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Effect of Freeze-dried *Bacillus Thuringiensis* Starter on Cocoa Fermentation

Lamine SAMAGACI*, Hadja OUATTARA, Victoria KADET, Michael ABIHI, Honoré OUATTARA, Sébastien NIAMKE

Biotechnology Laboratory, UFR Biosciences, Félix Houphouët-Boigny University, Abidjan, Côte d'Ivoire
*Corresponding author: salamine1983@gmail.com

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Abstract The overall objective of this study is to evaluate the impact of Bacillus on the cocoa fermentation process to facilitate the establishment of a microbial cocktail to control cocoa fermentation. Microbial starters were revived in their respective media and subsequently used in fermentation tests conducted in 1L plastic vessels. Fermentation parameters and quality determinants of cocoa beans were measured every 24 hours during six days of fermentation. The results obtained show that the presence of the Bacillus strain in the microbial cocktail appears to increase the temperature of the cocoa fermentation mass compared to the control. In addition, a fermentation index of 1 was obtained with the cocktail containing Bacillus after 4 days of fermentation, compared to the control. The improvement of the cocoa fermentation process by the Bacillus strain resulted in an increase of more than 7% in well-fermented cocoa beans (43 ± 2.5%) compared to the trial with the cocktail without Bacillus (36 ± 3). These results suggest that the inclusion of the Bacillus strain in the cocoa fermentation starter cocktail could significantly improve the fermentation process and cocoa beans quality.

Keywords: *fermentation, cocoa, starter, freeze-drying, Bacillus thuringiensis*

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1. Introduction

The cocoa tree (*Theobroma cacao* L.) is a plant native to the tropical rainforests of the Amazon Orinoco Basin [1]. The beans extracted and fermented from the fruits of this tree play an important role in the economy of several countries, including Côte d'Ivoire. In fact, cocoa contributes to almost 15% of the Gross Domestic Product and represents 40 % of Ivorian export earnings [2] and the sector employs nearly 700 000 farmers [3].

However, the poor quality of cocoa beans is causing huge economic losses in producing countries. The poor quality of cocoa beans is related to post-harvest processing, including pod opening, fermentation, drying and storage [4]. Of these treatments, fermentation is the one that most affects bean quality and plays an essential role in the production of chocolate flavour precursors [5,6]. However, cocoa fermentation is carried out spontaneously by important microorganisms such as yeasts, acetic and lactic acid bacteria and Bacillus sp. In addition, the variable quality of cocoa beans is related to the spontaneous nature of cocoa fermentation, hence the need for control [7].

Several studies have been carried out worldwide to control cocoa fermentation. These studies have allowed the isolation and identification of the microorganisms involved in cocoa fermentation in the producing countries [8,9,10,11].some studies have also proposed microbial starters consisting of yeast strains alone or in combination with acetic and lactic acid bacteria [12,13]. The use of microbial starter cultures in cocoa fermentation processes tested at laboratory, semi-pilot and pilot scale, has improved the quality of cocoa beans in most cases [14,15]. However, no studies have used a starter culture containing Bacillus sp. In fact, the use of *Bacillus sp* as a starter in cocoa fermentation has been strongly criticized by some scientists without any prior study. According to these researchers, *Bacillus sp* is responsible for the undesirable characteristics of the beans after fermentation [16]. However, *Bacillus sp* is present throughout the cocoa fermentation process [17,18] showed that the high production capacity of pectinolytic enzyme by the Bacillus sp involved in cocoa fermentation would contribute to the improvement of cocoa beans quality by degrading of the bean pulp. Also, the *Bacillus sp* involved in cocoa fermentation could degrade the citric acid contained in the cocoa beans pulp and produce aromatic compounds [19]. The aim of this study is to evaluate the impact of *Bacillus sp* on the cocoa fermentation process in the laboratory in order to facilitate the selection of starters cultures for the control of cocoa fermentation.

2. Materials and Methods

2.1. Materials

The cocoa pods used in this study were from the Agneby-Tiassa (region of Côte d'Ivoire). Also, seven (7) freeze-dried strains of microorganisms isolated from cocoa fermentation in Côte d'Ivoire were also used in this study. These potentials starters were proposed by researchers from the Biotechnology Research Unit of Felix Houphouët Boigny University. Among these strains, three (3) strains of different species of lactic acid bacteria (*Leuconostoc mesenteroides* (T8AB6), *Lactobacilus casei* (T10G5), *Lactobacillus plantarum* (T11G3)) involved in the fermentation of cocoa, two (2) strains of yeasts, one of wich high ethanol production capacity(*Saccharomyces cerevisiae* (T7GB10) and the other with pectinolytic enzyme (*Candida tropicalis* (D5P12)), one strain of acetic acid bacteria (*Acetobacter Pasteurianus* (T6HS14)) and one strain of *Bacillus thuringiensis* (T11I10).

2.2. Methods

2.2.1. Determination of the Microbial Load of the Starters Culture Used for Fermentation Test

The Thoma cell was used to determine the microbial load of the freeze-dried starters. A quantity of 0.1 g of each starter powder was resuspended in 0.9 mL of peptone water. A volume of 25 µL of each suspension and 25 µL of methylene blue were then applied to the Thoma cell. Microorganisms were enumerated under a microscope (G×40). The number of live cells per millilitre was then determined using the following formula:

$N = n \times 5 \times 10^{5} \times fd$

n: Average number of cells counted per square selected; N : Number of cells per milliliter ; fd : Dilution factor

2.2.2. Fermentation Tests Carried out with Microbial Starters in Plastic Vessels

2.2.2.1. Disinfection of the Cocoa Pods and Equipment Used During the Fermentation Test

Ripe and healthy cocoa pods were subjected to a sanitisation process. For this purpose, the pods were first immersed in sodium hypochlorite solution at 1.8° for 30 seconds and rinsed with sterile distilled water for 30 seconds. Then, the cocoa pods were immersed in a 75 % (v/v) ethanol solution for 2 min and finally rinsed with sterile distilled water for 30 seconds. Also, the fermenters (1 L plastic containers) have undergone the same disinfection step as the cocoa pods. Furthermore, the knives and spoons used for this study were wrapped in aluminum foil and sterilized in the oven at 150°C for 15 min [10,20].

2.2.2.2. Pod Opening and Cocoa Fermentation with Starter Powders

The cocoa pods were opened with a knife close to the flame of the Bunsen burner. The beans were scooped out with a spoon and weighed so that 1kg was in the fermenter (1 L plastic container), which had previously been placed on a scale. In addition, the cocoa beans (1 kg) contained in the fermenter (1 Liters plastic vessel) were inoculated with a mass of freeze-dried starter powder of 10^5 cells /g of cocoa beans according to the formula presented in 2.2.1. For microbial groups using more than one strain of microorganism, the load for each strain is obtained by dividing the load 10^5 cells by the number of strains inoculated. The different fermentation conditions are carried out as indicated below (Table 1) and the fermenters (1 Liters plastic vessels) are left on the benches of the laboratory. During the 6 days of fermentation, the temperature of the fermentation mass is measured every 24 hours as indicated below and, after mixing the fermentation mass with sterile knives, cocoa bean samples (20 g) are taken for the following tests.

Table 1. Different fermentation conditions used for cocoa fermentation in plastic vessels

Type of fermentation	Microorganism composition
Cocktail + bacillus	10^5 cells of yeasts (D5P12 and T7GB10) + 10^5 cells of acetic acid bacteria (T6HS14) + 10^5 cells of lactic acid bacteria (T8AB6, T11G3 and T10G5) + 10^5 cells of Bacillus (T11I10)
Cocktail without bacillus	10^5 cells of yeasts (D5P12 and T7GB10) + 10^5 cells of acetic acid bacteria (T6HS14) + 10^5 cells of lactic acid bacteria (T8AB6, T11G3 and T10G5)
Control	No microorganism added + beans and equipment (fermenter, spoon, knife) sterilized

2.2.2.3. Temperature of Cocoa Fermentation Mass

Temperature was measured with a thermometer according to the method described by [21]. The temperature measurement was measured every 24 hours during six days of fermentation. The thermometer was inserted at different points in the fermentation mass at a depth of about six centimeters and after stabilization to take the temperature. The temperature was read directly. The temperature of the fermentation mass was determined by averaging.

2.2.2.4. Acidity of the Cotyledon of Cocoa Beans

The cotyledons of the cocoa beans were ground using a blender. Then, two (2) grams of the ground material weighed on a precision balance was then added to 18 mL of distilled water. The mixture was homogenised and then filtered using a Whatman paper with a porosity of 0.45 µm. A volume of 5 mL of the filtrate is titrated with a NaOH solution (0.1 N), after the addition of two (2) drops of phenolphthalein, until a persistent pink colour is obtained. The following formula is used to determine the acidity of the cotyledon of fermented cocoa beans [22,23].

$$A(\%) = \frac{V \times Vt \times N}{Pe \times Vp} \times 100$$

N : NaOH Normality ; V : Volume (in mL) of NaOH poured ; Vt : total volume (in mL) of the sample ; meq : Citric acid equivalent (0,070); Pe : essay sample collection (2 g); Vp : Volume (in mL) taken for titration

2.2.2.5. Extraction of Water-soluble Sugars from the Cotyledon of Fermented Cocoa Beans

The extraction of water-soluble sugars was carried out

according the protocol described by [24]. Five (5) g of crushed cocoa bean cotyledon was added to a 200 mL volumetric flask containing 50 mL of distilled water heated to 60°C. The mixture was stirred until completely cooled and filtered using Whatman-type filter paper with a porosity of 0.45 µm. The resulting filtrate was collected in a 100 mL volumetric flask and made up to the calibration mark with distilled water.

2.2.2.6. Determination of Reducing Sugars in the Cotyledon of Fermented Cocoa Beans

The reducing sugar content of cocoa beans cotyledon was determined according to the method described by [25]. A volume of 100 µL of water-soluble sugar extracted from cocoa cotyledon was introduced into test tubes to which 200 µL of DNS was added. The mixture ~~is~~ was vortexed and then heated in a boiling water bath at 100 °C for 5 minutes. After cooling, 2 mL of distilled water was added, and the optical density of the solution was read against a control using a spectrophotometer at a wavelength of 540 nm. The sugar-free, water-soluble control was treated under the same conditions as the tests. A standard series was prepared under the same conditions from a stock glucose solution with a concentration of 1 mg/mL. The amount of reducing sugars in each sample is obtained from the equation of the regression line established from the standard range.

2.2.2.7. Fermentation Index of Fermented Cocoa Beans

The fermentation index was determined according to the method described by [26]. A mass of 0.5 g of cocoa cotyledon previously ground using a blender, was added to 5 mL of a mixture of methanol (97%) and hydrochloric acid (3%). The mixture was homogenised and then refrigerated at 8 °C for 18 hours. The absorbance of the filtrate was read with a spectrophotometer at 460 nm to measure oxidized anthocyanins, and then at 530 nm for non-oxidized anthocyanins. The fermentation index (IF) of the sample is obtained by calculating the ratio of absorbance at 460 nm to that at 530 nm according to the following formula:

$$IF = \frac{A(460)}{A(530)}$$

2.2.2.8. Cut test of Fermented Cocoa Beans

The cut-test is method of assessing the physical quality of cocoa beans. This assessment is based on the proportion of fermented beans with a brown coloured cotyledon. The principle is to cut the beans longitudinally to expose the inner part of the cotyledons [27]. Thus, one hundred (100) beans were randomly selected and dried. The cut was made with a cutter. Visual inspection of the internal parts of the two half beans allowed the beans to be classified into 3 groups (brown, brown-purple and purple).

2.2.2.9. Statistical Analysis of Data

All experiments were performed in triplicate and the results were averaged ± standard deviation. Statistical analyses of the data obtained were performed using the XLSTAT 2016 software. The Duncan test at 5% level was used to determine significant differences between the means.

3. Results

3.1. Temperature of the Fermentation Mass of Cocoa

The temperature of cocoa mass change over time during fermentation conducted at the laboratory scale. The curves of temperature evolution show the same profile. Indeed, from 0 to 120 hours, we observe an increase in temperature values from 28.7 ± 0 °C to 35.16 ± 0.35 °C. While after 120 hours of fermentation, the temperature values decrease for the fermentation carried out with microbial starters. In addition, the temperature curve of the fermentation carried out with cocktail + bacillus gives higher temperature values for 120 hours than the control and fermentation with cocktail without bacillus (Figure 1).

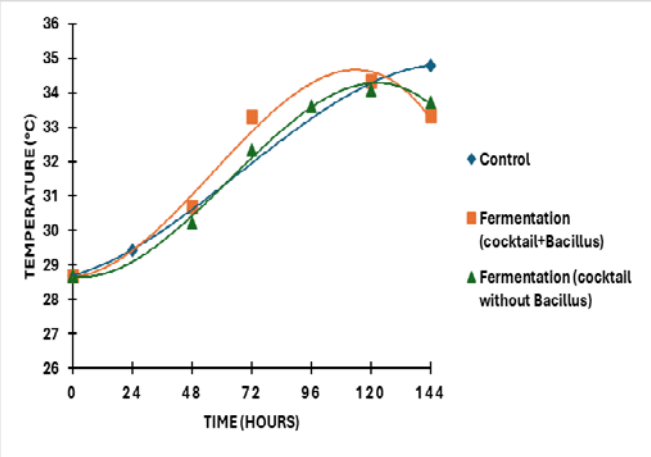

Figure 1. Temperature of cocoa mass during the fermentation

3.2. Evolution of Acidity of Cotyledon During Fermentation Tests

The acidity curve of the cotyledon shows an upward trend (Figure 2). In addition, the acidity curves of the fermentation carried out with cocktail + bacillus and without bacillus strain are mixed up with values ranging from (0.094 ± 0.00 and 2,313 ± 0,06 %). While the control gives the lowest acidity values ranging from (0.094 ± 0.00 and 0,87± 01%).

3.3. Evolution of Reducing Sugar Content of Cotyledon During Cocoa Fermentation Tests

The reducing sugar content of the cotyledon increases during cocoa fermentation. Indeed, the lowest levels of reducing sugar are observed with the control and these values are between 0.006 and 0.05 mg/100 g during 44 h of fermentation. In addition, the fermentation test carried out with the cocktail+ bacillus strain gave the highest concentrations between 0.044 and 0.075 mg/100 g during the fermentation process. Whereas the fermentation carried out with the cocktail without bacillus gave reducing sugar contents ranging from 0,005 and 0,062 mg/100g (Figure 3).

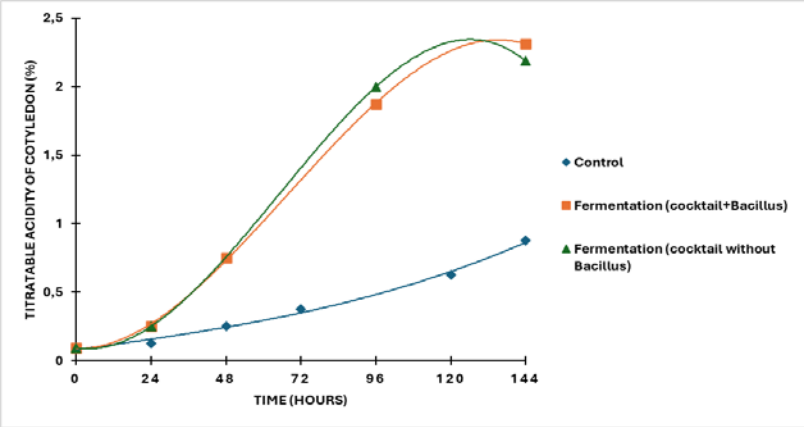

Figure 2. Acidity of the cotyledon of cocoa beans during fermentation tests

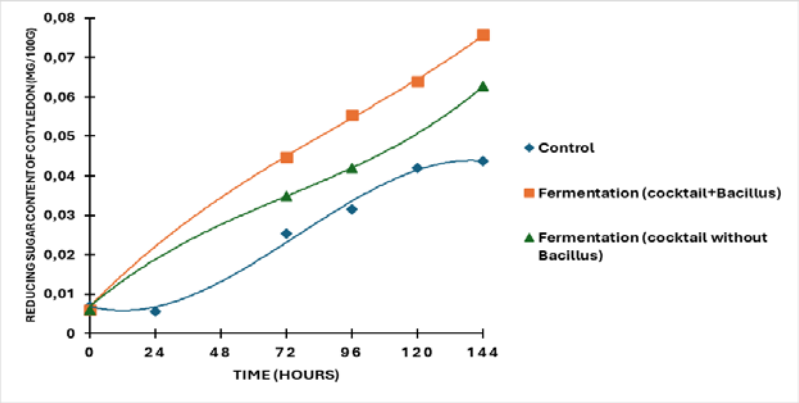

Figure 3. Reducing sugars content in the cotyledon of cocoa beans during cocoa fermentation in laboratory scale

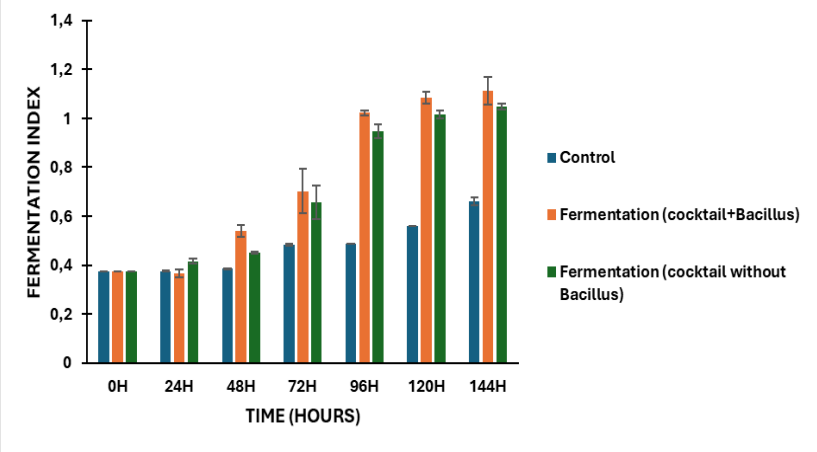

Figure 4. Fermentation index of cocoa beans during laboratory fermentation tests

3.4. Fermentation Index of Cocoa Beans

Figure 4 shows the evolution of the fermentation index of cocoa beans during the fermentation tests. During 48 h of fermentation, the fermentation index changed slightly for all the tests with values below 0.6. Whereas the fermentation test carried out with the cocktail + bacillus gives a fermentation index of 1 after 96 hours. Such a value of the fermentation index (1) is obtained with the fermentation test carried out with the cocktail without the bacillus after 120 hours of fermentation. While the control did not reach fermentation index 1 during the six days of fermentation.

3.5. Quality of Fermented and Dried Cocoa Beans

The results of the cut-test show more than 14% of well-fermented beans (brown) with the fermentation carried out with the cocktail + bacillus (43 ± 2.5%) compared to the fermentation control (29 ± 3%). In addition, fermentation with the cocktail without the bacillus strain (36 ± 3%) yielded less than 7% of well-fermented beans compared to the fermentation test with t cocktails + bacillus (Table 2).

Table 2. Percentage of well-fermented cocoa beans (brown colour) during fermentation tests

Type of fermentation	Percentage of Brown beans (%)
Control	27 ± 0.5
Fermentation (Cocktail + *bacillus)*	43 ± 2.5
Fermentation (Cocktail without *bacillus)*	36 ± 3

4. Discussion

The spontaneous nature of cocoa fermentation has led the scientific community to develop microbial starters capable of controlling cocoa fermentation. However, attempts to control cocoa fermentation have been made worldwide without using Bacillus strains in microbial cocktails [11,20,28]. However, the Bacillus strain seems to play an important role in the fermentation process of cocoa [17,18,29]. Furthermore, some researchers, without scientific evidence, consider them undesirable for cocoa fermentation [16]. Through this study we will show the impact of bacillus on cocoa fermentation in the laboratory. This study shows the effect of Bacillus on cocoa fermentation in the laboratory.

During this study, the profiles of the temperature, acidity, and sugar content curves are similar to those obtained by researchers working on cocoa fermentation around the world [30,31,32,33,34]. The results also show that the presence of Bacillus strain in the microbial cocktail seems to increase the temperature of the cocoa fermentation mass. Indeed, the temperature increase is thought to be related to the activity of yeasts, which oxidise the sugars in the pulp to ethyl alcohol and acid acetic bacteria, which that convert the alcohol produced by the yeasts to acetic acid during fermentation [12]. According to [16], these exothermic reactions would cause the embryo to die and activate the endogenous enzymes that produce the precursors of cocoa flavour. The drop in temperature at the end of fermentation is due to the decrease in ethanol content and thus the decrease in the activity of acetic acid bacteria [21].

In addition, the increase in acidity of the beans cotyledons in the starter trials compared to the control could be related to the microbial activity of these starters. Indeed, during fermentation, the carbonaceous substrates contained in cocoa pulp are converted into ethanol and organic acid by yeasts, lactic acid, acetic acid and Bacillus bacteria [6]. These metabolites, which diffuse into the cotyledon and acidify the cotyledon are thought to lower the pH and thus increase the acidity of the cotyledon [35]. The acidification of cotyledons also allows the oxidation of polyphenols, the hydrolysis of glycosides and anthocyanin pigments, leading to the disappearance of cocoa astringency and the development of the brown colour characteristic of well-fermented cocoa [33,34,36,37].

Unlike the acidification of cocoa beans, where the Bacillus strain appears to have no impact, the addition of Bacillus to the microbial cocktail promotes an increase in reducing sugars in the cocoa beans cotyledon. This is very important for improving the quality of cocoa beans. Indeed, the precursors of chocolate aroma are developed through the Maillard reaction between the reducing sugars and amino acids contained in cocoa beans. According to [38], increasing the content of reducing sugars in the cotyledon allows for good aromatic quality.

In addition, the fermentation index is a ratio between the oxidised and non-oxidised forms of polyphenols [39]. In addition, a fermentation index of 1 indicates good fermentation of cocoa [40,41]. However, the presence of Bacillus in the microbial cocktail makes it possible to obtain a fermentation index of 1 during 4 days of fermentation. Where the cocktail without the Bacillus strain gives 1 in 5 days. Where the cocktail without the Bacillus strain gives 1 in 5 days. This suggests that the Bacillus strain may contribute to the improvement of cocoa fermentation. The improvement of the cocoa fermentation process by the Bacillus strain allows an increase of more than 7% in well-fermented cocoa beans (43 ± 2.5%) compared to trial with cocktail without bacillus (36 ± 3).

Conclusion

During the fermentation of cocoa in the laboratory, the Bacillus strain appears to increase the temperature of the cocoa fermentation mass and reducing sugar content of the cocoa beans cotyledon. Also, the presence of Bacillus in the microbial cocktail reduces the time required for good fermentation and increases the number of well-fermented beans.

Competing Interests

Authors have declared that no competing interests exist.

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