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WINE PRODUCTION FROM SOURSOP USING GENETICALLY-IDENTIFIED *Saccharomyces Cerevisiae* ISOLATED FROM PALM WINE

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Keywords: *Wine production, Soursop, Genetically-identified Saccharomyces Cerevisiae, Palm wine*

Abstract: *The need to indigenize the raw material base for wine production in Nigeria especially the source of juice and yeast starter cultures cannot be overemphasized. Studies were carried out on wine production from soursop fruit using genetically-identified Saccharomyces cerevisiae isolated from palm wine. Soursop juice (1,000ml) was used to produce wine through the following procedures; extraction, fermentation, racking, stabilization, pasteurization, ageing and maturation. The juice was fortified with 100g of granulated sugar to raise the sugar level. Sodium metabisulfite (2.0g) was added to treat the must against microbial attack. Fermentation commenced after pitching 1,000ml of sterile juice with 150ml of yeast biomass. Primary fermentation and secondary fermentation lasted for 7 and 14 days respectively. Physicochemical, microbiological and organoleptic analyses were carried out on the wine sample produced. The pH, specific gravity and sugar level of the fresh "must" prior to fermentation was 4.2, 1.069 and 17°Brix, respectively. The result of the physicochemical analysis of the wine sample showed that the specific gravity, %alcohol (v/v), total acidity as acetic acid, pH and sugar level were 0.9800, 9.5%V/V, 0.8945%, 3.6 and 1.8°Brix, respectively. The result of bacteriological analysis showed colony count of the isolates as $2.0x10^{-3}$cfu/ml. Organoleptic evaluation result of the wine sample after one month storage showed that the appearance of the wine sample ranked more than its taste and aroma and it was generally accepted at 95% confidence level. These results show that wine of acceptable organoleptic quality can be produced from soursop fruits.*

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INTRODUCTION

Wine is an alcoholic beverage produced from juices of variety of fruits by fermentative action of microorganisms either spontaneously or seeding with a particular strain mainly of yeast species to adopt a particular quality of wine (Mbaeyi-Nwaoha and Ajumobi, 2015). Wine is one of the most recognizable high value-added products from fruits. Most commercially produced wines are usually made from fermented grapes; this fermentation process is not done by introducing any chemicals or sugar but by adding different species of yeast to the crushed grapes (Akpomie *et al.,* 2015).

According to Benjamin (2013), the process of fermentation is basically feeding sugars and nutrients in solution to yeast, which return the favour by producing carbon dioxide gas and alcohol. This process goes on until either all the sugar is gone or the yeast can no longer tolerate the alcoholic percentage of the beverage (Akubor *et al.,* 2013). Different yeasts produce different results, and have different tolerance levels. Fermentation is a process of deriving energy from the oxidation of organic compounds, such as carbohydrates, and using an endogenous electron acceptor, which is usually an organic compound (Fleet, 2013) as opposed to respiration where electrons are donated to an exogenous electron acceptor, such as oxygen, via an electron transport chain (Amoa-Awua *et al.,* 2016). The risk of stuck fermentation and the development of several wine faults can also occur during this stage which can last from 5 to 14 days for primary fermentation and potentially another 5 to 10 days for a secondary fermentation. Fermentation may be done in stainless steel tanks, which is common with many white wines like Riesling, in an open wooden vat, inside a wine barrel and inside the wine bottle itself as in the production of many sparkling wines (Legras *et al.,* 2017).

Fermentation is a cheap and energy efficient means of preserving perishable raw materials such as pineapple juice (Clement-Jimenez *et al.,* 2015). Harvested fruits may undergo rapid deterioration if proper processing and storage facilities are not provided, especially in the humid tropics where the prevailing environmental conditions accelerate the process of decomposition (Duarte *et al.,* 2010). Although there are several options for preserving fresh fruits, which may include drying, freezing, canning and pickling, many of these are inappropriate for the produce and for use on small-scale in developing countries. For instance, the canning of fruits at the small-scale has serious food safety implications and contamination especially botulism (Elijah *et al.,* 2010). Freezing of fruits and vegetables is not economically viable at the small-scale.

Yeast has the capability of converting grapes into an alcoholic compound and removing the sugar content in it for the production of different types of wines. Sometimes wines are produced from different types of fruits like; paw-paw, mango, pineapple, banana, lemon, soursop, watermelon etc., here the wine so produced bears the name of the

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fruit or fruit mixture used in its production (Ezeogu and Emeruwa, 2013).

Soursops (*Annona muricata*) are important tropical fruits that contribute to the economic growth of some tropical countries, including Vietnam. Soursop is prized as very pleasant, sub-acid, aromatic and juicy fruit (Imade *et al.,* 2013). The soursop pulp is widely used for manufacturing various juice blends, nectars, syrups, shakes, jams, jellies, preserves and ice creams (Nguyen, 2015).

Palm wine is a refreshing alcoholic beverage widely consumed in southern Nigeria, Asia and southern America (Elijah *et al.,* 2010). Palm wine is presented in a variety of flavours, ranging from sweet (unfermented) to sour (fermented) and vinegary (Chilaka *et al.,* 2010). It is produced by a succession of microorganisms, Gram-negative bacteria, lactic acid bacteria and yeasts as well as acetic acid bacteria (Amoa-Awua *et al.,* 2016). Yeasts isolated from palm wine have been identified as coming from various genera such as *Saccharomyces*, *Pichia*, *Schizosaccharomyces, Kloekera*, *Endomycopsis*, *Saccharomyeoides* and *Candida* which find their way into the wine from a variety of sources including air, tapping utensils, previous brew and the trees. Hence, palm wine serves as a source of single cell protein and vitamins (Adedayo and Ajiboye, 2011). The major fermentation is undertaken by about twenty indigenous strains of *Saccharomyces cerevisiae* which are genetically different from the strains used to make wine from grapes and have the capability to survive and continue fermentation process up to ethanol concentration of 18%, making them ideal for producing ethanol (Ezeogu and Emeruwa, 2013).

Statement of the Problem

- The postharvest losses of our local fruits due to the apparent lack of preservation facilities are worrisome; it therefore becomes imperative to utilize our indigenous fruits for wine production to reduce the huge economic losses and wastage of fruits.
- Establishment of wine production industries will create employment opportunities to our teaming Nigerian graduates and also earn us foreign exchange if the wines are exported.
- Wines are shelf stable commodities and can remain microbiologically stable for years when compared with fruits which has very poor shelf life

Aim and Objectives of the Study

The aim of the study is to produce wine from soursop fruits using *Saccharomyces cerevisiae* isolated from palm wine.

Specific Objectives

1. To isolate and molecularly identify the palm wine yeast for wine production
2. To evaluate the physicochemical properties of wine produced from soursop.
3. To determine the quality of the wine produced from soursop using One-way Analysis of Variance (ANOVA) at $P < 0.05$.

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4. To make recommendations based on the results of the study.

MATERIALS AND METHODS

Materials

The raw materials that were used for this research include: soursop fruits, distilled water, yeast (*Saccharomyces* species i.e., wine yeast), potato dextrose agar and D-glucose.

Equipment

The equipment that were used for this work include: glass fermenter, refrigerator, pH meter, hydrometer, alcoholmeter, Brix refractometer, knife, measuring cylinder, Autoclave, thermometer, plastic container, electric blender, electric weighing balance, petridishes and pipette.

Source of Materials

Three kilograms (3kg) of ripe soursop fruits were procured from Shoprite Mall, Enugu state and was used to produce soursop wine. The palm wine yeast used for the study was sourced from palm wine tapper at Nchantacha Nike Emene, Enugu East local government, Enugu state. The microbiological media were procured from Conraws Nigeria limited

Processing of Fruit Must

The fruits were peeled after washing with 1% brine solution and sliced into cubes with a sterile knife. Juice was extracted from the fruits using juice extractor (Rowenta KA-60, Germany). The juice (1.0 litres) was sterilized at 80^{o}C for 10 minutes using a thermostatically controlled water bath and allowed to cool to 30^{o}C.

Isolation of *Saccharomyces* spp from Palm Wine

Potato Dextrose Agar (PDA) was used to isolate *Saccharomyces* spp. A total of 500mg of amoxicillin was added to inhibit growth of bacteria in 200ml of potato infusion mixed with 4g D-glucose and 3.0g of agar-agar powder, the mixture was sterilized at 121^{o}C at a pressure of 15 PSI for 15 minutes, after sterilization and cooling to 45^{o}C, the molten medium was dispensed into sterile petridishes.

Inoculation

A loopful of palm wine yeast dreg was inoculated on the PDA medium using streak plate technique; the PDA plates were incubated at 30^{o}C for 72 hours prior to molecular and cultural analyses.

Preparation of yeast biomass

Ten (10) ml of genetically identified palm wine yeast slurry was measured into 500ml conical flask containing 200ml of distilled water together with 10g of D-glucose and 1.0 gram of Yeast extract (as source of nitrogen) and the solution was subjected to shake flask fermentation to produce yeast biomass at 30^{o}C for 4 hours.

Molecular Analysis of Palm wine Yeast isolate

The processes involved in molecular identification of the yeast isolates were extraction of the organism genomic DNA, Polymerase chain reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE), sequencing of the PCR products (amplicons) and BLAST (Basic local alignment search tools).

Extraction of organism genomic DNA

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The genomic DNA of each of the isolate was extracted following the boiling method as described by Maria *et al.* (2008).

Procedure

Four hundred microlitres (400µl) of sterile distilled water was measured into 1.5ml Eppendorf tube each and 3 loopfuls of each of the isolate was added into the tube, covered and vortexed to dissolve. The tubes containing the isolates were put into the wells of Accublock heater (Dri-Block Techne, model: FDBO3DD R, Mbby Scientific Ltd. USA) and boiled at 100^{o}C for 10 minutes. After boiling, the tubes were cooled in ice and centrifuged at 15,000 rpm for 5 minutes. The supernatant of each isolate was decanted into fresh eppendorf tubes and used as clean DNA extracts which were stored at -20^{o}C for PCR (polymerase chain reaction) amplification.

Polymerase chain reaction

PCR Mix Components

The PCR mix was made up of 12.5µL of Taq 2X Master Mix from New England Biolabs (M0270); 1µL each of 10µM forward (ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'and reverse (ITS4 5' TCCTCCGCTTATTGACATGS 3') primer; 2µL of DNA template and then made up with 8.5µL Nuclease free water.

Cycling Conditions for PCR

Initial denaturation at 94˚C for 5mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 54˚C for 30secs and elongation at 72˚C for 45sec. Followed by a final elongation step at 72˚C for 7 minutes and hold temperature at 10 ˚C forever.

Procedure for Gel Electrophoresis of DNA products

- One (1.0) gram of agarose (for DNA); 2g of agarose for PCR were measured
- The agarose powder was mixed with 100 mL 1xTAE in a microwavable flask.
- And microwaved for 1-3 min until the agarose is completely dissolved (but not over boiled)
- The agarose solution was cooled down to about 50 °C (about when you can comfortably keep for about 5 mins.
- 10µL EZ vision DNA stain was added, EZ vision binds to the DNA to allow the DNA visualized under ultraviolet (UV) light.
- The agarose was poured into a gel tray with the well comb in place
- The newly poured gel was allowed to solidify at 4 °C for 10-15 mins

Loading Samples and Running an Agarose Gel

- loading buffer was added to each DNA sample or PCR product
- After solidifying, the agarose gel was placed into the gel box (electrophoresis unit).
- The gel box was filled with 1xTAE (or TBE) until the gel is covered.
- Molecular weight ladder was carefully loaded into the first lane of the gel.
- The DNA samples were carefully loaded into the additional wells of the gel.
- The gel was run at 80-150 V for about 1-1.5 hours

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- Power was put off and the gel was removed from the gel box.
- The DNA fragments or PCR products were visualized under UV transilluminator equipment.

Morphological Characterization of the Yeast Isolates

Morphological identification was done based on the appearance on the plates: colour pigmentation, mycelia growth, texture, structure and shape.

Biochemical Characterization of the Yeast Isolates

Germ Tube Test

A Total of 0.5ml of human serum was added into test tube and yeast cell colony was also added into the test tube containing the serum using sterile Pasteur pipette then it was incubated at 37°C for 4 hours. After that a drop of the suspension was placed and smeared on the sterilized slide and examined under microscope using x40 eye piece objective for the presence of germ tube which was negative. This test was used to differentiate *Candida albicans* from other yeasts.

Fortification

Granulated sugar of 100g were added to the soursop fruit must to increase the sugar level to 17°Brix.

Must analysis

The following are some of the analyses that were carried out on the fruits juice. These parameters are original gravity, pH, sugar level and temperature.

Determination of Original Gravity (°ρ)

The original gravity was determined using a hydrometer and measuring cylinder. The must was gently poured into the measuring cylinder up to 100ml, and then the hydrometer was inserted into the measuring cylinder and allowed to float. The reading was taken based on principle of floatation.

Determination of Temperature

The temperature was determined using a thermometer. The thermometer was inserted into the conical flask containing the "must" and then the value was taken.

Determination of pH of the must

A pH meter was used for this analysis. The pH meter first was dipped in a buffer solution to standardize it. It was removed from the buffer solution and then inserted into the conical flask containing the "must". The pH value was noted and recorded.

Determination of Sugar level (°Brix)

The percentage of the must samples was determined using Brix refractometer. A drop of each must sample was placed on the glass prism of the equipment and the sugar level was read from the eye piece lens.

Inoculation/Fermentation

Exactly 150ml of the yeast biomass was pitched into 1000ml of the soursop must to commence primary fermentation which last for 7 days.

Racking

The wine sample was racked two times during fermentation, firstly at the end of primary fermentation to remove the lees (unstable particles including dead yeast cells) at the bottom of the fermenting vessel using a muslin

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cloth and secondly at the end of secondary fermentation. Clean wine was obtained after final racking and dispensed into sterile bottles.

Maturation

After primary fermentation, the virgin wine was filtered to remove the yeast proteins and polyphenols and then subjected to secondary fermentation to allow biochemical reactions which was necessary for aroma and taste development to take place. Maturation was allowed for 14 days (2 weeks). As secondary fermentation is taking place, ten grams (10g) of gelatin was added to the wine to clarify the wine by forming heavy coagulation with polyphenol, proteins, enzymes and remnant yeast which sediment and filtered-off after maturation.

Krausening

Sucrose sugar (50g) was added to the fermenting wine to increase the alcoholic content of the wine.

Preservation of the wine

Five (5.0) gram of sodium metabisulphite was added to 1,000ml of the wine and further pasteurized at 60^{o}C for 30 minutes to protect the wine from microbial spoilage

Organoleptic analysis

The wine sample was subjected to organoleptic evaluation using taste, aroma, appearance and overall acceptability as sensory attributes. The wine sample was evaluated using nine-point hedonic scale where 1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely.

Statistical analysis of data

The data collected from organoleptic analysis of the wine sample was subjected to a one-way analysis of variance (ANOVA) at 95% confidence level and the means that differed will be considered significant at $P < 0.05$.

Physicochemical analysis of wine samples

The wine sample was subjected to the following physicochemical analysis after shelf stability test for one month: specific gravity, % alcohol, % sugar, total acidity as acetic acid and pH.

Specific gravity

The specific gravity of the wine was determined using a hydrometer. One hundred (100ml) of wine was measured into a measuring cylinder and a hydrometer was dipped into the wine to read the specific gravity based on principle of floatation.

Percentage alcohol (v/v)

An alcoholimeter was dipped into 100ml of the wine in a measuring cylinder and the percentage alcohol (v/v) was read-off from the alcohol meter.

Percentage sugar

The residual sugar percentage of the wine sample was determined using Brix refractometer. A drop of the wine sample was placed on a glass prism of the equipment and the sugar level was read from the eye piece lens.

Determination of the total acidity of the wine samples

The total acidity of the wine sample was determined by titrating 0.1NaOH in a burette against 10ml of the sample in a conical flask with the addition of 5 drops of phenolphthalein indicator. The total acidity was calculated based

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on the volume of base used in neutralizing the acid in the wine sample.

%T.A (as acetic acid) = $\frac{V}{10}$ x 0.75

Where v = volume of base used for neutralization

0.75 = constant

pH determination

The pH which is the hydrogen ion concentration to base 10 was determined by dipping the electrode of a pH meter (Hannah instrument, Italy) into 50ml of wine sample. The pH reading was taken after one minute.

Microbiological Analysis

After one-month shelf storage, the wine sample was subjected to bacteriological analysis. A total of 5.4g of Nutrient Agar powder was dissolved in 200ml of distilled water and mixed to homogenize. The media was autoclaved at 15psi at 121°C for 15 minutes. The wine sample was inoculated on nutrient agar medium using pour plate technique and incubated at 35°C for 24–48 hours.

4.0 RESULTS AND DISCUSSION

4.1 Juice (Must) Analysis

Table 4.1 shows the result of the soursop "must" analysis before fortification. The original gravity of the soursop juice before it was fortified was 1.040° ρ ; sugar level of 9.99°Brix and pH of 5.20. Table 4.2 shows the result of soursop juice analysis after fortification with 50g of granulated sugar giving original gravity 1.069°ρ, sugar level 17°Brix and pH 4.80.

Table 4.1: Juice (Must) Analysis

Parameters	Original gravity (°ρ)	Sugar level (°Brix)	pH	Temperature (°C)
Soursop Juice	1.040	9.99	5.2	27

Table 4.2: Analysis of Juice "Must" after Fortification

Parameters	Soursop Juice
Original gravity (°ρ)	1.069
Sugar (°Brix)	17
pH	4.8

4.2 Bacteriology of wine sample

The result of bacteriological analysis showed that there was no growth of microorganisms after one month shelf stability test

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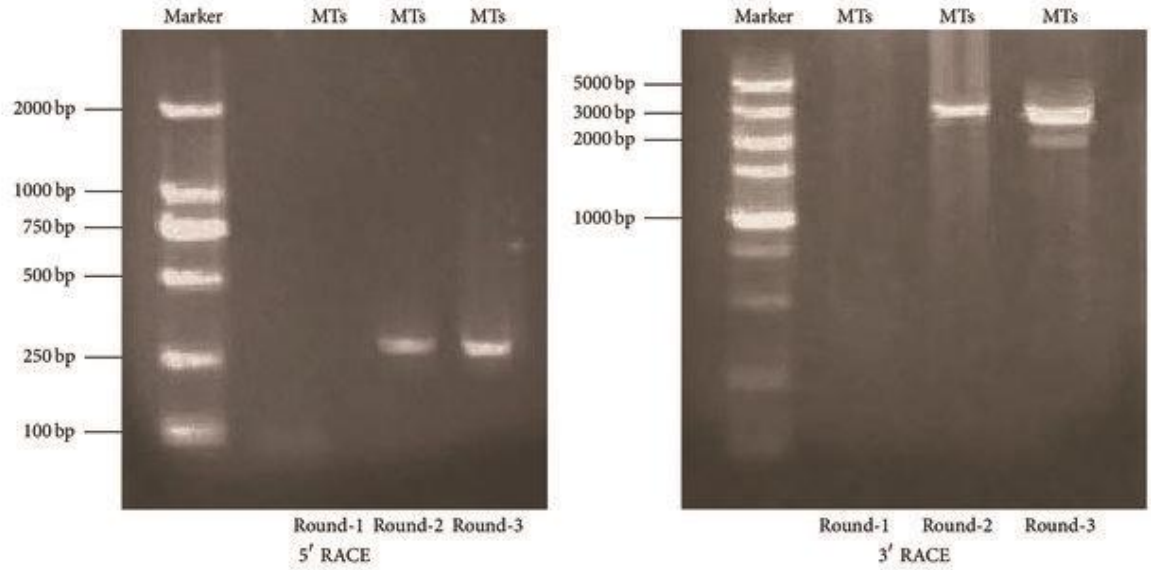

Gel images of *Saccharomyces cerevisiae* variants (GPR1 5`RACE 250bp and GPR1 3` 3000bp)

Table 4.3: Organoleptic Evaluation of the Wine Sample Using ANOVA (P≤0.05)

Sensory Attributes	Sensory Score
Taste	6.5
Aroma	7.0
Appearance	7.50
Overall acceptability	7.0

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4.4 Physicochemical Analysis of Wine Sample

The result of the physicochemical analysis of the wine sample showed that the specific gravity, %alcohol (v/v), total acidity, pH and sugar content was 1.007, 8.25%V/V, 0.8945%, 3.60 and 1.8°Brix respectively (Table 4.6).

Table 4.4: Physicochemical Analysis of Wine Sample

Parameters	Results
Specific Gravity	1.007
% Alcohol (v/v)	8.25
Total Acidity (as acetic acid)	0.8945
pH	3.60
Sugar (°Brix)	1.8

4.6 Effects of Fermentation Time on Sugar Content and Alcohol Content of the Wine.

The result of the effect of fermentation time on the residual sugar and alcohol content of wine showed that the residual sugar decreased during fermentation while the produced alcohol increased with time as seen in Figure 4.1.

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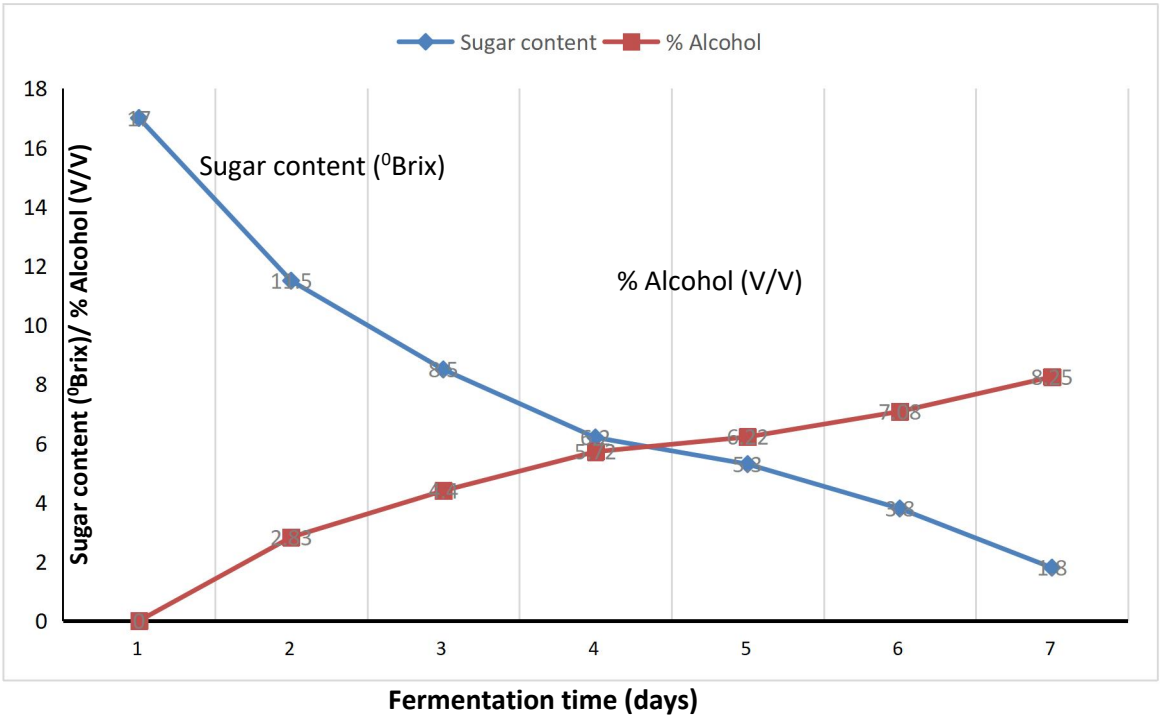

Figure 4.1: Effects of Fermentation Time on Sugar Content and Alcohol Content of the Wine.

4.2 DISCUSSION

Studies were carried out on the production of wine from soursop fruits using *Saccharomyces* spp isolated from palm wine. The result of must analysis showed that the sugar content (9.99⁰Brix) of the soursop juice was low but with fortification of sucrose sugar (50g), a juice with sugar content of 17⁰Brix was obtained (Idise, 2012).

In order to supplement the sugar content of the musts, sucrose was part of the additives. Reports have shown that the major problems associated with the use of tropical Fruits in wine production are their low sugar content (Chilaka *et al.*, 2010).

The soursop wine had flocs present by the 5th day. The production of the flocs might be due to improper storage of the wine. Remarkable amount of alcohol was produced from the fruit wines during fermentation. Results of the analysis conducted during the fermentation of "must" after pitching with the culture yeast showed that the alcoholic content of the wine increased as the fermentation

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progressed. The specific gravity and sugar content decreased throughout the fermentation process. This was as a result of activities of the fermenting yeast. The yeast converts the sucrose to glucose sugars which is being utilized for growth and subsequently produces ethanol and CO_2 (Okafor, 2007).

This study revealed that there was a continuous drop in pH and sugar content of the wine as fermentation progressed due to yeast metabolism while the alcohol content increased. Studies have shown that during fermentation of wine, low pH is inhibitory to the growth of spoilage organisms but create conducive environment for the growth of desirable organisms. Also, low pH is known to give fermentation yeast comparative advantage in natural environment (Reddy and Reddy, 2005). This result agrees with the reports of Anita *et al.* (2018).

The optical density decreased during the fermentation process which is plausibly due to the elimination or reduction of microorganisms that could not withstand the new environment of low pH and high alcohol content. This confirms with the report of Idise (2012) that as fermentation proceeds there is succession and reduction in the number and type of microorganisms in the fermentation liquor.

The result of microbiological analysis of the wine samples after one-month shelf stability showed negative growth of bacteria and fungi. The stability of the wine can be attributed to the effectiveness of sodium metabisulphite and pasteurization at 60^{0}C for 30 minutes.

Acetic acid bacteria such as *Gluconobacter and Acetobacter* species. could be found in wine that is not properly preserved. This observation was in agreement with the report of Okafor (2007) and Aniaku, (2018) that acetic acid bacteria were the major contaminants of alcoholic beverages where they metabolize ethanol to acetic acid under aerobic or microaerophilic condition.

Table 4.3 shows that the sample of wine produced from soursop fruit is acceptable when compared with a commercially available grape wine. The table showed that the appearance of the wine sample ranked more than its taste and aroma and it is generally accepted.

During fermentation, the results obtained as shown in Table 4.6, the changes in specific gravity and sugar (^{0}Brix) of the wine sample decreased from the initial day to the end of fermentation, this could be due to microbial succession, available nutrients, sugar and alcohol resulting in the production of acid. This result agrees with the reports of (Okafor, 2007 and Robinson, 2006).

The result of the effect of fermentation time on the residual sugar and alcohol content of wine showed that the residual sugar decreases during fermentation while the produced alcohol increases with time. The changes in sugar (^{0}Brix) of the wine sample decreased from the initial day to the end of the fermentation. This result agrees with the reports of Akpomie *et al.* (2015); Imade *et al.* (2013) and Okafor (2018). It was observed that the alcoholic content decreased from the initial day to the end of the fermentation. This is due

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to production of ethanol during fermentation process through metabolic activities. These results agree with the reports of Akubor *et al.* (2013). The observed reduction in sugar from 17°Brix and the resultant increase in alcohol concentration during fermentation show the efficiency of the yeast cells isolated from palm wine.

The interaction of wine component that occurred during the ageing process helped in the development of bouquet, special flavor as a result of volatile and non-volatile constitutions of wine (Robinson, 2006). Robinson, 2006 stated that alcoholic contents of wine play a role in their microbial stability. Thus, it can be stated that the pH, alcoholic content gives the wine added microbial stability.

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The results of the research project have proven that it is possible to produce wine with acceptable organoleptic qualities as well as good physiological qualities from local soursop fruit sold in Nigerian market with *Saccharomyces* spp yeast isolated form palm wine. The wine produced from soursop showed no significant ($P \leq 0.05$) difference with a commercial wine sample using colour, taste, appearance and overall acceptability as test attributes at 95% confidence level. This result also showed that *Saccharomyces* species yeasts isolated from palm wine can efficiently ferment soursop fruit juice to produce acceptable wine. This study also gave an insight into the efficiency and role of local yeast (palm wine yeast) during alcohol fermentation of fruit juice.

5.2 Recommendations

If government can support and sponsor local wine production using our local fruits even on a small scale, it will reduce the unemployment problem in the country and drastically reduce the amount of money spent on the importation of wine into the country. It will also ensure the reduction of post-harvest losses experienced by farmers on fruits. Furthermore, researchers should put in more efforts and intensive research on this fruit (soursop) to bring the importance and economic visibility if commercialized. Commercial agricultural practice (Agronomy) involving the planting of soursop should be encouraged, to make available this fruits for immediate consumption and also for further use as a raw material for wine and fruit juice production.

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