growth of both LAB and acetic acid bacteria leading to the increase in the ethanol lactic acid, and acetic acid concentration and finally improved the fermentation index indicating that it can shorten the fermentation process. Those lactic acid bacteria were able to inhibit the growth of fungi that could potentially produce mycotoxin. *Lactobacillus plantarum* isolated from fermentation of cocoa has potential as anti-fungi for *Aspergillus niger*. The role of LAB starter on the improvement of cocoa quality could be determine based on the result of the cut test and lactic acid content of the dry cocoa bean, and others parameters according to the standard.

Introduction

Cocoa (*Theobroma cacao* L) is one of the leading commodities of strategic plantations that can increase people's income, generate foreign exchange for the country, provide jobs for the community and help conserve environmental functions. Indonesia is the third largest cocoa producer in the world after Ivory Coast and Ghana with 701,229 tons of cocoa production by 2015 (Directorate General of Plantation, 2015), so it become an important agricultural commodity for Indonesian national economy.

Cocoa becomes a source of foreign exchange, regional development, driving the development of agribusiness and agro-industry. As a high commercial commodity, quality is a very important factor in world cocoa market competition. Therefore, processing facilities, quality control and the application of technology in all phases of the processing cocoa beans must be considered. However, post-harvest processing of cocoa in particular at the farm level has not been properly done: cocoa beans are often found mixed with foreign substances, are not properly dried, and mostly unfermented. Thereby in the international market, Indonesian cocoa beans are often priced low and even imposed discounts (Kresnowati and Febriami, 2015). Therefore, it is necessary to develop fermentation technology to improve the quality of cocoa beans.

The process of fermentation of cocoa beans is an important step to obtain good quality cocoa beans. Cocoa bean fermentation involves microorganism such as yeast, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). The principal objectives of fermentation are the removal of mucilage to provoke aeration of the fermenting seeds and to facilitate drying later on, and to provide heat and acetic acid necessary for killing (preventing germination) and curing the seeds. A change in the dynamics of microbial population may alter the overall activity and may impact the fermentation process. The existence of mold in cocoa bean fermentation is undesired because it defects the quality of cocoa product and potentially produces mycotoxin (Ho et al., 2013). Dried cocoa beans often found myotoxin-producing fungi, namely *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Penicillium* sp, *Fusarium* sp, *Trichoderma* sp., *Rhizopus* sp., *Mucor* sp. and *Verticillium* sp (Asrul, 2009). One of the efforts to inhibit the growth of mold and the formation of mycotoxins in cocoa beans is by using lactic acid bacteria as starter in the fermentation process.

Effect of Lab Starter Addition to the Cocoa Bean Fermentation Performance

Cocoa bean fermentation is usually initiated by indigenous microorganisms that naturally present in the cocoa bean pulp including yeast, lactic acid bacteria and acetic acid bacteria, bacillus and fungi (Guehi *et al.,* 2008) Yeasts, lactic acid bacteria, and acetic acid bacteria are the dominant microorganisms in cocoa beans fermentation. Yeast and lactic acid bacteria dominate the first 2-3 days of fermentation, followed by acetic acid bacteria which dominate until day 4-5. Later on fungi and Bacillus may appear and this indicates that the beans are over fermented (Schwan and Wheals, 2004).

Schwan *et al*. (1998) found more than 40 species of microbes that grow during fermentation of cocoa. The species of microorganisms involved in the cocoa bean fermentation process vary with the geographical location of the plantation (Ardhana and Fleet, 2003; Papalexandratou, 2001). Sugary compounds, the main components of the mucilage, are fermented, releasing heat and yielding various metabolic products, among others are ethanol, lactic acid, and acetic acid. The combined effects are the death of the beans and the development of various precursors for the cocoa flavour formation. It also turns the colour of the beans to brown-black, reduces the bitterness, improves the cocoa and nutty flavor, and hardens the cocoa bean shell (Biehl, 1986; Sulistyowati dan Yusianto, 1998; Thompson *et al.,* 2000). Fermentation index measures the colour change of cocoa that can be used as the indicator of completeness of the fermentation (Gourieva and Tserevitinov, 1979).

The dominant yeast species is *Saccharomyces cerevisiae* and *Candida tropicalis* with a high survival rate, 10^7 cfu/g during 36 hours (Ardhana and Fleet, 2003). Typical lactic acid bacteria significantly present during the cocoa bean fermentation process is *Lactobacillus plantarum*. While typical acetic acid bacteria actively presents in the first 24 hours of fermentation is *Acetobacter aceti*.

Schwan (1998) studied the use of cocktail inoculum consisted of a yeast, *Saccharomyces cerevisiae* var. *chevalieri*, two lactic acid bacterial species, *Lactobacillus lactis* and *Lactobacillus plantarum*, and two acetic acid bacterial species, *Acetobacter aceti* and *Gluconobacter oxydans* subsp. *Suboxydans*, for the cocoa fermentation were performed in wooden boxes under the following four experimental regimens: beans naturally fermented with wild microflora; aseptically prepared beans with no inoculum; and beans inoculated with a defined cocktail containing microorganisms at a suitable concentration either at zero time or by using phased additions at appropriate times. Result showed that the natural fermentation mimicked exactly the conditions in 800-kg boxes on farms. The aseptic box remained largely free of microflora throughout the study, and no significant biochemical changes occurred. With the zero-time inoculum the fermentation was almost identical to the natural fermentation. The fermentation with the phased-addition inoculum was similar, but many changes in parameters were slower and less pronounced, which led to a slightly poorer end product. The data show that the nearly 50 common species of microorganisms found in natural fermentations can be replaced by a judicious selection and concentration of members of each physiological group. *S.cerevisiae* var. Chevalieri was chosen in particular, considering its pectinolytic activity that may improve pulp degradation.

Kustyawati and Setyani, (2008) report the effects of the addition of mixed inoculums consisted of *Saccharomyces cerevisiae, Lactobacillus lactis and Acetobacter aceti* on the chemical and microbial changes during cacao fermentation. The addition of this mixture consisting in the early stages of fermentation (day 0) can optimize the fermentation process. Microbial inoculums added at the first or second day of fermentation are not recommended.

Lefeber *et al.* (2010) indicates that, by setting the initial conditions, defined inocula of yeast, LAB, and AAB can now be considered mixed-strain starter cultures that should lead to better controlled and more reliable cocoa bean fermentation processes and hence good-tasting and flavorfull chocolates. A mixture of *L. plantarum* 80, *L. fermentum* 222, and *A. pasteurianus* 386B can now be considered a mixed-strain starter culture for better controlled and more reliable cocoa bean fermentation processes

Lefeber *et al.,* (2011) found the result for the actual control of cocoa bean fermentations by the use of LAB strains as appropriate starter cultures is under investigation. This kinetic study contributes to a better understanding of the functional behavior of cocoa-specific LAB strains to be used as interesting starter cultures for controlled cocoa bean fermentations. In addition, the cocoa-specific *L. fermentum* strains can be categorized as the ones best adapted to the cocoa pulp ecosystem and show interesting functional roles for the development of a defined starter culture. They fermented glucose to lactic acid and acetic acid, reduced fructose to mannitol, and converted citric acid into lactic acid and 2,3-butanediol.

The polyphasic selection study allowed to construct a better picture of the physiology and ecology of the indigenous yeast, LAB, and AAB strains. Some strains from these three major groups were selected as potential starter cultures. In particular, *L. fermentum* UFLA CBE8.12 (citric acid fermenting, lactic acid producing, and tolerant to heat, acid, lactic acid, and ethanol), *S. cerevisiae* UFLA CYC7.04 (ethanol producing and acid, heat, and ethanol tolerant), and *A. tropicalis*UFLA CBE16.01 (ethanol and lactic acid oxidizing, acetic acid producing, and tolerant to acid, heat, acetic acid, and ethanol) were selected as candidates for a mixed-strain starter cocktail that should lead to better-controlled and more-reliable cocoa bean fermentation processes (De Melo Pereira *et al.,* 2012).

Considering the role of microbial population during the fermentation, a change in the dynamics of microbial population may alter the overall activity and may impact the fermentation process. The addition of *Lactobacillus plantarum* ITB CC 188 starter into cocoa bean fermentation modify the microbial composition during the cocoa bean fermentation and thus accelerate the growth of both lactic acid bacteria and acetic acid bacteria, leading to the increase in the ethanol, lactic acid, and acetic acid concentration finally improved the fermentation index indicating that it can shorten the fermentation process. The effect of *Lactobacillus plantarum* ITB CC 188 starter addition to the cocoa bean fermentation with concentration 10^3CFU of LAB per gram cocoa beans could be used to improve the fermentation process (Kresnowati, *et al.,* 2013).

Kresnowati and Febriami (2015) reported that combinations of *S.cerevisiae* var. Chevalieri, *A.aceti*, and *L.plantarum* as starter culture for cocoa bean fermentation improved the fermentation performance through modification of microbial population, acceleration of sugar reduction and metabolites production and improvement of fermentation index. These indicated that with addition of mix starter culture in the beginning of fermentation, the execution of cocoa bean fermentation can be shortened. Further, the addition of starter culture modified microbial composition during the fermentation and thus directed he fermentation towards the targetted fermentation mechanism, regardless the initial cocoa bean conditions. The best result was obtained from the addition combination of yeast and lactic acid bacteria in the starter culture, giving more sugar degradation and more primary metabolites production which leading to the fermentation index of 1.38 ± 0.07 by the end of fermentation.

The addition of the two starter culture mixtures, one composed of *S. cerevisiae* H5S5K23, *L. fermentum* 222, and *Acetobacter pasteurianus* 386B, and another composed of *L. fermentum* 222 and *A. pasteurianus* 386B to the fermenting cocoa pulp-bean mass accelerated the cocoa bean fermentation process regarding citric acid conversion and lactic acid production through carbohydrate fermentation. For the production of a standard bulk chocolate, the addition of a yeast/LAB/AAB starter culture was necessary. This enabled an enhanced and consistent ethanol production by yeasts for a successful starter culture-added cocoa bean fermentation process. This study showed possibilities for the use of starter cultures in cocoa bean fermentation processing to achieve a reliably improved fermentation of cocoa pulp-bean mass that can consistently produce high-quality fermented dry cocoa beans and flavourful chocolates produced thereof (Lefeber *et al.,* 2012).

Effect of Lab Starter Addition to the Cocoa Bean Quality

Sulistyo *et al.,* (2014) reported that one of the opportunities to improve the quality of cocoa is through development on fermentation and preservation technology of cocoa beans, using cultures of lactic acid bacteria (LAB) isolated from palm sap-based fermented products. Results of assay on their antimicrobial activities showed that only *L. fermentum* and *L. plantarum* were effective on inhibiting against the growth of some microbial contaminants in cocoa beans. Strain of *L. plantarum* was able to produce as much as 2.05% lactic acid and hydrogen peroxide as much as 24.87 µg/ml, but did not produce bacteriocins. Strain of *L. plantarum* was also able to reduce the presence of microbial pathogens *S. Typhimurium* and *Aspergillus flavus* by 2 log units at concentrations of 10^7-10^9 CFU / ml, so that it can meet the quality standards of cocoa that has been established.

Submersion cacao beans isolate L.plantarum NL-249 after fermentation using was able to reduce *S. Typhimurium* by 3 log units (Sulistyo *et al.,* 2014). The ability of *L. plantarum* NL-249 to reduce *S*. Typhimurium in cocoa beans may be caused by accumulation of organic acid and production of H_2O_2 by NL-249 during submersion (Sulistyo *et al.,* 2014). Organic acids produced by NL-249 will passively diffuse into the microbial cells in undissociated form, and then there will be a separation of anions and protons which will penetrate cell membrane and will affect the integrity of the cytoplasmic membrane, resulting in cell acidification and denaturation of proteins, and therefore the cytoplasmic membrane will be damaged. This will cause a disruption in the metabolic system, such as the inhibition of transport of substrate, energy production and synthesis of macromolecules (Lazarova, 1994)

Submersion of cocoa beans in a suspension containing isolates *L. plantarum* NL-249 at 10^7 CFU/ml could reduce *A. flavus* by 1 log unit. Concentration of isolates *L. plantarum* NL-249 could be increased up to 10^9 CFU/ml for 2h submersion to reduce the number of *A. flavus* as amount of 2 log units, (Sulistyo *et al.,* 2014) however impact on the decrease in pH to 4.8 was not desired since requirements for the quality cocoa should has a lowest pH at pH5 (Amin, 2005). Submersion of cocoa beans resulted the pH of dry beans could still be maintained above pH 5, and it still meets the quality requirements of cocoa beans.

The isolate *L. plantarum* NL-249 exhibited its ability to reduce *A. flavus* in cocoa beans (Sulistyo *et al,* 2014) due to production of metabolites which inhibited germination of spores and growth, such as phenyllactic acid, phydroxy phenyl lactate, cyclic dipeptide such as cyclo (Gly-L-Leu), cyclo (L-Phe-L-Pro), and cyclo (L-Phe-trans-4-OH-L-Pro), benzoic acid, methyl hydantoin, mevalonolactone and short chain of fatty acids (Corsetti, 1998; Lavermicocca *et al,* 2000; Storm, 2002).

Application of *Pediococcus* A19 in cocoa fermentation as starter inhibited the growth of each of the OTA-producing species. At the end of fermentation in boxes inoculated with *Pediococcus* A19, *A. niger* was not detectable while *A. carbonarius* concentration was found to be 2 Log

CFU/g of wet beans. The assessment of the ochratoxin produced during fermentation of cocoa inoculated with *A. carbonarius* indicated that the use of *Pediococcus* A19 as starter could reduce their level of growth so as to have only a toxin production of 0.0012 ± 0.0005 µg/kg after 40 days of storage, while this was 2.45 ± 0.35 µg/kg of fermented and dried cocoa beans in the absence of *Pediococcus* A19 (Ngang, 2015)

Hernani and Haliza (2013) reported that the cocoa beans quality could be enhanced by the fermentation process with the addition of microbial cultures. The microbes used were *S. cerevisiae* 10g / kg, *L. plantarum* 1 ml / kg or about 10^9CFU / ml and *A. aceti* of 1ml / kg sample or as much as 10^9 CFU / ml.

The NL-249 isolates was identified as *Lactobacillus plantarum* showed the highest inhibitory activity against *S. Typhimurium*. This was allegedly due to presence of lactic acid which was a main product of *L. plantarum* NL-249 as homofermentatif LAB (Jay 1996). This was supported by a statement of Nousianen *et al* (2003) that organic acids and H2O2 which were produced by LAB showed inhibitory activity against Salmonella. According to Jacobsen (1999), LAB has been declared to have antimicrobial activity, if it has a minimum inhibitory area of 1 mm, and was stated to has a positive inhibitory activity (+) when the area of inhibition between 2mm to 5 mm, and has a high inhibitory activity (++).

Conclusions

The addition of LAB starter to the cocoa bean fermentation increased the fermentation performance by: accelerate the growth of LAB, AAB and leading to the increase their product and finally improved the fermentation index. The addition of LAB starter to cocoa fermentation can improve the quality of cocoa beans, among others, by inhibiting the growth of mushrooms that have the potential to produce OTA and the growth of pathogenic bacteria.

Conflict of Interest

All the authors declare that they have no conflict of interest.

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