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Behavior of Fermentable Sugars in the Traditional Production Process of Cassava Bioethanol

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Abstract: The aim of study is to evaluate the ferment ability of cassava must in the ethanol production process from cassava in Congo. Three traditional methods of ethyl fermentation were tested: spontaneous fermentation, fermentation with yeast inoculation and fermentation led with yeasting and sugaring. Consumption of fermentable sugars was further in the case of directed fermentation with yeast inoculation (3° Brix residual extract from 48 h) compared to spontaneous fermentation without yeast inoculation (3.8° Brixresidual extract from 120 heures). Total sugars have been consumed only partially (66.7% of limit attenuation), while reducing sugars have been almost completely (about 91%). The addition of yeast in the cassava wort have led to a lower assessment of dextrins (2.7% glucose equivalent) compared to spontaneous fermentation (3.6%). It have also assured a better overall ethanol productivity (P_{TE} = 0.83 g ethanol/L.h) than sugaring proceeding (P_{TE} = 0.61 g/L.h) and without yeast additional (P_{TE} = 0.32 g/L.h). Among the fermentable sugars developed in the cassava mash there are reducing sugars, such as glucose and maltose. Non-fermentable sugars represent a significant slice of stock of soluble carbohydrate (on average 3.24% dextrose equivalent) of the must in the three cases of fermentation tested.

Keywords: Bioethanol, cassava mash, fermentable sugars, fermentation, reducing sugars

INTRODUCTION

Bioethanol is ethanol from agricultural origin produced by fermentation of carbohydrates biomass (World Bank, 1980) and distillation. Traditionally, ethanol is involved in the preparation of beer, wine, potable alcohol as is the case *Lungwila* and Congolese *Boganda* (Luziétoso *et al*., 2000; Diakabana *et al*., 2007), Scottish whisky and Russian vodka. In recent years, many countries are involved in the production of bioethanol to meet industrial, chemical and pharmaceutical needs as well as energy perspectives.

According to studies conducted in the Congo Basin, bioethanol has long been locally produced from the traditional process of ethyl saccharification and fermentation of the dough cooked cassava added grist corn (Diakabana *et al*., 2008) or other cereals (Delaude *et al*., 1993; Maïdou and Kamayem, 1994), with or without addition of yeast and with or without sweetening (sugar cane). In some practices, saccharification is improved by the use of the roots of a shrub *Eminia polyadenia* Hauman, locally named *Munkoyo* in Democratic Republic of Congo (Delaude *et al*., 1993).

All carbohydrates of cassava wort almost come from starch. In the traditional process of ethanol production, two distinct biochemical phases occur simultaneously, the saccharification of starch and conversion of fermentable sugars into ethanol (Diakabana *et al*., 2007). The mechanisms of this transformation remain to be elucidated.

In order to optimize the saccharification process in the ethyl traditional fermentation of cassava must, the content of fermentable and non-fermentable sugars, the reducing sugars and overall ethanol productivity were tested.

MATERIALS AND METHODS

Fermentation methods of boiled cassava: In the study of fermentation, flour of root cassava of a local cultivar *Liyia* was used. Three methods of fermentation of mash cassava commonly used in traditional settings were used: spontaneous fermentation, fermentation with yeast inoculation and fermentation led with yeasting and sugaring (Fig. 1).

Preparation of the porridge of cassava flour: Five hundred gram of dry matter of cassava flour was gelatinized by heating at 100±2°C with 1.5 L of water. The cooked slurry was allowed to stand overnight at room temperature and fluidized manually the next day in a bowl by adding of 1.5 L of water to get the porridge of cassava flour cooked.

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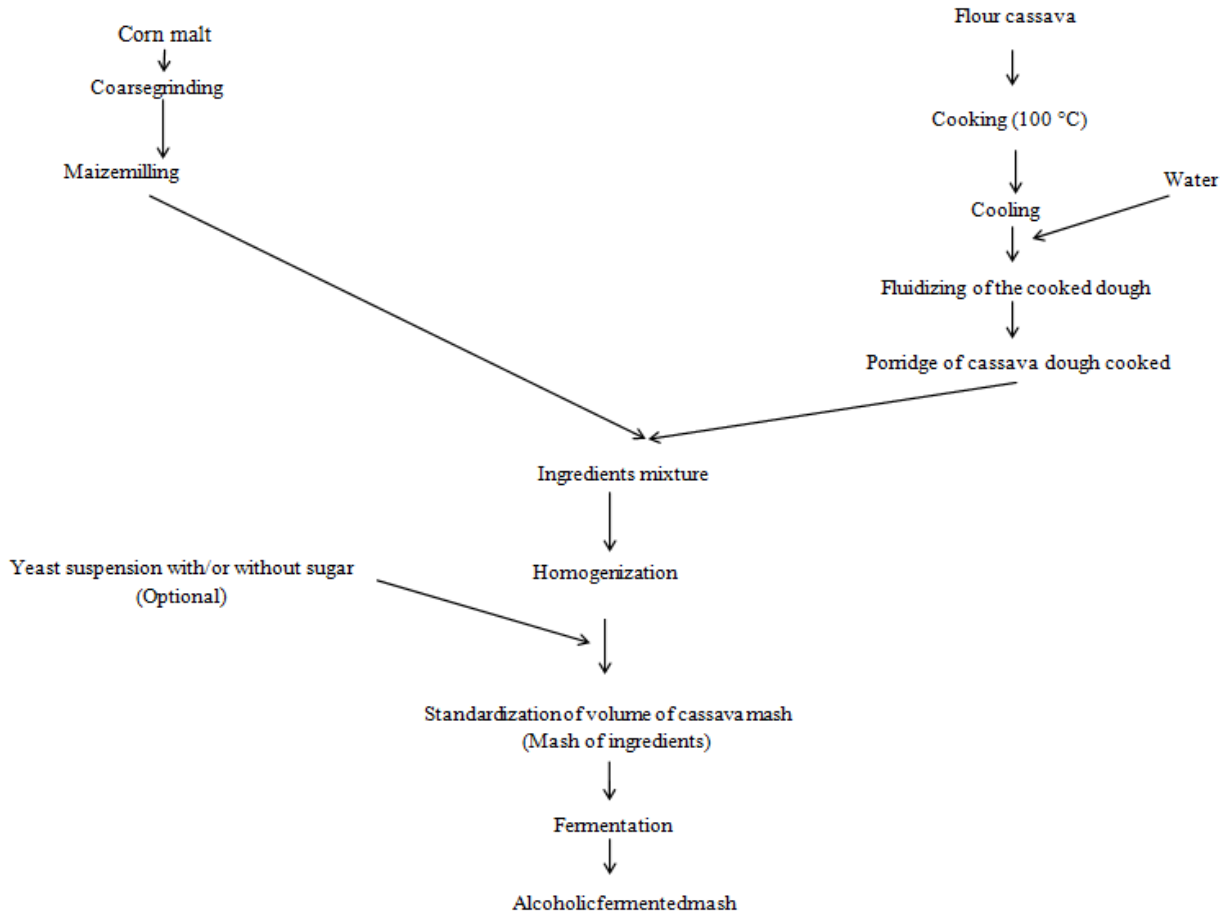

Fig. 1: Diagram of alcoholic fermentation of cassava flour

Launching of fermenting of mash of ingredients: Immediately before fermenting, 280 g of malt of maize (*Zea mays* L.) of a local cultivar named *Accros* were coarsely ground. Coarse grinding obtained was added to the porridge of cassava dough cooked and mixed together. The volume of the mixture was standardized to 4 L with water cooled at 10°C and the resulting mash was homogenized: it was the launching of spontaneous fermentation.

The launching of the test of fermentation led by yeasting was carried out by addition of 4.8 g of *Saccharomyces cerevisiae* (Gosselin *et al*., 2000) to 4 L of mash of ingredients (Fig. 1).

For the test of fermentation led with yeasting and sugaring, in the mixture of ingredients 4.8 g of *S. cerevisiae* and 800 mL of cooled sugar solution containing 90 g of sugar cane (11.25%) were added and the volume of this mixing was also standardized at 4 L.

Methods of analysis:

Evaluation of saccharification: A diluted iodine test was performed on aliquots of the must to appreciate the level of fermentation by using the following method (E.B.C., 1975; Ugboaja *et al*., 1991).

A drop of fermented mash was placed on a porcelain plate and a drop of 0.05 N iodine solution was immediately added there. Saccharification was considered complete when the color obtained after mixing was light yellow and was incomplete when the color was blue or dark brown.

Determination of soluble extract: For the determination of sugar content in degrees Brix (wt/wt) during fermentation, the method by refractometry has been used (Diakabana *et al*., 2013).

Determination of total fermentable sugars and determination of the apparent attenuation: During the different tests of fermentation of must of cassava, fermentable extract was determined and the apparent attenuation calculated by using the formula EBC (1987) as follows:

$$[\frac{P-m}{p}] \times 100$$

with p = primitive extract of wort in % (g/100 g) and m = final apparent extract of wort also in %. The apparent attenuation value indicates the percentage of fermentable sugars in the wort.

Determination of total reducing sugars: For the determination of total reducing sugars in the wort, the method of Fehling's solution was used with adaptation (Diakabana *et al*., 2007).

Appreciation of the amount of non-fermentable sugars in suspension into the cassava wort: Dosing dextrins (non fermentable sugars) in the wort after apparent attenuation during fermenting has been achieved by acid hydrolysis and the reducing sugars formed before and after hydrolysis were dosed. This difference represents the glucose formed by the dextrins.

Procedure: The wort obtained at the end of fermentation was tested. In a flask of 300-400 mL were introduced: 25 mL of 10% sugarcanesolution prepared for the determination of the test, 200 mL of distilled water and 20 mL of concentrated HCl (density = 1.19). The ball was topped with a cap traversed by a glass tube about a meter long and 6-8 mm diameter to condense vapors. The tube was cut beveled at its lower end to facilitate the fall of drops of condensation.

Raised 3 h in boiling bain-marie, the contents of the flask was cooled, neutralized with concentrated NaOH solution to litmus paper, brought to 500 mL. Determination of reducing sugars was performed by the method of Fehling's solution (Diakabana *et al*., 2007).

The amount of reducing sugars found is expressed as dextrose equivalent (Scriban, 1984) measured before and after inversion; the difference multiplied by 0.9 gives the percentage of dextrin in glucose equivalent.

Evaluation of the ethanol content of the fermenting wort: The ethanol content of the must during fermentation tests performed was evaluated according to the pycnometry method described by Heineken (1986).

Determination of total ethanol productivity: The total ethanol productivity P_{TE} was determined by using the following equation (Diakabana *et al*., 2013):

$$PTE = \frac{Ef}{tM} \text{ x } Ef$$

where,

E_f : The maximum concentration of ethanol (g/L) evaluated at the end of fermentation

t_M : A total fermentation cycle (h) duration and P_{TE} (in g ethanol/L.h)

The total ethanol productivity was chosen as a performance criterion to evaluate different fermentation trials tested by taking the time as one of economic constraints (Engasser, 1988).

Isolation and identification of fermentable sugars by Haas and Fleischmann: The isolation and identification of fermentable sugars (glucose, maltose) were carried out by thin chromatography layer TLC on silica gel and taking pure sugaras witnesses. The methods described by Préaux (1963) and Jong *et al*. (1999) have been used with adaptation.

Before use, the TLC plate silica gel on aluminum support was dried by baking at 110°C for 10 min. Twenty micro litter aliquots of diluted samples tested containing 0.1-1% soluble extract with 10 µL of standard solution of pure sugars (0.1% wt/v) were deposited at least 5 times as spot at a specific location on the starting line located at 1.5 cm from one end. The distance of 1 cm from the outside edges of the plate was respected. Four samples were tested at a time on the same plate, the distance of 1 cm was also observed between two points of application. The standard solution of pure sugars dissolved in ethanol at 90° GL (0.1% wt/v) consisting of glucose and maltose was used as reference.

The upward-dimensional chromatography was used. The solvent system used for the separation of sugars was composed of mixture of glacial Acetic Acid/Ethyl Acetate/96° Ethanol/Water in the ratio 3/1/2/1 (v/v/v/v) and separation conducted for 60 min.

Revelation: After development, the chromatograms were dried vertically in the air, the starting line downwards to avoid leaching of tasks by the excesses of the mobile phase (solvent of migration). Tasks (spots) were colorless, they were revealed (visualized) in two ways:

- The detection of sugars on dried plates by using UV fluorescence at 254 nm
- By spraying the plates with a solution diluted to 50% H_2SO_4 in methanol, then heated at110°C for 10 min by steaming

After the revelation of the tasks, the plates were photographed to illustrate the results. The identification of tested sugars samples was made by comparison of reference sugars as external controls (glucose and maltose).

Statistical analysis: For the purpose of the characterization of the kinetic study of ethanol fermentation of cassava must, the following statistical values were considered: mean, standard deviation S.D. and confidence interval (mean±S.D.). The modified statistical method based on the law of Gauss-Laplace in bell was used (Larrieu, 1988).

RESULTS

All the results presented are the mean values of three repetitions for every trial.

Appreciation of term of fermentation: The end of the duration of fermentation of cassava mash was highlighted when the saccharification of starch was

Table 1: Duration of the fermentation of the must

	Samples tested		
Parameters	E1	E2	E3
0.05 N iodine test	Negative	Negative	Negative
Time of fermentation (h)	120	48	96
Stage of fermentation	End	End	End

E1: Test of spontaneous fermentation without additional yeast; E2: Fermenting test led with yeasting; E3: Test of fermentation led with additional yeast and sugaring; Negative 0.05N iodine test was visualized by the formation of the yellow color of the must

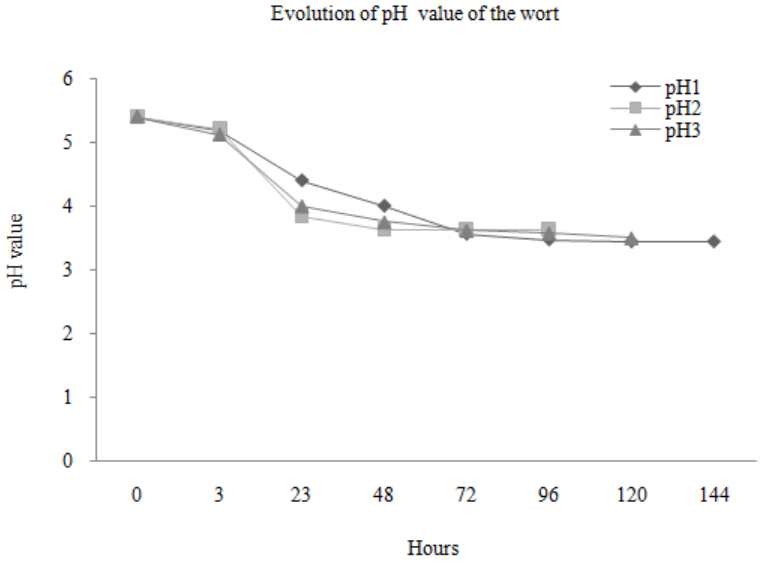

Fig. 2: Evolution of pH value during fermentation
pH1: Wort without yeast addition; pH2: Wort with yeast addition; pH3: Wort with yeast inoculation and sugaring; Incubation time (h)

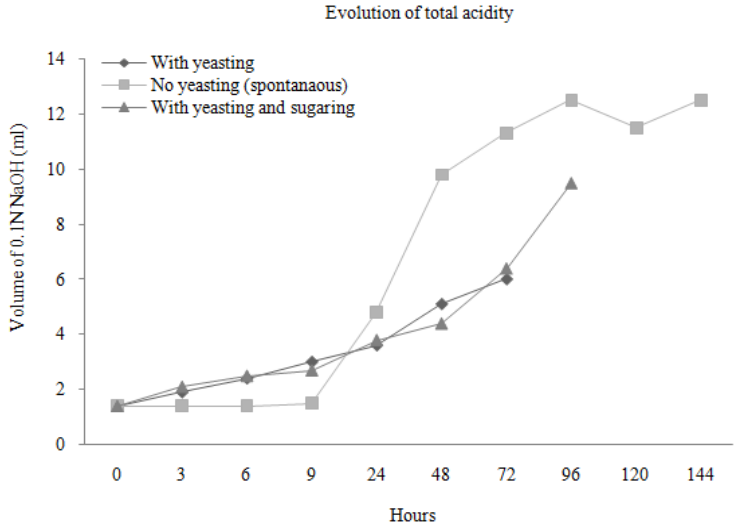

Fig. 3: Evolution of total acidity (in mL of 0.1 N NaOH) during the fermentation of boiled cassava with or without addition of yeast and with or without sugaring

deemed complete by the negative 0.05 N iodine test (Table 1).

Changes in pH: The evolution of the pH of the wort has steadily declined in the three cases of fermentation. However, the pH of the wort with yeast inoculation decreased faster than the wort without yeast inoculation (Fig. 2).

Evolution of the total acidity of the must during fermentation of various tests: The total acidity of the must increased more rapidly from 24 h of spontaneous

fermentation without yeast inoculation (4.8 mL of 0.1 N NaOH) compared to the other two cases (about 3.6 mL of 0.1 N NaOH for the wort with yeasting) (Fig. 3).

Behavior of the soluble extract from the mash during fermentation: In all three cases must fermentation of cassava (Fig. 4), the profile of the disappearance of the soluble extract was similar to a final limit on each case (final extract content Es1 = 3.9% at 120 h for the control without addition of yeast and Es2 = 3% to 48 h for the test with addition of yeast into wort).

Profile of consumption of fermentable sugars in the must of cassava: The consumption profile of fermentable sugars presented the same pace for all three fermentation tested (Fig. 5). The attenuation limit was reached faster in the case of wort with yeast addition (from 48 h) compared to the case of mash without the addition of yeast (from 120 h).

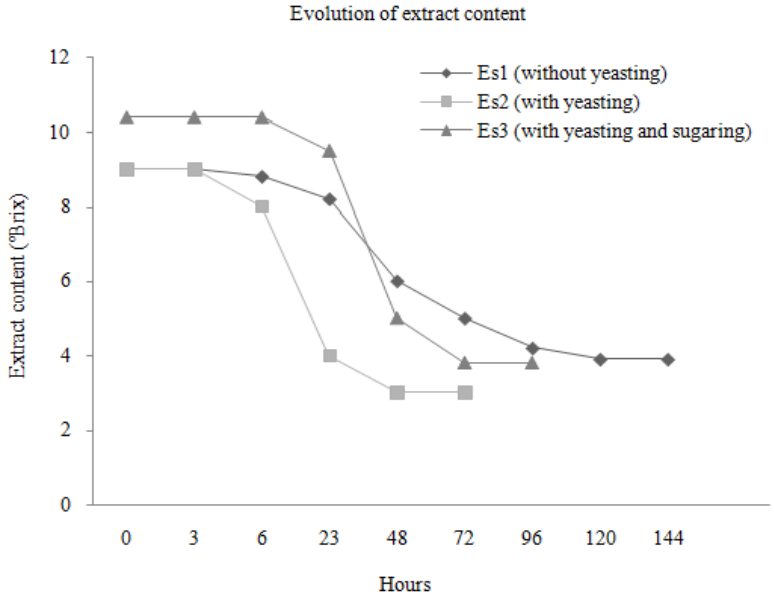

Fig. 4: Evolution of the extract content ES (°Brix) in different
Fermentation tests: Es1: Without adding yeast, Es2: With yeasting, Es3: With yeasting and sugaring

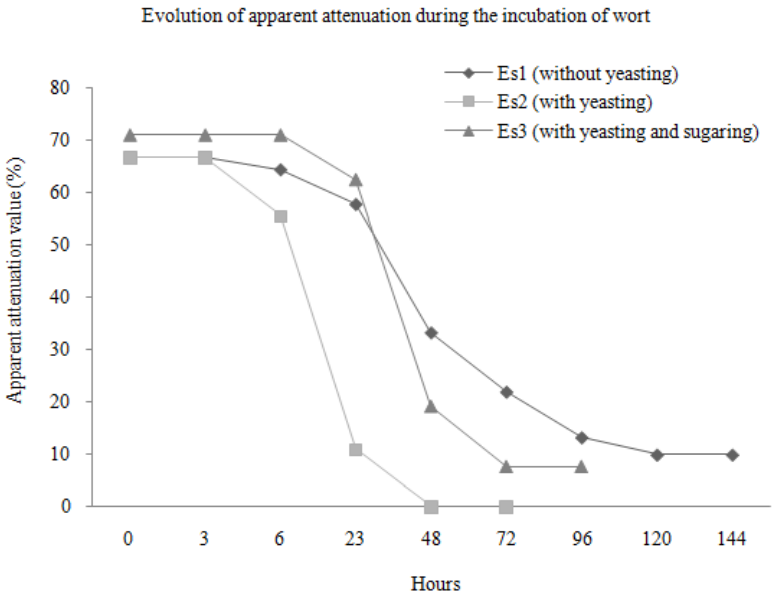

Fig. 5: Evolution of the apparent attenuation attap during different fermentation tests
Attap 1: Percentage of reduction for the test without addition of yeast; Attap 2: Percentage of reduction for the test with addition of yeast; Attap 3: Percentage of reduction for the test with addition of yeast and sugaring

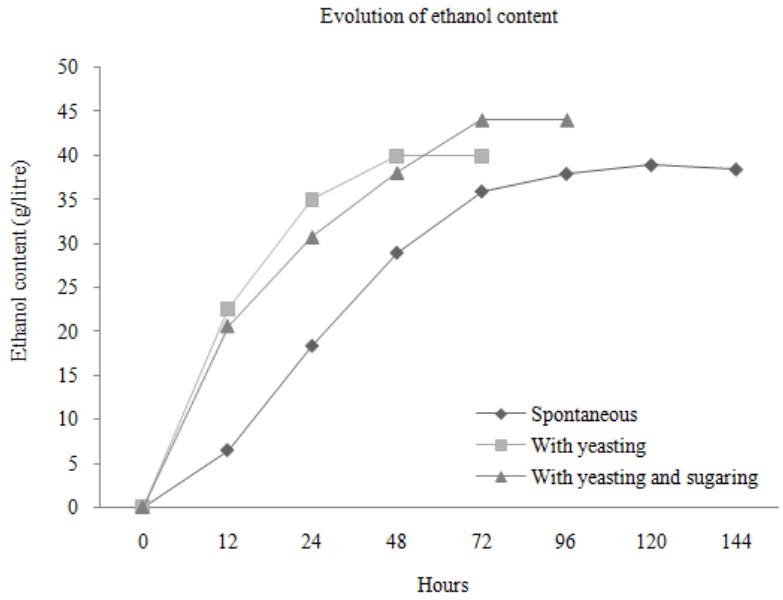

Fig. 6: Evolution of ethanol (g/L) during different fermentation tests (spontaneous, with or without the addition of yeast and sugaring)

Table 2: Relationship between the amount of ethanol and dextrin (residual sugar) at the end of fermentation

	Samples tested		
Parameters	Without additional yeast	With additional yeast	With additional yeast and sugaring
Dextrins (%glucose équivalent)	3.60	2.70	3.42
Ethanol (g/100 mL)	3.60	4.00	4.40
Total ethanol productivity (g ethanol/L.h)	0.32	0.83	0.61

Evolution of the ethanol content in different modes of fermentation tested: Ethanol production had evolved following an ascending profile in the three fermentation methods tested (Fig. 6). It grew significantly faster in the case of wort with yeast inoculation compared to the case of spontaneous fermentation without yeast inoculation at 24 h. It then stopped at 48 h for fermentation of cassava mash with addition of yeast (44 g of ethanol formed/L) and 120 h in the case of spontaneous fermentation without addition of yeast (38 g of ethanol/L).

Relationship between amount of ethanol formed and dextrins in wort after fermentation: The results in Table 2 show that the addition of yeast in cassava wort helped lowering dextrins and promoting the production of ethanol (2.7% glucose equivalent of dextrin with 4 g/100 mL of ethanol produced) compared to the spontaneous fermentation carried out without addition of yeast (3.6% glucose equivalent of dextrin and 3.6 g/100 mL of ethanol).

The results showed (Table 2) a higher total productivity for ethanol fermentation test with yeast inoculation (0.83 g ethanol formed/L.h) compared to the test with addition of yeast and sugaring (0.61 g ethanol/L.h).

Behavior of fermentable sugars: Fermentable sugars in the wort cassava were revealed by TLC, among them

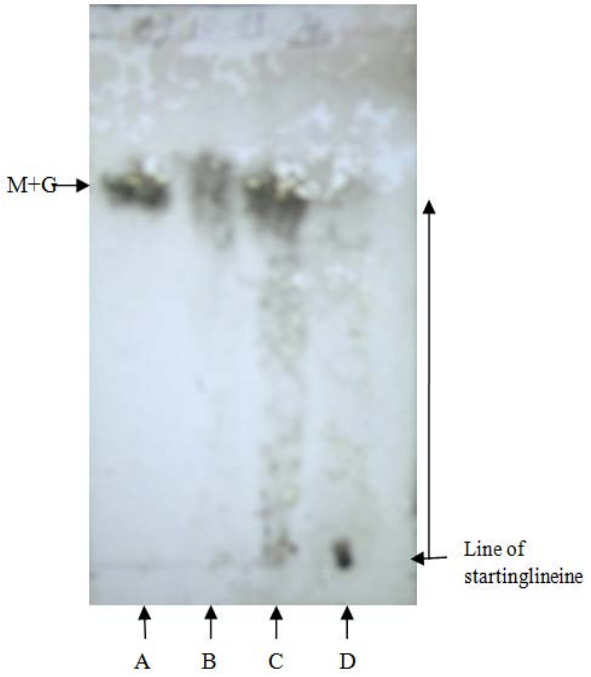

Fig. 7: TLC analysis of sugars in the fermentation of cassava mash
A: Reference sugars (M+G: mixture of glucose G and maltose M); Stages of fermentation: B: 6 h, C: 23 h, D: 48 h

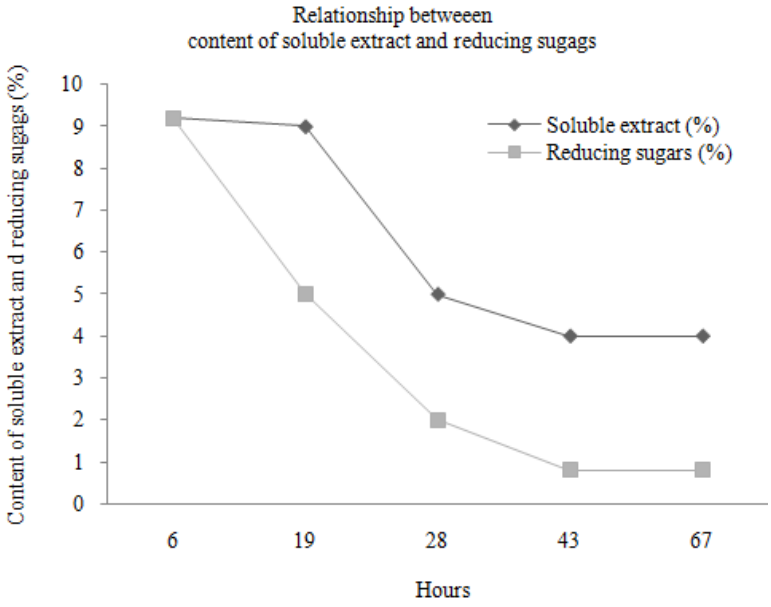

Fig. 8: Relationship between content ES soluble extract and reducing sugars during fermentation test with yeast inoculation

are glucose and maltose (Fig. 7). However, they have been used at different times during the fermentation.

Compared with the reference standard (Spot A), glucose and maltose were present (spots in B and C) as well as 6 to 23 h of incubation. However, the observation of spot in D revealed that these two sugars were gone at 48 h of incubation. In addition, the chromatogram also revealed (Fig. 7) the presence of a spot in the D position on the line of starting, showing so that the soluble extract residual in the final wort.

Relationship between total soluble extract and total reducing sugars: Consumption of total soluble extract of cassava wort had evolved inparallel to total reducing sugars from 19 h of incubation. Compared to the total soluble extract of wort (Fig. 8), reducing sugars were consumed more quickly and almost completely (about 0.8% of residual reducing sugars compared to 4% of total soluble extract at the end of fermentation).

DISCUSSION

Traditional bioethanol producers face many difficulties related to the control process of fermentation of cassava must (Diakabana *et al*., 2008). Investigations conducted during the fermentation showed that the total acidity of cassava mash was higher at the end of the process for the case without adding yeast (12.5 mL of 0.1 N NaOH at 144 h) compared to fermentation directed with yeast inoculation (5.1 mL of 0.1 N NaOH at 48 h). The significant decrease in pH in the three cases of fermentation tested (pH = 3.5 on average) would be favorable for the preservation of the environment against the acid-sensitive pathogens which cannot exert their negative effects (Stewart *et al*., 2007; Keke *et al*., 2009).

The results of this study showed that the fermentable sugars have a low enough attenuation (66.7% of sugars processed). These fermentable sugars come from, in large part, progressive enzymatic hydrolysis of starch of cassava mash starched as reported in our works in connection with the production of *Boganda* of Congo (Diakabana *et al*., 2008).

Fermentable sugars present in the initial cassava must, including glucose and maltose were consumed at different times during the fermentation (De Clerck, 1984; Stewart, 1985). The use of selective methods of identification of fermentable sugars in the cassava must elucidate, partly, the origin of insufficiency of fermentation. Reducing sugars including maltose and glucose being almost completely consumed (approximately 91% at 43 h) compared to the total extract (about 56.5%), are therefore fermentable.

The sugaring of the cassava mash, by adding sugarcane, does not seem to increase the total ethanol productivity, probably because of the mismatch to maltose by yeast after consuming a certain amount of sucrose (Marc, 1982; Fernández *et al*., 1985; Stewart, 1985).

The rapid disappearance of glucose and maltose (Fig. 7) explains their preferential consumption by yeast and the inhibition of saccharifying α-amylase activity by the ethanol formed (Fernández *et al*., 1985; Boivin, 2001).

During the fermentation of boiled cassava, dextrins escaped from total degradation of polysaccharides by endogenous amylases. These dextrins, composed of glucose residues, represent a residual amount of soluble sugars in the wort, but are not available for the yeast strains implicated (Marc, 1982).

CONCLUSION

The presence of dextrins in significant amounts (3.6% dextrose equivalent) in the cassava mash at the end off ermentation reveals the absence of debranching enzyme activity of amylopectin. Thus the addition of exogenous enzyme having debranching amylolytic activity in the cassava must could lead at the improvement of the yield of saccharification of starch and therefore increase the production of ethanol in the traditional fermentation.

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