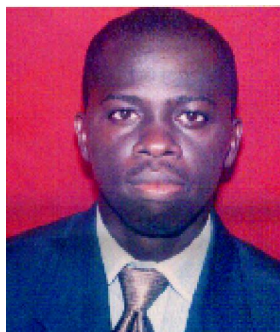


**COMPARISON OF BIOCHEMICAL CHANGES DURING ALCOHOLIC
FERMENTATION OF COCOA JUICE CONDUCTED BY SPONTANEOUS
AND INDUCED PROCESSES FOR THE PRODUCTION OF ETHANOL****Anvoh KYB*¹, Guéhi TS¹, Beugré GAM¹, Kinimo JM¹ and D Gnagri¹****Koutoua Anvoh**

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ABSTRACT

Ivory Coast (Côte d'Ivoire) is the biggest cocoa producing country in the world with an average 1405 000 metric tons of cocoa beans. It also produces several quantities of cocoa mucilage juice during cocoa harvesting. In spite of its high sugar and over minerals content, cocoa juice is currently abandoned in farms. This study aims to compare the nature of products resulted from a spontaneous fermentation conducted by uncontrolled or indigenous yeasts to those from a controlled fermentation made by using commercial bakery yeast such as *Saccharomyces cerevisiae* at a rate of 30g/hL during 3 to 5 days of cocoa mucilaginous juice. To conduct this study, fermentations were held at different temperatures 28, 30, 35 and 41°C. Chemical analyses related to the pH by pH-meter, total sugar content using standard method and to the measurement of the yield of ethanol using a gas chromatography (CPG), microbial count were carried out. Analyses showed that yeast, mould, aerobic mesophilic and lactic acid bacteria were isolated in almost all samples. Cocoa shield was shown to contain the highest number of yeast and mould at 1.6×10^4 CFUcm⁻², aerobic mesophilic flora at 1.5×10^5 CFUcm⁻² and lactic acid bacteria at 2.1×10^4 CFUcm⁻². Whatever the type of fermentation, yields of ethanol was highest at 30°C. Fermentation conducted with controlled yeast gave a better yield of ethanol with 8.4 % than spontaneous fermentation that yielded only 4.3 %. There were significant differences (p 0.05) between both alcohol productivity and yield at spontaneous and controlled fermentation. Total sugar content decreased drastically during the fermentation process but a residual content was observed at the end due to the incomplete fermentation. Residual sugar content was highest with values ranging from 35 to 80 g/L at 35°C and 41°C in the controlled fermentation. Production of ethanol was associated with increase in pH value ranging from 4.5 to 4.9.

Key words: Yeasts, Cocoa juice, Fermentation, Ethanol

INTRODUCTION

The wine fermentation is a complex biochemical process, during which yeasts metabolize sugars and other constituents as substrates for their growth by converting them into ethanol [1]. Several ingredients rich in sugars such as fruits (grape, apple) and certain juices such as sugar cane, cocoa beans can be used as raw material to produce alcohol.

Tropical fruits (pawpaw, banana) were used successfully for wine production. In the same vein, a traditional beverage such as koutoucou is produced using palm wine [2]. During cocoa post harvest treatment such as pods opening, a viscous and acidic solution is produced from the mucilaginous pulp surrounding beans. For example, one litre of juice could be produced from 10kg of fresh cocoa beans. Cocoa producing countries in West Africa such as Côte d'Ivoire and Ghana, and in some Asian countries such as Indonesia and Malaysia produce the largest amount of cocoa beans. Côte d'Ivoire, Ghana and Indonesia produced 1,405, 000, 736,000 and 41500 metric tons respectively during year 2003-2004 [3]. Each year, the amount of pulp solution produced by these countries is estimated 550 000m³. Unfortunately, only small quantity of this sweet cocoa juice is consumed as beverage and the major part is abandoned in farms. The valorization of cocoa juice could produce additional financial benefits for small farmers and other storekeepers in the cocoa production and supply cocoa network and so reduce the negative drawbacks due to the drop in cocoa beans price at the international market.

The microflora which ferment cocoa juice at farm level contained several and uncontrolled strains of micro-organisms of yeasts, bacterias and moulds [4, 5, 6]. These micro-organisms can ferment cocoa bean juice and degrade its sugars into alcohol and a panel of several secondary products like organic acids.

Assays on cocoa bean juice transformation into wine have been successfully conducted but these authors have added sugars to the cocoa juice and obtained 12.5 % as yield of ethanol [7]. Current fermentations in winery and other alcohol distilleries use high biotechnology process with selected yeasts at each step during the operation. These fermentations were conducted with *Saccharomyces cerevisiae* because of its high performance and its better yield of alcohol (17 %).

Despite the high performance and ethanol yield of controlled fermentations, spontaneous fermentations with uncontrolled microflora which may contain yeasts, bacteria and moulds are still used in some winery [8]. Some winemakers prefer uncontrolled yeasts for alcoholic fermentation because of their low costs. Indeed, the variability of the strains involved in spontaneous fermentation would favor the production of more important aromatic complexities of wine and of more undesirable products [9]. Moreover, spontaneous fermentation would lead to obtaining distinct styles of wines reflecting the diversity of a specific region of vintage [10]. Consumers seem to appreciate the wines or other alcoholic beverage depending on their origin or the producing district.

The present work aims to compare the dynamic of uncontrolled yeasts involved in spontaneous fermentation on the performance of controlled yeast such as *Saccharomyces cerevisiae* used in alcoholic fermentation at several temperatures.

MATERIALS AND METHODS

Material

Cocoa bean juice was collected immediately after cocoa pods opening from three industrial farms around Abidjan (South of Côte d'Ivoire) in September 2008. Fresh cocoa beans were packed in a basket at about a meter up from the ground. The basket was slightly tilted in order to collect the juice in bottles. Juice was immediately stored in coolbox at 4°C during the transport to the laboratory before storing it in a freezer at low temperature (-22°C).

Alcoholic fermentation process

Before beginning the fermentation process, fresh cocoa juice was analysed to determine certain chemical characteristics such as pH, moisture content, titratable acidity, sugar content and inorganic component. In order to conduct spontaneous fermentation, fresh cocoa juice was put at different stable temperatures (28, 30, 35, 41°C) in a stirred bain-marie.

Controlled fermentation was carried out with commercial and dry *S. cerevisiae* used in bakery industry. Fresh cocoa juice was pasteurised at 70°C for 15 minutes in a water bath then reactivated *S. cerevisiae* [2] in a 10% solution of saccharose for one hour at ambient temperature of between 28 and 30°C then added to the juice with 30 gL⁻¹ as inoculation ratio. Biochemical and physical characteristics of fresh and pasteurized cocoa juice before fermentation were determined (Table 1).

Biochemical analysis

The pH was measured with a pH-meter (107 Consort, Belgium) using an agitator (JP Selecta, Spain) [11]. Sugar and organic acid contents were determined by HPLC. HPLC system (shimadzu corporation, Japan) used was equiped with a pump (shimadzu LC-6A liquid chromatographic detector) and integrator (Shimadzu, C-R 6A Chromatopac). The column used was tansgenic organic acids (ICSep ORH-801), mobile phase was sulphuric acid (0,004 N) at 0.8ml/min debit. Detection was made at 210 nm at 35°C.

Ethanol concentration was measured by using Shimadzu GC-12A gas chromatography with a chromosorb 105 column and a flame ionization detector (Shimadzu Seisaku Sho, Japan). Then, nitrogen was used as carrier gas at flow rate of 50 ml/min.

Enumeration of yeasts and bacteria in cocoa juice, cocoa shield and banana leaves

Cocoa juice sample was shaken by hand in the stomacher bag and 10ml pipeted into 9ml sterile Salt Peptone Solution containing 0-1% peptone (Difco0118-17; Becton Dickinson & Co., Sparks, MD,USA) and 0-85% NaCl with pH adjusted 7.2 as the

1:10 dilution. After serial dilution, yeasts and mould were enumerated on Sabouraud agar medium with chloramphenicol (Biorad Marnes la Coquette, France) incubated at 30°C for 5 days. Aerobic mesophiles were enumerated on Plate Count Agar medium (Merck Dramstadt, Germany) incubated at 30°C for 4 days. Lactic acid bacteria were enumerated on MRS agar (De Man Rogosa Sharpe, Biokar Diagnostic, Beauvais, France) containing 10 mgml⁻¹ cycloheximide (ICN 100183; ICN Biomedicals Inc., Aurora, OH, USA) and incubated in an anaerobic jar with anaercult A (Merck) at 30°C for 5 days [12].

RESULTS

Microbial population present on banana leaves, cocoa shields and cocoa juice are presented in Table 2. Yeast and mould count averaged 5.2.10⁴ CFU.ml⁻¹ in cocoa juice, 1.6.10⁴ CFU.cm⁻² on cocoa shield and 1.3.10² ± 0.4 CFU.cm⁻² on banana leaves. Aerobic mesophilic germs were encountered at concentration of 2.5x10³ CFU.cm⁻² on banana leaves, 8.1x10⁴ CFU.ml⁻¹ in cocoa juice and 1.2.10⁵ CFU.cm⁻² on cocoa shield. The population of lactic acid bacteria was 1.4x10² CFU.cm⁻² on banana leaves, 2.7x10⁴CFU.ml⁻¹ in cocoa juice and 1.4.10² CFU.cm⁻² on cocoa shield. Cocoa shield contained more microbial population than banana leaves. The microbial population count in cocoa juice is slightly similar to that encountered on cocoa shield.

Alcohol Production during cocoa juice fermentation

The evolution of production of ethanol during cocoa juice fermentation is presented in figures 1 and 2. Alcohol was produced more at low temperature range between 28 and 30°C in both of the studied processes of alcohol fermentation. However, alcohol yield was higher (8.3 %) (v/v) in controlled fermentation than in spontaneous fermentation process. At 35 and 41°C alcohol yield did not reach 0.5 % (v/v) particularly in natural fermentation processing.

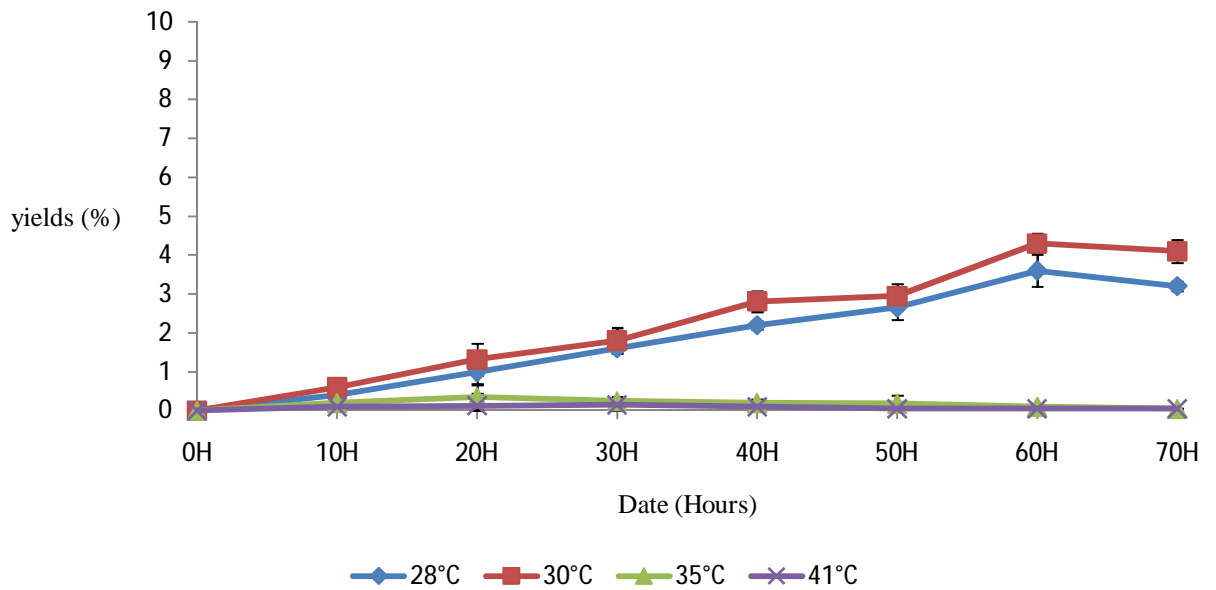


Figure 1: Yields of ethanol produced during spontaneous alcohol fermentation of juice at different temperatures

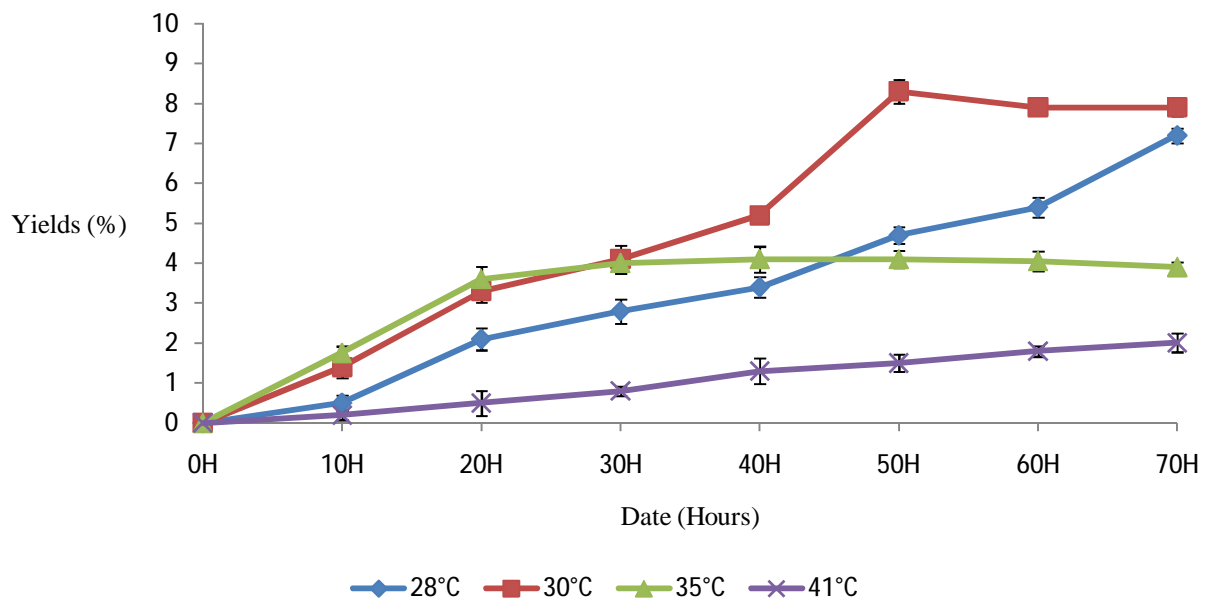


Figure 2: Yields of ethanol produced during controlled alcohol fermentation of cocoa juice at different temperatures

During spontaneous fermentation, pH values were variable depending on fermentation processing temperature (Figure 3). At lower temperature such as 28 and 30°C, pH values grew regularly from 4.24 to 4.9. But at 35°C, pH was constant during 20 hours before decreasing to 4 at the end of fermentation. At 41°C, pH value increased during 20 hours and reached 4.8 and after a short stabilization it decreased to 3.9 at the end of fermentation.

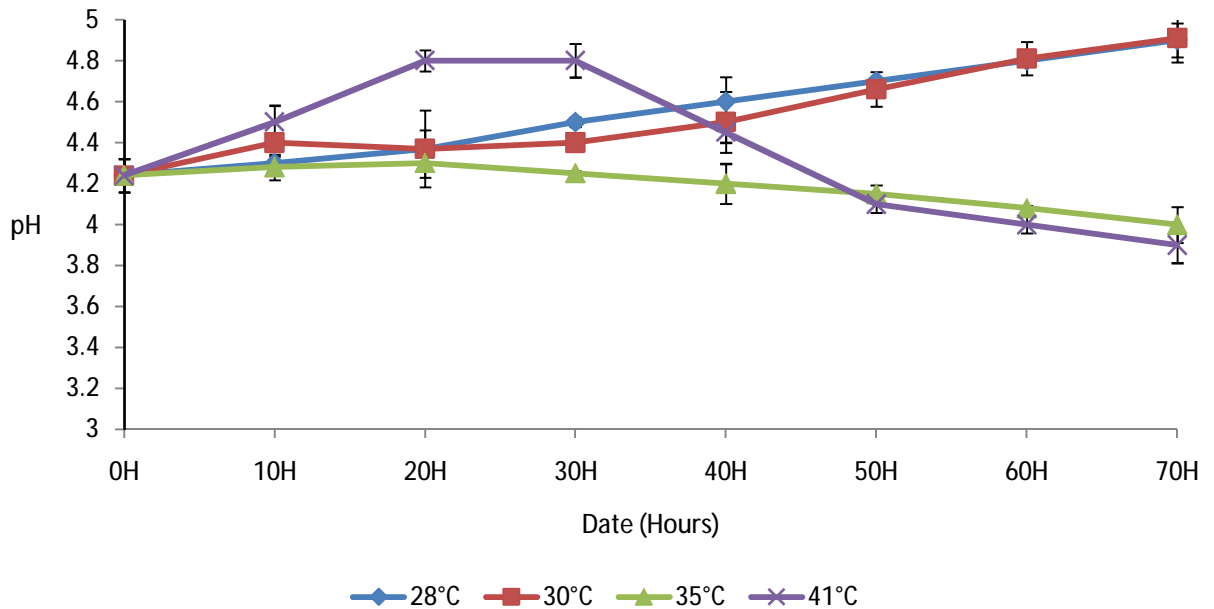


Figure 3: Variation in pH in spontaneous fermentation of juice at different temperatures

Figure 4 shows the variation in pH value during the process of fermentation. Values of pH decreased slightly at lower temperature (28 and 30°C) and reached 5 in both types of fermentation processes. We observed a fast decrease in pH from 4.19 to 3.93 at 35 °C after 40 hours during controlled fermentation. At 41°C, pH value increased slightly to 4.5 before decreasing rapidly to 3.8.

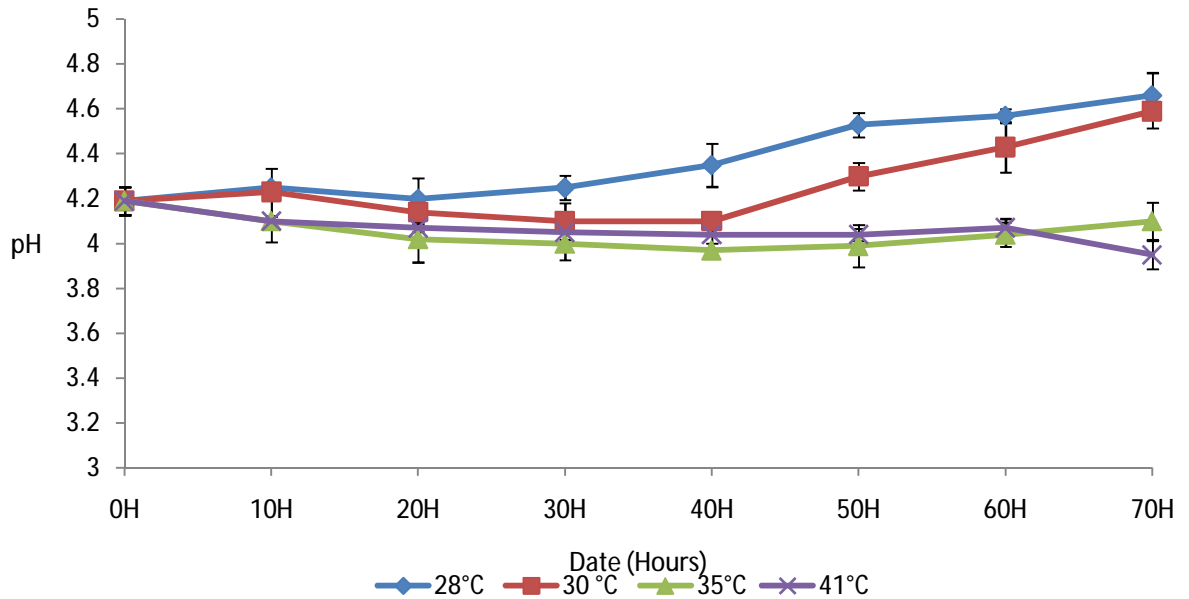


Figure 4: pH variation in controlled alcohol fermentation of juice at different temperatures

Total sugar consumption in fermentation is presented in figures 5 and 6. Total residual sugar content remained low during the process of spontaneous fermentation (17-35 g/L) than in controlled fermentation (17-67 g/L). The concentration of sugars was lowest at 30°C. At 35 and 41°C, residuals sugars were higher (35-80 g/L). At 28°C, consumed sugar was 120 g/L and 161.5 g/L in controlled and spontaneous fermentation respectively. These values were lower than those observed at 30°C which ranged from 170 to 182 g/L.

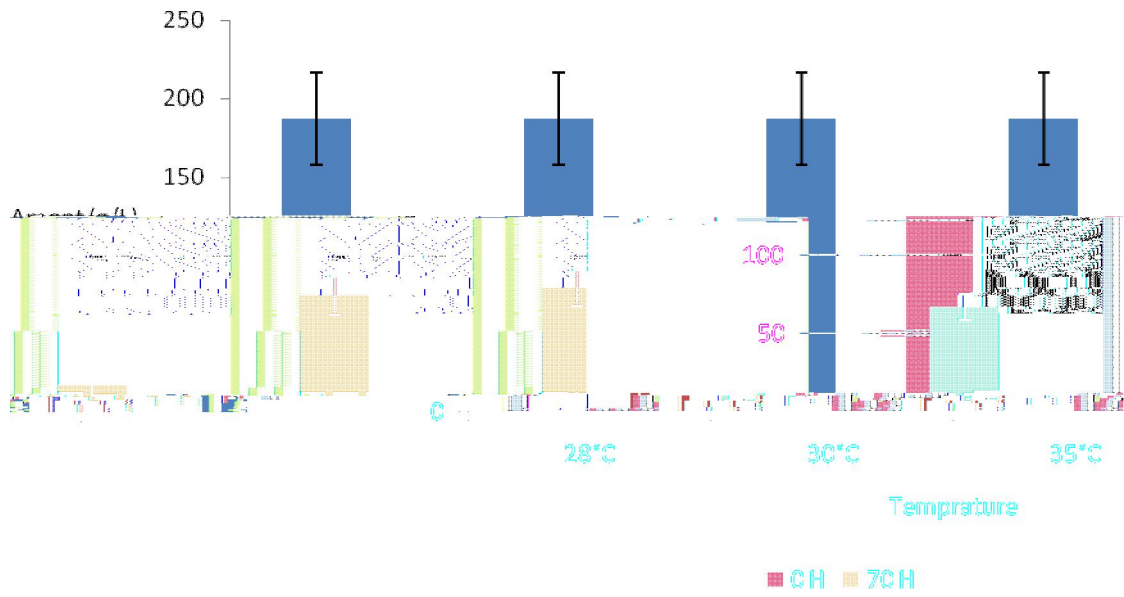


Figure 5: Total sugar content at the beginning and at the end of controlled alcoholic fermentation of cocoa juice at different temperatures

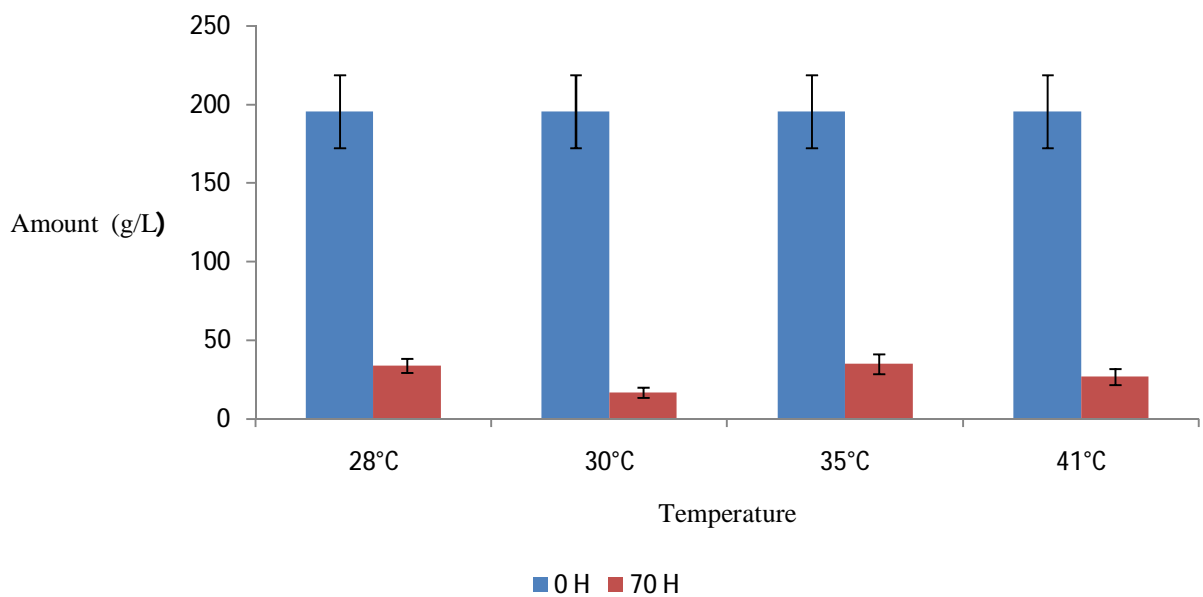


Figure 6: Total sugar content at the beginning and at the end of spontaneous alcoholic fermentation of cocoa juice at different temperature

DISCUSSION

Alcohol fermentation of fresh cocoa juice lasted between 3 and 4 days, respectively in spontaneous and controlled fermentation. The biochemical parameters varied according to the used process of fermentation. They are influenced by multiple factors such as composition of the juice, temperature under which the alcohol fermentation process was conducted. At higher temperatures (35-41°C) the duration of the fermentation process was shorter than those held at lower temperature (28 and 30°C). This phenomenon could be explained by the faster degradation of sugars at higher temperature [13]. It seems to hold up considerably the metabolism of yeasts which corresponded to the optimal growth temperature of between 28 and 30°C [14]. At these temperatures, yeast cells probably produced a lot of enzymes such as glycosidase which swiftly broke down sugars and converted them into the alcoholic secondary metabolites.

At temperatures above 28-30°C, yeast growth could be disturbed, and the utilization of sugars became probably slow. This trouble induced by higher temperatures could lead to low speed of citric acid consumption and ethanol production; but these phenomena were favourable to secondary products (acetic acid) production during spontaneous fermentation and lactic acid during controlled alcoholic fermentation particularly at 35 °C [12].

In spontaneous alcoholic fermentation held at 41°C, savage yeasts seem not to be all inhibited by higher temperatures as the residual sugar content was low (27 g/L). These results seem to confirm the observation which concluded that optimal growth temperature of yeast is sometimes higher like *Brettanomyces claussenii* (45 °C) [15].

Changes in sugar content during alcohol fermentation of cocoa juice

In our experiments, the substrate was incompletely used. Residual sugar amount in all samples was higher than that was previously reported ranging from 2 to 3g/l [12]. That means an advanced end of fermentation which explains that fermentation was incomplete. In fact, sugar amount decreasing during cocoa fermentation was previously described. More than 80% of total sugar was consumed during spontaneous cocoa juice fermentation. Only 17 % of residual sugar remained at the end of cocoa fermentation as previously demonstrated [6]. The decrease of sugar content from 180 to 140g/L after 72 hours previously described in grape juice fermentation is in agreement with these results. Higher sugar consumption was observed in spontaneous fermentation than in controlled process. This fact could be due to the higher level of microorganisms in cocoa juice such as 10^4 - 10^5 CUF/ml bacillus and 10^7 - 10^8 UFC.ml⁻¹ other bacteria [16]. The higher residual content of sugar in some samples may probably be related to initial high calcium concentration (171,5mg/L), which could strongly inhibit alcoholic fermentation [17]. Indeed, cocoa juice contains high calcium content averaging 171.5 ± 34.02 mg/L and this concentration could disturb the growth of yeast [17]. These metallic ions are important in wine production. Unlike magnesium, calcium was revealed to be toxic at more than 25 Mm for yeasts growth [18, 19, 20, 21]. The inhibitory effect of calcium

in cocoa bean juice could be reduced by dilution as earlier described in wine production with cocoa mucilage juice [7].

Production of alcohol during fermentation of cocoa juice

Alcohol yield produced during spontaneous fermentation of cocoa juice was lower (4.3 %) than that produced during controlled fermentation (8.4 %). The low yield of ethanol during spontaneous fermentation was due to the high diverse micro organisms concentration found in the juice. It has previously been reported that this diversity establishes antagonist action namely *Kloeckera apiculata*, which inhibits yeast by producing acetic acid and acetaldehyde. The main cause of lower alcohol production was its oxidation to acetic acid by *Acetobacter pasteurianus* and *Acetobacter aceti* [22]. It could help in original wine production. It was found that the maximum ethanol content obtained in controlled fermentation was lower than that obtained in winery, averaging 13.1 %; but was higher than that produced with tropical fruits (6.5%, [23]), and could be considered as satisfactory.

Considering the volume of 550,000 m³ of juice produced each year, the rate of ethanol more than 44,000,000 litres could be produced by main cocoa producing countries each year. That could increase the income of smallholders of cocoa producing chain by creating distilleries for alcohol production from cocoa juice. This perspective could reduce the utilization of tropical fruits such as orange and pineapple in ethanol production and allow diversifying the local fermented beverages [24].

CONCLUSION

The results obtained in this study have demonstrated that efficient ethanol production from cocoa juice is possible even without any pre-treatment of the juice. The present work represents both qualitative and quantitative approach of cocoa juice fermentation with savage yeasts and *Saccharomyces cerevisiae*. Controlled alcoholic fermentation held at 30°C with backer yeasts allows for production of the highest alcohol yield. The production of alcohol from cocoa mucilaginous juice is a significant finding that can constitute a valuable way of using derivative products from cocoa beans at farm level.

Table 1: Biochemical and Physical characteristics of fresh and pasteurized cocoa juice before fermentation

Characteristics	Fresh juice	Pasteurized juice
Moisture (%)	85.7	85.7
pH	3.9	4.1
Titratable acidity (%)	2.5	2.3
Total soluble solids (°Brix)	17.1	16.4
Total sugar content (g/L)	203.1	198.4
Mineral matters concentration (%)	3.7	3.7

Table 2: Enumeration and count of yeasts, moulds enterobacteria and lactic acid bacteria isolated from banana leaves, cocoa shield and cocoa juice

Vegetal material	Aerobic mesophilic germs count (CFU.cm ⁻² or CFU ml ⁻¹)	Yeats and moulds count (CFU.cm ⁻² or CFU ml ⁻¹)	Lactic Acid Bacteria count (CFU.cm ⁻² or CFU ml ⁻¹)
Banana leaves	2.5.10 ³	1.3.10 ²	1.4.10 ²
Cocoa shield	1.5.10 ⁵	1.6.10 ⁴	2.1.10 ⁴
Cocoa juice	8.1.10 ⁴	5.2.10 ⁴	2.7.10 ⁴

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