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Isolation and Identification of Yeasts from Fermenting Indigenous Fruit and Beverage Drinks Sold In Awka, Nigeria

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Abstract
Background: *Yeasts are among the important microorganisms useful to man. Some are applied in food because of their protein and are used in food industries such as brewing, baking etc. This Study aims to isolate and characterize yeast strains from "burukutu", Shaddock juice and "fura" (millet meal) as well to determine the efficacy of the isolated yeast strains compared to the conventional commercial bakers' yeast.*
Materials and Methods: *The extraction of the juice from the respective raw materials as well as the enrichment procedures were carried out using standard procedures. The isolated yeasts were tested for their flocculation abilities, their ability to ferment glucose, sucrose, maltose, fructose, ability to produce carbon dioxide, their temperature tolerance, fermentative capacities as well as their survival under various stress conditions. The isolated yeasts were designated as Yeast from shaddock fruit (SY), yeast from burukutu (BY), yeast from fura (FY) and Control commercial yeast (CY).*
Results*: The commercial bakers' yeast (CY) had the highest colony count followed by SY, FY and BY respectively. Microscopic observation of isolated yeast isolates indicates effective budding capability and an indication of active fermentation. All the yeast isolates were able to grow intensively in medium containing 10% (v/v) of ethanol. All yeast isolates had the ability to flocculate moderately and were also able to ferment all sugars provided.*
Conclusion: *This study showed that yeast strains that can be used in place of baker's yeasts can be isolated from local materials such as "burukutu", "fura" and shaddock juice due to the fact that all the yeast isolates served a good purpose in their Flocculation abilities, their ability to ferment glucose, sucrose, maltose, fructose, ability to produce carbon dioxide, their temperature tolerance, fermentative capacities as well as their survival under various stress conditions. Since these fruit and beverage drinks are always available, they will make for a cheap and inexpensive source of yeasts for various industrial production processes.*
Keywords: *Alcoholic Beverages, Awka, Fermentation, Indigenous, Saccharomyces cerevisiae.*

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I. Introduction

Yeast is known as sugar-utilizing fungus and can be found naturally from the surrounding [1]. According to [2], fruits, vegetables, drinks and other agricultural products are very important microhabitats for various yeast species. A succession of yeast populations in agricultural products are involved in a variety of biochemical processes which utilize complex sugars present to simple sugars. The yeast which is the main organism responsible for alcoholic fermentation usually belong to the genus *Saccharomyces*.

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 [3], as opposed to respiration where electrons are donated to an exogenous electron acceptor, such as oxygen, via an electron transport chain. According to [4], the process of fermenting is basically feeding sugars and nutrients in solution to yeast, which return the favor 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. Different yeasts produce different results, and have different tolerance levels.

Burukutu is an alcoholic beverage with 1.0-3.3% alcoholic content [5]. It is found in bars and areas dispensing alcoholic beverages, either in their own right or as an accompanying meal. The production of

burukutu is by uncontrolled fermentation by its indigenous yeast species. It is one of the indigenous alcoholic beverages produced mainly from grains of guinea corn (*S.vulgare* and *S.bicolor*). Burukutu is important to most rural Nigerian population who could not afford the price of a lager beer and other beverages.

The shaddock juice is a beverage drink obtained from the shaddock tree. It contains a wide microbial biodiversity such as fungi and bacteria [6]. The shaddock is tropical or near-tropical and flourishes naturally at low altitudes close to the sea [6]. On the salty mud flats, farmers dig ditches and create elevated beds of soil for planting the trees. The salt content of the water varies throughout the year but may be as high as 2.11 % at times.

Fura (from millet) is a semi-solid dumpling millet-based meal [7] or cereal porridge. 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 [8,7]. 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.

This study was undertaken with the view to isolating, characterizing and identifing yeast strains from "burukutu", Shaddock juice and "fura" (millet meal) as well to determine if the yeast strains isolated compete favorably with the conventional commercial bakers' yeast in bread production.

II. Materials And Methods

Collection of Samples

Commercial bakers' yeast (STK; *Saccharomyces cerevisiae*), flour and other ingredients were purchased from a retail shop in Eke-Awka, Anambra State.

Millet meal (Fura) was purchased from Eke Awka market and placed in a sterile plastic container and transported to the laboratory. "Burukutu" (local sorghum beer) was obtained from a local seller at Aromma junction Awka and with a sterile plastic container and immediately transferred to the laboratory. Shaddock fruits were harvested from a farm at Umuzocha Village, Awka, Anambra State and was identified in the Department of Plant Science and Biotechnology, University of Nigeria Nsukka. The shaddock fruits were washed with water containing sanitizers and peeled. The juice was extracted with the aid of a sterilized hand juice extractor, the extracted juice was then filtered with the aid of a muslin cloth and the filtrate collected in a sterile plastic container.

Isolation and Microscopic Observation of the Yeasts

The enrichment procedure to detect and isolate fermenting yeast species from the collected samples was carried out by adding 1 ml of each sample into high-sugar medium (grape must, pH 3.2, with sugar added to a final concentration of 27%, w/v) using the method of [9]. All of the micro-fermentations were carried out at 27°C in 100ml Erlenmeyer flasks containing 50 ml sterilized grape must [10] and incubated for 3 days.

After incubation, 1ml of each of the enriched samples was serially diluted in triplicates ranging from 10^1 to 10^4 and 1ml of each dilutions was inoculated onto Sabouraud dextrose agar (SDA) medium containing chloramphenicol (to avoid bacterial growth)by a pour plate method and subsequently incubated for 3 days. The colonies were then counted and selected according to their morphological characteristics as described by [11] and the colony count for the yeast isolates obtained. Tentative yeast isolates from the SDA plates with $10^2$and 10^4 dilutions were then sub- cultured onto Yeast Peptone Dextrose (YPD) medium (10 g /L Yeast Extract, 20 g/L Peptone, 20 g/L dextrose and 20g/L agar) and the plates incubated at 30°C for 3 days. Representative colonies were picked from the plates and their pure cultures preserved in slants, labeled as SY (shaddock yeast), BY (burukutu yeast), FY (fura yeast) and stored in the refrigerator at 4^0C. The commercial baker's yeast was reconstituted, sub-cultured, labeled CY and also preserved in the refrigerator as others.

A loop full of each colony of the isolate was supplemented in a drop of sterile distilled water placed on glass slide and smeared until the smear dry off. The smear was then stained using diluted methylene blue dye, air dried and observed under light microscope at 100 x magnification [10].

Ethanol and Temperature Tolerance of the Isolated Yeasts

The ability of the isolated yeast strains to grow in higher ethanol concentrations was tested by growing them in Yeast peptone glucose(YPG) broth containing 3 different concentration of ethanol, 10% , 13% and 15% (v/v), respectively and incubated at 30ºC for 72 hours [10]. A loop full of each isolates was inoculated into a freshly prepared YPG broth containing different concentrations of ethanol 10%, 13% and 15% (v/v) respectively and observed after 72 hours.

The ability of the yeast to grow at higher temperatures was verified by plating the yeast isolates onto YPG medium and incubated at 3 different temperatures i.e. 27, 37 and 45ºC for 72 hours [10]. A loop full of each isolate was streaked on a freshly prepared and dried YPG medium and incubated at three different temperatures of 27^{o}C, 37^{o}C and 45ºC and observed for 72 hours.

Flocculation Ability of the Isolates

In this test, isolates were inoculated in 10 ml of freshly prepared YPG broth and incubated at 30ºC for 3 days. After incubation, tubes were agitated to observe the flocculation formed [10]. A loop full of each isolates was inoculated into a freshly prepared YPG broth and observed for 72 hours. After 72 hours, the tubes were agitated to observe the flocculation formed.

Fermentative Capacity of The Isolated Yeast Strains

In this test, the fermentative capacity media was prepared and the test was conducted as described by [12]. Prior to yeast cells growth into a freshly prepared Yeast fermentation broth (YFB) (Peptone 7.5 g/L, yeast extract 4.5g/L; 1ml of 1.6% (w/v) bromothymol blue as an indicator), 6% (w/v) glucose, sucrose, fructose and maltose were autoclaved separately. The yeast cells were grown at 30^{o}C for 3 days. The YFB was added with respective sugar, then yeast cells were examined on the fermentative ability on different carbon sources. The Durham tubes were also placed into the media to trap the carbon dioxide released, and the indicator to determine acid production by a colour change (green to yellow) if the yeast cells have the ability to ferment the respective sugar.

Stress Exclusion Test

Stress exclusion test was conducted as described by [10] The continuously growth of stress exclusion test was overall done for 15 days' incubation onto different media. The ability to grow under different stress conditions were conducted by growing the yeast isolates onto Yeast Peptone Glucose (YPG) (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar) medium and incubated at 30ºC for 3 days. A single colony was then transferred and continuously grown on YPG medium and incubated at 37^{0}C for another 3 days, before further subculture of the isolated yeast colony on YPG medium containing 8% (v/v) ethanol and incubated at 30ºC for 3 days. A single isolated colony on YPG with 8% ethanol was further sub cultured on YPG supplemented with 20% (w/v) glucose and incubated under the same conditions as above. Finally, the yeast cells were transferred on YP (10 g/L yeast extract, 20 g/L peptone) medium supplemented with 2% (w/v) sucrose and 8% (v/v) ethanol and incubated under the same conditions as above.

Reconstitution of the Yeast Isolates

Different yeast isolates were inoculated separately in sterilized peptone broth containing 25% (w/v) glucose in 100ml conical flask and incubated at 30°C for 8 days. The culture was centrifuged at 10,000 rpm for 15 min. The pellet of yeast cells were collected and washed with cold sterile distilled water and re-suspended in 20 ml sterile distilled water and used to inoculate the dough [13].

Viability Test of the Reconstituted Yeast Isolates

Viability of reconstituted yeast isolates was determined with the viable count method according to [14]. Four test tubes were filled with 9ml of buffer phosphate solution and 1ml each of the reconstituted yeast suspension was mixed thoroughly with the buffer solution. One milliliter (1ml) of the mixture each was pipette into a second set of tubes containing 9ml of the buffer solution and mixed thoroughly. Thereafter 1ml from the second set of tubes was taken and placed into the third sets of tubes containing 9ml of the buffer solution. The process was repeated until the fourth sets of tubes was inoculated with the yeast suspension from the third test tube, 1ml each from the four sets of dilutions was inoculated onto freshly prepared YPG medium by a spread plate method and incubated at 30^{O}C for 72 hours. The average of the counts and viability of the colonies formed was calculated. To get the viability in the form of cells unit the average value obtained was multiplied with 10^4 units.

Statistical Analysis

The data generated were analyzed statistically using SPSS software (IBM Incorporation New York, USA) and the results obtained were subjected to one-way analysis of variance (ANOVA) and difference in means identified at $p<0.05$.

III. Results

Microscopic examination of the cell morphology of isolates CY, BY, SY and FY showed the presence of ellipsoid to ovoid cells with multipolar buds and ascospores (clusters of four small spheres within a small sac or ascus).

The colony morphology of the yeast isolates on the YPD medium as shown in Table 1 indicated that isolates SY, BY and FY showed fluffy and rough colonies while isolate CY showed a creamy and smooth colony.

The yeast colony count on the SDA medium as shown in Table 1 indicated that the isolates SY, BY, CY and FY had the following values 3.0 $x10^5$cfu/ml, 2.5 $x10^5$cfu/ml, 3.8 x 10^5cfu/ml and 2.7 x 10^5cfu/ml respectively.

Table 1: Colony morphology and count of the yeast isolates on YPD and SDA after3 days incubation

Isolates	Creamy	Fluffy	Smooth	Rough	Yeast counts (cfu/ml)
SY	-	+	-	+	3.0 x 10^5
BY	-	+	-	+	2.5 $x10^5$
CY	+	-	+	-	3.8 $x10^5$
FY	-	+	-	+	2.7 $x10^5$

Key: (+) positive, (-) negative

The ethanol tolerance of the isolates in yeast culture broth containing ethanol concentrations of 10%, 13% and 15% respectively as shown in Table 2 indicated that all the isolates showed intensive growth at 10% ethanol concentration, at 13% ethanol concentration SY and CY showed intensive growth while BY and FY showed moderate growth and at 15% ethanol concentration all the isolates showed moderate growth.
The flocculation ability of the isolates in yeast culture broth as shown in Table 2 indicated that all the isolates showed a moderate response on agitation of the culture broth.

Table 2: Ethanol tolerance and flocculation ability of the isolates in yeast

Isolates	Ethanol concentration (v/v)			Flocculation
	10%	13%	15%	
SY	$+++^{TB}$	$+++^{TB}$	$++^{TB}$	++*
BY	$+++^{T}$	$++^{T}$	$++^{T}$	++*
CY	$+++^{TB}$	$+++^{B}$	$++^{B}$	++*
FY	$+++^{T}$	$++^{T}$	$++^{T}$	++*

KEY: (+++) intensive growth,(++)moderate growth,(+)low growth,(-)no growth.
(TB) top and bottom growth, (T) top growth,(B) bottom growth.
KEY: (+++*) rapid response,(++*)moderate response,(+*)low response,(-)no response

The temperature tolerance of the yeast isolates in the YPG medium at 27^{o}C, 30^{o}C, 37^{o}C, and 45^{o}C respectively as shown in Table 3 indicates that at 27^{o}C all the isolates showed an intensive growth, at 30^{o}C isolates SY, CY and FY showed intensive growth while BY showed moderate growth, at 37^{o}C isolates SY and FY showed intensive growth while CY and BY showed moderate growth, at 45^{o}C isolates SY, CY and FY showed moderate growth while BY showed low growth

Table 3: Temperature tolerance of the isolated yeasts

Isolate	Temperature (^{o}C)			
	27	30	37	45
SY	+++	+++	+++	++
BY	+++	++	++	+
CY	+++	+++	++	++
FY	+++	+++	+++	++

KEY: (+++) intensive growth, (++) moderate growth, (+) low growth, (-) no growth.

The ability of the isolates to ferment different sugars in the fermentation broth as shown in Table 4 indicates as follows; in glucose broth, all the isolates produced acid and gas, in fructose broth, all the isolates produced acid and gas, in maltose broth, all the isolates produced acid and gas, and in sucrose broth all the isolates produced acid and gas.

Table 4: Fermentative capacity of the isolated yeasts strains

Carbon source	Isolates			
	SY	BY	CY	FY
GLUCOSE	++	++	++	++
FRUCTOSE	++	++	++	++

MALTOSE	++	++	++	++
SUCROSE	++	++	++	++

KEY: (++) acid and gas production,(+)acid production

A combination of the temperature tolerance and cell osmotic pressure tolerance in high concentration of ethanol and sugar medium as shown in Table 5 indicated as follows; at 30^{o}C all the isolates showed intensive growth, at 37^{o}C all the isolates showed intensive growth, in 8% ethanol all the isolates showed intensive growth, in 20% glucose isolates SY, BY and FY showed intensive growth while CY showed moderate growth, and in 20% sucrose+8% ethanol isolates SY and FY showed intensive growth while BY and CY showed moderate growth.

Table 5; Stress Exclusion test properties

Isolates	**Growth onto different media**				
	YPG		YPG	YPS (20% v/v sucrose+	
	30^{O}C	37^{O}C	+Ethanol (8% v/v)	+glucose(20% v/v)	8% v/v ethanol)
SY	+++	+++	+++	+++	+++
BY	+++	+++	+++	+++	++
CY	+++	+++	+++	++	++
FY	+++	+++	+++	+++	+++

KEY: (+++) intensive growth,(++)moderate growth,(+)low growth,(-)no growth

Viability enumeration of the reconstituted yeast suspension showed that isolates SY, CY, BY and FY had the following average values; 30 x 10^4, 26 x 10^4, 21 x 10^4 and 23 x 10^4 cells units respectively as shown in Table 6.

Table 6: Viability of reconstituted yeast isolates

Replication	**CY(x10^4) cells unit**	**SY(x10^4) cells unit**	**BY(x10^4) cells unit**	**FY(x10^4) cells unit**
1	15	18	12	15
2	25	28	22	24
3	30	35	24	26
4	32	39	25	28
Average	26	30	21	23

IV. Discussion

In this work, three local materials namely shaddock juice, "burukutu" and "fura" were used as sources of *Saccharomyces cerevisiae* and after the enrichment and isolation the strains were observed to have some qualities better than the commercial yeast. The three isolated yeast strains were designated as SY from shaddock juice, BY from burukutu and FY from fura, while the commercial baker's yeast was termed CY.

The control bakers' yeast (CY) had the highest colony count followed by SY, FY and BY respectively (Table 1). These variations in colony count could be attributed to the effects of perishable parameters of the local food materials such as storage, processing, freeze-thaw, osmo-tolerance resistance and colour [15]. The various colony counts are indicative of the isolates' viability [16].

Result also showed that the isolates SY, BY and FY have rough morphology of fluffy colonies while that of the commercial yeast (CY) showed smooth creamy colonies (Table 1). These characteristics had been reported by other researchers like [17]. [18] reported that the colonies formed by cells of different yeast genera can be smooth, fluffy, rough, and creamy, depending on the ability of the particular yeast to form capsules or other extracellular matrix material, as well as on the capability of the cells to enter different stages of the life cycle for example, mating, sporulation or pseudohyphal growth.

From the microscopic observation, the ellipsoid or ovoid shapes with multipolar buds present in all the yeast isolates indicates effective budding capability of the various isolates and an indication of active fermentation [19]. During the fermentation process, yeasts produce carbon dioxide, ethanol and other secondary metabolites which contribute to flavor and aroma [10].

As shown in Table 2, all yeast isolates were able to grow intensively in a medium containing 10% (v/v) of ethanol. At 13% (v/v) of ethanol concentration, the isolates BY and FY grew moderately in the respective broth medium. In 15% (v/v) of ethanol, all the local yeast isolates (SY, BY and FY) including the commercial yeast (CY) also grew moderately. High concentration of alcohol is reported to be toxic to the yeast by inhibiting the cells growth due to the destruction of the cell membrane [20]. In the experiment carried out by [20], the

highest concentration of ethanol that the commercial yeast strain was able to survive was at 15% (v/v).This was in conformity with the study carried out in this work as all the isolates grew moderately in the same ethanol(15%(v/v)) concentration.

The flocculation abilities were also tested on the yeasts strains. According to [21] and [22], yeast cells which have ability to flocculate caused by cell adhesion process is an interesting characteristic in bread making and brewing industry. Results showed that all yeast isolates had the ability to flocculate moderately (i.e. moderate response)(Table 2). The flocculation characteristic was determined by yeast cells sticking together and provides easy separation from the broth medium. This phenomenon has an economic effect on the production of yeast biomass as it can reduce the energy cost during biomass centrifugation [23]. In addition, flocculation properties of *S. cerevisiae* ensure a high cell density and large volume of harvested cells and also able to raise the ethanol productivity during the fermentation process [24].

The isolated yeasts were also tested on their ability to ferment glucose, sucrose, maltose and fructose and to produce carbon dioxide (Table 4). Results showed that all isolates were able to ferment all sugars provided and releasing carbon dioxide gas as observed in durham tube. This could be an important indication of invertase activity.

Temperature tolerance and fermentative capacity tests were carried out on the different isolates in order to have a better understanding of their behavior. The temperature can affect the fermentation process and the metabolism of the yeasts. Table 3 illustrates the growth and the inhibition of the isolates at different growth temperature. All the isolates were able to grow at 37°C. SY, CY and FY showed more resistant at higher temperature of 45^{o}C compared to BY isolates (Table 3). Those yeast isolates which were able to survive at high temperature indicated that they may be used in bread making to speed up the proofing process, increased carbon dioxide production and formation of flavor and aroma may be enhanced.

The survival of yeast isolates under various stress conditions could provide useful information on their ability to grow and carry out fermentation as impaired yeast. The impaired yeast growth [25] during the fermentation usually does not grow in optimal conditions and continuously exposed to several stresses especially to osmotic and ethanol stress [26].

The statistical analysis of the mean values in the Tables obtained 95% confidence level illustrates no significant difference ($p<0.05$) between SY, BY and FY isolates with the commercial isolate (CY).

In the overall experiments, it was noticed that from the three (3) local yeast isolates, SY (shaddock yeast), showed better fermentative ability than *S. cerevisiae* commercial strains (CY), while isolates BY (burukutu yeast) and FY(fura yeast) showed equal or slightly less fermentative ability when compared with the commercial yeast isolates(CY). It was evident from this finding that the local materials which include "fura", shaddock and "burukutu" could be sources of new bakers' yeasts trains which can be potentially used as dough leavening agent for bread making and other commercial purposes.

The results obtained from this work lends support to the previous report that the abundant prevalence of wild yeast in local food materials such as indigenous alcoholic beverages [5] and fruits has provided one of the basic requirements in fermentation industries.

V. Conclusion

This study showed that yeast strains that can be used in place of baker's yeasts can be isolated from local materials such as "burukutu", "fura" and shaddock juice due to the fact that all the yeast isolates served a good purpose in their Flocculation abilities, their ability to ferment glucose, sucrose, maltose, fructose, ability to produce carbon dioxide, their temperature tolerance, fermentative capacities as well as their survival under various stress conditions. This will make for a cheap and inexpensive source of yeasts for various commercial production processes.

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Competing Interests

Authors have declared that no competing interests exist.

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