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Yeast Diversity Associated with Fermenting Nigerian Beverages, Burukkutu and Fura

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Abstract
Yeasts had been known to man to be very important in everyday life. They can be associated with ethanol production, beer brewing, bread baking and many other applications in different industries. Researchers had postulated the presence of yeasts in fermenting beverages such as palm wine, grapes, *burukutu* and many other agricultural products. This work is aimed at determining the diversity of yeasts from two fermenting Nigerian important beverages, *burukutu* and *fura*. Using pour plate method, serially diluted samples of the fermenting drinks were cultured on Sabouraud dextrose agar plate and incubated at 28±2^{O}C for 48hrs, discrete colonies were sub-cultured on Potato dextrose agar for 72hrs and the isolates identified and characterized. Results obtained showed the colony count for *burukutu* to be 7X10^{3}cfu/ml and *fura* 6.8X10^{3}cfu/ml. On sub-culturing the discrete counted colonies, five and four isolates were identified from *burukutu* and *fura* respectively and designated as YB1 to YB5 and YF1 to YF4. Isolated yeasts YB1, YB2, YB3, YF2 and YF3 were identified as *Saccharomyces sp*; Yb4 and YF1 were *Rhodotorula sp* while YF4 is *Candida sp.* All the isolates survived temperature of 25 to 35^{O}C, ethanol concentration of 8 to 10%, YB2 and YF4 assimilate nitrate, YB2, YB4 and YF4 produce hydrogen sulphide while YB5 and YF4 did not form flocculation. This work confirms the local beverages, *burukutu* and *fura* as rich sources of yeasts that can be useful in different industries.

Keywords: a. Students' Learning, b. Future Consequences, c. Leaders' Intervention, d. Impactful Significance and e. Satisfactory Output.

Introduction

Background

Much biodiversity of microorganisms of economic importance inhabit natural habitats such as soil, food, water and other agricultural products. Most agricultural products can easily undergo fermentation and the predominant microorganisms that are isolated from them are bacteria and yeasts (Awasthi *et al.*, 2022).

Yeasts are actually microbial eukaryotes which belong to Ascomycetes that are good source of vitamin B and protein. They are plant-like unicellular fungi thriving on every living organism. As living organism fungi require warmth, water, albumen or nitrogenous material and sugars to survive (Adesokan *et al.*, 2020). Yeasts cells are typically spherical, oval or cylindrical in shape. The conditions required for their growth are warmth (optimum 25-30 °C), moisture and food (starch plus a small amount of sugar) (Lawrence, 2004). Refrigeration slows down the growth so that yeast can be kept for a limited period of time under refrigeration.

Yeast cells contain some enzymes that act as natural catalysts in the fermentation process such as maltase, invertase and zymase complex. Maltase has the ability to alter maltose, which is formed by starch degradation by alpha and beta-amylases, to glucose and acts when the supply of simple sugars has been bushed. Invertase converts sucrose to glucose and fructose, while the zymase complex converts glucose, fructose and other simple sugars into carbon dioxide and ethanol. Carbon dioxide thus formed can raise the dough during fermentation (Madigan *et al.*, 2003).

The earliest reported traditional African fermented product is the production of beer from cereal extracts invented in Egypt some 5000 to 6000 BC. Apart from beer, other fermented beverages have been produced and consumed by many people. For centuries the production of these beverages has gradually evolved as an art of craft which is passed on from one

generation to another, without proper understanding of scientific basis of the art. The traditionally fermented alcoholic beverages from cereal grains, such as guinea corn (*Sorghum vulgaris*, *Sorghum bicolor*) and millet (*Panicum miliacum L.)* had gained popularity over the years.

Burukutu (Sorghum beer) is an alcoholic beverage drink popularly produced and consumed in Nigeria. Its production is by uncontrolled fermentation by its indigenous yeast species. It is one of the indigenous alcoholic beverages from grains of guinea corn (*S. vulgare* and *S. bicolor*). It has about 1.0-3.3% alcoholic content when allowed to ferment over night and the alcoholic content may increase after prolonged fermentation (Ogbonna, 1983; Umeh *et al.,* 2015). It is found in bars and can be hawked by women around towns and areas dispensing alcoholic beverages. It can be consumed alone or accompany meal. *Burukutu* is important to most rural Nigerian population who could not afford the price of lager beer and other beverages because it is cheap. Most people take this beverage to add variety to their diets and for stimulating effects. Since this beverage is obtained from guinea corn, it is good source of essential minerals e.g. calcium, iron, zinc and copper. It has high content of suspended solids (Yabaya and Jatau, 2009).

Fura (millet meal) is a semi-solid dumpling millet-based meal or cereal porridge (Jideani *et al*., 2001). It is a traditional staple food in West Africa particularly in Nigeria, Ghana and Burkina Faso produced mainly from millet blended with spices and water, compressed into dough balls and cooked (Kordylasi, 1990; Jideani *et al*., 2001). The cooked dough balls are broken up and made into porridge by mixing with yoghurt (*nunu*), fresh milk or water (Kordylasi, 1990). Sugar may be added to taste. The mixture of fermented milk and cooked spiced millet (*fura denunu*) is almost a complete food with milk serving as a source of protein while the cooked spiced millet provides energy. The sour taste is known to be particularly suited for quenching thirst. The fermentation process in traditional *fura* processing, like many other traditional fermentation processes occurs spontaneously with the help of its indigenous yeast species and difficult to control. The process is not predictable in terms of length of fermentation and quality of product.

These two beverages had been reported to harbour yeast species during the fermentation processes (Umeh *et al.,* 2017, Umeh *et al.,* 2019). This work is therefore carried out to determine the assorted yeast species that can be associated with their fermentation.

Materials and Methods

Already prepared *burukutu* and *fura* were purchased from retailers at the Aroma Motor Park Awka and transported to the Applied Microbiology and Brewing Department Laboratory. Media, chemicals and reagents were purchased from a dealer at the Onitsha Bridge Head Drug Market and were of analytical grade.

Yeast isolation

Yeasts isolation was done using the method applied by Sergei (2020). One millilitre of each sample was serially diluted using peptone water and the third dilution cultured on Sabouraud dextrose agar (SDA) plate with added antibacterial agent for elimination of bacteria. The developed colonies were counted and discrete colonies sub-cultured on Yeast peptone dextrose (YPD) agar plates. The colonies that grow were sub-cultured on slant in bijou bottles and preserved for identification.

Yeasts identification and characterization Yeast microscopy

This was done using the method used by Camara and Sant'Ana (2021). A loop full of each of the developed colonies was smeared in a drop of sterile distilled water on a glass slide, dried and stained with dilute methylene blue dye. The dried smear was viewed under the microscope at 100X magnification and observations recorded.

Physiological and Biochemical screening

The morphological and physiological properties of the isolates were determined using the method of Nouroll *et al.,* (2013) modified by Adesokan *et al.,* (2020). The following test parameters; cell and colony morphology, viable cell and colony count, temperature tolerance test, nitrate assimilation test, hydrogen sulphide production test, ethanol tolerance test, fermentative capacity test and stress exclusion test were determined.

Flocculation test

Helm's flocculation test was done according to D'Hautcourt and Smart (1999) modified by Agwuna *et al.,* (2019). A suspension of yeast cells was centrifuged at 3000 rpm for 5 minutes. The pellets were washed 3 times in distilled water. The cells were re-suspended in distilled water and diluted to a cell concentration of $1x10^8$cells/ml. One millilitre of cell suspension was aspirated into different six Eppendorf tubes grouped into two portions of three tubes each. One group was labelled A while the other group was labelled B.

Treatment of group A: The Eppendorf tubes were centrifuged at 3000 rpm for 4 minutes and the supernatant removed. One millilitres of 0.05M EDTA was added and re-suspended by vortexing for 15 seconds. The tubes were inverted five times and left to sediment for 20 minutes. The top 100 µl of the suspension was carefully removed using a pipette and transferred into a cuvette. Nine hundred microliter of distilled water was added and the optical density (OD_{600}) measured using spectrophotometer zeroed with distilled water.

Treatment of group B:

The Eppendorf tubes were centrifuged as in A above and 1 ml of a washing solution, Helms A (0.51 g/l $CaSO_4$) was added to the cell pellets and re-suspended by votexing for 15 seconds. The tubes were centrifuged again and the supernatant discarded. The cell pellets were re-suspended in 1 ml of Helms solution B (0.51 g/l $CaSO_4$ in solution with 6.8 g/l Sodium acetate, 4.05 g/l Glacial acetic acid, 4% Ethanol in 1 litre of deionised water). Different sugar solutions (mannose, glucose or maltose) were added where appropriate to achieve sugar inhibition profiling. The tubes were inverted 5 times and left for 20mins to sediment. The top 100 µl was treated as in A above. Percentage flocculation was calculated determining the mean OD_{600} of A and B, them applying the formula;

$$\% \text{ Flocculation} = \frac{(A-B)}{A} \times 100$$

Sugar fermentations

The method used Umeh *et al,* (2019) and modified by Camara and Sant'Ana (2021).

Molecular identification and characterization

The isolates were further identified and characterized by the methods of molecular analysis, gene sequencing methods for 16S RNA sequence analysis and genomics.

Results and Discussion

In this work, yeasts were isolated from two fermenting Nigerian beverage drinks, *burukutu* and *fura*. The results obtained from the research work were presented in this section. Colony counts on the culture plates were $7X10^3$cfu/ml and $6.8X10^3$cfu/ml for *burukutu* and *fura* respectively. Nine yeasts were isolated, characterized and identified. They were designated as yeasts from burukutu (YB) and yeasts from fura (YF) with acronyms as YB1, YB2, YB3, YB4, YB5, YF1, YF2, YF3 and YF4. Five isolates (YB1, YB2, YB3, YB5 and YF3) were characterized as *Saccharomyces cerevisiae*, one isolate (YF2) with a slight different in the characters of *Saccharomyces cerevisiae* was labelled *Saccharomyces* sp. Two strains of yeast (YB4 and YF1) were identified as *Rhodotorula* sp while the other isolate (YF4) was identified as *Candida utilis*. Their microscopic and morphological characteristics were presented inTable1.

Table 1: Morphological and budding characteristics of the isolated yeasts.

Yeast isolates	Colony shape and colour	Surface and appearance	Vegetative morphology, appearance and cell arrangement	Budding ability
YB1	Light, creamy, spherical	Smooth, flat	Single budding, spherical	+
YB2	White to creamy, spherical	Smooth, hairy	Multi polar buds, spherical	+
YB3	White to tan, spherical	Glaborous, smooth	Single budding, spherical	+
YB4	Creamy to Tan, spherical	Hairy, smooth, flat	Multi polar buds, spherical	+
YB5	Creamy, spherical, raised	Smooth and shiny	Single budding, raised	+
YF1	Pink, convex	Smooth and globose	Multi polar buds, entire	+
YF2	Light to cream, oval	Smooth, shiny, hairy	Multi polar buds, entire	+
YF3	Creamy, spherical, convex	Smooth, glaborous, hairy	Multi polar buds, spherical	+
YF4	White to creamy, spherical	Smooth, globose	Single to multi budding, pseudohyphae	+

Key: + (Ability to develop buds)

The isolates depicted the microscopic and morphological characteristics of ideal yeasts as found by many researchers Mir and Mohammed, (2014), Umeh and Okafor, (2016), Sergi (2020). All the isolates were budding yeasts developing buds ranging from single, multi polar and budding pseudohyphae (Table 1).

Temperature tolerance and nitrate assimilation tests results were presented in Table 2. The isolates were able to survive certain degrees of temperature and two were able to assimilate nitrate incorporated in the medium. All the yeasts isolated grew well in temperature range of 25-35^oC but YB2, YB3, YB4, YF1, YF3 and YF4 survived scantly at 37^oC. None grew at temperatures above this. The finding is in consonance with the findings of Mir and Mohammed, (2014) who found that yeasts isolates from fruits and other fermenting substrates did not grow at temperature of 40 ^oC. The finding did not agree with the findings of Agwuna *et al.*, (2019) that found isolated yeasts from sucrose enriched and non-sucrose enriched palm wine can survive even temperatures of 45 ^oC. Ability of yeasts to tolerate high temperatures makes them suitable to be used in food industries without being denatured or mutated (Umeh and Okafor, 2016).

Two isolates, YB2 and YF4 assimilate nitrate incorporated in their medium but other isolates did not (Table2). Yeasts that assimilate nitrate reduce the protein content of the final products they are used to produce. For example, if such yeasts were employed in baking, the protein content of the produced bread will be reduced by the yeasts (Umeh and Okafor, 2016).

Table 2: Temperature tolerance and Nitrate assimilation ability of the yeasts.

Yeast isolates	Varying temperature degrees (^oC)					Nitrate assimilation
	25^oC	27	30	35	37	
YB1	+++	+++	+++	++	-	-
YB2	+++	+++	+++	++	+	+
YB3	+++	+++	+++	++	+	-
YB4	+++	+++	+++	++	+	-
YB5	+++	+++	+++	++	-	-
YF1	+++	+++	+++	++	+	-
YF2	+++	+++	+++	++	-	-
YF3	+++	+++	+++	++	+	-
YF4	+++	+++	+++	++	+	+

Key: +++ (Intensive growth), ++ (Moderate growth), + (Scanty growth), - (No growth)

Ethanol tolerance, Hydrogen sulphide test and flocculation abilities of the isolates were presented in Table 3. The isolates were able to tolerate different concentrations of ethanol contained in growth media. The nine isolated yeasts

tolerated ethanol concentrations of 8% and 10%. YB1, YB4 and YF2 grew moderately at ethanol concentrations of 15% while YB5 and YF4 did not grow at that temperature (Table3). YB1, YB2, YB3, YB4 and YF2 show scanty growth in medium with 20% ethanol. This is in agreement with the findings of Mir and Mohammed (2014) and Umeh *et al.*, (2019) who found out that yeasts isolated from natural sources can tolerated high ethanol concentrations of up to 20%. Yeast isolates especially *Saccharomyces cerevisiae* that tolerate high ethanol concentrations can be applied in bread and beer production if other parameters are in right order (Umeh and Okafor, (2016), Agwuna *et al.*, (2019). Four isolates, YB5, YF1, YF3 and YF4 did not show any growth at ethanol concentration of 20%. High concentrations of ethanol generally inhibit growth of yeasts as is toxic to their cells. As concentration of ethanol increases in media, a reduction in growth is generally observed (Mir and Mohammed, 2014; Umeh *et al*., 2019).

Six isolates did not produce hydrogen sulphide in Lead acetate media, but three isolates did. Hydrogen sulphide production is not a good attribute of yeasts that can be employed as baker's and brewer's yeasts (Agwuna *et al.*, (2019), Umeh *et al.*, (2019). The isolates were able to flocculate producing different intensities of flocculates except YB5 and YF4 (Table 3). Useful industrial yeasts are the ones that are able to flocculate. They can be employed in the production of products like bread and beer (Agwuna *et al.*, (2019), Umeh *et al.*, (2019).

Table 3: Ethanol tolerance, Hydrogen sulphide production and Flocculation abilities of the isolated yeasts.

Yeast isolates	Ethanol conc. (v/v)				H_2S production	Flocculation (%)
	8 %	10%	15%	20%		
YB1	+++	+++	++	+	-	96.65^{a}±0.5
YB2	+++	+++	+	+	+	97.0^{d}±0.1
YB3	+++	+++	+	+	-	65.4^{b}±0.4
YB4	+++	+++	++	+	+	88.3^{a}±0.2
YB5	+++	+++	-	-	-	-
YF1	+++	+++	+	-	-	94.3^{b}±0.4
YF2	+++	+++	++	+	-	90.2^{b}±0.5
YF3	+++	+++	+	-	-	72.2^{a}±
YF4	+++	+++	-	-	+	-

Key: +++ (Intensive growth), ++ (Moderate growth), + (Scanty growth), - (No growth)

Stress inclusion test for growth at different temperatures and cell osmotic pressure in high concentration of ethanol and sugar were presented in Table 4. The isolates were able to survive the different stress conditions depicting different growth responses from profuse to scanty growth. Yeast isolates from local materials especially fermenting beverages are good sources of yeasts that can withstand stress, tolerate high ethanol concentration and withstand changes in degrees of temperatures (Mir and Mohammed, 2014; Camara and Sant'Ana, 2021). Stress tolerance is an attribute that makes yeasts and other microorganisms adapt to changing conditions and environments. For yeasts that tolerate stress; aerations, agitations and vigorous mixings will not denature them.

Table 4: Stress inclusion test of the isolates on YPG medium under different stress conditions and sub-culture for consecutive 15 days.

Isolates	YPG at 28^{o}C	YPG at 30 ^{o}C	YPG + 8% v/v ethanol	YPG + 20% w/v glucose	YPS (20% w/v sucrose + 8% ethanol
YB1	+++	+++	+	+	-
YB2	+++	+++	++	++	+
YB3	+++	++	+++	+	-
YB4	+++	++	++	++	-
YB5	+++	+	+	++	+
YF1	+++	+	++	+++	++
YF2	+++	+++	++	++	+
YF3	+++	++	+	++	+
YF4	+++	+++	++	+	-

Key: +++ (Intensive growth), ++ (Moderate growth), + (Scanty growth), - (No growth)

Results of the fermentative ability of the isolates of different sugars were as shown in Table 5. One isolate (YF4) ferment only two sugars, sucrose and raffinose, used. It did not ferment other sugars and from other identification techniques identified as *Candida utilis* (Table5). Two isolates that ferment Lactose were identified as *Rhodotorula spp*. Other isolated yeasts were identified as *Saccharomyces cerevisiae* except Yf2 (*Saccharomyces spp)* which was identified using other characteristics but it did not ferment melibiose sugar but ferments other sugars as *Saccharomyces cerevisiae* (Table 5).

Table 5: Sugar fermentation and utilization abilities of the isolated yeast strains.

Isolates	Glucose	Sucrose	Lactose	Fructose	Melibiose	Raffinose	Galactose	Probable isolates
YB1	+	+	-	+	+	-	+	*Saccharomyces cerevisiae*
YB2	+	+	-	+	+	-	+	*Saccharomyces cerevisiae*
YB3	+	+	-	+	+	-	+	*Saccharomyces cerevisiae*
YB4	+	+	+	+	-	-	+	*Rhodotorula spp*
YB5	+	+	-	+	+	-	+	*Saccharomyces cerevisiae*

YF1	+	+	+	+	+	-	+	*Rhodotorula spp*
YF2	+	+	-	+	-	-	+	*Saccharomyces spp*
YF3	+	+	-	+	+	-	+	*Saccharomyces cerevisiae*
YF4	-	+	-	-	-	+	-	*Candida utilis*

Key: + (Able to ferment the sugars) - (Unable to ferment the sugars)

Yeasts are used in food and beverage production due to their capability of utilizing the carbohydrate substrate to produce ethanol for example in beer production. They can also aid in carbon dioxide production in the production process as in bread making, where they can utilize sugars and other carbohydrate substrates to leavening the dough. Different strains of yeasts can also be used in variety of industries like food, pharmaceutical, cosmetics and other important industries. So harnessing these yeasts and preserving them will enhance our industries and reduce the task of importation of yeasts from other countries.

Conclusion

Based on the research done, *burukutu* and *fura* are good sources of useful industrial yeasts. These yeasts from indigenous beverages are going to boast production in our various industries and will reduce the importation of conventional commercial yeasts that drain the economy and make productions easier and affordable.

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