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Effects of Starter Addition on Microbiology and Sugars Produced during Cocoa Bean Fermentation

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Abstract

The significance of cocoa in the beverage industry cannot be underscored, as its fermentation by microorganisms contributes to better yield. Natural fermentation as well as controlled fermentation of the cocoa bean with defined starter groups of *Saccharomyces cerevisiae, Lactobacillus plantarum* and *Acetobacter pasteurianus* was carried out. The physiological characterization of the prevailing species was carried out. The dynamics in microbial population and time course were determined by microbial count method. The physico chemical properties of the cocoa beans as well as the sugar production were determined by titration while the chemical content of the beans was determined through proximate analysis. The physiological characterization of the dominant isolates revealed that *Saccharomyces cerevisiae* grew at temperature of 25 and 35°C and at pH 2.5, 3.5 and 5 as well as ethanol concentration of 5, 10 and 15%. *Lactobacillus plantarum* grew at temperature of 25, 35 and 45°C, pH of 3.5 and 5 but not at 2.5 and grew at ethanol concentration of 5 and 10, but not at 15%. Again, *Lactobacillus plantarum* grew at the temperature of 25, 35 and 45°C, pH of 3.5 and 5, and ethanol concentration of 5 and 10, but not at 15% while a luxuriant growth was recorded at the temperature of 25 and 35°C for *Acetobacter pasteurianus*, pH 5.0 and 3.5 and ethanol concentration of 5 and 10%. The dynamics in microbial arrangement showed that the Yeast population, LAB and AAB populations increased gradually and extended to a maximum of 6.1 x 10^7, 4.4 x 10^7 and 5.4 x 10^7 cfu, respectively, at days 3 - 4 for the natural fermentation, while they increased sharply reaching a maximum of 7.2 x 10^7, 6.0 x 10^7 and 6.1 x 10^7 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 quicker sugar exhaustion in the controlled than in the naturally fermented beans, leading to greater metabolite content in the controlled than in the natural fermentation. At the cessation of the fermentation on the 5th day of the controlled fermentation, about 12.33 mg/g, 0.91 mg/g and 5.99 mg/g of glucose, sucrose and fructose respectively were left when compared with 12.29 mg/g, 3.83 mg/g and 16.55 mg/g glucose, sucrose and fructose respectively at day 5 of the natural fermentation. In 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. It also accelerated the depletion of sugary compounds in the cocoa pulp thus increasing the metabolic product development and bean seeds with required trade value.

Keywords: *Cocoa Bean; Fermentation; Starters; Sugars*

Introduction

*Theobroma cacao L. (*Cocoa) belongs to the family *Sterculiaceae* and is very important in the beverage industry. Three major groups distinguished are - *Criollo, Trinitario* as well as *Forastero as* described by Amoye [1] and Chagas., *et al* [2]. Nigeria is one of the cocoa ex-

porting countries in the whole world [3]. After drying, the beans are used for the manufacture of products such as chocolate, cocoa powder and cocoa butter according to Mota-Gutierrez., *et al* [4]. The processes involved during chocolate production results in the peculiar chocolate flavor going by the report of Vinicius., *et al* [5]. The cocoa pods when exposed to the activities of different microorganisms in the environment during fermentation, lead to a lot of transformations [6]. Different volatile and non-volatile compounds such as alcohols and sugars are produced as a consequence of the microbial activities during the cocoa fermentation process. Also, the removal of the pulp that surrounds the fresh beans and the production of indispensable metabolites are also achieved; these include pectin depolymerisation, fermentation of sugars to ethanol, citric acid, lactic acid, acetic acid and mannitol.

The cocoa beans fermentation naturally takes about a week and involves the activities of microbes on the beans, its pulp and the cotyledons [7]. The microbial interactions are marked by lactic acid bacteria (LAB), yeast as well as acetic acid bacteria (AAB). Again, other organisms can also contribute to the quality and flavor achieved during the fermentation [7]. And different metabolites and sugars are released during these microbial interactions [8,9]. These are converted to products such as mannitol, ethanol, acetic acid, citric acid and lactic acid [5,10]. The sugars come as a result of the hydrolysis of sucrose which releases glucose and fructose and from glycosides by pectinase enzyme activity [11]. These activities create an increased acidic environment in the cotyledons with a raised cocoa bean temperature which results in the formation of aroma enhancers and the breakdown of pigments by inherent proteins or enzymes [12]. The sugars released in the course of the fermentations may alter the quality of the beans as well as the metabolites produced [2]. Therefore, the study of the sugars obtained in the course of the fermentation is necessary in the choice of microbial starter that will help in faster metabolism of the sugary complexes in the bean seeds and a good knowledge of the various microbes involved in the fermentation is essential for the accomplishment of this objective.

Materials and Methods

Cocoa beans collection

A hundred (100) ripened cocoa pods were bought from (Eke Awka) market in Anambra State and taken to Nnamdi Azikiwe University, Awka Laboratory for fermentation.

Fermentation of the cocoa beans

The beans were processed and 5 kg beans were placed in a local fermenter for a period of six days for fermentation as given by Ouattara., *et al* [13].

Determination of temperature and pH of fermentation

The temperature and pH were determined at various time schedules using a thermometer and a pH meter (Metler Toledo MP120) respectively according to Cheesbrough [14].

Drying of the fermented beans

The beans were dried in a temperature controlled forced air oven for 24 hours at a temperature of 45 - 50°C by spreading on a tray as described by Okonkwo and Igwilo [15]. They were intermittently agitated at (24h) intervals with a turner for uniform drying, as applied by Hamdouche., *et al* [16].

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 used by Lefeber., *et al* [17]. For the isolation, the beans were removed every 12h and cultured on Nutrient agar NA, Sabouraud

dextrose agar SDA, Mann Rogosa Sharpe agar MRS, and formulated medium containing (Peptone - 3 g/l, Yeast Extract - 5 g/l and Glucose - 25 g/l; pH 7.2 ± 0.2). The media were supplemented with 0.05 mg/ml nystatin and chloramphenicol to prevent yeast and bacterial growth respectively. The plates were incubated at 28 ± 2°C for 48h and 37°C for 24 - 48h, respectively. The isolates were simulated on new SDA, MRS, GYPA and NA medium respectively, to obtain 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 later identified using biochemical tests according to the method described by Cheesbrough [14] and Sarbu and Csutak [18].

Characterization of the isolates

Nutrient broth (NB), Sabouraud Dextrose broth (SDB), De Man Rogosa and Sharpe broth (MRSB) (Merck, Darmstadt, Germany), Glucose yeast extract peptone broth (GYPB) containing 20 g/l D-glucose, 5 g/l yeast extract and 5 g/l peptone were used to characterize the isolates respectively adopting the method of Lisdiyanti., *et al* [19].

Test for pH tolerance

The broths were adjusted to pH 2.5, 3.5 and 5.0 with 5M HCl.

Test for ethanol tolerance

The broths were enriched with 5%, 10% or 15% (v/v) ethanol.

Test for heat tolerance

The broths were adjusted to pH 5.5 and incubated at 25, 35 and 45°C. All the tubes were then inoculated with 1 ml culture from the different media developed at 30°C for 24h. Growth was determined by accessing the OD_{600nm} post incubation at 30°C for 5 days.

Improved fermentation (Controlled fermentation)

The washed and cleaned cocoa pods were opened and 10 kg of the beans injected aseptically with cultures from the natural fermentation and allowed to ferment on sterile foil as described by Schwan and Fleet [20]. The isolated colonies were cultured in MRS, SD and AAB broth respectively at 28°C in a 100 ml flask and incubated in a shaker at 200 rpm 48h. The cells were recovered by centrifuging at 3000 rpm for 20 minutes. The biomass were later washed with saline (0.85% NaCl w/v) and used as inoculum for the controlled fermentation. Also, a natural fermentations protocol was also set up and incubated for 6 days for comparison after which they were evaluated at different time intervals (24, 48, 72, 96, 120 and 144h).

Microbial count of natural and controlled fermentations

The fermenting beans were collected every 12h and cultured by pour plate method on SDA, MRS and mineral salt medium (consisting of 50 g/l of D-glucose, 10 g/l of yeasts extract, 1 g/l of peptone, 20 g/l of glycerol, 15 g/l of potato and 40 g/l of ethanol), for AAB. The agar plates were then incubated at 30°C for 3 days after which the number of colony- forming units (CFU) were noted by Visintin., *et al* [21]. All the investigations were repeated in triplicates.

Determination of pH and titratable acidity

The pH of the cocoa bean was determined using a pH meter (Metler Toledo MP120) while the titratable acidity was determined by adopting the method of Nazarudin., *et al.* [22] by titration using a further 5 ml filtrate obtained during the pH determination. The data was stated as mole of g/100 ml sample. The study was done in triplicate and the mean values recoded.

Sample preparation for sugar analysis

A 10g of the pulverized cocoa sample was saturated in 100 ml of distilled water over 24h and then clarified with the aid of a whatman paper. The filtrate was employed for the sugar analysis.

The fructose obtained during the natural and the controlled fermentation of the cocoa beans was determined using the method of Edewor and Theresa [23]. Sucrose and glucose were evaluated with Dinitrosalicylic acid (DNS) and calculated (mg/g) from the normal curve plot based on the absorbance at 510 nm Finley and Fellers [24].

Statistical analysis of data

The various results from the study were subjected to statistical analysis using One-Way Analysis of Variance (ANOVA) and Duncan Multiple range test in statistical package for social sciences (SPSS) software (version 20).

Results and Discussions

In this study, the diversity of microorganisms explained the variability of cocoa beans quality from Nigeria and identified key species needed for Nigerian cocoa fermentation. The yeast species isolated from the natural fermentation was *Saccharomyces cerevisiae*, which is dominant as a result of its prompt growth at a slightly raised pH, pectinolytic activity, ethanol and heat tolerance as reported by Daniel., *et al.* [25], Papalexandratou [26] and De Vuyst [27], Hamdouche., *et al*. [16], Odilon., *et al*. [28] and Qauttara., *et al*. [13] had given a report of diversity of yeast species implicated in the natural fermentation of cocoa beans; the restricted types documented in this study could be as a result of laboratory-based fermentation.

The isolates obtained from MRS agar showed that the fermentation was dominated by *L. plantarum*. The prevalence of homofermentative LAB strains was also reported by other studies-Kostinek., *et al*. [29], Qauttara., *et al*. [13], Liliane., *et al*. [30] as homofermentative LAB strains convert sugars almost entirely into lactic acid while heterofermentative strains yield lactic acid and ethanol and thus, compete with yeast for nutrients there by hindering their growth, slow down fermentation and weaken their ability to produce ethanol Thomas., *et al* [31]. Hence, homofermentative strains are most desirable.

The isolates from GYPA agar showed that the process was controlled by *Acetobacter spp* which has a renowned acidification capacity needed for manufacture of cocoa beans and chocolate quality as reported by Schawn and Wheal [7], Romero-Cortes., *et al*. [32], Liliane., *et al*. [30], Ouattara., *et al*. [13] obtained *Acetobacter* spp as well as *Gluconobacter* species. The variances in the species isolated may be because of laboratory-based fermentation. The high incidence of *Acetobacter spp* species was also documented by De vuyst [27], Pereira., *et al*. [33], Samagaci., *et al*. [34] and Odilon., *et al* [28].

The outcome of the adaptation of the isolates showed that *Saccharomyces cerevisiae, L. fermentum, L. plantarum and A. pasteurianus* continued through the fermentation as a consequence of their ability to grow in the cocoa bean environment during fermentation (Table 1). They tolerated acid, ethanol and heat. The results obtained were comparable to those documented by Lisdiyanti., *et al.* [19] and Romero-Cortes., *et al*. [32] who stated 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.

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	+	+	+	-	+	+	+	+	-

Table 1: *Physiological characterization of isolates.*

Key: +: Positive, -: Negative.

The temperature and pH values of the cocoa beans fermenting mass for both fermentations (Table 2 and 3) were significant ($P < 0.05$). Temperature and pH are useful in evaluating the progress of the fermentation [35]. 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 as reported by Okonkwo and Igwilo [15] 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 [7], Sandhya., *et al*. [36], Ooi., *et al* [37].

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

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

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

The breakdown of increased sugars by yeasts and citrate by lactic acid bacteria caused a rise in the pH [38]. The present studies stated that controlled fermentation indicated different pH values as a result of extreme release of acetic acid. Sandhya., *et al*. [36] revealed that increased pH (5.9 - 7.2) indicates poorly fermented cocoa bean seed. The pH got in this study is similar to that recorded by Sandhya., *et al*. [36], who specified that 4.3 pH in 72h showed well-fermented cocoa beans. It was also perceived in another work that 72h led to good fermented cocoa beans [32,37].

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

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

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

The effect of the pH study 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 noted in this study was comparable to those achieved by Graziani de Farinas., *et al.* [39], who recorded a pH of 4.75. This could be credited to the porousness of the bean pulp to acetic acid, which has a tendency to destroy the embryo leading to a drop in the pH [40]. Afoakwa., *et al.* [40] 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.

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

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

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

A substantial 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 differed from the one reported by Rodriguez., *et al.* [41] and Hernández., *et al*. (2019) [42], who presented

that the total acid increased considerably on days 2 and 4 only. The acidity of the cocoa bean was least with controlled fermentation at (0h) 0.68 ± 0.01 g/mol. and rose to a peak of 2.48 ± 0.01 g/mol after 144h of fermentation. Rivera., *et al.* [43] stated that acid produced during microbial fermentation usually lead to an increased acidity and subsequent reduction in the pH. These agreed with the report of Pedro., *et al*. [44], who documented a pH of 2.37 for fermented cocoa.

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

Table 5: *Titratable acidity of the cocoa bean during natural and controlled fermentation.*

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

The amount of ethanol reported in this study (Table 6 and 7) was comparable to the report by Leharian and Patterson [45], who documented 9.6 to 7 mg/g for alcohol after 48 hours, and in contrast to reports by Camu., *et al*. [42], Papalexandratou., *et al*. [26], Lefeber., *et al*. [17], Sandhya., *et al*. [36], and Mota-Gutierrez., *et al*. [4] who recorded value as high as 30 to 60 mg/g; this variation may be accredited to the differences in microbial ecology in the fermentation environment. Highest lactic acid and acetic acid production were attained in 48h (day 2) for the controlled fermentation (Table 7). This may be associated with the breakdown of ethanol and subsequent lactic acid production by yeast and LAB strains. This is comparable to a previous finding by [21,36].

The results obtained with the sugars produced during both the controlled and natural fermentation table 6 and 7 were statistically different ($p < 0.05$). The natural fermentation recorded a decrease in sugar concentration from 19 mg/g in day 1 to 10.50 mg/g and 15.58 to 9.85 on the sixth day for both glucose and fructose respectively while the controlled fermentation led to a decrease in sugar from 18 mg/g in day 1 to 6 mg/g and 13.23 to 1.97 m/g on the sixth day for both glucose and fructose (Table 6 and 7). The decrease in reducing sugars was attributed to the inocula level and stimulation of metabolite production by the strains during fermentation. Glucose and fructose were exploited during fermentation, both in the natural and controlled fermentation. The consumption of glucose and fructose in the controlled fermentation was higher than in the natural fermentation, since the growth of *S. cerevisiae, L. plantarum* and *A. pasteurianus* were higher. Sandhya., *et al.* [36] reported a decline in the amount of reducing sugars, whereas Kresnowati., *et al*. [46] reported that 20 mg/g sugar was still left after day 5 of controlled fermentation. In all, the mucilage of cocoa bean exhausted faster with the controlled fermentation. The reducing sugars can also differ as a result of differences in sugars in the pods, maturity of the harvested pods and natural features of cocoa fermentation [36].

Day	Glucose	Sucrose	Fructose
1	19.00 ± 1.00	5.68 ± 1.00	15.58 ± 1.00
2	17.00 ± 1.00	4.10 ± 0.10	15.12 ± 0.02
3	15.00 ± 1.00	2.34 ± 0.01	14.67 ± 0.01
4	12.00 ± 1.00	2.16 ± 0.01	14.22 ± 0.01
5	10.30 ± 1.00	0.58 ± 0.00	13.77 ± 0.01
6	10.50 ± 1.00	0.25 ± 0.01	9.85 ± 0.01

Table 6: *Variation in sugar content of cocoa bean during natural fermentation in mg/g.*

Day	Glucose	Sucrose	Fructose
1	18.00 ± 1.00	5.16 ± 0.00	13.23 ± 0.00
2	16.00 ± 1.00	4.19 ± 0.00	11.93 ± 0.01
3	13.00 ± 1.00	2.82 ± 0.01	10.64 ± 0.01
4	11.00 ± 1.00	1.45 ± 0.01	6.74 ± 0.01
5	9.00 ± 1.00	0.13 ± 0.01	2.84 ± 0.01
6	6.00 ± 1.00	0.08 ± 0.01	1.97 ± 0.01

Table 7: *Variation in sugar content of cocoa bean during controlled fermentation in mg/g.*

Conclusion

The addition of microbial starter into cocoa bean fermentation as well as environmental factors (pH and temperature) accelerated the depletion of sugary compounds in the cocoa pulp especially glucose and fructose during the period of six days thus increasing the metabolic product development, specifically ethanol, lactic acid and acetic acid. This has resulted to bean seeds with required trade value. The addition of microbial starter has been found to be the ultimate for the enhancement of cocoa bean fermentation quality, validating additional use of these strains as starters during fermentation.

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