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IMPROVEMENT OF *BORASSUS AKEASSII* WINES QUALITY BY CONTROLLED FERMENTATION USING *SACCHAROMYCES CEREVISIAE* STRAINS

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ABSTRACT

Palm wine produced traditionally and consumed by many people around the world and specifically in Burkina Faso posed health risks because of questionable quality of wine produced by mix culture fermentation and the use of antiseptics for the stabilization. In order to improve its quality, *Saccharomyces cerevisiae* strains isolated from *Borassus akeassii* wines and identified by amplification and RFLP analysis of the 5-8S-ITS region were used for in vitro fermentation of unfermented palm sap. The physicochemical characteristics of the sap were measured before and after fermentation process by High-Performance Liquid Chromatography (HPLC) and the microbiological quality were also performed. HPLC analysis showed that glucose and fructose concentration in palm sap were 37.0 and 27.6 g/L respectively, ethanol content was ranged between 2.76 and 5.31 % (g/mL) for controlled fermentation and 2.20 % (g/mL) for spontaneous fermentation. Lactic and acetic acids were ranged between 0.1 and 0.3 g/L and 1.5 and 1.6 g/L for controlled fermentation versus 2.5 and 3.1 g/L and the spontaneous fermentation respectively. Coliforms and *Staphylococcus aureus* were detected only in the unfermented palm sap and the wine fermented spontaneously.
Principal component analysis showed a good separation between spontaneous and controlled fermentation. Sterilization and controlled fermentation of the unfermented sap with palm wine *Saccharomyces cerevisiae* strains led to the improvement of palm wine quality.

Keywords: *Borassus akeassii* wine, Fermentation, improvement, quality, *Saccharomyces cerevisiae*, RFLP, HPLC

INTRODUCTION

Palm wine is an alcoholic beverage from the sap of various species of palm tree such as *Palmyra* and coconut palm (**Adeleke and Abiodun, 2010**). It is a sweet alcoholic beverage widespread in African, American and Asian tropical regions and which is obtained by spontaneous fermentation of sap tapped from palm trees such as *Elaeis guineensis* , *Raphia hookeri*, *Raphia vinifera* (**Ezeronye and Legras, 2005**) and *Borassus akeassii Bayton* (**Bayton et *al.*, 2006 ; Bayton and Ouédraogo, 2009**). Palm wine contains 300 calories/L, 0.5-2.0 g of proteins, considerable of vitamins, a major component of which is vitamin A, C and K helps consumers eye sight, protects and improves the eye sight (**Santiago-Urbina and Ruíz-Terán, 2014)**. Many components of palm wine have been found previously in conventional wines. This wine is colorless and very sugary (**Obahiagbon and Osagie, 2007**) until sugars are fermented into alcohol then organic acids spontaneously. According to producers and consumers, palm wine obtained by mix culture (spontaneous) fermentation, gets inconsumable after 3 days (**Ouoba *et al.*, 2012**). Natural uncontrolled fermenting process led to unstableness and easy spoilage of this product quality (**Ngọc *et al.*, 2014**). This beverage produced traditionally is unstable, therefore exposed to alteration if fermentation is not controlled. The acidification and instability of palm wine during its fermentation need to be controlled in order to ensure its quality. Different antiseptics such as sorbic acid, diethylpyrocarbonate (DEPC) and sodium metabisulfite have been already used for stabilisation of palm wine (**Okafor, 1975 a**). Even though use of these antiseptics would be the efficient means for the stabilization of palm wine, they can pose health risks. In a former work, coliforms and *Staphylococcus aureus* were detected in natural palm wine (**Tapsoba et *al.*, 2011; 2014**). According **Olawale *et al.* (2010)**, sterilization and use of purified *Saccharomyces cerevisiae* in fermentation of palm sap could confer a more quality and hygienic palm wine. Because of questionable quality of palm wine produced by mix culture fermentation and the use of antiseptics for the stabilization towards the world and specifically in Burkina Faso, we proposed an improvement of this beverage quality by controlled fermentation of unfermented sap with selected strains of *Saccharomyces cerevisiae* isolated from palm wine. It has been to use active *S. cerevisiae* strains isolated from *Borassus akeassii* palm wine for fermentation of the unfermented sap, evaluate the microbiological and biochemical quality of palm wine produced secondly and to compare controlled fermentation and spontaneous.

MATERIAL AND METHODS

Sampling of palm crude sap

Palm crude sap of the same *B. akeassii* species was purchased in South-West of Burkina Faso where palm wine is largely tapped and very consumed. Two samples of 1 L of fresh palm sap were transferred in sterile plastic containers which were immediately immersed in an isothermal box, and brought to the laboratory and maintained at 4° C before the analysis.
Before fermentation, 10 mL of the unfermented palm sap were used for microbiological analysis and 15 mL were filtered and stored at -20° C for further analysis

Palm wine fermentation process

Preparation of the inoculums for fermentation

We used *Saccharomyces cerevisiae* strains YBPW7, YBPW13 and YBPW25 isolated from *Borassus akeassii* wines and identified by amplification and RFLP analysis of the 5-8S-ITS region (**Esteve-Zarzoso *et al.*, 1999**). Each strain was overnight grown aerobically in shake 10 mL flasks at 30 °C in YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose).

Fermentation process assays

The unfermented palm sap was subjected to flowing vapor sterilization as method described by **Clément (2012)**. The fermentation assays were carried out using *Saccharomyces cerevisiae* strains YBPW7, YBPW13 and YBPW25 isolated from palm wines to inoculate 250 mL palm sap collected. The Overnight culture each of strain was used to inoculate 250 mL of sterilized *Borassus akeassii* crude

sap at a density of 10^6cells / mL. Spontaneous (Mix culture) fermentation was at the same time carried out with the endogenous microorganisms (natural microflora). The experiments were performed at 30° C for 72 hours.
Palm wines produced were designed CF7, CF13, CF25 and SP respectively. Sterile samples were collected at 24 hours time intervals for further analysis.
Wine was filtered using cheese cloth as method described by **Kumar *et al.* (2012)** and stored at 4°C. Then 10 mL were used for microbiological analysis and 15 mL were stored at -20 ° C for analysis of physico-chemical parameters. Glucides, organic acids, glycerol and ethanol were measured by HPLC in the supernatant. The pH was also measured using a pH-meter (WTW 82362) at 25° C.

Biochemical and microbiological analyses of palm wines

Sugars, ethanol and organic acids analyses by High-Performance Liquid Chromatography (HPLC)

Glucose, ethanol, glycerol and organic acids in palm wines were determined by High-Performance Liquid Chromatography (HPLC-1 Agilent 1260) equipped of a degasser G132A, a quaternary pump G1311 A, a passor of samples G131 A, a furnace G131 A, a detector UV (G131A) to the variable wavelength, a refractometer G1382 A and a column Phenomenex-Rezex ROA-Organics Acid H^+ (Size 300 x 7.8 mm). For analysis, column effluents were monitored by an UV detector (G131A) set at 210 nm and a refractometer (G1382A). The mobile phase (0.0005 N H_2SO_4) filtered through a 0.2 µm Millipore membrane filter was used at a low rate of 0.6 ml/min and 25 µl of the prepared sample were automatically injected.
The detection of targeted compounds was performed by refractometry for glucose, fructose, ethanol, glycerol and succinate and by UV spectrophotometry at a wavelength set at 210 nm for pyruvate and acetate.

Analysis of microbiological quality of produced palm wines

In order to control the quality of palm wine produced, coliforms bacteria and *Staphylococcus aureus* counts were performed (**Norme ISO 7218, 2007**). Ten (10) mL of each sample were mixed with 90 mL of sterile peptone solution. Serial dilution was performed with the sterile peptone solution and 100 µL of decimal dilutions were plated in duplicate on Petri dishes. Chapman's Agar medium (Sigma Aldrich, USA) were used for *Staphylococcus aureus* counts and Violet Red Bile Agar (VRBL) (Biokar, France) for coliforms bacteria. Plates were incubated for 48 hours at 37±2°C *Staphylococcus aureus* counts and VRBL plates that were incubated at 30±2°C for total coliforms and 44±2°C for thermo-tolerant coliforms for 48 hours. Counts were expressed as colony forming units per mL (cfu/mL).

Principal Component Analysis (PCA) of different palm wines

The comparison of different process was performed by Principal Component Analysis using the package FactoMineR of Rcommander of R 3.2.3.

Data analysis

Data analyses were performed with R 3.2.3. Data obtained were subjected to an analysis of variance (ANOVA) using the Fisher's least significant difference (LSD) test to determine significant differences between each sample (wine or sample) ($P \leq 0.05$). Principal Component Analysis (PCA) was performed to compare the different types of fermentation by using the package FactoMineR of Rcommander.

RESULTS AND DISCUSSION

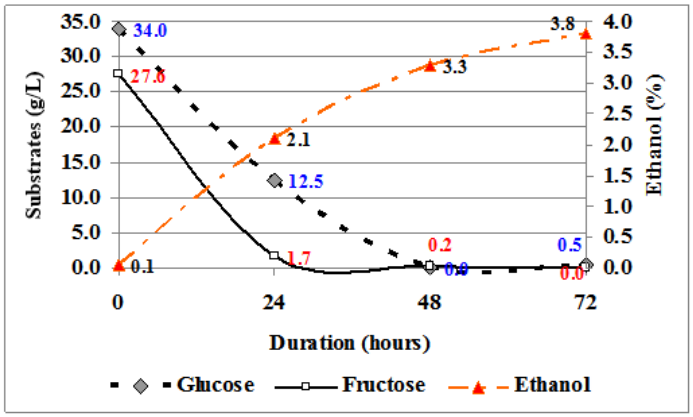
Strains used in this study were YBPW7, YBPW13 and YBPW25, isolated from *Borassus akeassii* wine. As presented in table 1, strains were identified as *S. cerevisiae* species by amplification and RFLP analysis of the 5-8S-ITS region (**Esteve-Zarzoso *et al.*, 1999**).

Table 1 Size (bp) of the PCR products and the restriction fragments

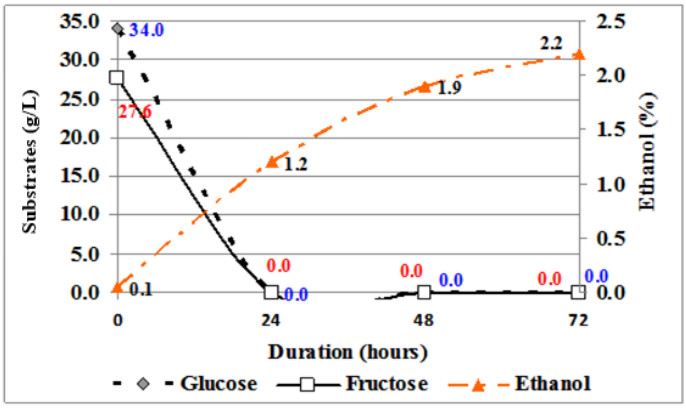
Strains	Fragments size (pb)	Restricted fragments size (pb)		Identification
		Hae III	Hinf I	
YBPW7	880	140-360	120-180-220-300	*S. cerevisiae*
YBPW13	850	140-380	120-180-220-300	*S. cerevisiae*
YBPW25	850	140-380	120-180-220-300	*S. cerevisiae*

Many authors reported that *S. cerevisiae* was the species as responsible for the fermentation and aroma of the wine (**Amoa-Awua *et al*,. 2007; Stringini *et al*., 2009; Ouoba *et al*., 2012**).

The kinetics of fermentation of palm sap by *S. cerevisiae* was presented in figure 1.



a. Controlled fermentation with *Saccharomyces cerevisiae* strains



b. Mix culture fermenation of *Borassus akeassii* sap by endegenous microorganisms

Figure 1 Kinetics of mix culture and controlled fermentation of *Borassus akeassii* palm wine

Substrates (glucose and fructose) detected in palm sap were consumed completely at 24 hours in the spontaneous (mix culture) fermentation as presented in figure 1a. According to **Opara et *al.* (2013)**, four micro-organisms were found to be frequently present during the mixed culture fermentation of palm sap. These micro-organisms in the order of succession are: Yeasts, *Micrococcus*, Lactic Acid bacteria and *Leuconostoc spp*.
We have analyzed substrates and metabolites present in palm sap and palm wines and the results are presented in table 2.

Table 2 Physico-chemical characteristics of crude sap and wines of *Borassus akeassii*

Samples	pH	Glucose (g/L)	Fructose (g/L)	Ethanol (%)	Glycerol (g/L)	Succinate (g/L)	Acetate (g/L)	Malate (g/L)	Lactate (g/L)	Pyruvate (g/L)
PWS	6.5	34.0	27.6	0.05	0.10	0.8	1.0	1.2	0.1	ND
SP	3.5	0.50	0.0	2.20	1.50	1.1	3.1	2.0	2.5	0.1
CF7	4.2	0.0	0.0	5.31	3.0	2.4	1.6	0.6	0.2	0.2
CF13	3.9	0.0	0.0	3.34	3.0	2.5	1.5	0.6	0.3	0.5
CF25	4.5	0.0	0.0	2.76	2.6	2.5	1.6	0.4	0.1	0.2

Legend: PWS: Palm wine sap; SP: Spontaneous fermentation; CFX: Controlled fermentation with strain YVPWX; ND: Not detected

Ethanol was also found in all samples that indicating the alcoholic fermentation. Ethanol content was ranged between 2.76 and 5.31 % for controlled fermentation versus 2.20 % for spontaneous fermentation. Glucose and fructose were about 34.0 g/L and 27.6 g/L respectively but sucrose was not detected in unfermented palm sap. There was an important production of glycerol in the controlled fermentation. Glycerol concentration was ranged between 2.6 and 3.0 g/L versus 1.5 g/L in spontaneous fermentation process. Glycerol was the major fermentation by-product of *Saccharomyces cerevisiae*, which indirectly contributes to the sensory character of wine (**Yalçin and Özba, 2006**).

The comparison of physicochemical characteristics of palm wines shows that the wine produced by mix culture fermentation is more acidic that those obtained by controlled fermentation. It has also been shown that the mix culture fermentation process is acidic as it progresses and there is proliferation of micro-organisms depending on the condition of the medium (**Opara *et al.*, 2013**). **Naknean *et al.* (2010)** reported that when fructose is available in wine and lactic acid bacteria are able to grow, they can produce equimolar amounts of lactic and acetic acids from fructose and this could constitute a serious source of acetic acid in wine.

The results of microbiological quality of palm sap and wine were presented in table 3.

Table 3 Microbiological characteristics of palm sap and wines of *Borassus akeassii*

Samples	TC (10^4)	FC (10^2)	*S. aureus* (10^5)
PWS	3.75	1.5	3.4
SP	1.75	0.75	1.5
CF7	ND	ND	ND
CF13	ND	ND	ND
CF25	ND	ND	ND

Legend: PWS: Palm wine sap; SP: Spontaneous fermentation; CFX: Controlled fermentation with strain YBPWX; ND: Not detected; TC: Total coliforms; FC: Thermotolerant coliforms

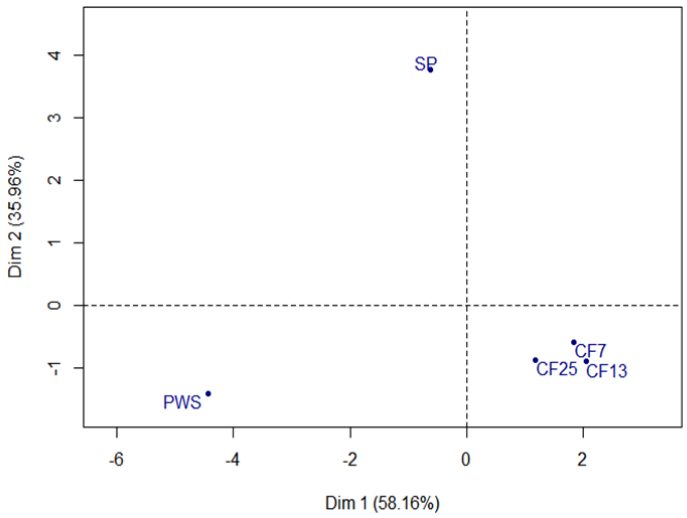
In the palm wine obtained by mix culture fermentation and the crude sap, we have detected coliforms and *Staphylococcus aureus* while in the controlled fermentation, they were not detected. The presence of these microorganisms revealed that unfermented palm sap used is collected under unhygienic condition (**Olawale *et al*,. 2010; Tapsoba et *al*., 2014**).

Bacteria and yeasts usually contaminate the juice as it is tapped and there are changes in biochemical composition of the palm wine (**Olawale *et al.*, 2010**). Normally, palm sap is a raw material to produce palm sugar syrup. Some factors affected the quality of palm sugar syrup such as processing method and quality of palm sap (**Phaichamnan *et al.*, 2010**).

Naknean *et al.* (2009) studied the effect of processing method on quality of palm sugar syrup. Flowing vapor sterilization could be important before fermentation of palm sap because the presence of potential endogenous microorganisms is avoided. The microbiological quality of palm sap becomes important to obtain a more quality and hygienic palm wine.

According to **Olawale *et al.* (2010)**, the sterilization and the use of purified *Saccharomyces* in fermentation of palm sap led to a more quality and hygienic palm wine. Of the yeasts responsible for palm wine fermentation, the predominant and best alcoholic fermenter was *Saccharomyces cerevisiae* (**Stringini *et al.*, 2009**).

Fermentation processes were compared using principal component analysis (PCA) as presented in figure 2. This analysis showed a good separation between fermentation processes. The PCA gives also an overview of the differences between the mix culture fermentation and controlled fermentation.



Legend: PWS**:** Palm wine sap; SP: Mixed culture fermentation; CFX: Controlled fermentation with *S. cerevisiae* strain X

Figure 2 Principal component analysis of palm wine fermentation

The wines produced by controlled fermentation (CF7; CF13 and CF25) were grouped together in the bottom right of the figure 2. The wine produced by mix culture fermentation (SP) was at the top of the figure while the unfermented sap (PWS) was shown in the bottom left. This analysis shows that there is a difference between the unfermented sap, the wine produced by mix culture fermentation and the wine obtained by controlled fermentation.

Figure 3 provides the flow chart for controlled fermentation for improved palm wine. Indeed, this diagram provides a wine, different of the wine obtained by mix culture fermentation. It enables the improvement of microbiological and physicochemical quality of palm wine.

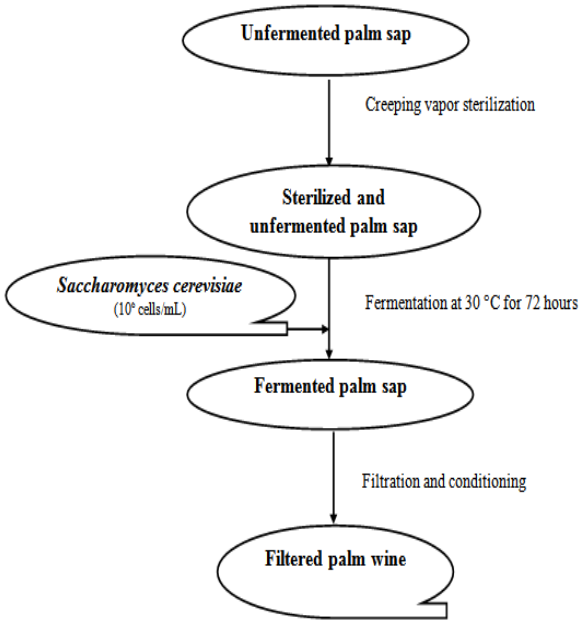


Figure 3 Flow chart (diagram) for controlled fermentation of palm wine

Flowing (Creeping) vapor sterilization eliminates undesirable microorganisms that could contaminate raw sap during its extraction process. According to the care applied in the collection of the crude sap, a first hypothesis on the presence of potential endogenous microorganisms in the sap was emitted (**Ben Thabet *et al.* 2010**).

CONCLUSION

Three *Saccharomyces cerevisiae* strains isolated from *Borassus akeassii* wines and identified by amplification and RFLP analysis of the 5-8S-ITS region were used for in vitro fermentation of unfermented palm sap.

This work confirmed that the use of active *Saccharomyces cerevisiae* strains for fermentation of palm sap gave a more quality and hygienic palm wine. Flowing vapor sterilization used during the production of conventional wines, can be used for the improvement of palm wine quality.

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