Original article

Elaboration of a fruit wine from cocoa (*Theobroma cacao* L.) pulp

Disney Ribeiro Dias,[1] Rosane Freitas Schwan,[1]* Evandro Sena Freire[2] & Rogério dos Santos Serôdio[3]

1 Biology Department, Federal University of Lavras, Cx. Postal 37, 37.200-000, Lavras, MG, Brazil
2 Departamento de Ciências Exatas e Tecnológicas, Universidade Estadual de Santa Cruz, Km 16 da Rodovia Ilhéus/Itabuna, BA. 45650-000, Ilheus, BA, Brazil
3 Cocoa Research Centre, CEPLAC, Km 22 Rodovia Ilhéus, Itabuna, 45.600-000, Itabuna, BA, Brazil

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Summary The objectives of this study were the selection of a strain of *Saccharomyces cerevisiae*, the elaboration of a fermentative process using cocoa (*Theobroma cacao* L.) fruit pulp, and the assessment of the acceptance of the elaborated beverage. Three *S. cerevisiae* strains (CA116, CA1162 and CA1183) were assessed while growing in a fruit pulp medium at different temperatures. The ethanol:biomass and glycerol:biomass ratios showed that there were no significant differences among the three strains at different temperatures. However, the strain CA1183 reached a higher ethanol production and yield and it was chosen as a starter to produce the cocoa beverage. The concentration of higher alcohols, methanol, esters and acetaldehyde found in the elaborated beverage was in accordance with the standards established for table wine. Sensory analysis revealed a high degree of acceptance amongst the great majority of tasters. It can be concluded that pulp processing into an alcoholic beverage is a realistic additional way of utilisation of the cocoa fruit.

Keywords Alcoholic fermentation, cocoa, fruit wine, *Saccharomyces*, *Theobroma cacao*.

Introduction

Cocoa (*Theobroma cacao* L.) is world-wide known for its beans used in the manufacture of chocolate. For a long time the production and commercialisation of cocoa has been the basis for the economy of some Brazilian states, mainly Bahia. From 1990, because of the financial crisis in the international market of cocoa and the emergence of the 'witches broom' disease caused by the pernicious fungus *Crinipellis perniciosa* in Bahia (Brazil), the agro-industry of cocoa declined quickly, causing a decrease in production and exportation of cocoa beans. In this period producers started to use and commercialise the pulp of the fruit as an income source, which was almost completely discarded at the height of the exportation of beans. This alternative is one of the current forms of commercialisation of the cocoa in Brazil. The cocoa pulp is a substrate rich in nutrients, which can be used in industrial processes for by-product manufacture (Freire *et al*., 1999; Schwan & Wheals, 2004).

*Correspondent: Fax: + 55 (35) 3829 1100;
e-mail: rschwan@ufla.br

Present address: Disney Ribeiro Dias, Unilavras – Centro Universitário de Lavras, Rua Pe. José Poggel 506, 37.200-000, Lavras, MG, Brazil.

Cocoa pulp can be readily fermented by yeasts such as *Saccharomyces cerevisiae*, producing an alcoholic beverage. *Saccharomyces cerevisiae* has been used in fermentative processes for thousands of years, according to the first historical stories of the production of beer and wine (Rose, 1977; Hansen & Kielland-Brandit, 1996; Demain, 2000; Ostergaard *et al*., 2000). Because of commercial importance of this microorganism, strains with good fermentative characteristics have been selected and commercialised in dehydrated form and/or lyophilised to be employed in breweries, wineries and other industries (Fleet & Heard, 1993; Colagrande *et al*., 1994; Ubeda-Iranzo *et al*., 2000; Cappello *et al*., 2004). A range of environmental factors influences the production of metabolites and survival of yeasts during industrial fermentations. The main factors are temperature, pH, sugars concentration and acidity of fruit juice (substrate). In case of yeasts, temperature and tolerance of ethanol have an important influence on their performance (Heard & Fleet, 1988; Walker, 1998). Winemaking is a good example of the biotechnological evolution in the beverage production, passing from art- to science-based technology. As the grape, several other fruits can be used for forming musts that can be submitted to alcoholic fermentation. Experiments with fruits for fermented beverage production have been carried out in the last two decades, using musts of

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banana, coconut water, pineapple and tomato (Garcia *et al*., 1976), mango (Akubor, 1996), pineapple (Ayogu, 1999), pupunha (Oliveira *et al*., 2001), orange (Selli *et al*., 2003), yellow mombin (Dias *et al*., 2003) and carambola (Vidya & Neela, 2004). However, because of the great difference of composition between the fruits, there is a necessity for more studies for the production of these beverages notably about the strain to be used, the ideal temperature of fermentation and the type of treatment that has to be applied to the must of the fruit, or the proper fruit, in the pre-fermentative phase and during the fermentation.

World-wide Brazil is one of the countries with a large production of fruits and it is also one of the biggest exporting countries. In relation to the world-wide production of cocoa, the country occupies the fifth position (Schwan & Wheals, 2004; FAOSTAT Data, 2005) after Ivory Coast, Ghana, Indonesia and Nigeria. However, not all of the harvest finds its destination in the market, because of overproduction or damage. New forms of use or processing of the production excess must be developed, in order to prevent losses and to generate profits. One of the alternatives is its use in fermented beverage production.

The purpose of this study was to use cocoa pulp for the production of a fermented alcoholic beverage. This was carried out under three steps: selection of strains of *S. cerevisiae*, elaboration of a flow diagram for the process of production and evaluation of the chemical and sensory properties of the final beverage.

Materials and methods

Yeast strains

The three *S. cerevisiae* strains have previously been isolated from natural cocoa and sugar cane fermentations and selected because of their differences in fermentative behaviour in synthetic fermentation medium (Schwan, 1988; Schwan *et al*., 2001). The strains belong to the Microbial Physiology laboratory at DBI/UFLA and are codified as CA116, CA1162 and CA1183. These strains were cultured for 48 h at 30 °C on fresh YPD medium (dextrose 20 g L^{-1}; bacteriological peptone 20 g L^{-1}; yeast extract 10 g L^{-1}) solidified with 2% agar when required, before they were used in the fermentations.

Fermentation experiments

Batch fermentations were carried out in 500 mL Erlenmeyer flasks containing 450 mL of fruit fermentation medium (FFM) containing glucose 10.0 g L^{-1}; KH_2PO_4 4.5 g L^{-1}; $(NH_4)_2SO_4$ 3.0 g L^{-1}; yeast extract 1.0 g L^{-1}; $MgSO_4{\cdot}7H_2O$ 0.25 g L^{-1} and $CaCl_2$ 0.25 g L^{-1}; at pH 5.0 and cocoa pulp in order to obtain a final sugar concentration of 200 g L^{-1} and covered with sterile hydrophobic cotton cap. The initial yeast inoculum was 1.0×10^6 cells mL^{-1} from FFM overnight cultures. Cultures were evaluated about their fermentative ability in three different temperatures of incubation: 18, 22 and 25 °C without shaking. At several time points during the fermentation process, bottles were gently shaken and samples were taken for optical density (OD_{600}) and viability measurements, determination of residual sugars (Miller, 1959) and chemical analysis. Each batch fermentation was carried out at least four times. After 24 h or when no residual sugars were left, the supernatant culture medium (FFM) was descarted and a fresh culture medium was added to the cells. This procedure was carried out three times in a row. Sugar consumption was plotted against time. The maximum fermentation rate was derived from the slope in the inflexion point of the curve and expressed as g of sugar consumed per litre per day.

Viability determination

For viability measurements, 100 μL of appropriate dilutions of the cultures were plated (in triplicate) on YPD plates. Plates were incubated at 30 °C until the appearance of colonies (1–3 days), and the number of colony-forming units per millilitre of cell culture was determined. Microscopic cell viability was measured using methylene blue.

Budding index

Budding index, defined as the fraction of all cells with visible buds, was measured for about 300 cells using a microscope. This measure has been shown to be a good index for determining the rate of cell proliferation (Lord & Wheals, 1980).

Dry mass

The dry cell weight of 10 mL culture samples was determined using 0.45 μm membrane filters and a microwave oven (180 W, 15 min) (Olsson & Nielsen, 1997).

Alcoholic beverage production

Fruit pulp

The cocoa pods were from Bahia State in the Northeast region of Brazil. The fruits were washed and broken up to extract the seeds and processed in the CEPEC/CEPLAC (Cocoa Research Centre, Itabuna, BA, Brazil). The fruits were analysed in relation to total weight, pod:pulp and seed:pulp weight ratios. The pulp was extracted using an automatic depulping machine (ITAMETAL 0.5 DS, Itabuna, BA, Brazil). Samples were taken from cocoa pulp and were characterised in relation to total soluble solids and starch (AOAC,

1990a), total sugars (Yemn & Willis, 1954), reducing sugars (Miller, 1959), total titratable acidity and pH (Instituto Adolfo Lutz, 1985), pectins (McCready & McComb, 1952) and phenolics (Reicher *et al*., 1981). The cocoa pulp was frozen in plastic bags and stored at −20 °C.

Cocoa must

To prepare the fermenting must, cocoa pulp was defrosted at room temperature. The cocoa pulp had an average sugar level of 16°Brix and a pH value of 3.2. The cocoa pulp was diluted with sucrose solution to reduce the turbidity and to adjust the sugar concentration to 22°Brix. $CaCO_3$ was added to increase the pH value to 4.0. Pectinolytic enzyme (Ultrazym® AFP-L; Novo Nordisk Ferment Ltd, Fuglebakken, Denmark) was also added to aid the clarification of the juice. The enzyme concentration was used as recommended by Novo catalogue (Novo Nordisk Ferment Ltd, 1998). Sulphur dioxide was added: up to 200 mg of $K_2S_2O_5$ per litre, to inhibit bacteria proliferation.

Fermentation conditions

Fermentation experiments were carried out in stainless steel vats of 5 and 20 L. The fermentation temperature for cocoa wine production was approximately 22 °C and no stirring was performed during any stage of the fermentation. Inocula of the selected *S. cerevisiae* were prepared as follows: one colony from a fresh YPD plate was inoculated into 200 ml of YPD broth and grown at 30 °C until a cell density of approximately 10^7 cfu mL^{-1} was reached. The cells were counted and an equal amount of cells per strain was resuspended in the same medium as used for the fermentation. Each vat was then inoculated with 10 mL of this suspension, corresponding to a final cell density of 1×10^5 cfu mL^{-1}, unless otherwise stated. The maximal fermentation rate was determined by the maximum ethanol production and decrease of sugar content and the fermentation was considered complete when the Brix level was stable. The CO_2 production was observed during the fermentation process. Samples were taken at the indicated points and microbiologically and chemically analysed.

At the end of the fermentation, the vats were transferred to a 10 °C incubator to aid the sedimentation of solid material from cocoa pulp. After 10 days at this temperature, the wine transfer was carried out with some aeration and the beverage was incubated at 10 °C for another 30 days. After that period, another transfer without aeration was carried out and the fruit wine was left for another 10 days at 10 °C before filtration. The cocoa wine was then filtered through diatomaceous earth and cellulose filters. The beverage was storage at 8 °C in glass bottles fully filled to avoid oxygen entrance.

Chemical analysis

Analysis performed were pH, density, ethanol content, volatile acidity, total acidity, total dry extract, free and total SO_2, determined according to the methodology proposed by Brasil (1988) and AOAC (1990b).

Substrates and metabolites

Alcohols (ethanol and glycerol), organic acids (acetic acid, lactic acid, malic acid, succinic acid, tartaric acid and citric acid) and carbohydrates (glucose, sucrose and fructose) were obtained from pulp extracts and analysed (Schwan *et al*., 2001). Analyses were carried out with a high-performance liquid chromatography system (Shimadzu, model LC-10Ai; Shimadzu Corp., Kyoto, Japan), equipped with a dual detection system consisting of a u.v. detector and a refractive index (RI) detector (RID – 10A SPD-10Ai, Shimadzu Corp., Kyoto, Japan). A Shimadzu ion exclusion column (Shim-pack SCR-101H, Shimadzu Corp., Kyoto, Japan) operated at a temperature of 40 °C was used to achieve chromatographic separation. Water-soluble acids, sugars (fructose, glucose and sucrose), and ethanol were eluted with 100 mM of perchloric acid at a flow rate of 0.8 mL min^{-1}. The acids were detected both by u.v. absorbance (210 nm) and by RI, while sugars and ethanol were detected only by RI detector. Individual sugars, acids and ethanol were identified and their concentrations were determined by comparison with retention times and amounts of authentic standards. All samples were examined in triplicate. The coefficient of variation was less than 5% in each case.

Higher alcohols (1-propanol, isobutanol, 1-butanol, isoamyl alcohol, amyl alcohol and hexanol), acetaldehyde, methanol and esters (ethyl acetate and methyl acetate) were analysed by gas chromatography using a Chromopack, model 511, equipped with flame ionisation detector using a capillary column of silica Carbowax 57 CB (50 m × 0.22 mm, Wasw, model 52 CB, Chrompac International, Milddelburg, the Netherlands) (Boscolo *et al*., 2000). One hundred microlitres of each sample (non-distilled) were diluted 20-fold in milli-Q water and filtered using a nitrate-cellulose membrane (0.20 μm pores) before injection in the gas chromotograph (GC). Operating conditions were as follows: oven temperature was kept at 55 °C for 5 min, programmed to 100 °C at 2 °C min^{-1}, kept at 100 °C for 3 min, programmed to 190 ° C increasing 5 °C min^{-1}, then kept at 190 °C for 30 min and then programmed to 220 °C in 15 min, injector and detector temperature at 250 °C, and carrier gas (N_2) was at a flow rate of 1.2 mL min^{-1}. The identification of the volatile compounds was carried out by comparing their retention times with those of standards. One sample which contained the internal standard and the standard compounds at similar concentration as found in the wine, was also treated in the same way as the wine

samples and the final calculations are described on the basis of the concentration of this reference solution. Determination of the different compounds was made in triplicate.

Sensory evaluation

The final beverage was evaluated by 50 panellists, males and females of 18–55 years of age (staff and students of the Universities UNILAVRAS and UFLA). The panellists were selected for participation on the basis of their preference for dry (< 5 g L^{-1} of sugar) beverage, interest and availability. Randomised refrigerated (10 °C) samples, of 20–25 mL, were served in clear, 170 mL, tulip-shaped glasses; these were marked with three digit random numbers and covered with plastic Petri dishes. Distilled water was provided for rinsing of the palate during the testing. Evaluations took place in the mornings between 9:00 and 10:00 a.m. and were conducted at room temperature of 20–22 °C under white light.

The cocoa wines were evaluated for taste, clarity, colour and general acceptability according to the Hedonic scale (Moraes, 1993). This scale consists of the comparison, punctuation, and classification of foods and beverages of the same class or origin according to their qualities and defects. A card of six parameters (visual examination, smelling intensity, smelling quality, taste intensity, taste quality and harmony) was proposed, accompanied by a scale of nine categories: dislike extremely = 1, dislike much = 2; dislike moderately = 3; dislike slightly = 4, neither dislike nor like = 5, like slightly = 6; like moderately = 7; like much = 8, like extremely = 9 (Moraes, 1993). The sensoric analysis was carried out in two sensory sessions, each lasting 1 h. The wines were evaluated in duplicate in each session and the mean score of wines for each quality attribute was computed. The results were statistically analysed using a non-parametric test of Chernoff and the Statistica programme (Statsoft Inc., 1995).

Results and discussion

Yeast selection

The three isolates of *S. cerevisiae* were selected because of their differences in fermentative behaviour in laboratory scale alcoholic fermentations and they were evaluated in relation to ability to grow and ferment under different temperatures. The isolates were pregrown in YPD medium and then cultivated in FFM medium until a density of 10^7 cells mL^{-1} was reached. The cells were inoculated into fresh FFM media and incubated at 18, 22 and 25 °C for 64 h. All three strains showed growth when incubated at 18 °C (Fig. 1). The CA116 growth rate was higher than that of the other two yeasts and its population reached 1.8×10^8 cells mL^{-1} after 12 h of growth. After this time viability decreased and a loss of viability of about 26% was measured after 48 h in the CA116 culture. The CA1162 isolate presented an increase in the population during the first 12 h of incubation and then it remained almost constant at a population of 10^8 cells mL^{-1} (Fig. 1). The CA1183 isolate showed 12 h of adaptation (lag phase) and then started to grow at a slower rate than the other two isolates. Although the growth was slower, the CA1183 isolate reached a maximal population similar to CA1162, i.e. about 10^8 cells mL^{-1} (Fig. 1). The isolates CA1162 and CA1183 had a delay in growth compared with CA116,

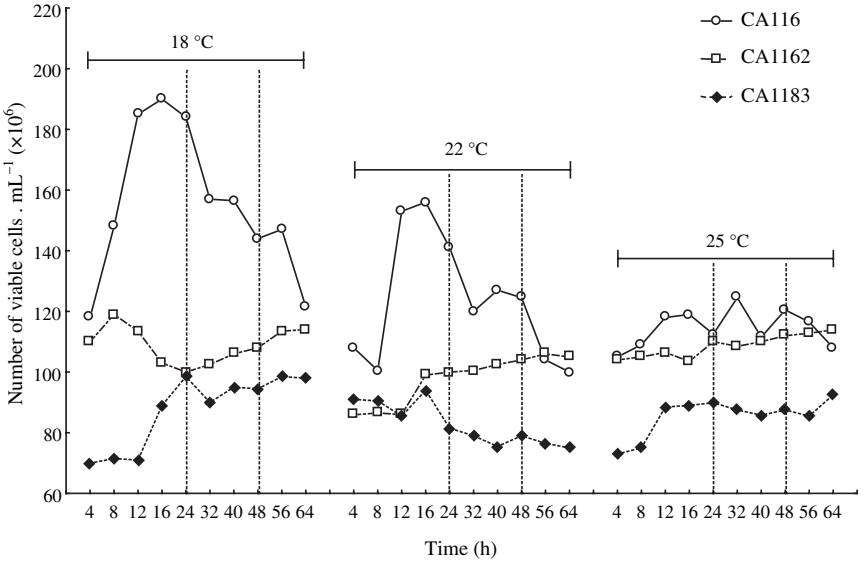

Figure 1 Number of viable cells of three strains of *Saccharomyces cerevisiae* incubated at different temperatures of 18, 22 and 25 °C. Cells were transferred to a fresh FFM medium every 24 h. The vertical lines indicate the transfer to fresh fermentation medium.

however, once the maximal population was reached it remained at high values for the whole process, i.e. there was a long stationary phase and no declining phase. No increase in the population was observed when the fermentation broth was renewed after 24 h, however, there was a small increase in the number of cells after 48 h; this increase was coincident with the second renewal of fermentation broth.

After 72 h of incubation at 18 °C, the cultures were left for 2 h at room temperature to sediment before the supernatant was removed, and subsequently the cultures were further incubated at 22 °C. The pattern of viable cells of CA116 was similar to the one observed when cells were incubated at 18 °C. The CA116 strain reached maximum population of 1.5×10^8 cells mL^{-1} after 16 h of incubation and then a dramatic decrease in viability was observed over a 16 h period (Fig. 1). It was also observed that strain CA1183 had a similar pattern of cell density as CA116 when incubated at 22 °C but with lower numbers of cells at the end of the process (7.5×10^7 cells mL^{-1}) CA1162 showed a lag phase of 12 h and then the cell densities increased up to 1.1×10^8 cells mL^{-1} (Fig. 1). As was observed at 18 and 22 °C, strain CA116 showed an increase in the population in the first hours of incubation at 25 °C and then a decline in cell viability (Fig. 1). CA1183 had a small phase of adaptation and after little growth the concentration of cells remained at 9.2×10^7 cells mL^{-1} (Fig. 1). The numbers of cells of strain CA1162 steadily increased during the 72 h of the fermentation process reaching 11.4×10^8 cells mL^{-1} (Fig. 1). Although CA116 showed a rapid increase in cell numbers in the three temperatures tested, it was also observed that this strain showed a high decrease in cell viability as the temperature increases (Fig. 1). The differences in the population of the strains CA1162 and CA1183 were not significant at 5% Tukey test in any of the temperatures analysed. At 25 °C, similar population numbers of the strains CA116 and CA1162 were observed.

The temperature of incubation may affect not only growth and rate of fermentation but also yeast metabolism, which will determine the chemical composition of the final beverage. According to Jones *et al.* (1981), the ideal temperature for yeast fermentation is about 5–10 °C above the optimum growth temperature, which is in general between 25 and 30 °C. However, there is a difference if the final product is fermented or fermented and distilled. To produce distilled beverage from sugar cane, the optimum fermentation temperature is approximately 30 °C and the fermentation lasts 24 h (Schwan *et al.*, 2001). However, for wine fermentation the temperature is usually below 25 °C and the fermentation process takes more than 10 days (Kunkee & Goswell, 1977; Vogt *et al.*, 1986; Torija *et al.*, 2003).

As expected, the yeast growth and cell viability varied according to the temperature of incubation. Our results agree with those of Fleet & Heard (1993) who reported that the temperature affected the development of wild-type yeast strains and suggested that various strains showed differences in the ability to ferment over a wide range of temperatures.

The budding index shows that there was approximately 45–65% of bud cells during all the experiments for the three strains of *S. cerevisiae* analysed. These values indicated that the fermentation conditions were suitable for cell growth. In the CA1183 culture supernatant, after 10 min at rest, a decrease of more than 85% of the OD was detected. The CA1183 strain showed a visible flocculation after the first change of the culture medium.

The yeast CA1183 showed flocculation behaviour 24 h after the cells were transferred to a fresh culture medium. This strain grew and fermented as single cells, but showed a flocculation mechanism when the concentration of fermentable sugars was low. It is known that yeast flocculation is under genetic control and dominant and recessive flocculation genes and flocculation suppressor genes have been described (Stratford, 1992, 1994; Teunissen & Steensma, 1995). It has been suggested that the genes that regulate flocculation might be related to nutrient availability and metabolites produced by yeasts during growth and fermentation (Soares & Vroman, 2003; Soares *et al.*, 2004; Sampermans *et al.*, 2005). Our results suggested that the shortage of fermentable carbon sources after 24 h growth, combined with the presence of ethanol, which has a positive effect on yeast flocculation (Soares *et al.*, 2004), may be the signal compounds that induce the onset of flocculation. Flocculation favours mass sedimentation, recovery of the final product and cells and facilitates the batch fermentation system.

In all experiments the production of ethanol and glycerol by the three strains of *S. cerevisiae* were evaluated. There was no significant difference in the ethanol production by the strains (Table 1) at the temperatures evaluated. Glycerol is a non-volatile compound, which has no aromatic properties, but which significantly contributes to wine quality by providing sweetness and fullness (Amerine & Cruess, 1960; Eustace & Thornton, 1987; Fleet & Heard, 1993). Glycerol and succinic acid are the main secondary products in alcoholic fermentations led by *S. cerevisiae*. Glycerol amounts were approximately 10% of the total ethanol produced by strain CA1183 in all temperatures evaluated (Table 1). Glycerol production was slightly increased with a temperature rise from 18 to 25 °C for all three strains tested, although the strains CA116 and CA1162 showed lower production of glycerol than strain CA1183. No significant differences were observed in the values

Table 1 Results (mean ± SE) of chemical compounds and pH value produced by three strains of *Saccharomyces cerevisiae* during fermentation at different temperatures in FFM medium

Temperature of incubation	CA116			CA1162			CA1183		
	18 °C	22 °C	25 °C	18 °C	22 °C	25 °C	18 °C	22 °C	25 °C
Ethanol (g L^{-1})	89.0 ± 0.1155	87.0 ± 0.1528	78.0 ± 0.1764	92.0 ± 0.2000	91.0 ± 0.1453	90.5 ± 0.2082	92.5 ± 0.1463	92.0 ± 0.2333	90.5 ± 0.2423
Glycerol (g L^{-1})	5.53 ± 0.0590	6.09 ± 0.0546	7.00 ± 0.0721	7.90 ± 0.0797	8.10 ± 0.0321	7.90 ± 0.0504	9.00 ± 0.0902	8.90 ± 0.0643	9.00 ± 0.0623
Acetic acid (g L^{-1})	0.08 ± 0.0018	0.10 ± 0.0015	0.10 ± 0.0012	0.12 ± 0.0025	0.14 ± 0.0044	0.16 ± 0.0023	0.05 ± 0.0009	0.07 ± 0.0017	0.07 ± 0.0015
Succinic acid (g L^{-1})	0.92 ± 0.0219	0.79 ± 0.0153	0.71 ± 0.0186	0.89 ± 0.0176	0.77 ± 0.0208	0.70 ± 0.0145	0.95 ± 0.0176	0.84 ± 0.0145	0.79 ± 0.0153
Acetaldehyde (g L^{-1})	0.05 ± 0.0011	0.05 ± 0.0012	0.04 ± 0.0006	0.09 ± 0.0015	0.08 ± 0.0009	0.09 ± 0.0015	0.03 ± 0.0007	0.02 ± 0.0003	0.02 ± 0.0002
pH	4.1 ± 0.0343	3.9 ± 0.0882	3.6 ± 0.0667	4.0 ± 0.0284	3.8 ± 0.0422	3.7 ± 0.0333	4.0 ± 0.0577	3.9 ± 0.0326	3.7 ± 0.0234
Ethanol yield*	44.5 ± 0.0577	43.5 ± 0.0764	39.5 ± 0.0882	46.0 ± 0.1000	45.5 ± 0.0726	45.3 ± 0.1041	46.3 ± 0.0726	46.0 ± 0.1167	45.3 ± 0.1267
Time of maximum population (h)	12	16	16	12	16	16	24	16	12

Results are the mean of four replicates and did not vary more than 3%.
*Ethanol yield = Ethanol produced (g L^{-1}) × 100/initial sugars (g L^{-1}).

of yield of ethanol for all strains analysed (Table 1), however the strain CA1183 showed highest ethanol production. Several authors have reported on the influence of fermentation temperature on the concentrations of volatiles produced during grape fruit fermentation and its interference in the quality of wines (Killian & Ought, 1979; Fundira *et al.*, 2002).

Based on the data obtained in our work strain CA1183 was selected as inoculum for elaboration of cocoa wine because of its high fermentation speed, fully using up all the sugars present in the medium, its flocculation ability, its lower loss of viability, its better yield of ethanol, its low production of volatile acids and other fermentation by-products such as ethyl acetate and isoamyl alcohol.

Elaboration of the beverage

Chemical characterisation of the pulp

The cocoa pulp used in this work has been removed by mechanical processes and chemically characterised before initiating the fermentation process. The averages of the results in triplicate are to be found in Table 2. The cocoa pulp had on average pH value of 3.2, which characterised it as an acid fruit. The concentration of total and reducing sugars was 17.4% and 10.7% respectively (Table 2). With these amounts of sugars the cocoa pulp could be compared with other tropical fruits with potential for use in the beverage industry (Morton, 1987; Franco, 1999; Alves *et al.*, 2000). The total soluble solid concentration in the pulp of cocoa, determined as its Brix value, was 20.5 (Table 2). As the determination of °Brix by refractometry indicates total soluble solids, which are not necessarily constituted of sugars only, the final alcohol yield might appear to be low if based on the Brix value. The presence of endogenous pectinases is relatively common in fruits (Grassin & Fauquembergue, 1996), however, any natural activity of pectinases was not detected in the cocoa pulp. Therefore, the addition of the enzymatic complex was necessary to reduce the viscosity of the pulp.

Table 2 Physico-chemical characterisation of mature cocoa pulp

Characteristics	Mean ± SE
Total weight (g)	543.0 ± 4.5826
Pulp (%)	26.4 ± 0.2216
Pod (%)	50.5 ± 0.3512
Seed (%)	23.1 ± 0.2186
Size- length (mm)	169.1 ± 1.3077
Diameter (mm)	94.6 ± 0.8386
Total soluble solids (°Brix)	20.5 ± 0.1528
Total titratable acid (mEq L^{-1} in malic acid)	1.0 ± 0.0088
°Brix:acidity ratio	20.5 ± 0.1880
pH	3.2 ± 0.0167
Total sugars (%)	17.4 ± 0.1155
Reducing sugars (%)	10.7 ± 0.0577
Starch (%)	0.16 ± 0.0012
Total pectin (%)	0.57 ± 0.0023
Pectin fractionated (% in relation to alcohol insoluble solids)	
High methylation	4.04 ± 0.0186
Low methylation	1.49 ± 0.012
Protopectin	1.03 ± 0.0070
Phenolics soluble in water (%)	0.17 ± 0.0012
Phenolics soluble in methanol (%)	0.15 ± 0.0011
Phenolics soluble in methanol 50% (%)	0.13 ± 0.0009

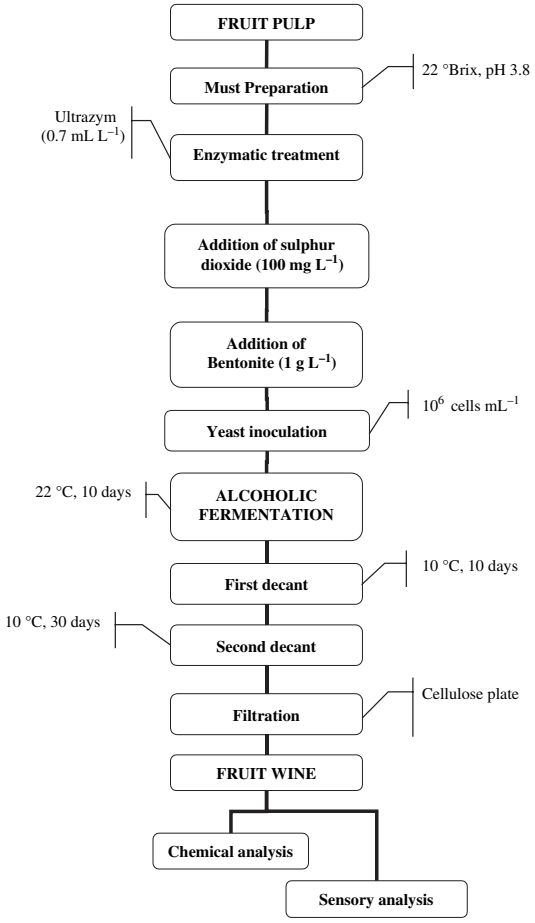

Figure 2 Flow diagram of the process of alcoholic beverage production from cocoa pulp.

Alcoholic fermentation

The cocoa pulp was processed to get a fermentable must from which the alcoholic beverage was produced. The flowchart represented in Fig. 2 shows the different stages to which the must of the fruit was submitted up till the final product. Before the beginning of the fermentative process, sucrose, pectinolytic enzymes, sulphite and bentonite had been added to the cocoa pulp. To attain 22°Brix, 10 L of sucrose solution (350 g L^{-1}) had to be added to an equal volume of 10 L of cocoa pulp. The addition of the enzymatic complex Ultrazym® AFP-L in the concentration of 0.7 ml L^{-1} favoured the process of clarification of the wine of cocoa. Gomez-Ruiz *et al.* (1988) had obtained similar results in the clarification of apple juices treated with endopolygalacturonases. To limit the bacterial growth 100 mg of SO_2 was added per litre of must (about 200 mg of $K_2S_2O_5$). These values, although considered high to initiate the fermentation, had been adopted because of low pH value of the cocoa must (Hashizume, 2001). During the counting of the population of viable cells, in the period of fermentation, the presence of bacteria was not observed, indicating that the use of metabisulphite was efficient in the control of the bacterial growth during the fermentative process (data not shown). Another evidence of absence of contamination, in this case for lactic acid bacteria was that lactic was not detected, whereas malic acid was found in the final beverage of cocoa (Table 3). However, as a result of the SO_2 concentration used for the elaboration of the cocoa wine a delay in the beginning of the fermentation of about 12 h was observed. These observations agree to those described by Gerbaux & Meurgues (1995). These authors had a delay in the beginning of the fermentation when they treated the grape must with concentrations of SO_2 higher than 80 mg L^{-1}. It was also observed that once the fermentation had started, its course was not affected by sulphite, as was also described by Gerbaux & Meurgues (1995).

To facilitate the non-fermentable solids to sediment, 1% of bentonite was added to the must. The bentonite was suspended in water in a 10% concentration to facilitate the dispersion of the clay in the must. The main effect of the bentonite was the precipitation of proteinaceous material by adsorption and neutralisation, involving denaturation of oxidative enzymes (Manfredini, 1989). The bentonite introduced to the cocoa must in the pre-fermentative phase provided a better clarification of the beverage by facilitating the sedimentation of the solids of the must, thereby improving decanting and filtration in later stages.

The fermentation temperature selected in the previous phase was 22 °C and strain CA1183 was chosen as inoculum for the fermentation process. The inoculum of *S. cerevisiae* CA1183 of 10^6 cells mL^{-1} showed to be efficient as the ethanol concentration after 10 days of fermentation was close to 9.6% w/v (12% v/v). These results could be compared with the ones reported by Oliveira *et al.* (2001), who found a value of 7.5% w/v when fermenting pulp of pupunha with an initial 24°Brix. The fermentation process took 50 days and after this period the beverage was filtered and stored in glass bottles at 4 °C.

Chemical analyses of the beverage

The chemical analyses of the final beverage produced from cocoa pulp showed that the beverage produced is similar to dry table wines (Table 4). Although cocoa and grape are different fruits, the steps to produce the alcoholic beverage were very similar. All the steps required good hygiene conditions and the results showed that they were satisfactory in relation to the purposed methodology.

Sucrose, glucose or fructose were not detected in the final beverage of cocoa wine (Table 3), which charac-

Table 3 Concentration (mean ± SE) of organic compounds determined with HPLC and GC (acids, sugars and alcohols) in the fermented beverage from cocoa pulp

Compounds	Concentration in cocoa wine
HPLC analyses (g L^{-1})	
Carbohydrates	
Sucrose	n.d.
Glucose	n.d.
Fructose	n.d.
Alcohols	
Ethanol	12.3 ± 0.0110
Glycerol	4.6 ± 0.0068
Acids	
Acetic	1.1 ± 0.0058
Citric	5.5 ± 0.0306
Lactic	n.d.
Malic	1.4 ± 0.0137
Oxalic	n.d.
Succinic	2.0 ± 0.0120
Tartaric	0.7 ± 0.0067
GC analyses (mg L^{-1})	
Aldehydes	
Acetaldehyde	n.d.
Esters	
Methyl acetate	n.d.
Ethyl acetate	350.7 ± 0.1342
Alcohols	
Methanol	n.d.
Higher alcohols	
Propanol	44.8 ± 0.0338
Isobutanol	90.4 ± 0.0153
Butanol	n.d.
Isoamyl alcohol	498.8 ± 0.1337
Amylic alcohol	n.d.
Hexanol	n.d.
Total of higher alcohols	634 ± 0.1193

n.d., not detected.

terised it as a dry beverage (Brasil, 1988). Methanol was not detected either in the samples of must or in the final product. Despite the high concentration of SO_2 in the beginning of the fermentation of the cocoa must (100 mg L^{-1}), it could be observed it was eliminated during the fermentative process and decanting, not leaving residual concentrations, which could disqualify the flavour of the final beverage.

Tables 3 and 4 present analyses of compounds that are important with respect to the flavour and the aroma of fermented beverages. The results show that the total amount of higher alcohols for the cocoa beverage was about 0.6 g L^{-1} (Tables 3 and 4). This level was higher than that reported by Vogt *et al.* (1986), who showed a variation between 0.1 and 0.3 g L^{-1} of higher alcohols in wines. The amount of these alcohols in wines is influenced by the presence or absence of SO_2, as reported by Herraiz *et al.* (1989). Although these authors described a positive correlation between the

Table 4 Analytical values found in alcoholic beverage from cocoa

Indicators	Limits*		Cocoa alcoholic beverage (mean ± SE)
	Maximum	Minimum	
Ethyl alcohol % (v/v)	13.0	10.0	12.5 ± 0.0667
Methyl alcohol (g L^{-1})	0.35	(–)	n.d.
Total acid (mEq L^{-1})	130.0	55.0	98.5 ± 0.8192
Volatile acids (mEq L^{-1})	20.0	(–)	6.5 ± 0.0500
Total sulphate, as K_2SO_4 (g L^{-1})	1.0	(–)	n.d.
Total chloride, as NaCl (g L^{-1})	0.20	(–)	n.d.
Total SO_2 (g L^{-1})	0.35	(–)	0.06 ± 0.0006
Total sugars			
Dry wine (g L^{-1})	5.0	(–)	n.d.
Sweet wine (g L^{-1})	(–)	20.1	(–)
pH	(–)	(–)	3.6 ± 0.0173

n.d., not detected; (–) not established.
*Source: Brasil (1988).

presence of the SO_2 during the fermentation and a high acetaldehyde concentration in the wine, this did not occur in the cocoa beverage, where the presence of acetaldehyde was not detected (Table 3). The absence of the acetaldehyde could have been due to the low pH (3.8) value of the must, as also observed by Cleto & Mutton (2004). The values of ethyl acetate found in this work (Table 3) are higher than those described by Berry & Watson (1987) and are similar to values found by Cleto & Mutton (2004) in fermented beverage from sugar cane, orange and grape. The glycerol concentration, about 4.6 g L^{-1}, was close to the values of 6 and 10 g L^{-1}, suggested by Vogt *et al.* (1986) as characteristic to confer body and texture to the beverage. In Table 3 there are some remarkably high values of isoamyl alcohol (about 499 mg L^{-1}) and ethyl acetate (about 351 mg L^{-1}) compounds, which contribute to the fruity flavour in the final beverage.

Sensory analysis of the beverage

After the chemical analyses, the beverage was submitted to sensory analysis to assess its acceptance among the consumers. Table 5 presents notes attributed to the beverage by 50 untrained tasters, designated in the hedonic scale of nine points. Similar averages were recorded for the four evaluated attributes whereas the aroma is the one with slightly higher value, followed by general appearance, taste and overall aspects, with respective notes of 7.7, 7.6, 7.5 and 7.5 (Table 5). The aroma was also responsible for the biggest maximum note frequency, getting 16 votes (35.5% of the tasters) (Table 5). The attribute of overall aspects, which corresponds to the global acceptance of the beverage by part of the tasters, reached a higher frequency (19 tasters, that is, 42.2% of the total) in note 8, what meant

Table 5 Frequency and average notes for the attributes of sensory analysis

	Frequency and average notes									
Attribute	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Mean ± SE**
Appearance	–	–	–	–	2	5	14	12	12	7.6 ± 0.17
Aroma	–	–	–	3	–	1	14	11	16	7.7 ± 0.20
Taste	–	2	–	1	–	5	9	17	11	7.5 ± 0.24
Overall	–	–	–	1	2	4	11	19	8	7.5 ± 0.17

1 = dislike extremely; 9 = like extremely.

'like very much' the beverage (Table 5). The taste of the beverage reached the lesser average (7.5) what reflected that the tasters 'had moderately liked' the beverage. According to Rapp & Mandery (1986), the trend is that the beverage loses sensorial qualities when the total concentration of higher alcohols is over 400 mg L^{-1}. In the evaluation of the cocoa beverage, according to the methodology used, the 633.0 mg L^{-1} of high alcohols had not brought about a negative influence on aroma, as aroma was the most appreciated attribute.

The expression of acceptance and non-acceptance of the beverage is presented in Table 6, in function of the grades attributed by the tasters. It can be observed that in relation to the appearance, none of the tasters was displeased with the beverage and the percentage of acceptance of this attribute (they had at least 'liked very much') was of 53%. For the attributes aroma and flavour, about 7% of the tasters were displeased with the beverage, while, also for both, about 60% 'liked it much'. Table 6 also shows that, in relation to the general aspects, 60% of the tasters had liked the beverage much, while 2% was displeased. A non-parametric test known as faces of Chernoff was carried out to establish the approval of the beverage with the tasters (Fig. 3). This test made clearer the differences reached among the attributes of the beverage by using a visual recognition. The values attributed to the cocoa beverage had been compared with a standard scale 'normal'. From the presented diagrams a homogeneity in conferred grades to each one of the attributes and in relation to the global acceptance of the beverage was observed (overall aspects).

Table 6 Percentage of acceptance and rejection of the fermented beverage from cocoa

Attribute	**1–4 (rejection) (% of tasters)**	**8–9 (acceptance) (% of tasters)**
Appearance	0.0	53.3
Aroma	6.7	60.0
Taste	6.7	62.2
Overall	2.2	60.0

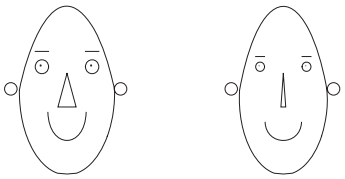

Figure 3 Representation of the acceptability of cocoa fermented beverage through Chernoff's face where size of the face is overall rating, width of the nose is aroma, curvature of the mouth is taste and size of the eyes is appearance.

Conclusions

From the data reported in this experiment, comparing three strains of *S. cerevisiae* (CA116, CA1162 and CA1183), it could be concluded *S. cerevisiae* strain CA1183, selected in this work, appeared to be capable to ferment all the sugars of the cocoa must and it also possessed the flocculation characteristic. The processes normally used in fermented beverage manufacture could be adapted for the elaboration of the fermented cocoa pulp. The dilution of cocoa pulp with sucrose solution, to improve the fluidity of must, diminished the concentration of marker compounds of the beverage's aroma, not intervening with the production of ethanol, as the formation of ethanol follows a different pathway. The use of the enzymatic complex and the solution of bentonite during all the fermentative process brought satisfactory results for the clarification of the beverage, as well as the SO_2, that in the concentration applied inhibited the growth of bacteria and did not interfere with the quality of the final beverage. The sensory analysis revealed a good acceptance, mainly when taking into account the fact that the tasters were not familiar with the cocoa-derived beverage. The methodology described here is simple, and can be implanted in a relatively small space and without high cost. It was observed that, from the acceptability of the beverage, this technology can be one of the alternatives for the use of the cocoa pulp, or a new industrial outlet for this fruit.

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