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COCOA FERMENTATION: STARTER ADDITION EFFECT

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ABSTRACT

The importance of cocoa in the beverage industry cannot be over-emphasized, and its fermentation by microorganisms gives rise to better yield. Natural fermentation and controlled fermentation of the cocoa bean with *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, and *Acetobacter pasteurianus* were carried out, and the physiological characterization of the dominant species was determined. The dynamics in microbial population and time course were examined by the microbial count method. The physicochemical properties of natural and controlled fermented cocoa beans and the metabolite production were determined by titration. We figured out the chemical contents of naturally fermented and controlled fermented cocoa beans through proximate analysis. The physiological characterization of the dominant isolates showed that Saccharomyces cerevisiae grew at 25 and 35°C and pH 2.5, 3.5, and 5, as well as ethanol concentrations of 5, 10, and 15%. Also, Lactobacillus plantarum grew at 25, 35, and 45°C, pH of 3.5 and 5, and ethanol concentration of 5 and 10, but not at 15%. We recorded a luxuriant growth at 25 and 35°C for *Acetobacter pasteurianus*, pH 5.0 and 3.5, and ethanol 5 and 10%. The dynamics in microbial composition showed that the Yeast population, LAB and AAB populations increased slowly and reached a maximum of $6.1x10^7$, $4.4x10^7$, and $5.4x10^7$ cfu, respectively, at days 3-4 for the natural fermentation, while they increased abruptly and reached a maximum of $7.2x10^7$, $6.0x10^7$, and $6.1x10^7$ cfu, respectively at day 2-3 for the controlled fermentation. The controlled fermentation of the cocoa mass led to the highest temperature and pH after 72h of fermentation. There was faster sugar depletion in the controlled fermented beans than in the naturally fermented beans, which led to higher metabolite content in the controlled fermentation than in the natural fermentation. Thus, the fermentation of cocoa beans with starter addition produced fermented beans with higher chemical quality and reduction in the time of fermentation to 3 days, compared to 6-7 days obtainable during natural fermentation.

***Keywords*:** Natural and controlled fermentation, dynamics in microbial population and time course, metabolite

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1. INTRODUCTION

Cocoa also known as *Theobroma cacao L* .is found in the family *Sterculiaceae* and is essential as a result of its important seed used in the beverage industry. There are three main groups of cocoa which include – *Trinitario, Forastero and Criollo as reported by* (Beckett 2000; Awua 2002; Amoye 2006; Vinicius et al. 2020; Chagas Junior et al. 2021). The world's major producers of cocoa include Ivory Coast, Ghana, Nigeria, Cameroun, Indonesia and Brazil with Nigeria being the fourth leading exporter of cocoa in the world (IFPRI 2010). Dried cocoa beans serve as main source of foreign exchange in Nigeria while a minor proportion are used to produce cocoa butter, chocolate products as well as cocoa powder (Adeyeye et al. 2010; Mota-Gutierrez et al. 2018).

The process of fermentation of the cocoa beans during chocolate preparation is responsible for characteristic flavor of chocolate (Afoakwa 2010; Vinicius et al. 2020). The microbial multiplicity of cocoa bean fermentation methods has been explored in the last decade in detail to better understand microbial succession and their influences to improving cocoa fermentation (Figueroa-Hernández et al. 2019; Santos et al. 2020). The reaped cocoa pods are subjected to impulsive fermentation out of varied natural microflora from environments such as handling staff, knives, transport containers, pod surfaces, etc. (Jespersen et al. 2005).

The fermentation of raw cocoa beans which lasts 6-7 days usually involves two processes: the microbial interactions inside the pulp and on the surface of the beans as well as other interactions inside the cotyledons (Schwan and Wheals 2004). The microbial activities in the cocoa pulp are characterized by yeast, lactic acid bacteria (LAB) and later by acetic acid bacteria (AAB). Also, other bacterial species including fungi may also be encountered during the fermentation all of which may have impact on the quality of the beans as well as the flavor of the cocoa (Schwan and Wheals 2004).

Diverse metabolites are formed as a result of the microbial activities as the fermentation progresses. (Schwan and Wheals 2004; dos Santos et al. 2021). Different volatile and non–volatile compounds such as alcohols and

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sugars are produced because of the microbial activities during the cocoa fermentation process (Rodriguez-Campos et al. 2012; Pelicaen et al. 2019). During cocoa bean fermentation, the role of microorganisms includes the removal of the pulp that surrounds the fresh beans and the production of indispensable metabolites (Schwan and Wheels 2004; Afoakwa et al. 2007; Santos et al. 2020). These include pectin depolymerisation, fermentation of sugars to ethanol, citric acid, lactic acid, acetic acid and mannitol. (Camu et al. 2008; Vinicius et al. 2020; Rahayu et al. 2021).

The sugars come from sucrose and its hydrolysis products, glucose and fructose, by both cotyledon and pulp pectinase activity, in addition to being released from glycosides (Figueroa-Hernández et al. 2019). These activities result in the death of the bean due to penetration of main ethanol and acetic acid through the husk into the cotyledons, and the creation of an environment, i.e., a decrease of internal pH from 6.5 to 4.8 and increased bean temperature up to 50 °C for development of flavor precursors and pigment degradation by endogenous enzymes, such as invertase, glycosidases, proteases and polyphenol oxidase (Camu et al. 2008; dos Santos et al. 2021). The microbial metabolites produced during cocoa bean fermentations may be responsible for the variations in the cocoa beans quality (Maura et al. 2016; Chagas Junior et al. 2021). This may be responsible for the call to try the use of starter culture to access these metabolites for the improvement of the fermentation processes. These different metabolites improve cocoa fermentation process and the product quality, but results obtained are still not enough for process standardization. Thus, the study of the metabolites produced during cocoa bean fermentation is essential to select appropriate microbial starter that will aid in rapid fermentation of the sugar compounds in the pulp and a proper understanding of the variety of microbial strains associated with cocoa fermentation is important to achieve the aim.

2. MATERIALS AND METHODS

2.1. Cocoa Beans Collection

One hundred (100) ripened cocoa pods were purchased from the local market (Eke Awka) in Awka, Awka South Local Government Area of Anambra State and were transported in sacs to the Microbiology Laboratory of Nnamdi Azikiwe University, Awka for fermentation. The cocoa pods have characteristic size, peel, pulp, 16.32cm in length and 9.75cm in diameter with yellowish bark and 24-27 seeds per pod.

2.2. Spontaneous Fermentation of Cocoa Beans

The cocoa beans were separated from the placenta and five kilo gram beans were introduced into a box fermenter and allowed for six days fermentation as described by Ouattara et al. (2008).

2.3. pH and Temperature of Fermentation

The temperature was taken at various times during the fermentation with thermometer while the pH of the fermenting heap was measured with a pH meter (Metler Toledo MP120) using the method described by Cheesbrough (2006).

2.4. Drying of the Fermented Cocoa Beans

The fermented beans were dried in a temperature controlled forced air oven for 24 hours at a temperature of 45-50^{o}C by spreading on a tray. They were occasionally stirred (24 h) with a turner for even drying, using the method of Hamdouche et al. (2015).

2.5. Isolation and Identification of Isolates

The isolation and identification of the various microbial species involved in cocoa fermentation was carried out according to the method of Lefeber et al. (2012). The beans were aseptically removed every 12h during the fermentation and plated using spread plate method on Nutrient agar (NA), Sabroaud dextrose agar (SDA), Mann Rogosa Sharpe agar (MRS) and formulated medium containing (peptone: 3g/L, yeast extract: 5g/L and glucose: 25g/L; pH 7.2±0.2). The media except the SDA were supplemented with 0.05mg/mL nystatin to prevent yeast growth while the SDA medium was supplemented with 0.05mg/mL chloramphenicol to prevent bacterial growth. The SDA plates were incubated at 28±2°C for 48h and other plates at 37°C for 24-48h. Different isolated colonies were replicated on fresh plates of SDA, MRS, GYPA and NA medium respectively, for pure cultures of the isolates. The colonies detected were expressed as the number of colony forming units in terms of $\log^{10}$ cfu/g. The isolates were identified by biochemical tests using the method described by Cheesbrough (2006) and Sarbu and Csutak (2019).

2.6. Physiological Adaptation of the Isolates

Nutrient broth (NB), Saboraud Dextrose broth (SDB), De Man Rogosa and Sharpe broth (MRSB) (Merck, Darmstadt, Germany), glucose yeast extract peptone broth (GYPB) containing 20g/L D-glucose, 5g/L yeast extract and 5g/L peptone were used for the characterization of the physiological adaptation of the NA, SDA, MRS and GYPM isolates respectively using the method described by (Lisdiyanti et al. 2000). To test for pH tolerance, the different broths were attuned to pH 2.5, 3.5, and 5.0 with 5M HCl. To test for ethanol tolerance, the different broths were enhanced with 5%, 10%, or 15% (v/v) ethanol. To test for heat tolerance, the different broths were adjusted to pH 5.5

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and were incubated at 25, 35 and 45°C. All tubes were inoculated with 1ml of cultures from the different media developed at 30°C for 24h. Growth was confirmed by measuring the OD_{600nm} after incubation at 30°C for 5 days.

2.7. Improved Fermentation by Starter Culture Addition (Controlled Fermentation)

The cocoa pods were washed with distilled water and cleaned with 90% ethanol and broken open with a sterilized knife; ten (10) kg of cocoa beans was inoculated aseptically with cultures from the spontaneous fermentation and fermented on sterilized foil (foils were sterilized in an oven for 24h at 50°C) using the method described by Schwan and Fleet (2014). The isolate consortia were cultivated in MRS, SD, and AAB broth respectively at 28°C in a 100mL conical flask and placed in a shaker for 48h at 200rpm. The biomass was later harvested by centrifugation at 3000 rpm for 20 min, washed with sterile saline (0.85% NaCl w/v) and used as starter for the controlled fermentation. For comparison, fermentations without starter addition (natural fermentation) were also performed and incubated for 6 days after which they were analyzed. The fermented cocoa beans were harvested at different time intervals (24, 48, 72, 96,120 and 144 h).

2.8. Microbial Count of Isolates During Natural and Controlled Fermentation of Cocoa Bean

The fermenting beans were harvested every 12h and plated using the pour plate method on SDA, MRS and acetobacter medium (consisting of 50g/l of D-glucose, 10g/l of yeasts extract, 1g/l of peptone, 20g/l of glycerol, 15g/l of potato and 40g/l of ethanol), for AAB. The agar plates were incubated at 30°C for 3days. After the incubation period, the number of colony- forming units (CFU) was recorded. Colonies were picked at random in a number equal to the square root the total colonies present on the counted plate, seeking to ensure that all different colony morphologies were represented in each case (Senguna et al. 2009; Visintin et al. 2017). All samples of microbial analysis were done in triplicates.

2.9. Physicochemical of Natural and Controlled Fermented Cocoa Bean pH and Titratable Acidity

The pH of the cocoa bean was measured using a pH meter (Metler Toledo MP120). The acidity was also measured using the method of Nazaruddin et al. (2006) by titrating a further 5mL filtrate obtained during the pH determination. The data was reported as mole of g/100mL sample. The analysis was done in triplicate, and the mean values were reported.

2.10. Sample Preparation for Metabolite Analysis

Ten (10) grams of the pulverized cocoa sample was soaked in 100mL of distilled water overnight and then filtered using Whatman paper. The filtrate was used for metabolite analysis. Ethanol was estimated using potassium dichromate ($K_2Cr_2O_7$) oxidation method and spectrophotometry (College of Science, University of Canterbury, 2005). The quantification was done with standard curve and expressed as (mg/g). The production of lactic acid and acetic acid was determined by titration using the methods described by the College of Science, University of Canterbury (2005) and Danzer (2007), respectively.

2.11. Statistical Analysis

The results of the pH, titer-able acidity, and metabolites of natural and controlled fermentation from the study were subjected to statistical analysis using One-Way Analysis of Variance (ANOVA) and Duncan Multiple range test in a statistical package for social sciences (SPSS) software (version 20).

3. RESULTS AND DISCUSSION

The diversity of microorganisms was studied to explain the variability of cocoa beans quality from Nigeria and the need for important starters for Nigerian cocoa fermentation. The yeast species were mostly isolated as a result of its acidophilic nature as well as its stability to both heat and increased ethanol concentration and pectinolytic activity (Daniel et al. 2009; Papalexandratou and De Vuyst 2011; Hamdouche et al. 2015; Ooi et al. 2020; Moreira et al. 2021). Odilon et al. (2017), Ouattara et al. (2008) and Chagas Junior et al. (2021) reported a diversity of yeast species implicated in the spontaneous cocoa fermentation, the limited species recorded in this study could be because the fermentation was performed in the laboratory.

The isolates obtained from MRS agar showed that the fermentation process was controlled by *L. plantarum*. The predominance of homofermentative LAB strains was also reported by other studies (Kostinek et al. 2008; Ouattara et al. 2008; Liliane et al. 2015; Viesser et al. 2020). It is well known that homofermentative LAB strains convert sugars into lactic acid while heterofermenters liberate lactic acid and ethanol and thus, compete with the yeast for nutrients, thereby inhibiting their growth, slowing down fermentation, and impairing the production of ethanol (Thomas et al. 2002; Vinicius et al. 2020). Therefore, homofermentative strains producing only lactic acid may be more interesting and desirable.

The isolates obtained from GYPA agar indicated the fermentation process was dominated by *Acetobacter spp*, which has a high acidification capacity necessary cocoa beans and chocolate quality (Schwan and Wheal 2004;

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Romero-Cortes et al. 2013; Liliane et al. 2015). Ouattara et al. (2008) isolated both *Acetobacter spp* and *Gluconobacter* species. The differences in the isolated species recorded could be because the fermentation was on a Lab-scale. The high occurrence of *Acetobacter* species was also previously recorded (Daniel et al. 2009; De vuyst et al. 2010; Pereira et al. 2012; Samagaci et al. 2016; Odilon et al. 2017).

The result of the physiological adaptation of the isolates showed that *Saccharomyces cerevisiae, L. fermentum, L. plantarum, and A. pasteurianus persisted throughout* the fermentation as a result of their physiological ability to thrive in the cocoa bean environment during fermentation (Table 1). *They were* tolerant to acid, ethanol and heat. The results obtained were similar to those recorded by Lisdiyanti et al. (2000), Romero-Cortes et al. (2013), Ooi et al. (2021) and dos Santos et al. (2021), who reported the ability of S. *cerevisiae, L. fermentum, L. plantarum, and A. pasteurianus to* grow at temperatures of 45°C, pH 3.5 and 10% ethanol.

Table 1: Physiological characterization of isolates

Isolates	Temperature (°C)			pH			Ethanol concentration (%)		
	25	35	45	2.5	3.5	4.5	5	10	15
S. cerevisiae	+	+	-	+	+	+	+	+	+
L. plantarum	+	+	+	-	+	+	+	+	-
A. pasteurianus	+	+	+	-	+	+	+	+	+
L. fermentum	+	+	+	-	+	+	+	+	-

Key: + positive, - negative.

The temperature and pH values of the cocoa beans fermenting mass both fermentations (Table 2 and 3) were significant (P<0.05). Temperature and pH are useful in evaluating the progress of the fermentation (Wood and Lass 2001). The above temperature and pH values gave a precise fermentation pattern in the controlled fermentation process. The oxidation of ethanol produced by the yeast to acetic acid by acetic acid bacteria is an energy releasing process which led to a rise in temperature of up to 47°C or higher in the fermenting mass. This was in consonance to the earlier reports by (Schwan and Wheels 2004; Sandhya et al. 2016; Ooi et al. 2021a).

Table 2: Variation in temperature (°C) of cocoa beans fermenting heap during natural and controlled fermentation

Time (hours)	Fermentation		
	Natural	Controlled	SEM
0	28.40	28.20	±1.50
12	31.40b	33.20a	±1.50
24	37.20b	39.60a	±1.50
36	38.40b	42.60a	±1.50
48	42.30b	44.70a	±1.50
60	42.40b	43.40a	±1.50
72	44.40b	47.60a	±1.50
84	41.50a	40.10b	±1.50
96	38.20b	39.20a	±1.50
108	36.40b	37.40a	±1.50
120	34.60b	35.60a	±1.50
132	31.60b	34.70a	±1.50
144	30.40b	34.60a	±1.50

Means bearing different alphabets across the row are significantly (P<0.05) different from each other.

Table 3: Variation in pH of cocoa beans fermenting heap during natural and controlled fermentation

Time (hours)	Fermentation		
	Natural	Controlled	SEM
0	3.70	3.72	0.15
12	3.80	3.84	0.15
24	3.90a	3.70b	0.15
36	4.00a	3.71b	0.15
48	4.10a	3.72b	0.15
60	4.40a	3.74b	0.15
72	4.91a	4.20a	0.15
84	5.42a	5.10b	0.15
96	5.80a	5.30b	0.15
108	6.00a	5.22b	0.15
120	6.56a	5.42b	0.15
132	6.96a	5.60b	0.15
144	6.98a	5.70b	0.15

Means bearing different alphabets across the row are significantly (P<0.05) different from each other.

The breakdown of increase sugars by yeasts and citrate by lactic acid bacteria resulted to a rise in the pH (Galvez et al. 2007). The current studies specified that controlled fermentation showed different pH values as a result of excessive liberation of acetic acid. Sandhya et al. (2016) showed that high pH (5.9-7.2) indicates poorly fermented cocoa bean seed. The pH obtained in this study resembles that recorded by Sandhya et al. (2016), who stated that 4.3pH in 72h indicated well-fermented cocoa beans. It was also observed in another study that 72h led to good fermented cocoa beans (Romero-Cortes et al. 2013; Ooi et al. 2021a).

The outcome of the pH analysis of the cocoa bean seed for the natural and controlled fermentation for the first three days were found to be significant P<0.05, with the controlled fermentation having higher acidity (Table 4). No significant difference was recorded for both fermentations at days 4, 5, and 6 (Table 4). The pH recorded in this study was similar to those obtained by Graziani de Farinas *et al.* (2002), who recorded a pH of 4.75. This could be attributed to the permeability of the bean pulp to acetic acid, which tends to destroy the embryo leading to a decrease in the pH Afoakwa *et al.* (2007) stated that a pH of 5.5-5.8 is synonymous to poor fermentation while properly fermented cocoa bean usually has a pH of about 4.7-5.2.

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Table 4: pH of the cocoa bean during natural and controlled fermentation

Days	Fermentation		
	Natural	Controlled	SEM
1	3.88b	4.86a	0.01
2	4.15b	4.97a	0.01
3	4.47b	4.98a	0.01
4	4.99	4.89	0.01
5	5.20	5.16	0.01
6	5.66	5.60	0.01

Means bearing different alphabets across the row are significantly (P<0.05) different.

Table 5: Titratable Acidity of the cocoa bean during natural and controlled fermentation

Days	Fermentation		
	Natural	Controlled	SEM
1	0.88a	0.69b	0.01
2	1.24b	1.35a	0.01
3	1.82b	1.92a	0.01
4	2.16b	2.28a	0.01
5	2.45b	2.55a	0.01
6	2.72b	2.58a	0.01

Values (mean+SD) with different alphabets across the row are significantly (P<0.05) different.

A significant difference (P<0.05) was observed between the total titratable acidity of the natural and controlled fermentation from days 1, 3, 4, 5, and 6, respectively, no significant difference (P<0.05) was observed in the acidity of the natural and controlled fermentation at day 2 (Table 5). This result is different from that reported by Rodriguez-Campos et al. (2012) and Figueroa-Hernández et al. (2019), who showed that the concentration of the total acid increased significantly on days 2 and 4 only. The acidity of the cocoa bean was lowest with controlled fermentation at (0h) 0.68±0.01g/mol. and rose to a peak of 2.48±0.01g/mol after 144h of fermentation. Rivera et al. (2012) stated that acids products of microbial fermentation led to a rise in acidity and subsequent lowering of the pH. These agreed with the work of (Pedro et al. 2016), who recorded a pH of 2.37 for fermented cocoa.

Table 6: Variation in metabolites produced during natural fermentation of cocoa bean seed in mg/g

Days	Ethanol	Lactic Acid	Acetic Acid
1	0.35±0.01	0.01±0.01	0.66±0.00
2	1.21±0.01	0.09±0.00	1.36±0.00
3	1.75±0.01	0.25±0.01	1.94±0.00
4	1.41±0.01	0.16±0.01	1.86±0.00
5	1.06±0.01	0.09±0.00	1.44±0.00
6	0.00±0.00	0.01±0.01	0.94±0.00

Table 7: Variation in metabolites produced during controlled fermentation of cocoa bean seed in mg/g

Days	Ethanol	Lactic Acid	Acetic Acid
1	4.83±0.02	0.19±0.00	1.00±0.00
2	9.32±0.01	0.61±0.01	2.30±0.01
3	5.92±0.01	0.21±0.01	1.51±0.01
4	8.74±0.01	0.65±0.01	2.01±0.01
5	5.01±0.01	0.63±0.01	1.83±0.01
6	4.14±0.01	0.65±0.01	0.95±0.01

The amount of ethanol reported in this study (Table 6 and 7) was similar to the report by Lehrian and Patterson (1984), who recorded 9.6 to 7mg/g for alcohol after 48 hours and in contrast to works done by Camu et al. (2008), Papalexandratou and De Vuyst (2011), Lefeber et al. (2012), Sandhya et al. (2016) and Mota-Gutierrez et al. (2018) who recorded value as high as 30 to 60mg/g; this difference could be attributed to the microbial diversity in the fermentation environment. Maximum lactic acid and acetic acid production were attained in 48h (day 2) for the controlled fermentation (Table 7). This may be linked to the breakdown of ethanol and lactic acid production by yeast and LAB strains. This is similar to an earlier finding by Sandhya et al. (2016) and Visintin et al. (2017).

The increased level of metabolic products during the controlled fermentation led to an increase in the liberation of cocoa flavor enhancers, as was observed in the increase of yeast, LAB, and AAB. This measure indicated that the addition of yeast, LAB and AAB starters hastened the cocoa bean fermentation and thus lowered the fermentation time. The decreased fermentation time is a significant process improvement. Furthermore, the production of ethanol, lactic acid, and acetic acid in the pulp and beans was also greater in the controlled fermentation, resulting in larger populations of *S. cerevisiae, L. plantarum*, and *A. pasteurianus* during fermentation.

4. Conclusion

The addition of microbial starter into cocoa bean fermentation as well as environmental factors (pH and temperature) reduced the fermentation process from 6 to 3 days accelerated the depletion of sugary compounds in the cocoa pulp, thereby increasing the formation of the metabolic products. This results in seeds with higher desirable export quality. The addition of microbial starter proved to be the basis for the improvement of cocoa bean fermentation quality, demonstrating the further application of these strains.

Author's Contribution

Okonkwo IF conceived, designed, supervised and edited the work. CQ Igwilo conducted the experiment, analyzed the data and wrote the manuscript under the supervision of Okonkwo IF.

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