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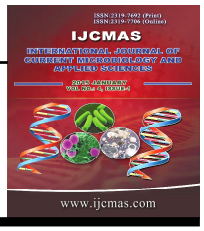

Original Research Article

Assessment of microbial growth and survival in fresh rafia palmwine from Umuariaga community, Ikwuano L. G. A. Abia State, Nigeria

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A B S T R A C T

The assessment of the presence, growth and survival of bacteria and yeasts in fresh raffia palm wine sample gave rise to the isolation of eight bacteria: *Staphylococcus aureus, Escherichia coli, Lactobacillus* spp, *Micrococcus luteus, Serratia marcessens, Acetobacter* spp, *Bacillus* and *Streptococcus* spp and four yeasts: *Candida* spp, *Saccharomyces uvarum, Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* The total bacterial count was in the range 6.08 x 10^3-3.48 x 10^3CFU/ml and the bacterial counts between 24-120 hrs of fermentation were not significantly different from the bacterial count in 0hr (fresh; $P<0.05$). Total coliform count was in the range 4.60 x 10^3 – 0 CFU/ml. There was an initial rise in total yeasts counts after 24hrs (7.38 x 10^3- 8.22 x 10^3CFU/ml) and then a gradual decrease to 4.10 x 10^3 CFU/ml. The yeast count after 120 hrs of fermentation was significantly different ($P<0.05$) from counts between 0-72hrs. The growth and survival pattern of the bacteria isolates from 0-120hrs showed that *S. aureus* was eliminated from the samples after 24hrs of growth while *E. coli, M. luteus, Lactobacillus* spp and *Streptococcus* spp were eliminated after 48hrs of growth. *Serratia marcesens* did not survive beyond 72hrs of fermentation while *Bacillus* and *Acetobacter* spp were present till the end of the fermentation. *S. cerevisiae*, S. *uvarum* and *S. pombe* survived from 0-120hrs of fermentation while *Candida* spp was eliminated after 48hrs of growth. All the physicochemical parameters tested varied with respect to time. The pH values decreased from 6.8 -3.8. Fermentation temperature dropped from 25°C to 21.5°C after 24hrs and then fluctuated between 21.4°C-21.5°C till the end of the fermentation. The alcohol values of the palm wine samples increased steadily (1.6% v/v-15.10% v/v) from 0-120hrs of fermentation. There was a gradual increase in the moisture level (96.49% - 97.55%) as fermentation progressed. Bacterial and fungal pathogens which were present from the beginning of the fermentation as handling and processing contaminants were eliminated after some hours as fermentation progressed.

Keywords

Bacteria, Growth, Palm wine, Pathogens, Survival, Yeasts

Introduction

Palm-wine is the collective name for alcoholic beverages prepared from fermented sap derived from various palm (Okafor, 1972). It is usually obtained from *Raphia rinfera, R. hookeri* and *Elaeis guineensis* by methods described by Bassir

(1962). Raphia palms usually yield more sap than oil palms although *Raphia* palms can only be tapped once in its life time because its terminal florescent is destroyed during tapping (Okafor, 1978). During fermentation, the sugars in the palm-sap are metabolized to alcohol and organic acids with the result that sap loses the sweetness (Okafor, 1975a). The type of bacteria present depends on the stage of fermentation and the composition of the sap (Bassir 1962; Okafor, 1977). Although alcohol production is common among yeasts, it is rare among bacteria (Ingraham and Ingraham 2004). Yeasts are used to make most alcoholic beverages. Palm-wine is consumed in parts of Africa, Asia and South America. In Nigeria, the two principal sources of sap for palm wine fermentation are the oil palm (*Elaeis guineensis*) and the *Raphia* palms (*Raphia* spp).

The quality of the wine is highly stable and depends among other factors on the genus of palm from which the sap is obtained. When Palmwine is examined under the microscope, a large number of yeast and bacteria are observed. One factor that influenced the palm wine is the nature of the yeast and other microorganisms it contains. The unfermented raphia Palm sap is clean, sweet, colourless syrup containing about 10-12% sugar which is mainly sucrose (Bassir 1962). Upon fermentation by the natural microbial flora, the sugar and other products (Obire, 2005), whereas the sap becomes milky-white due to the increase of microbial suspension resulting from the prolific growth of the fermenting organisms (Okafor, 1975ab). Palm wine is characterized by an effervescence of gas resulting from the fermentation of the sucrose content (Bassir, 1962), by the fermenting organisms. Previous studies on the microbiology of *E. guineensis* and *R. hookeri* have incriminated several bacterial and yeast flora to be involved in the fermentation process. The spp organisms have also been reported to originate from several sources which include tapping equipment, containers and the environment etc (Faparunsi and Bassir, 1972a).

The fermentation of *Raphia palm* wine is considered an inexpensive and effective means of food production in Nigeria, fresh palm sap is usually contaminate the juice as is tapped and there are changes in biochemical composition of the palm wine (Faparunsi and Bassir, 1972a). Palm wine loses its sweetness as the fermentation continues and the original colourless juice becomes milky. If not consumed or bottled within 24 hours of production, it gets sour due to prolonged fermentation and sublime malo-lactic acid fermentation by the bacterial microflora (Ezeronye, 2003). To produce palm wine, a succession of microorganisms occur mainly gram negative bacteria. The wine contains about 3% alcohol and since the bacteria and yeasts are consumed live, it is a source of single cell protein and various vitamins. The shelf life of palm wine is short, so, it is best consumed with about 48hours, various methods have been devised to preserve palm wine such as pasteurization, use of chemicals like Sorbate and Sulphite and other preservatives like *Alstonia boonei* (Egbu) and *Saccoglottis gabonensis* (Nche).

The main objectives for this study include, isolating and characterizing the microbial flora (and possible pathogens) in palm wine. To ascertain the survival pattern of microorganisms in palm-wine during the course of fermentation and to determine the physicochemical changes associated with palmwine fermentation and storage.

Sample collection

Fresh palm wine samples (200ml) from Raphia palm (*R. hookeri*) was collected

from traditional palm wine trapper from Umuariaga community, Ikwuano, Abia State, Nigeria. The freshly tapped samples were collected using 6 pre-sterilized labeled 100 ml capacity sample bottles with perforated screw caps corresponding to 0, 24, 48, 72, 96 and 120 hours of fermentation. The perforated screw caps were plugged with sterile non-absorbent cotton wool. The samples were transported to the laboratory in a cooler supported with packs of freezing mixture of salt and ice-block for analysis within 1 h of collection. This was to reduce considerably the rate of fermentation of the sample before the analyses began (Bassir, 1962; Obire, 2005).

Isolation of bacteria and yeast

One ml (1ml) of the palm wine sample was collected aseptically at 0, 24, 48, 72, 96 and 120 hours of fermentation and serially diluted in sterile peptone water and 0.1ml aliquots of suitable dilution was inoculated in duplicates by spread plate method (Cheesebrough, 1994) on MCA (for Total Coliform Count), Nutrient agar (NA) (for Total Bacterial Count) and Sabourand Dextrose Agar (SDA) for Total Yeast Count (Cruickshank *et al*., 1982). The inoculated plates were incubated aseptically at 30°C for 24hours for bacteria and 24-48 hours for the yeast at 22^{o}C. The recovered isolates were purified by sub-culturing and stored on agar slants at 4°C for characterization.

Identification of isolates

Isolates were Identified using standard morphological characteristics and identification keys (Barnett *et al.*, 1990; Kregger, 1987) for yeasts and Bergey and Holt (1993) for bacteria. The tests used in the identification of bacteria include morphology, gram reaction, spore production, Biochemical test, and sugar fermentation. The test used in the identification of yeasts includes, morphology, methylene blue staining, sugar fermentation.

Yeast viability staining

A smear of the yeast isolate was prepared, heat fixed and covered with methylene blue stain for 1 minute and then washed off with tap water. It was placed on a slide rack to drain and air dry and was later viewed with 40x objective lenses.

Sugar fermentation

The test described by Lodder (1971) was carried out using 1.0g each of glucose, lactose, fructose, galactose and sucrose. Both the inoculated and un-inoculated tubes (control) were then incubated for 48h at 37°C (for bacteria) and at 22^{o}C for 48-72 hours (for yeasts). A colour change to yellow showed acid production and was recorded as positive fermentation.

Spore staining

A smear of the organisms was made on a microscope slide and flooded with malachite green dye solution. It was steamed and allowed to stand for 3 minutes before the dye was washed off and the smear counter stained with 0.25% aqueous safranin and air dried. The slide was viewed under the microscope with ×40 objective lenses and then oil immersion objective lenses was used for clearer view. Retention of the test dye colour indicates the presence of spores, whereas vegetative cells will stain a red/ brown colour (Cheesebrough, 1994).

Physiochemical tests

pH determination

The pH of the fermenting palm wine samples was measured using a pH meter (Hanna, Model Hi 9810).

Temperature

The temperature of the fermenting samples was measured at room temperature using mercury-in-glass thermometer.

Moisture content

This was based on removal of water from the palm wine samples. A crucible was washed and dried in hot air oven at 100°C and later cooled in a desiccator. The crucible was weighed and 10 ml of the samples added to it and then transferred to hot air oven at 70-80°C for 2hrs and 100°C for the next 4 hours. After weighing, the moisture loss was measured at any given time interval.

Alcohol content

An alcohol meter was used in the determination of the alcohol content of the palm wine before and after fermentation by finding the differences between the two numbers (original gravity (OG) and terminal gravity (TG). Using the specific gravity, the density of the Palm wine was then determined. A hygrometer was used to measure the specific gravity of the solution after a temperating to the room temperature. The original gravity (OG) and the terminal gravity were then determined. The difference between the two infers how much alcohol is in the palm wine thus:

$$\% \text{ Alcohol} = \frac{(1.05 \times \{OG-TG) /TG)}{0.79} \times 100$$

Result and Discussion

The assessment of microbial presence, growth and survival in fresh palm wine samples gave rise to the isolation of eight bacterial genera: *Staphylococcus aureus*, *Escherichia coli*, *Lactobacillus* spp, *Micrococcus luteus*, *Serratia marcessens*, *Acetobacter* spp, *Bacillus* and *Streptococcus* spp and four yeasts genera: *Candida* spp, *Saccharomyces uvarum*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Tables 1 and 2). Agu *et al.*, (1993) stated that palm wine is rich in non-pathogenic microorganisms, so, the presence of *E. coli* (a pathogen) in fresh palm wine suggest faecal contamination through the untreated water that is traditionally used to dilute the palm wine and thereby increase the quantity for market sales. *Bacillus* spp and *S. aureus* have been reported to be mostly found as food contaminants and their presence in large amount could result to food poisoning (Whong *et al*., 2006). The fruit fly (*Drosophila melanogaster*) is an important factor in the contamination of the product *ab initio.* The insect habours yeasts on its body and transfers the same from ripped fruits to the palm wine which attracts its attention via the natural aroma of the fresh product. The microbial counts showed and initial bacterial count of 6.08 x 10^3 CFU/ml (0hr), but the count progressively decreased as fermentation moved on to 120hrs to a count of 3.48 x 10^3CFU/ml. However, the bacterial counts at 120 hrs, 96 hrs, 72hrs, 48hrs, and 24hrs of fermentation were not significantly different when compared to the bacterial count in 0hr (fresh; $P<0.05$). The same trend in microbial count decrease was noted for the coliform count beginning from 4.60 x 10^3 CFU/ml (0 hr). But there was zero count for the coliforms at the end of the 120hrs of fermentation. This showed that the *E. coli* was totally eliminated from the palm wine due to a mixture of parameters produced in the palm wine by the bacteria (acids; Fig. 1) and yeasts (alcohol, CO_2; Fig. 3). The coliform counts at various stages of the fermentation were significant ($P<0.05$) indicating that the various factors produced the palm wine which contributed to the killing of the coliforms had profound effect on the organisms. The complete elimination

of *E. coli* from the palm wine is very significant as it correlates with the fact that cases of ill healths associated with *E. coli* are very rare among the population that consumes palm wine. For the yeasts, there was an initial increase in the population count between 0-24hrs of fermentation (7.38 x 10^3- 8.22 x 10^3CFU/ml: Tab. 3) suggesting that the yeasts were best adapted to the nutritional status of the fresh palm wine. This supports the findings of Okafor (1990) which revealed that fresh palm wine contains several sugars (sucrose, fructose, raffinose) and various growth factors like vitamin C and $B_{12.}$ After the first 24hrs, there was a gradual decrease in the yeast count obviously due to the competition for nutrient, oxygen and space that must have ensued among the microorganisms as fermentation progressed. The yeast count after 120 hrs of fermentation was significantly different ($P<0.05$) from counts at the 0hrs, 24hrs, 48hrs and 72hrs. The high bacteria, yeast and coliform counts indicated inadequate hygienic conditions during collecting, dilution, processing and storing. Microbial contamination during and or post processing can also result in spoilage or persistence of some bacteria in palm wine. The presence of micro-organisms in palm wine can sometimes influence the stability of the product and its hygienic quality.

The genus *Saccharomyces* was the organism of importance in palm wine as revealed in this study, by its numerical predominance. Due to its superior fermentative ability, *Saccharomyces* may have adapted to growth in the special condition of the palm-sap (Faparusi and Bassir, 1991). Earlier, Faparusi (1973) and Okafor (1972) had reported *S. cerevisae* and *Schizosacchoromyces pombe* and *Saccharomyces uvarum* as the dominant yeasts in palm wine, whilst Fahwehinmi (1981) also reported the presence of *Saccharomyces chevalieri* and *Pichia membrabefaciens*. Van Pee and Swing (1971) reported *S. cerevisiae* and *Saccharomyces pastorianus* as the dominant yeasts in palm wine samples in Congo. It is possible that differences in the chemical composition of palm wine tapped from the felled and upright trees favour the complete domination of the yeast biota by *S. cerevisiae* in palm wine. According to Ayernor and Matthews (1971), the methods of palm wine tapping and collection of palm sap influence the microbial content of the sap. *Saccharomyces cerevisiae* has been confirmed in the present work as the dominant yeast species responsible for the fermentation of palm wine tapped from the felled palm trees (Table 2). In matured palm wine samples, only *S. cerevisiae* was isolated and this species appear to completely dominate the fermentation of palm wine in the felled palm trees. It was also reported that *Lactobacillus* spp were responsible for the sour taste of palm wine in this study. Earlier, Bassir (1968) had reported *L. plantarum* and *L. mesenteriodes*as being responsible for the souring of palm wine tapped from the live upright palm tree. The presence of *Lactobacilli* in palm wine samples in Nigeria have also been reported by Faparusi and Bassir (1971) and Okafor (1975).

Four (4) yeasts were isolated from this study, they include; *Candida* spp, *Saccharomyces cerevisae* and *Saccharomyces uvarum, Schizosaccharomyces pombe* all were positive to sucrose, maltose, glucose and fructose and negative to lactose respectively.

The growth and survival pattern of the bacteria isolates from 0-120hrs showed that *S. aureus* was eliminated from the samples after 24hrs of growth while *E. coli, M. luteus, Lactobacillus* spp and *Streptococcus*

spp were eliminated after 48hrs of growth. *Serratia marcesens* was not isolated again after 72hrs of fermentation while *Bacillus* and *Acetobacter* spp were present till the end of the fermentation.

S*accharomyces* cerevisiae, S. *uvarum* and *Schizosaccharomyces pombe* were present in the palm wine from 0-120hrs of fermentation and can be said to be of major importance in the fermentation of palm wine. *Candida* spp (a pathogen) was eliminated 48hrs of growth and activity possibly due to the increased production of alcohol by the S. *cerevisiae*, S. *uvarum* and *Schizosaccharomyces* pombe and organic acids by the bacteria. This could be seen from the fact that the pH and alcohol values of the palm wine increased after 48 hrs (Figures 1 and 3). Earlier, Faparunsi (1973) and Okafor (1972) reported *S. cerevisiae* and *Schizosaccharomyces* pombe as the dominant yeast in palm wine while Sanni and Lonner (1993) reported to have isolated *Saccharomyces cerevisiae*, *Saccharommyces uvarum* and *Candida* spp from palm wine which is in consonance with the findings of the research work.

All the physicochemical parameters tested varied with respect to time. The pH values decreased (increase in acidity, Fig. 1) with time from 6.8 -3.8 till 120hrs of fermentation and this is attributed to the activities of *Acetobacter* spp which is known to produce acetic acid (since Lactobacillus spp was eliminated after 48hrs of fermentation). The further production of acid by *Acetobacter* spp usually makes the palm wine very sour and unfit for human consumption as affirmed by Theivendirarajah and Chrystopher (1987).

During fermentation, the palm wine temperature dropped from 25^{o}C to 21.5^{o}C and then fluctuated between 21.4^{o}C-21.5^{o}C. The alcohol values of the palm wine samples increased steadily from 0-120hrs of fermentation due to the activities of Lactobacillus spp and Acetobacter spp. The lowest alcohol value (0hr) was 1.6%v/v while the highest level of alcohol recorded was 15.10% v/v and that at 120 hrs of fermentation. Normal palm-wine contains approximately 0.5% to 7. 1% ethanol depending on its stage of fermentation (Bassir, 1968; Okafor, 1978). Further increase in alcohol concentration beyond 10% resulted in inhibition and possible elimination of the bacterial and yeast isolates from the palm wine.

Moisture content expresses the amount of water present in the palm wine. Moisture content determines the shelf life of the sap. The lower the moisture content, the longer the expected shelf life, while the higher the moisture content, the shorter the expected shelf life. The gradual increase in the moisture level recorded in this work (96.49% - 97.55%: Fig. 4), must have been from the respiratory activities of aerobic bacteria found in the palm wine which led to the formation of water as oxygen is finally reduced to water in the electron transport system. Given the high moisture content of the palm wine analyzed here the palm wine will deteriorate rapidly.

From the result obtained, palm wine is a natural habitat for the growth of microorganism, it could be deduced from this study that the microorganisms associated with the fermentation of palm wine were mainly lactic acid bacteria, acetic acid bacteria, some enterobactericea and yeast. The increased rate of their isolation further proved them to be the most important in the fermentation of palm wine. Lactic acid bacteria were considered to be responsible for the rapid acidification of the product as the acetic acid bacteria were not

isolated in the palm wine sample on the first 3 days. *Saccharomyces* species has been confirmed as the dominant yeast species responsible for the fermentation of palm wine. The physicochemical parameters tested varied with respect to time.

Table.1 Identification of bacterial isolates from palm wine samples

S/N	Colonial morphology	Gram rxn	Cell shape	Catalase	Coagulase	Citrate	Indole	Spore	Lactose	Maltose	Sucrose	Fructose	Glucose	Oxidase	Isolate
1	Smooth, with entire margin +round and milky colonies on nutrient agar	+	Cocci in cluster	+	+	-	-	-	AG	AG	A	A	AG	-	*Staphylococcus aureus*
2	Round purple colonies with methallic green sheen on EMB	_	Short rod in singles	+	-	-	+	-	AG	AG	-	-	AG	-	*Escherichia coli*
3	Small and smooth with white colonies	+	Rod	-	-	-	-	-	A	A	A	A	A	-	*Lactobacillus* spp
4	Round and yellow colonies on Nutrient agar	+	Cocci in clusters	+	-	-	-	-	-	A	A	A	-	-	*Micrococcus luteus*
5	Round and mucoid red colonies on nutrient agar	_	Short rod in clusters	+	-	+	+	-	-	A	A	A	A	-	*Serratia marcescens*
6	Mucoid brown colonies	_	Short rod	+	-	-	-	-	-	-	-	A	A	-	*Acetobacter* spp
7	Raised, irregular white colonies on nutrient agar	+	Short rod in chains	+	-	+	-	+	-	-	A	-	A	-	*Bacillus* spp
8	Small colourless colonies	+	Cocci in chains	-	-	-	-	-	A	AG	-	A	AG	-	*Streptococcus* spp

Keys: A= acid; AG= acid & gas; + = positive; - = negative

Table.2 Identification of yeast isolates from palmwine samples

S/N	Morphology	Microscopic features	Glucose	Lactose	Sucrose	Maltose	Fructose	Isolate
1	Round and creamy colonies on SDA	Single oval cells were seen, some in pairs and elongate, Also budding was pronounced, spores seen	+	-	+	+	+	*Saccharomyces cerevisiae*
2	Whitish colonies, not well developed pseudomycellium	Single round cells seen, spores were absent	+	-	+	+	+	*Candida* spp
3	Creamy colonies with whitish edges. Pseudomycelium was absent.	Single oval cells were seen, some cells were elongated while others were in pairs	+	-	+	+	+	*Schizsaccharomyces pombe*
4	White colonies were seen Pseudomycellium	Cells were ovals with a pear shape	+	+	+	+	+	*Saccharomyces uvarum*

Keys: + = Positive; - = Negative

Table.3 Total microbial counts of palm wine samples (log10cfu ml-1)

Age of palmwine (hrs)	Bacterial count	Coliform count	Yeast count
0 (fresh)	6.08 ±33.8^{a}	4.60 ±24.2^{d}	7.38 ±14.9^{ab}
24	5.30 ±24.8^{a}	3.52 ±7.73^{ab}	8.22 ±12.3^{d}
48	5.12 ± 17.9^{a}	2.60 ± 7.78^{b}	7.76 ±20.7^{cd}
72	4.94 ±23.4^{a}	1.92 ±19.6^{bc}	6.02 ±14.7^{bc}
96	3.92 ±17.5^{a}	3.0 ±6.71^{cd}	5.20 ±8.57^{ab}
120	3.48 ± 14.9^{a}	0.00 ±0.00^{d}	4.10 ±4.0^{a}

a, b, c, d superscript have level of significance. Similar subscript means no significance difference while different subscript means significant differences

Table.4 Growth and survival pattern of bacterial isolates in palm wine (hrs)

Organism		24	48	72	96	120
Staphylococcus aureus	+	+	-	-	-	-
Bacillus spp	+	+	+	+	+	+
Escherichia coli	+	+	+	-	-	-
Serratia marcessans	+	+	+	+	-	-
Micrococcus luteus	+	+	+	-	-	-
Acetobacter spp	+	+	+	+	+	+
Streptococcus spp	+	+	+	+	-	-
Lactobacillus spp	+	+	+	-	-	-

Key: + = Presence; - = Absence

Table.5 Growth and survival pattern of yeast isolates in palm wine (hrs)

Organism	**0**	**24**	**48**	**72**	**96**	**120**
Saccharomyces cerevisiae	+	+	+	+	+	+
Candida spp	+	+	+	-	-	-
Schizosaccharomyces pombe	+	+	+	+	+	+
Saccharomyces uvarum	+	+	+	+	+	+

Key: + = Presence; - = Absence

Figure.1 pH of fermenting palm wine samples

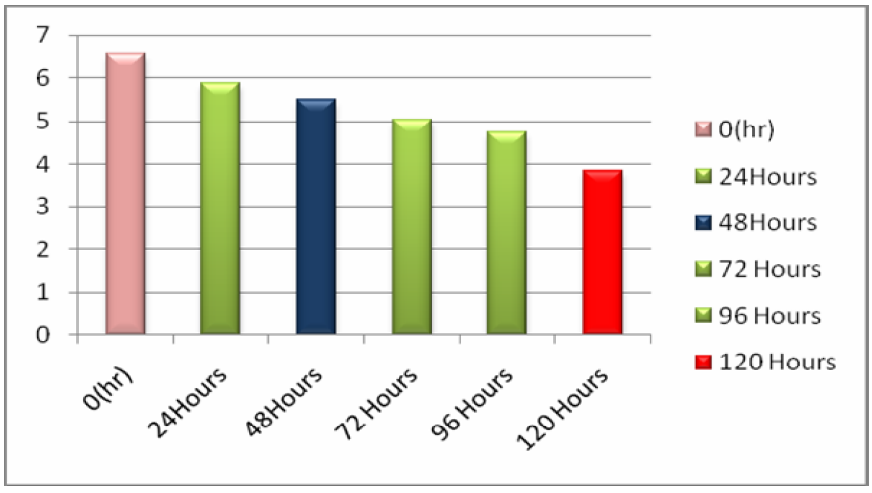


Figure.2 Temperature of palm wine samples (^{o}C)

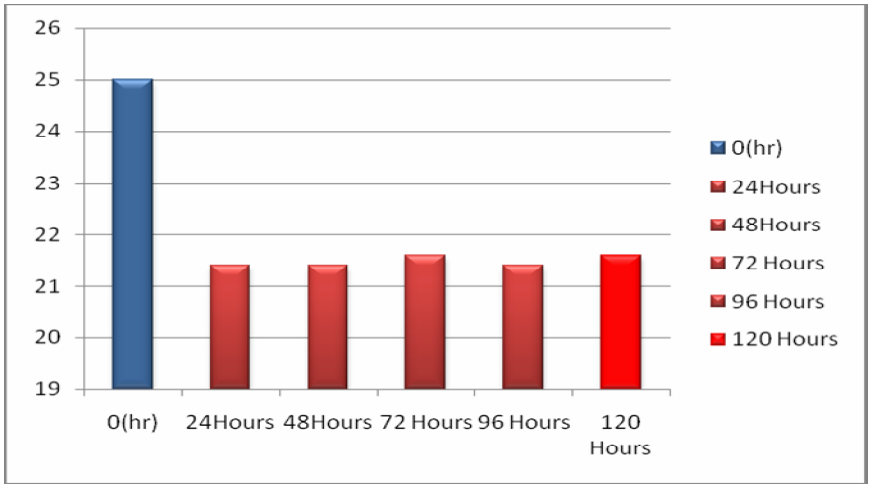


Figure.3 Alcohol content of palm wine samples (%)

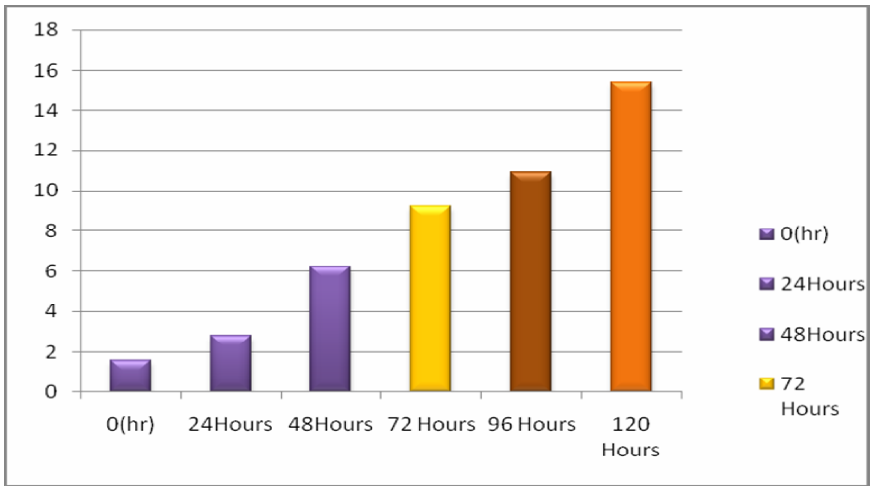
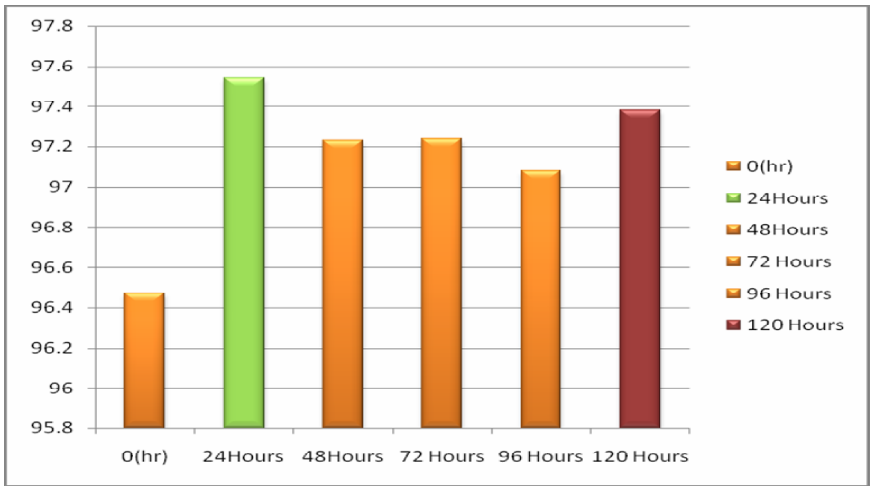


Figure.4 Moisture content of palm wine samples (%)



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