Wine production from *Hibiscus sabdariffa* calyxes using probiotics starter cultures

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**ABSTRACT**

Aqueous calyxes extract of Roselle, popularly known as Zobo drink in Nigeria is a non-alcoholic local beverage widely consumed by millions of people across different socio-economic classes. However, large-scale production of zobo is hindered by the rapid deterioration of the drink if not refrigerated. Thus the aim of this research was to extend the viability of the drink by fermentation into red wine using probiotic organism *Lactobacillus fermentum* and *Saccharomyces cerevisiae*. Both organisms produced wines of different physicochemical properties within 24 hours fermentation and with pH 3.47 – 2.76, titratable acidity; 0.236% – 0.252%; Vitamin C; 5.22 mg/100 – 2.44 mg/100 and total dissolvable solids; 11.95 Mg/L – 9.97 Mg/L for *Lactobacillus fermentum* and pH 3.48 – 2.8, titratable acidity; 0.2% - 0.225%, Vitamin C; 8.56mg/100 – 6.33mg/100 and total dissolvable solids; 13.32 Mg/L – 9.71 Mg/L for *Saccharomyces cerevisiae*. There was also a gradual alcohol increase during fermentation process and at the end, both wines showed alcohol content of 5.71% and 5.61% for *Lactobacillus fermentum* and *Saccharomyces cerevisiae* respectively. Except for the alcohol content, the results shows that the formulation with the brewers yeast produced a better wine at the 12 hour fermentation time. The sensory evaluation was also conducted using a 5 judge panel on a 7 point hedonic scale and the result shows that the wine produced from fermentation by *Saccharomyces cerevisiae* was moderately accepted (value 3) by the panel and the mean heterotrophic microbial load of 3±0.2 x 10³ cfu/ml indicated that formulated red wine is safe for human consumption. The outcome of this study will boost the economy of this country if adopted for large scale production as it will generate revenue that would have been lost on importation of wine.

**Keyword**: *Hibiscus sabdariffa*, Calyxes, Fermentation, Roselle, Zobo, Wine, Probiotics

1. **Introduction**

Roselle (*Hibiscus sabdariffa* L.) is a commonly grown plant and is extensively grown in Nigeria, particularly in the country's North-Eastern and middle belt areas [5]. It is an herb of economic importance as it can serve as a source of essential minerals and vitamins such as riboflavin, niacin, calcium and iron [18].

Zobo drink is a traditional refreshment normally created by either boiling or steeping the calyx of sorrel in a consumable, clean water and improved by sugar, which afterwards refrigerated before serving chilled to consumers [20]. The red and additionally purple succulent calyces are bubbled, sieved, improved with sugar, and flavoured with pineapple juice, ginger and lemon to deliver the drink. This drink has demonstrated its immense potential to be a great source of carbohydrate, vitamin C and protein which is the significant reasons for drinking juice [19].
However, Zobo drink has not been successfully produced at commercial scale. This is associated with its short shelf life attributable to microbial activity. This short shelf-life (approx. twenty-four hours following production if not refrigerated) of the drink is associated with degradation of the nutrient component which erodes its health benefits and antioxidant property. This has generated an interest in the possible fermentation of this drink by probiotic organisms to produce red wine that can impart additional nutrient and extend viability. Also in 2016, Nigeria spent about N9 billion on the importation of champagne (sparkling wine) alone [7]. However, high duty on imported wines has stimulated interest in the promotion of our local drinks (kunu and zobo) in the Presidential villa [8] and there was decline in the importation of wine by 24% [9]. Possible wine production from Zobo drink will provide health benefits to its consumers due to abundance of phenolic compounds coupled with low alcohol content. Thus, the objective of this work is to produce red wine from Hibiscus sabdariffa calyxes by fermentative activities of probiotics organisms *Saccharomyces cerevisiae* and *Lactobacillus fermentum* as well as determination of microbial, physico-chemical and sensory properties of the wine.

2. **Methodology**

2.1 **Sample collection**

The samples used for the isolation of the fermenting organisms and the production of wine were obtained from suppliers opposite Canaan land, Ota and Oja Ota in Ogun state. Samples acquired were corn gruel, yogurt and queen pineapple for isolation of the fermentation organism *Lactobacillus fermentum* and *Saccharomyces cerevisiae*. During the fermentation process, hibiscus calyxes were used to produce wine and sugar serve as a sweetener and carbon source.

2.2 **Isolation and identification of probiotic organisms**

De Man Rogosa and Sharpe Agar (MRS) and Potato Dextrose Agar (PDA) made according to manufacturer’s instructions were used for the isolation and characterization of the probiotic microorganisms. A volume of 0.1ml of the dilution factors 10<sup>-3</sup>, 10<sup>-5</sup> and 10<sup>-9</sup> were plated aseptically on MRS agar (for isolation of *Lactobacillus*) and PDA (for isolation of *Saccharomyces*). MRS agar plates were incubated in 5% CO<sub>2</sub> using an anaerobic jar while PDA plates were incubated at about room temperature of 25 °C for 3-5 days. The colonies on the agar plates were observed for morphology, pigmentation, shape and size upon incubation, and subsequently sub-cultured and incubated until pure colonies were obtained after which tests were performed to tentatively identify the organisms. Isolated organisms were identified based on their morphological, biochemical, cultural and physiological features. The tests carried out were Gram reaction, test for urease, oxidase, catalase, citrate utilization, indole, fermentation of different sugars test, endospore tests and milk coagulation test.

2.3 **Molecular identification**

Cultures of pure colonies grown on liquid medium were centrifuged at 4600 x g for 5 min. The resultant sediments were resuspended in 520 μl of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). 15 microliters of 20% SDS and 3μl of Proteinase K (20 mg/ml) were included. The resulting mixture was placed in an incubator for 60 minutes at around 37 °C, then 100 μl of 5 M NaCl and 80 μL of a 10% CTAB solution in 0.7 M NaCl were introduce and thoroughly mixed. The suspension was positioned in an incubator for 10 min at 65 °C and maintained on ice for 15 min. An exact volume of chloroform: isoamyl alcohol (24:1) was introduced, followed by ice incubation for 5 min and centrifugation at 7200rpm for duration of 20 min. The aqueous phase was decanted to a new tube, isopropanol in the ratio 1: 0.6 was included and DNA was precipitated out at –20 °C for 16 h. DNA was obtained by centrifugation at 7200rpm for 10 min, rinsed with 500 μl of 70% ethanol, dried in the incubator at 37°C for 30 minutes and finally dissolved in 50 μl of sterile distilled water as stock DNA. The stock DNA was checked on a 1.5% Agarose gel ran on a voltage of 120V for about 40min. This later viewed under UV light to confirm the presence of the bacteria DNA. The stock DNA was also quantified on Nanodrop spectrophotometer 2000 to determine the quantity and also purity of the samples before PCR. Working DNA solution was diluted at 1:50 for subsequent PCR assay.
2.4 Production of wine from hibiscus calyces

Fig 1: Flow diagram showing production line of red wine

2.5 Inoculum development
The isolates were developed using their respective medium for growth. De Man Rogosa and Sharpe broth for cultivation of *Lactobacillus fermentum* and Potato Dextrose broth for growing *Saccharomyces cerevisiae*. The yeast was introduced into the PD broth and later incubated at 27°C for 48 – 72 hours. After 72 hours, the broth was scaled up by 500 ml till a volume of 1.5 L was attained. After scaling up was complete, the inoculum was centrifuged at 4000 rpm for 15 minutes and the sediment was used for fermentation. *L. fermentum* was inoculated into MRS broth and incubated at 30°C to 35°C for 3 – 5 days. Scaling up was also done till a volume of 1.5 L was acquired and then centrifuged at 4000 rpm for 15 minutes and the sediment was used for fermentation.

2.6 Preparation for calyx extract fermentation
The Hibiscus calyces were sorted to remove dirt, after which 100 g were weighed in three places and wrapped in foil paper and sterilized. 100g of the sterile calyces were introduced to 1000 ml of sterile deionized water in triplicates and allowed to stand for 30mins to aid extraction. The suspension were filtered using the sterile muslin cloth and 100g of sugar was added as carbon source to each of the filtrate to aid fermentation. Three formulations A, B and C were adopted. Formulation A was sealed without inoculation while for Formulation B and C, 1 ml of the organisms were inoculated into their respective flasks and corked.
A: Calyx extract + sugar
B: Calyx extract + sugar + *Saccharomyces cerevisiae*
C: Calyx extract + sugar + *Lactobacillus fermentum*
The three formulations were incubated anaerobically at 25°C for 24 hours with 12 hour analysis of physicochemical properties from the time of preparation. The red wine obtained from the fermentation was clarified using gelatin

2.7 Physicochemical analysis
The formulations were shared into different sterile bijou bottles which were used to store the formulations for the different time zone (0 hours, 12 hours and 24 hours). The physicochemical parameters determined were the pH, total titratable acidity, total dissolved solids, alcohol content and vitamin C content. The pH was assessed by aid of pH meter in sample. Meter reading was recorded in triplicates and an average was calculated. For the estimation of total titratable acidity, 15 ml of the sample was measured and 100 milliliters of distilled water was added followed by 3 drops of phenolphthalein indicator and then titrated against 0.1N NaOH solution, until colour changed to pink. For total dissolved solids, a total of 50 cm³ of the sample was measured and filtered, then poured into cleaned, dried and pre-weighed evaporating dish and put in the oven at 105°C until the sample dried completely, cooled inside desiccator and weighed until the constant weight is recorded. Using the specific gravity method, the formula for calculating alcohol content was calculated thus as described by Biri [10]. Alcohol content by volume (%) = (Original Gravity-Final Gravity) × 131.25.

2.8 Total viable cell count
At intervals of 0 hours, 12 hours and 24 hours, microbiological culture was done on Potato Dextrose Agar and Deman Sharpe Rogosa agar to check for the variation in the growth of the organism used to achieve fermentation.

2.9 Organoleptic evaluation
According to a procedure reported by Maragatham & Panneerselvam (2011), organoleptic assessment was performed for all the formulations [11]. Wines were evaluated by a panel of 5 judges who were very familiar with wine. The judges were made up of 3 males and 2 females, ages between 23 to 30 years. Panelists were selected based on interest and availability and familiarity with wine (Drinks wine at least once in a week) lower values indicate greater acceptance on a 7-hedonic scale.

3. Results
The biochemical characteristics of organisms isolated from respective food source revealed the presence of *L. fermentum* and *S. cerevisiae* in table I. The identity is further confirmed with DNA sequencing and PCR amplification of the 16srRNA and the phylogenetic neighbor-joining tree is constructed based on the sequence (Fig. 2). During the experimental stage of this work, physicochemical analysis such as pH determination, temperature, titratable acidity, vitamin C content, total dissolved solids, specific gravity and alcohol content were analysed during the 0-24hours of fermentation (Table II) and after clarification (Table III). The sensory evaluation as judged by 5 panelist are elucidated in Table IV

### TABLE I: Biochemical characteristics of Isolates

|                      | *L. fermentum* | *S. cerevisiae* |
|----------------------|----------------|-----------------|
| **Colony morphology**| Rough, round   | Raised, Oval    |
| **Cell shape**       | Rod            | oval            |
| **Gram staining**    | +              | Nil             |
| **Catalase test**    | -              | +               |
| **Oxidase**          | -              | -               |
| **Urease test**      | -              | -               |
| **Citrate Utilization** | -             | -               |
Sucrose Utilization  +  +  
Lactose Fermentation  +  +  
Glucose Fermentation  +  +  
Gas production  +  +  

Spore staining  -  -  
Indole test  -  -  

**Fig 2:** The Neighbor-Joining method was used to deduce the evolutionary history [17].

**TABLE II:** Physicochemical properties of the three formulations of the drink at different time regime

| PARAMETERS | FORMULATION | pH | ACIDIT Y (%) | VITAMIN C (Mg/100) | TOTAL DISSOLVABLE SOLIDS (Mg/L) | ALCOHOL CONTENT (%) | TOTAL VIABLE COUNT (cfu/ml) |
|------------|-------------|----|--------------|-------------------|-------------------------------|-------------------|-------------------------|
| A 0  | 3.5  | 0.195  | 10.54  | 14.31  | 0  | 0  |
| 12  | 3.3  | 0.210  | 6.54  | 11.31  | 0  | 0  |
| 24  | 2.7  | 0.225  | 4.20  | 5.16  | 0  | 0  |
| B 0  | 3.4  | 0.2  | 10.5  | 13.31  | 0  | 2  |
| 12  | 3.3  | 0.215  | 6.34  | 12.31  | 2.7  | 3  |
### Table III: Physicochemical Analysis of Wine after Clarification

|        | pH | ACIDITY (%) | VITAMIN C (Mg/100) | TOTAL DISSOLVABLE SOLIDS (Mg/L) | ALCOHOL CONTENT (%) |
|--------|----|-------------|--------------------|---------------------------------|---------------------|
| A      | 2.5| 0.27        | 4.0                | 5.1                             | 0                   |
| B      | 2.6| 0.273       | 6.2                | 9.71                            | 5.65                |
| C      | 2.42| 0.29       | 2.38               | 9.97                            | 5.71                |

### Table IV: Sensory evaluation of wine

| Product         | Formulation A Sensory attribute | Formulation B Sensory attribute | Formulation C Sensory attribute |
|-----------------|----------------------------------|---------------------------------|---------------------------------|
| Aroma           | 3.2                              | 2.80                            | 3.00                            |
| Sweetness       | 3.0                              | 2.60                            | 2.60                            |
| Clarity         | 3.4                              | 3.60                            | 3.60                            |
| Alcohol content | 3.6                              | 3.00                            | 3.20                            |
| Overall acceptability | 3.30                        | 3.00                            | 3.10                            |
4. Discussion

There is a dearth of information on the fermentation of aqueous extract of *H. sabdariffa* calyces using *L. fermentum* and *S. cerevisiae* to produce red wine. The choice of these organisms were deliberate as both organisms have been implicated in the fermentation of grape for production of wine [12]. In several African cereal-based fermented food, *Lactobacillus fermentum* has been recognized as the predominant lactic acid bacteria (LAB) species that possess intrinsic functional features and can add to food quality and safety by providing one or more organoleptic, dietary or health benefits. The various biochemical characteristics were determined to identify both *Saccharomyces cerevisiae* and *Lactobacillus fermentum*. Sequencing of the isolates further confirm our organisms and the phylogenetic was constructed based on the obtained sequence which is elucidated in figure 2.

There were changes in the quality attributes and physicochemical properties of ‘zobo drink’ (formulation A) and formulation B and C following fermentation with *Saccharomyces cerevisiae* and *Lactobacillus fermentum* respectively (table II). The pH reduced from 3.50 to 2.79 over 24 hours. Titratable acidity which measures the amount of acid in a particular product [13] increased across the 24hours (0.195-0.225) in formulation A. This indicate and confirm the high acid content usually observed in Roselle drinks. Roselle is a naturally acidic fruit endowed in organic substances with low pH: Oxalate, tartarate, malate and succinic acid [14]. Formulation B and C showed gradual increment in their acidic content and a corresponding reduction in their respective pH value. These observations are similar to an account from Alobo and Oforony [6] on roselle wine in which the titratable acidity increased and pH reduced at the end of aging. The increase in titrable acidity and decrease in pH in formulation B and C can be attributed to the effect of the yeast and lactic acid bacteria in fermenting the sugars to produce organic acids [15]. Acidity is crucial in wine fermentation as it determines the quality of the wine and creates an environment that inhibit potential spoilage microorganisms but support the growth of desirable organisms [1]. Alcohol content at the onset of the fermentation was zero for the three different formulations (without any pre-inoculated starter culture-A; with *Saccharomyces cerevisiae* –B; and with *Lactobacillus fermentum* -C), but as time progresses, alcohol content gradually increased to 5.65 and 5.71 for formulation B and C respectively (Table II) while formulation A had no change due to the absence of microorganisms. These observations agrees with the findings of Archibong [1]. During fermentation, microorganism carry out metabolic activities which result in the release of metabolites such as organic acids. This may account for the increase in alcohol content recorded across the different formulations. Low alcohol content reported in this work is in tandem with Yokotsuka et al. (1997) [16] who reported that Hibiscus *sabdariffa* calyces contained small amounts of acetaldehyde, pyruvic acid, and alpha- keto glutaric acid, which normally react with SO₂ to form bisulphate complexes in fermentation, leading to the low amount of alcohol produced at the end of fermentation [16]. Total soluble solids measures the sugars present in the fermented must and wine. Total dissolvable solids (Table II) decreased across the different formulation with the formulation A having a range of 14.31-12.00, formulation B (13.31-9.71) and formulation C (11.95-9.97). The decrease can be linked to the activity of organisms that utilizes these sugars to produce acids. The total dissolvable solid for formulation B and C were more reduced compared to formulation A. This could be attributed to the pre-inoculated starter culture in both B and C formulations. This agrees with the report of Efionwewere and Eka [4] and also Nwafor and Ikemebobah [2]. There was a marked decrease in vitamin C content in all formulations. The loss of vitamin C is known to optimize with heat, light and oxygen exposure. Oxidation happens in fruit juices during storage and is extremely dependent on the presence of oxygen in the head room or dissolve in the samples could also be ascribed to the observed reduction. The sensory panelists comprised 60% males and 40% females between the ages of 23 - 30 years, though it is generally known that women have more developed olfactory and sensory system in wines than men [3]. Among the test wines, formulation B was the most acceptable wine to the panelists in terms of the sensory attributes analyzed among the test wines and also the most preferred in terms of the product preference.

5. Conclusion

In conclusion, the result shows *Saccharomyces cerevisiae* and *Lactobacillus fermentum* were able to ferment extract of red calyces of *H. sabdariffa* to produce red wine that is consumable with the absence of microorganisms due to sterilization methods ensured. This research work elucidates that zobo drink can be fermented by *Saccharomyces cerevisiae* and *Lactobacillus fermentum* to produce red wine. The composition of the low alcohol wines produced from *H. sabdariffa* calyces showed acceptable physicochemical properties with reduction in pH, increase in total titratable acidity, reduction in the carbohydrate value and decrease in the total soluble solid. This research shows that the most preferred probiotics to be used would be *Saccharomyces cerevisiae* as it has proved to be highly efficient in wine production from aqueous extract of *Hibiscus sabdariffa*. The outcome of this study, if adopted for large scale production, will boost the economy of this country as it will generate revenue that would have been lost on
importation of wine. It will also create job opportunity for the teeming Nigeria youth thereby reducing the rate of unemployment in the country.

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