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Isolation and possible usage of Yeast strains from Indigenous Drinks for Possible Application in Brewing.

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Abstract
Saccharomyces cerevisiae is an organism used in brewing industries for wine and beer fermentation. Brewers source it basically from foreign suppliers which make the price of beer and wine too high for local consumers. This study was aimed at isolating and identifying yeast strains from indigenous/local drinks, enriching and utilizing them in beer brewing as a substitute to the imported brewer's yeast. The isolates, PWY from palm wine and BBY from *burukutu*, were enriched and tested for the characters necessary for beer brewing and compared with the commercial brewer's yeast (CBY) which was used in top fermented beer brewing. The tested characteristics include ethanol production, sugar fermentation and flocculation ability, stress tolerance and hydrogen sulfide production during fermentation. Results showed that the isolates neither produced hydrogen sulfide gas nor ferment lactose and raffinose but were able to ferment the other sugars tested. Flocculation ability was high and they grew well in the presence of high ethanol in the fermenting mash even more than 15%(v/v). They were all viable at different degree of 96.6%, 83.0% and 91.0% for PWY, BBY and CBY respectively. The *Saccharomyces* spp isolated grew well between 37ºC and 45ºC for PWY and BBY respectively while CBY showed no growth at higher temperature of 45ºC. The three isolates showed good tolerance to different stress conditions of high temperature, ethanol and sugar conditions. These local drinks can be useful if utilized for *Saccharomyces cerevisiae* isolation and enrichment for use in brewing industries as a substitute to the imported brewer's yeast.

Keywords: Palm wine, Burukutu, Brewing industry, Beverages, *Saccharomyces cerevisiae.*

Introduction
Beer is a common drink used in many countries during ceremonies and entertainments. They are used majorly during celebrations such as traditional marriages, naming ceremonies etc. It is produced through a method of fermentation in which fungi especially yeast utilize sugars in the 'must', to yield alcohol and carbon dioxide gas (Michael, 2004). The yeast species used in beer brewing are *Saccharomyces cerevisiae* for ale beer and *Saccharomyces pastorianus* for lager beer. Several strains of indigenous yeasts capable of producing beer have been isolated from different local sources such as fermented foods and fermented pineapple juices, but in most of the studies, the preferred type for industrial production of beer has been *Saccharomyces cerevisiae* (Okunowo *et al.*, 2005, Umeh *et al*., 2015). These yeasts can produce beer with no contamination by other products present in the substrate (Brooks, 2008). Both species of the S. *cerevisiae* and commercial yeast strains are capable of transforming a carbohydrate source into alcohol and adding some essential sensory attributes such as aroma and taste the product beer (Michael, 2004).
Yeasts are groups of microorganisms in the fungal division of Ascomycota. They have been known for decades for their role in fermentation processes as in alcoholic beverages and in baking industries (Agwuna *et al.,* 2019). Scientists started the isolation of fungi from different sources and their application in different industries after their identification and isolation by Louis Pasteur (Umeh *et al*., 2015). In this recent time researchers have used that scientific knowledge to isolate, characterize and utilize them in industrial processes such baking, brewing etc.

Alcohol in beer and other beverages are beneficial to human health. It is encouraged that consumption of small quantity which can be obtained from beer can help in alleviating some health challenges such as cardiac disease, stroke and diabetes mellitus (Brooks, 2008, Agwuna *et al*., 2019). Beer is a very important drink in Nigeria where it is used in almost all ceremonies like weddings, entertainment of visitors, parties, child dedications etc. they are sold in restaurants, canteens, shops and other small eateries where they serve as employment and source of income to many.

Local beverages such as palm wine, *burukutu*, fura, pito, nunu etc are produced and consumed in very large quantities by many tribes in Nigeria (Agwuna *et al*., 2019). They contain nutritionally important nutrients including amino acids, proteins, vitamins and sugars (Odibo *et al*. 2002). This makes them nice nutrient and adaptive for consortium of microorganisms. Many researchers have indeed carried out studies aimed at isolating and exploiting yeasts from agricultural products in industrial processes like in bakery, ethanol production and single cell protein production (Rosa, 2000; Okoro, 2007). Brewer's yeast have certain qualities like ethanol and acid tolerant, stress resistant, low or no production of hydrogen sulfide and the ability to flocculate (Agu *et al*., 1997). If these yeasts from indigenous drinks can be enriched to poses these characteristics, they will compete well with the commercial yeasts and be sourced locally thereby reducing importation costs and promote the country's economic reserve.

Brewing industries in Nigeria depend mainly on imported yeast strains for their brewing purposes. These imported strains most times are not viable when reconstituted there by creating loss to the producers. It will be of utmost gain that isolates from specific regions be used in that region for easy adaptation to their own climate conditions. These specific strains may even have their associated characteristics which they can confer to the particular beer produced in that regions. The industry in Nigeria is also far, too expensive to manage using imported yeast strains and other materials making the product very expensive for most families.

In Nigeria, large quantities of fermented drinks that are perishable are produced daily. High rate of wastage of these drinks lead to a search for other means of diverting their usage. Nigerian economy demands that simple inexpensive processing techniques be carried out in the production of commodities to satisfy every household. If this is achieved in the production of beer, it will be cheap and affordable. Isolating the *Saccharomyces cerevisiae* from local materials will reduce the cost of brewer's yeast as well as reduce drastically the cost of beer.

The work is done to seek for a substitute to overcome the scarcity of brewer's yeast that is making beer produced in Nigeria costly. The two local drinks were purchased aseptically and the yeasts isolated from them. The yeasts were characterized and tested for their abilities to serve as brewer's yeast. From outcome of the research, they are confirmed to be useful in beer production.

Materials And Methods

Sample collection

Using sterile plastic bottles *Burukutu* (indigenous sorghum beer) was bought from a local seller at 82 Division, Palm wine was purchased from a local market in Ezagu, L.G.A. all in Enugu State. They were transported in an ice bag to the Department of Applied Microbiology and Brewing Laboratory of Nnamdi Azikiwe University, Awka. Commercial brewer's yeast (CBY) was collected from Pal Breweries Oko, Aguata LGA, Anambra State, Nigeria which offered them as a bottle of freshly produced Stout beer.

Preparation of inoculums

The method of (Agu *et al*., 1997) was used for the inoculums build-up. From fermenting beverages, held at 4^{o}C, 1 ml each was withdrawn from the upper layer and plated out on a semi-solid Sabouraud dextrose agar (SDA) plate using spread plate method. The method Umeh *et al.* (2013) was used to isolate the yeast strains from the drinks. From the growth on the spread plate, distinct colonies were streaked on SDA plate prepared from the media containing chloramphenicol (to inhibit bacterial growth). Incubation was done at room temperature for 2 days. The colonies from the SDA plate were streaked on fresh plates of Yeast Peptone Dextrose (YPD) agar to obtain their pure colonies. The pure cultures were stored on slant and stored at 4^{o}C. The method for reconstituting the control yeast (Commercial brewer's yeast) was the method used by Giusto *et al.* (2006). The yeast was isolated from a freshly produced bottle of stout after centrifuging in 4000 rmp for 20 minutes. The supernatant was discarded and the sediment streaked on SDA plate at 30^{o}C for 48 hours and a colony was further streaked on YPD agar plate to get a pure culture.

Characterization and Identification of the isolates

The isolated organisms were characterized and identified based on their colonial morphology, cell features, ascospore formation, vegetative structure, sugar fermentation and alcohol resistance. The organisms were further identified by comparing them with the structures in the Fungal atlas (Barnett *et al*., 2000).

Microscopic examination of the isolates

A drop of sterile distilled water was placed on a sterile grease free slide and one loop-full of each stored isolates was smeared until it dried off. The smear was stained with dilute methylene blue dye, air dried and viewed under light microscope using 100 x magnification (Thias *et al*., 2006)

Stress tolerance test

Ability to survive under stress was checked using the method described by Agwuna *et al*., (2019). This was carried out by continuously growing the isolates on different media at varying Temperature, Sugar and Ethanol conditions for three weeks.

Ability to survive in different ethanol concentrations and at varying temperatures.

The method of Thais *et al*., (2006) was used to check the growth of the isolates on the media with high ethanol concentrations, sugars and at varying temperatures in the fermenting mash.

Ability to produce Hydrogen sulfide.

The ability of the isolates to produce hydrogen sulfide (H_2S) was checked by culturing them on an agar plates containing lead acetate. The composition of the medium was 40 g/L of glucose, 5 g/L of yeast extract, 3 g/L of

peptone, 0.2 g/L of ammonium sulfate, 1 g/L of lead acetate and 20 g/L of agar. The medium components were mixed, autoclaved at the appropriate temperature and cooled. After pouring and setting, the plates were inoculated with the isolates and incubated at 30ºC for 10 days (Ono *et al*., 1991).

Ability to flocculate.

For this test, the isolates were inoculated into 10 ml YPG broth and incubated at 30ºC for 3 days. After incubation, tubes were agitated to observe the flocculates formed (Thais *et al*., 2006).

Sugar fermentation test

Sugar fermentation ability of the isolates was checked as described by (Thais *et al*., 2006). Yeast fermentation broth (YFB) made of Peptone 7.5 g/L, yeast extract 4.5g/L; 1ml of 1.6% (w/v) bromothymo-blue as an indicator, 6% (w/v) of each sugar solutions of glucose, sucrose, fructose, maltose, lactose and galactose prepared in different test tubes. Raffinose was mixed at a double concentration. Durham tubes were placed into each test tube to trap the carbon dioxide released, all were autoclaved at 100ºC for 10 minutes. After cooling, the isolates were inoculated into the test tubes and incubated at 30ºC for 72h. The fermented broth were green in color and turned to yellow (acidic) or blue (alkaline) if the yeast cells have the ability to ferment the respective sugar.

Results

From the result of stress exclusion test, the isolates PWY and BBY grew better in harsh environment than the control CBY (Commercial brewer's yeast) as shown in Table 1. The result of sugar fermentation test as shown in Table 2 indicated that the isolates fermented all the sugars tested on except lactose and raffinose.

The PWY and BBY showed better ethanol tolerance than the control CBY (Table 3). At temperatures between 25ºC to 37ºC the isolates show good growth but at 45ºC, the PWY showed moderate growth, the BBY showed low growth while the CBY had no growth (Table 3).

Morphological features of the isolates are as shown in Table 4, with PWY being creamy, BBY fluffy and CBY smooth. Also Table 4 showed positive result for flocculation and no yield of hydrogen sulfide. Colony count on SDA as presented in Table 4 showed that PWY has the highest colony count followed by CBY and then BBY.

Table 1: Stress tolerance tests of the isolates at varying temperatures, ethanol and sugar concentrations.

	Growth onto different media				
Yeast isolate	**YPG medium**	**Temperatures 37ºC**	**Ethanol (8%, v/v)**	**YPG + (Glucose 20%, w/v)**	**YPS (sucrose20%, w/v) + ethanol, 8% v/v)**
PWY	+++	+++	+++	+++	+++
BBY	+++	+++	+++	+++	+++
CBY	+++	+++	+++	+++	++

Key:
Intensive growth (+++), moderate growth (++), low growth (+), no growth (-)
YPG- yeast peptone glucose agar
YPS- yeast peptone sucrose agar

Table 2: Sugar fermentation abilities of the three isolates.

Yeast strain	glucose	maltose	fructose	sucrose	lactose	galactose	raffinose
PWY	+	+	+	+	-	+	-
BBY	+	+	+	+	-	+	-
CBY	+	+	+	+	-	+	-

Key: fermented the sugars (+); No fermentation (-)

Table 3: Ethanol and temperature tolerance test.

Yeast strains	Ethanol tolerance			Temperature (ºC)			
	10%	13%	15%	25	30	37	45
PWY	+++	+++	+++	+++	+++	+++	++
BBY	+++	+++	++	+++	+++	+++	+
CBY	+++	++	+	+++	+++	+++	-

Key:
Intensive growth (+++), Moderate growth (++), Low growth (+) and No growth (-)

Table 4: Morphological characteristics of the colonies, flocculation ability, hydrogen sulfide production and colony count of the isolates on SDA.

Colony characteristics				Flocculation	hydrogen sulfide	yeast count (cfu/ml)
Yeast strain	**Smooth**	**Fluffy**	**Creamy**			
PWY	-	-	+	+	-	3.7×10^{-6}
BBY	-	+	-	+	-	3.4×10^{-6}
CBY	+	-	-	+	-	3.6×10^{-6}

Key:
Response (+), no response (-)

Discussion

The two *Saccharomyces cerevisiae* isolated in this research work, PWY and BBY, were characteristically identical with the commercial brewer's yeast (CBY). The control yeast strain CBY (Brewer's yeast) had the yeast cell count of 3.6 x 10^{-6} cfu/ml; PWY had the highest count of 3.7 x 10^{-6} cfu/ml while BBY had the lowest count of 3.4 x 10^{-6} cfu/ml. The difference in colony counts may be due to processing technique and osmo-tolerance resistance (Pattison and Von-Holy, 2001). The low count of the BBY strain could also be as a result of the fermentative processing of the drink employed by the producer. The high plate counts of PWY and BBY in this study showed that they are viable and active in the beverages after production. The ability of isolating these yeast strains from the local drinks and their subsequent identification as *Saccharomyces cerevisiae* have confirmed earlier reports that fermenting agricultural products are good sources of *Saccharomyces cerevisiae* to be used in different food industries (Jideani *et al*., 2001, Umeh *et al*., 2013). The finding is also in agreement with the report Agu *et* al., (1997) who postulated that 89-92% of the total *Saccharomyces* species are *Saccharomyces cerevisiae* and that palm wine is its major source. PWY and BBY showed similar growth and biochemical characteristics to CBY except that in their colony morphology, PWY was creamy and BBY was fluffy while CBY was smooth. Similar features were found by Kevin, (2005) who observed that typical colonies of *S. cerevisiae* could be creamy, smooth or fluffy with regular shape. In another research carried out by Cavalieri *et al*. (2001) and Kuthan *et al*., (2003) it was shown that the appearance of some *S. cerevisiae* strains can be fluffy especially when they are isolated from wild sources. These differences in appearance are much dependent on the nature of substrate, environmental conditions and the shape of the cells within the growth colony (Querol *et al*., 2003). The isolated yeasts survived for three weeks in a continuous cultivation showing that they can tolerate stress. When the isolates will be used during beer fermentation, they will not find an environment of optimal conditions, because they had survived several stress conditions (Pataro *et al*., 2000). Also in this study, the isolated *Saccharomyces cerevisiae* were able to grow on the medium (YPG) containing up to 8% (v/v) of alcohol, 20% (w/v) of glucose and 37°C incubation temperature. The ability of the isolates to withstand stress can be attributed to the nature of the expressed gene which can lead to complications during the process of fermentation (Zuzuarregi and Olmo, 2004). Glucose as the required substrate in the growth of this yeast strains can antagonize the genes that code for enzymes (invertase) metabolism. This antagonistic reaction can be a better condition for the brewing of beer leading to beneficial activity of the enzymes (Gancedo, 1998, Zuzuarregi and Olmo, 2004). In the result of the fermentation of different sugars, the three colonies studied ferments all the sugar except lactose and raffinose (Table 2) showing that the two species has the same sugar fermentation capacity as the commercial strain. Ethanol stress is among the growth features checked in this research because of its high amount produced during the beer fermentation process (Stratford, 1992, Chi and Ameborg, 2000) and the isolated yeasts grow well and survived high concentrations of ethanol even in broth. Temperature affects the fermentation kinetic and the rate at which the organism metabolize its substrate. This helps in the determination of the beer chemistry, sensory characters and composition of the brew. The *Saccharomyces cereviciae* isolated from this research was capable of surviving temperature changes in the medium. Table 3 showed that the isolates were able to grow at varying concentrations of ethanol and different temperatures. This is among the desired qualities of brewer's yeast that produces acceptable beer.

Flocculation is the act of clumping together of the cells for easy separation after brewing with no further need to filter or centrifuge the beer after brewing. This is one of the reasons some industries prefer using yeasts entrapped to a support for brewing activities (Ribeiro and Horii, 1999, Cavalieri *et al*., 2001). All the isolates showed good flocculation ability as presented in Table (4) and therefore are suitable for fermentation of top-fermented beer (Ale beer). Yeasts that can produce hydrogen sulfide are undesirable for beer production because it gives undesirable beer flavor and other bad qualities in the beer that generate low quality drink (Kuthan *et al*., 2003; Cavalieri *et al*., 2001; Stratford, 1992). Thus the yeasts were tested for hydrogen sulfide production as shown in Table 4 and none of the isolates produced hydrogen sulfide, thus the isolates are suitable for use in the brewing of beer.

Conclusion

This study showed that *Saccharomyces cerevisiae* isolated from these indigenous/local drinks possess attributes necessary for beer production. The present study also provided information on the isolation and enrichment of *S. cerevisiae* from local drinks for industrial applications. Although this investigation cannot be considered to be exhaustive, the results obtained show that yeast from local drinks can compete favorably with brewer's yeast for application in beer production. The isolates from this study can be utilized in the production of ale (top- fermented beer) and not in the lager (bottom-fermented) beer because they were able to flocculate.

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