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MICROBIAL PROFILE OF FERMENTING RAFFIA PALM (*Elaeisguineensis*) SAP

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ABSTRACT

Raffia palm sap was analyzed for its microbial, pH, and alcohol components at different stages of batch fermentation for 96 hours. Micro organisms isolated during fermentation were *Gluconobacter sp*., *Lactobacillus sp*., *Candida albicans*, and *Saccharomyces cerevisae*. As fermentation progressed, pH decreased from 5.4 to 4.6 (from 0 hours to 96 hours), while alcohol contents increased from 1.8% v/v to 6.23% v/v (from 0 hours to 24 hours), and then started to decline. *Candida albicans* was not detected from 48 hours of fermentation; while *Gluconobacter sp*., *Lactobacillus sp*. and *Saccharomyces cerevisae* persisted till the end of the fermentation period studied. No significant statistical correlation ($p>0.05$) was observed: between alcohol production rates and specific microbial growth rates; and among specific microbial growth rates. Similarly, this study revealed no significant statistical correlation in population growth patterns among microbial isolates. Results obtained from this study showed that most of the alcohol produced in raffia palm sap fermentation occurred within the first 48 hours of fermentation; and though total microbial populations did not significantly change during fermentation, specific microbial populations were noticeably altered during fermentation.

Keywords: palm wine, microbial succession, alcohol tolerance, spontaneous fermentation.

INTRODUCTION

Palm wine is a generic name given to alcoholic beverages produced by the natural fermentation of sap obtained from various tropical plants of the *Palmae* family (Santiago-Urbina and Ruiz-Teran, 2014). In Nigeria, it is usually obtained from *Raffia rinfera*, *R. hookeri*, and *Elaeis guineensis*; with *Raffia* palms usually yielding more sap than oil palms (*Elaeis guineensis*) during the tapping period (Obi et al. 2015). Tapping is the process by which palm sap is obtained from palm trees. It involves a series of operations to stimulate the flow of sap (Aptutharajah et al. 1986). This involves the perforation of the trunk or inflorescence, to create a flow channel for the collection of the sap in a container (Ouoba et al. 2012).

The sap is a rich substrate for the growth of various micro organisms (Nwachukwu et al. 2006). The sap undergoes spontaneous fermentation converting the sweet substrate into several metabolites mainly ethanol, lactic acid and acetic acid. The yeasts converts the sugars to alcohol, hence the physicochemical properties of palm wine is a function of the metabolic activities of the inherent yeasts in palm wine (Ukwuru and Awah, 2013). The biochemistry of palm wine fermentation consists of: initial lactic acid fermentation, a middle alcoholic fermentation, and final acetic acid fermentation (Amoa-Awua et al, 2007). Decreased pH by the production of organic acids due to the activities of lactic acid fermentation probably enhances the growth and invertase activities of the yeasts (Naknean et al. 2010). The alcohol produced by the yeasts in turn serves as a substrate for the acetic acid production by acetic acid bacteria (Aptutharajah et al. 1986). The dominant yeast species associated with palm wine fermentation is *Saccharomyces cerevisiae* (Amoa-Awua et al. 2007). However, other yeasts such as: *Kloechera apiculata*, *Candida species*, *Pichia species*, and other *Saccharomyces species* have also been isolated (Santiago-Urbinna and Ruiz-Teran, 2014). Bacteria usually associated with palm wine fermentation include among others, species of *Lactobacillus*, *Leuconostoc*, *Bacillus*,

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Serratia, *Streptococcus*, *Micrococcus*, *Brevibacterium*, *Klebsiella*; and *Zymomonas mobilis* (Opara et al. 2012).

In this study, fermenting raffia palm sap was screened for culturable yeasts and bacteria during 96 hours, at 24 hours intervals. The main objective of this study is to identify and enumerate yeasts and bacteria present at different stages of raffia palm sap fermentation.

MATERIALS AND METHODS

Sample collection and batch fermentation

Twelve samples of freshly tapped raffia palm sap were collected from different palm wine tappers in Obinze community in Owerri North local government area of Imo State. These samples (100 ml each) were collected in sterile containers and transported immediately on ice to the laboratory for further analysis within two hours of tapping. Transportation on ice wasto forestall fermentation of the samples before the commencement of analysis (Obi et al, 2015). In the laboratory the samples were pooled together and homogenized, and then re-distributed into100 ml perforated screw capped sterile plastic containers, which were labeled 0, 24, 48, 72, and 96 hours corresponding to different fermentation periods. The perforated screw caps were plugged with sterile non-absorbent cotton wool. Samples were allowed to stand (ferment) at prevailing ambient room temperatures.

Culturing bacteria and yeast

One milliliter (1 ml) of homogenized palm sap/wine samples were collected aseptically at 0, 24, 48, 72, and 96 hours of fermentation, and serially diluted in sterile peptone water using tenfold dilution. Then 0.1 ml aliquot of each dilution was inoculated in triplicates using spread-plate technique on nutrient agar (for total heterotrophic bacterial count), Lactobacillus MRS agar (for total *Lactobacillus spp*. count), and potato dextrose agar (for total heterotrophic fungi count), and incubated at ambient room temperatures for 24 hours, 18-48 hours, and 24-48 hours, respectively. Lactobacillus MRS plates were incubated in a 5: 95% carbon dioxide: hydrogen atmosphere as described by Collins et al (2004).

Enumeration, isolation, and identification of isolates

Culture plates with best colony numbers were counted and recorded. Distinct colonies were sub-cultured to get axenic cultures. Pure isolates were stocked on solid sterile trypticase agar slants. Stock cultures were stored at 4°C until required. Bacteria isolates were characterized and identified using the schemes outlined by Bergey's Manual of Determinative Bacteriology (Holt et al, 1994). Pure isolates were determined for their microscopic colonial appearance. Gram staining and spore staining techniques as described by Wistreich (2003) were used to determine their cellular morphologies, Gram reactions, and the presence of spores. Other biochemical tests were also carried out.

Yeast isolates were characterized and identified using the methods described by Collins et al (2004). Pure isolates were examined for microscopic colonial appearance. Microscopy was used to determine cell shape, presence or absence of mycelia, pseudomycelia, arthrospores, chlamydospores, and capsules. Fermentation and assimilation tests were also done.

Determination of pH

The pH of palm sap/wine samples were determined by the method of Aneja (2018), using a calibrated digital pH meter (Hanna, model H196107).

Determination of alcohol content using alcoholometer

The percentage alcohol by volume (% v/v) was determined as described by Aneja (2018), by taking readings of a calibrated digital alcoholometer that was dipped in a measured column of palm wine samples.

Statistical analysis

Triplicate data obtained from experiments were statistically processed and analyzed using Microsoft Office Excel 2007 and Minitab v.17 software applications.

RESULTS AND DISCUSSION

Fermenting palm sap was screened for yeasts and bacteria, pH, and alcohol components at 0,

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24, 48, 72, and 96 hours of fermentation. Two bacteria were isolated – *Gluconobacter sp.* and *Lactobacillus sp.*; while two yeasts were also isolated – *Candida albicans* and *Saccharomyces cerevisae*. *Gluconobacter sp.* and *Lactobacillus sp.* persisted throughout the period of fermentation studied, while *Candida albicans* was only isolated at 0 and 24 hours of fermentation (Table 1).

The population of *Gluconobacter sp.* progressively diminished from 25.4 x 10^6 cfu/ml (at 0 hours) to 0.17 x 10^6 cfu/ml (at 96 hours). *Lactobacillus sp.* progressively increased from 0.14 x 10^6 cfu/ml (at 0 hours) to 19.05 x 10^6 cfu/ml (at 72 hours), and then dropped to 10.9 x 10^6 cfu/ml (at 96 hours). *Saccharomyces cervisae* progressively increased from 0 cfu/ml (at 0 hours) to 12.0 x 10^6 cfu/ml at 72 hours, and then dropped to 10.95 x 10^6 cfu/ml at 96 hours (Table 2).

Table 1. **Microbial isolates detected at different stages of palm sap fermentation.**

	Fermentation time in hours				
Organism	0	24	48	72	96
Gluconobacter sp.	+	+	+	+	+
Lactobacillus sp.	+	+	+	+	+
Candida albicans	+	+	-	-	-
Saccharomyces serevisae	-	+	+	+	+

+ = present, - = absent.

Table 2. **Microbial populations at different stages of palm sap fermentation**

Time of fermentation (hours)	Population (x 10^6 in cfu/ml)			
	Gluconobacter sp.	*Lactobacillus sp.*	*Candida albicans*	*Saccharomyces cerevisae*
0	25.4	0.14	0.11	0
24	8.85	4.25	0.39	8.05
48	6.45	10.65	0	11.1
72	0.54	19.05	0	12.0
96	0.17	10.9	0	10.95

Figure 1 shows that the pH of the fermenting palm sap progressively dropped from 5.4 (at 0 hours) to 4.6 (at 96 hours).

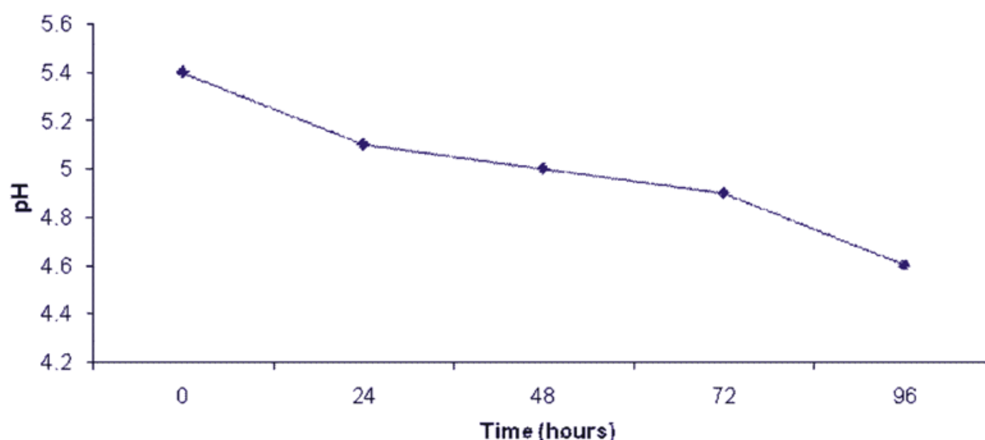

Figure 1. pH of fermenting palm sap over time.

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The specific growth rates of *Gluconobacter sp.*, *Lactobacillus sp.*, *C. albicans*., and *S. cerevisae* are shown in Figure 2. The highest specific growth rates for *C. albicans* and *S. cerevisae* occurred between 0 - 24 hours of fermentation, while their lowest specific growth rates occurred between 72 - 96 hours of fermentation, respectively. For *Gluconobacter sp*. and *Lactobacillus sp*., their highest specific growth rates occurred between 72 - 96 hours and between 48 - 72 hours respectively, while the lowest specific growth rates were between 0 - 24 hours and between 72 - 96 hours, respectively.

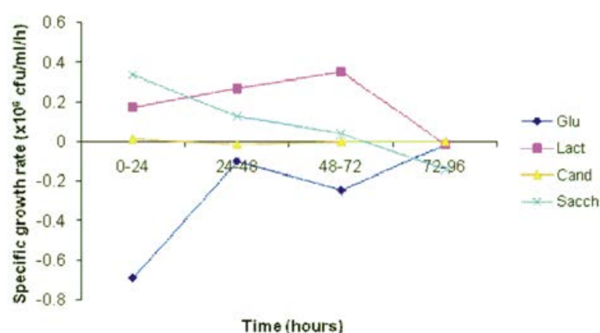

Figure 2. Specific growth rates of microbes associated with palm sap at different stages of fermentation.

At 0 hours, 1.8% v/v alcohol was detected. Alcohol concentration sharply increased to 6.23% v/v at 24 hours, gradually decreased to 6.19% v/v at 72 hours, and then sharply declined to 2.7% v/v at 96 hours. Figure 3 shows that the rate of alcohol production during fermentation was highest between 24 - 48 hours and lowest between 0 - 24 hours.

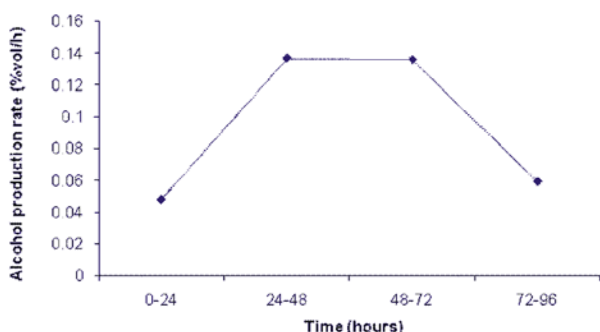

Figure 3. **Alcohol production rates during palm sap fermentation.**

The isolation of the yeasts *S. cerevisae* and *C. albicans* (with *S. cerevisae* dominating), is similar to the reports of Okafor (1978), Amoa-Awua et al. (2007), Ukwuru and Awah (2013), and many other workers. The issue of *C. albicans* not being detected from 48 hours of fermentation is also is similar to reports of Obi et al. (2015), where *Candida spp*. was not isolated beyond 48 hours of fermentation. *C. albicans* may have been inhibited by progressively lower pH or secondary metabolites produced by other microbes, or both. *Saccharomycescerevisae* has been fingered by some authors as the dominant yeast responsible for fermentation of palm sap (Okraku-Offei, 1968; Owusu, 1982; Ezeronye and Okerentugba, 2000). However, the presence of alcohol (1.8%) at 0 hours fermentation when *S. cerevisae* was not even detected in this study may suggest that another or other organisms (apart from *S. cerevisae*) may be implicated in the generation of alcohol. Some reporters, such as Uzochukwu et al (1999) and Obire (2005) have suggested that other species of *Saccharomyces* (such as *S.uvarum* and *S. chevalieri*) and even the bacterium *Zymomonasmobilis* are also associated with alcohol production during palm wine fermentation. On the other hand, some authors have listed *Candiaspp*., *Pichiaspp*., and *Zygosaccharomycesspp*., but have not been able to link these yeasts to alcohol production in palm wine fermentation (Santiago-Urbina and Ruiz-Teran, 2014). In their work, however, Mishra et al (2012) reported that some strains of *C. albicans* have been implicated in relatively low levels of alcohol production from fruit juice. The inability to detect the growth of *S. cerevisae* at 0 hours of fermentation may be as a result of inhibition due to a relatively high pH. This perceived inhibition on *S. cerevisae* may have also contributed to the low alcohol content at 0 hours. The fact that *Gluconobacter sp*. decreased in population as *S. cerevisae* increased may be due to the fact that *Gluconobacter* strains prefer sugar enriched environments in contrast to *S. cerevisae* which has a high preference and tolerance for an alcohol enriched environment (Ukwuru and Awah, 2013). On the other hand, the

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elimination of *Candidaalbicans* during fermentation may be due to its relatively low alcohol tolerance, when compared to *S. cerevisae* (Fleet, 2008).

Lactobacillus spp. in palm wine fermentation is responsible for pH decrease via production of organic acids. These organic acids give palm wine its characteristic sour taste (Ouoba et al. 2012). Lactic acid bacteria also control the growth of undesirable microbes such as enterobacteria, by the production organic acids and hydrogen peroxide (Alcantara-Hernandez et al. 2010; Naknean et al. 2010). *Gluconobacter species* is implicated in acetic acid production which constitutes part of the aroma volatiles. However, high acetic acid contents render palm wine unacceptable to consumers. Though *Gluconobacter species* may contribute to acidification and inhibition of undesirable micro organisms, it is often classified as a spoilage organism of palm wine (Ouoba et al. 2012).

Pearson's correlation analysis showed no significant statistical correlation ($p>0.05$) in population growth patterns among microbial isolates in this study, namely, *Gluconobacter sp*., *Lactobacillus sp*., *Candida albicans*, and *Saccharomyces cerevisae*. Similarly, no significant statistical correlation was seen between alcohol concentrations and any microbial population. This may seem absurd, because as *Gluconobacter sp*. decreased, there was a corresponding increase in alcohol content; similarly alcohol content increased as both *Lactobacillus sp*. and *S. cerevisae* increased in their populations (from 0 hours to 72 hours of fermentation). Though Pearson's correlation showed strong correlations ($r \geq 0.80$) for these variables, their correlations were not statistically significant ($p>0.05$). This simply implies that there is lack of sufficient evidence that the variables in question are closely associated. Though they may appear to have common trends, they may not necessarily be associated statistically (Sanders and Smidt, 2000). Further computations revealed that the coefficient of determination for these associations (r^2) were all less than 0.70. The coefficient of correlation values (r) though strong ($r>0.80$), but not statistically significant, may also have been caused by the small sample size ($n = 5$) of fermentation intervals investigated (Sullivan and Feinn, 2012). Similarly, no significant statistical correlation was observed among the specific growth rates of *Gluconobacter sp*., *Lactobacillus sp*., *Candida albicans*, and *Saccharomyces cerevisae*; nevertheless, a very strong inverse correlation ($r = -0.903$) was recorded between the specific growth rates of *Gluconobacter sp*. and *Saccharomyces cerevisae*. There were fairly strong correlations between alcohol production rate and specific growth rates of *Gluconobacter sp*. and *Saccharomyces cerevisae*, respectively. Another reason for a strong but non-statistically significant correlation may be due to the fact that the measured output (alcohol concentrations) may not have been caused by just a single input factor (microbial growth) but rather a combination of input factors (some of which may not have been determined in this study). Such other factors may include sucrose, fructose, glucose, and raffinose contents or consumptions. In other words alcohol contents may have been determined by interaction effects of different input factors, rather than just the main effect of a single factor.

Analysis of variance, however, revealed statistical significant differences ($p<0.05$) in specific growth rates between: *Gluconobacter sp*. and *Saccharomyces cerevisae*; *Gluconobacter sp*. and *Lactobacillus sp*.; and *Lactobacillus sp*. and *Candida cerevisae*.

CONCLUSION

This study showed that *Gluconobacter sp*., *Lactobacillus sp*., *Candida albicans*, and *Saccharomyces cerevisae* were present during the fermentation of the raffia palm sap investigated. During fermentation of palm sap, pH was observed to drop progressively, while alcohol content progressively increased only between 0 – 24 hours. However, most of the alcohol produced in raffia palm sap fermentation occurred between the first 48 hours of fermentation. ANOVA revealed that there were statistical significant differences in specific growth rates between: *Gluconobacter*

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sp. and *Saccharomyces cerevisae*; *Gluconobacter sp*. and *Lactobacillus sp*.; and also *Lactobacillus sp*. and *Candida cerevisae* during fermentation; suggesting that these microbial associates may have had different degrees of metabolic affinity for the same substrate.

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