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Novel Sources of Saccharomyces Species as Leavening Agent in Bread Making

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ABSTRACT
Mega biodiversity of Nigerian's flora and fauna which include microorganism could be conserved and served as alternative source for baker's yeast, the leavening agent in bread making. This study was conducted in attempt to exploit the potential of *Saccharomyces cerevisiae* strains isolated from two local beverages ("burukutu" and "fura") and a tropical fruit (shaddock) as a leavening agent in bread making. The enrichment was carried out by fermenting the samples (burukutu, fura and Shaddock juice) in medium containing sterilized grape must at 30°C for 3 days followed by isolation of tentative yeasts at 30°C for 3 days. The samples tested showed the presence of yeasts and thirty (30) isolates from the three samples was selected for identification of *S. cerevisiae* strains through biochemical and physiological tests. Of the thirty yeast strains identified, 12 strains were chosen as baker's yeast *due* to their potential to tolerate ethanol concentration of up to 13 % (v/v), grow at temperature of 30 - 45°C and low or no hydrogen sulphide production. They are able to flocculate intensively, grow at high stress levels and even showed better fermentative performance compared to commercial baker's yeast (CY). These attributes thus indicate that the local beverages and fruits could be potential sources of indigenous *S. cerevisiae* strains for leavening agent in bread making and for other confectionary industries.

Keywords: *Grape fruits, Burukutu, Fura, Shaddock, Bread making, Saccharomyces cerevisiae, leavening agent*

INTRODUCTION

As a predominant species it is well known that *Saccharomyces cerevisiae* plays an important role as a leavening agent in bread making. The leavening step is essential in the fermentation of dough. It does not only induce and increase the volume of dough through gas incorporation but helps creating the desired flavor and texture (Fleury *et al*., 2002). Previous researcher have argued that *S. cerevisiae* does not live in other environment except nature, but can be found only in vinery environment as a wild or domesticated species (Martini *et al*., 1996). In many instances, the strains are clearly specialized for use in the laboratory (Mortimer and Johnston, 1986) and in the production of wine (Kunkee and Bisson, 1993), beer (Hammond, 1993) and bread (Rose and Vijayalakshmi, 1993). This has supported the common view that *S. cerevisia*e is a domesticated species that has continuously evolved in association with the production of alcoholic beverages (Mortimer, 2000; Martini, 1993; Naumov, 1996). Nowadays there are controversial arguments regarding the nature of *S. cerevisiae*. Kurtzman and Fell (1998) reported that fruits, vegetables, drink and agriculture products are among important microhabitats for yeast species in the wild. Adequate work has been reported to look at the potential of indigenous *S. cerevisiae* as leavening agent during bread making from local sources.

The leavening agents (yeasts) currently used by bakery industries in Nigeria are mostly imported from Europe and America. Thus, in this work the presence of yeasts from Nigerian local beverages and fruits are exploited, thus S. *cerevisiae* strains isolated from them was characterized to be used as baker's yeast as leavening agent in bread making.
"*Burukutu"* and "*Fura"* are two cheap beverage drinks that are produced by rural women using their local technology. These beverages are produced and consumed all year round and are always available. They are produced from local sorghum and millet (Sorghum beer and Millet meal) respectively. They cannot be preserved for a long time even in the refrigerator due to their perishable nature. On the other hand, shaddock fruits are wild fruits from wild grape that has no recognizable use attributed to it. It ripes and wastes in the forests. Due to the report of Kurtzman and Fell (1998) that fruits, vegetables, drink and agriculture products are among important microhabitats for yeast species in the wild, the shaddock fruits were harvested, peeled and their juice extracted and fermented to check if they contain wild yeasts specifically *Saccharomyces cerevisiae.* Having isolated the yeasts from these local sources, they were enriched and tested for their ability to serve as baker's yeast. The isolated yeasts were compared with the attributes of commercial baker's yeast and found to be very close and even batter than the imported yeasts. They are fresh and directly isolated but the imported yeasts had stayed for so long before reaching the bakers. This work has recommended the isolation and enrichment of yeasts from indigenous products to be used in our baking industries as this will encourage our local production of bread and other confectionaries.

MATERIALS AND METHODS

SAMPLE COLLECTION

Millet meal (Fura), Sorghum beer (Burukutu), Commercial bakers' yeast (*Saccharomyces cerevisiae*), flour and other baking ingredients were purchased from Eke-Awka market, Anambra State using sterile plastic containers and were transported to the laboratory. Shaddock fruits were harvested from a farm at Umuzocha Village Awka and identified by a botanist in the Department of Botany, Nnamdi Azikiwe University Awka, Anambra State, Nigeria. Other chemicals and reagents used were obtained from the Department of Applied Microbiology and Brewing of the same institution and are of analytical grade.

PREPERATION OF SAMPLES

The shaddock fruits were washed with water containing sanitizers and peeled. The juice was aseptically extracted using a hand juice extractor, filtered with muslin cloth and the filtrate collected in a sterile plastic container.

ISOLATION 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). All the micro-fermentations were carried out at 27°C in 100ml Erlenmeyer flasks containing 50 ml sterilized grape must (Thais *et al.,* 2006) 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 dilution was inoculated onto Sabouraud dextrose agar (SDA) 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 Martini *et al.* (1996). Tentative yeast isolates from the SDA plates of $10^2$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 incubated at 30°C for 3 days. Representative colonies were picked from the plates and their pure cultures preserved in slants and stored in the refrigerator at 4^0C.The yeast isolates were coded as follows;

- **SY** (shaddock yeast)
- **BY** (burukutu yeast)
- **FY** (fura yeast) and the commercial baker's yeasts were designated as **CY**.

MICROSCOPIC OBSERVATION OF THE YEASTS MORPHOLOGY

A loop full of each colony of the isolates 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 with dilute methylene blue dye, air dried and observed under light microscope at 100 X magnification according to Thais *et al.,* 2006.

ETHANOL TOLERANCE OF THE ISOLATES

The ability of the isolates to grow in higher ethanol concentrations was tested by growing them in Yeast peptone glucose(YPG) broth containing three different concentration of ethanol (Thais *et al.*, 2006). A loop full of each isolate 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.

TEMPERATURE TOLERANCE OF THE ISOLATED YEASTS

The ability of the yeast isolates to grow at higher temperatures was verified by plating the yeast isolates onto YPG medium and incubated at three different temperatures (Thais *et al.,* 2006). A loop full of each isolate was streaked on a freshly prepared and dried YPG medium and incubated at three different temperatures of 27°C, 37°C and 45ºC and observed for 72 hours.

HYDROGEN SULFIDE PRODUCTION TEST

The ability of the yeast to produce hydrogen sulphide (H_2S) was examined by growing the yeast isolates on lead acetate medium (40 g/L glucose, 5 g/L yeast extract, 3 g/L peptone, 0.2 g/L ammonium sulfate, 1 g/L lead acetate and 20 g/L agar) and incubated 30ºC for 10 days (Ono *et al*., 1991).

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 (Thais *et al.,* 2006). A loop full of each isolates was inoculated into a freshly prepared YPG broth and observed for 72 hours after which the tubes were agitated to observe flocculation formation.

STRESS EXCLUSION TEST

Stress exclusion test was conducted as described by Thais *et al.,* (2006). The ability of the isolates to grow at different stress conditions was done for 15 days' incubation onto different media. This was done by first 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 at 30ºC for 3 days, then on YPG medium containing 8% (v/v) ethanol, YPG with 20% (w/v) glucose and then in YPG medium supplemented with 2% (w/v) sucrose and 8% (v/v) ethanol and incubated under the same conditions.

FERMENTATIVE CAPACITY OF THE ISOLATED YEAST STRAINS

In this test, the fermentative capacity media was prepared and the test was conducted as described by Atlas and Parks (1996). Prior to yeast cells growth into a freshly prepared Yeast fermentation broth (YFB) containing Peptone 7.5 g/L, yeast extract 4.5g/L; 1ml of 1.6% (w/v) bromothymo blue as an indicator, 6% (w/v) each of glucose, sucrose, fructose, galactose, raffinose, lactose and maltose were autoclaved separately. The yeast cells were grown at 30°C for 3 days. The YFB was added with respective sugar, and then yeast cells were examined on the fermentative ability of the different carbon sources. The Durham tubes were also placed into the media to trap the carbon (iv) oxide 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.

RESULTS

The following results were obtained from the study. Table 1 showed the effect of ethanol concentrations, different temperatures, flocculation ability and Hydrogen sulphide production of the isolated yeasts revealing their potential as leavening agent. Table 2 presents the ability of the isolates to grow on different media and Table 3 showed the fermentative capacity of the yeast on different carbon sources.

Table 1: Effect of ethanol concentrations, temperatures, flocculation and hydrogen sulfide production of *Saccharomyces cerevisiae* strains

Yeast Strains	Ethanol Concentration (V/V)			Temperature °C				Flocculation	Hydrogen Sulfide Production	Potential To Be Employed As Leavening Agent
	10%	13%	15%	25	30	37	45			
BY2	+++	++	-	+++	+++	+++	+++	+++	-	Yes
BY3	+++	++	-	+++	+++	+++	+++	+++	-	Yes
BY6	+++	++	-	+++	+++	+++	+	+++	+	Yes
BY8	+++	++	-	+++	+++	+++	+	+++	+	Yes
FY4	+	+	-	+++	+++	++	-	+++	+	Yes
FY7	++	+	-	+++	+++	++	-	+++	+	Yes
FY8	++	+	-	+++	+++	++	-	+++	+	Yes
FY9	+++	++	-	+++	+++	++	+++	+++	-	Yes
SY1	+++	++	-	+++	+++	++	++	+++	-	Yes
SY3	+++	+++	-	+++	+++	+++	-	++	+	Yes
SY6	+++	+++	-	+++	+++	+++	-	++	+	Yes
SY10	+++	+++	-	+++	+++	+++	+++	++	-	Yes
CY	+++	++	-	+++	+++	+++	+	++	-	Yes

Key: **Intensive response** (+++);**moderate response** (++);**low response** (+);**no response** (-);**CY**- *Saccharomyces cerevisiae* commercial as control **YPG** – yeast peptone glucose medium **YPS** – yeast peptone sucrose medium **Yes** - potentially use as leavening agent **No** – could not being use as leavening agent

Table 2: Stress exclusion tests on isolated yeasts for temperature and cell osmotic pressure in high concentration of ethanol and sugar

Yeast strains	Source	Growth on different Media Yeast strains					
		YPG	Temperature 37°C	Ethanol (8%, v/v)	YPG (20%, v/v)	YPS (20%, w/v) + ETOH (8%,v/v)	Potentially use as leavening agent
BY2	Burukutu	+++	+++	+++	+++	++	Yes
BY3	Burukutu	+++	+++	+++	+++	+++	Yes
BY6	Burukuru	+++	+++	+++	+++	+++	Yes
BY8	Burukutu	+++	+++	+++	+++	++	Yes
FY4	Fura	+++	+++	+++	+++	+++	Yes
FY7	Fura	+++	+++	+++	+++	++	Yes
FY8	Fura	+++	+++	+++	+++	++	Yes
FY9	Fura	+++	+++	+++	+++	+++	yes
SY1	Shaddock	+++	+++	+++	+++	++	Yes
SY3	Shaddock	+++	+++	+++	+++	+++	Yes
SY6	Shaddock	+++	+++	+++	+++	++	Yes
SY10	Shaddock	+++	+++	+++	+++	+++	Yes
CY	Commercial yeast	+++	+++	+++	+++	+++	yes

KEY: **Intensive response** (+++);**moderate response** (++);**low response** (+);**no response** (-);**CY**- *Saccharomyces cerevisiae* commercial as control **YPG** – yeast peptone glucose medium **YPS** – yeast peptone sucrose medium **Yes** - potentially use as leavening agent **No** – could not being use as leavening agent

Table 3 Fermentative capacity of the isolated yeasts

Yeasts strains	Car Glucose	bon sources Maltose	and carbon dioxide relea Fructose	Sucrose	se in each sugar fermentationa Lactose	Galactose	Raffinose	Yeast strains identified as Saccharomyces cerevisiae
BY2	+	+	+	+	-	+	+	Yes
BY3	+	+	+	+	-	+	+	Yes
BY6	+	+	+	+	-	+	*+	Yes
BY8	+	+	+	+	-	+	+	Yes
FY4	+	+	+	+	-	+	*+	Yes
FY7	+	+	+	+	-	+	+	Yes
FY8	+	+	+	+	-	+	+	Yes
FY9	+	+	+	+	-	+	+	Yes
SY1	+	+	+	+	-	+	+	Yes
SY3	+	+	+	+	-	+	+	Yes
SY6	+	+	+	+	-	+	*+	Yes
SY10	+	+	+	+	-	+	+	Yes
C.Y cerevisiae	+	+	+	+	-	+	+	Yes

Key: [a]Assimilate carbon and simultaneously release of carbon dioxide indicated (+); did not assimilate carbon source and not release carbon dioxide indicated as (-) Acidic fermentation (Broth turn into yellow), *Alkaline fermentation (Broth turn into blue), **Yes** - potentially use as leavening agent , **No** – could not being use as leavening agent

DISCUSSIONS

Baker's yeast is one of the most important ingredients in baking industries. Without it the fermentation and leavening of the dough will be impossible. The yeasts are imported into Nigeria from foreign countries and are most times costly. Since researcher had found out that our indigenous beverages and ripe fruits are mostly fermented by yeasts and other organisms, it is very needful that those yeasts to be isolated and tested for use in different food industries.

Two local beverages and a fruit sample were examined for the presence of indigenous *Saccharomyces cerevisiae*. Since the media used to isolate the yeast strains were selective for isolation of these specie only 30 colonies of tentative yeasts was isolated. The isolation of *S. cerevisiae* in the study was carried out using sterilized grape must (GM) broth and followed by sub culturing in SDA. Biochemical and physiological qualities are the important attributes of yeast strains to be used as baker's yeasts. During the course of this study, the isolated yeasts were characterized and enriched for their suitability as baker's yeasts. The 30 colonies of yeasts isolated possess the morphological features of *S. cerevisiae* species but 12 showed the attributes of baker's yeast as shown on the tables of results above. Of the 30 yeast colonies, 18 isolates showed opaque, smooth, fluffy, regular colony and creamy in colour while yeast strain isolated from BY2 and BY3 showed similar characteristics except they were rough compared to the others and the commercial *S. cerevisiae*. Similar finding was observed by Kevin (2005) who reported that typical *S. cerevisiae* colonies can be creamy or rough and regular in shape. Colony formed by cells of wild *S. cerevisiae* showed a fluffy morphology as reported by Kockova (1990), Cavalieri *et al*. (2001) and Kuthan *et al*. (2003). The morphological differences of yeasts colonies were very much depending on the absence of an extracellular matrix, the compactness of colony and the differences of cell shape within the colony (Kuthan *et al*., 2003).

The cells morphology of thirteen yeast colonies observed under a light microscope with 100X magnification showed an ellipsoid or ovoid shape which conformed to the characteristic of *S. cerevisiae* isolated by Greame and Nia, (2005). Ten of the yeast colonies have similar capability of budding (budding ascus formed), however, yeast strains of SY1 and SY10 (isolated from shaddock) showed extra capability of budding which indicated a higher growth rate when compared to other strains including the commercial yeast but lack other characteristics of bakers yeasts. With these characteristics shown, these strains should be able to perform a vigorous fermentation, according to Hough *et al*. (1971).

A suitable concentration of alcohol is needed in bread making in order to achieve the preferred flavor. As shown in Table 1, all yeast strains were able to grow in a medium containing 10% (v/v) of ethanol and 13% (v/v) of ethanol concentration, while all the yeast strain including the commercial yeast strain showed no growth at 15% ethanol concentration. 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 (Ingram and Buttke, 1984). In this experiment, the highest concentration of ethanol that the commercial yeast strain was able to survive was at 13% (v/v). Those strains which were capable to grow in similar concentration were expected to have ability to produce similar quality of bread as commercial strain.

The selected yeast strains were also tested for their growth at high temperature (Table 1). Most of the yeast strains could tolerate the temperature up to 37°C. Yeast strains of SY1 and SY10 (isolated from shaddock), FY9 (isolated from fura), BY2 and BY3 (isolated from burukutu) and CY (commercial yeast strain) were able to grow at higher temperature of 45°C. Therefor it can be concluded that the yeasts isolated from the beverages and fruit were able to grow at high ethanol concentration (13%) and high temperature of 37°C and 45°C.

The flocculation abilities were also tested on the yeasts strains. According to Amri *et al*. (1982) and Miki *et al*. (1982), yeast cells which have ability to flocculate caused by cell adhesion process is an interesting characteristic in bread making and brewing industry. Results showed all the chosen strains isolated from "burukutu" and "fura" showed intensive flocculation, those from shaddock and the commercial yeast strain (CY) exhibited moderate flocculation as shown in Table1. 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 because it can reduce the energy cost during biomass centrifugation (Iraj *et al*., 2002). In addition, flocculation properties of *S. cerevisiae* ensure a high cell density and large volume of harvested cells and also able to rise the ethanol productivity during the fermentation process (Kevin, 2005).

The production of hydrogen sulfide by twelve isolated *S. cerevisiae* strains was determined during fermentation of lead acetate medium (LA). Henschke and Lee (1994) reported that hydrogen sulfide production property was not useful for wine yeasts. Yeasts with elevated production of hydrogen sulfide are undesirable for bread making because it confers flavor and taste that compromise the quality of the bread. However, in this study all yeast strains produced hydrogen except commercial yeast (CY), BY2, BY3, FY9, SY1 and SY10 (Table 2). Since their hydrogen sulphide production were of low response and other attributes in bread making are acceptable, they can be used in the industry without their effect being noticed.

In stress exclusion test, thirteen yeast colonies including commercial baker's yeast were examined in order to select the strains which were able to adapt in bread making conditions. Using strains which were not effectively adapted to stress condition could be a mistake due to impossible settlement during fermentation process (Thais *et al*., 2006). In all stress analyses, the yeast cells were initially grown on YPG medium to ensure yeast were in the similar state condition, followed by growing onto medium YPG incubated at 37^{O}C, 8% (v/v) of ethanol, 20 % (w/v) of glucose and 20% (w/v) of sucrose with 8% (v/v) of ethanol. The yeast cells were grown continuously for 15 days to observe the cell viability due to each stress condition. Strain survival of baker's yeast under various stress conditions could provide useful information on its ability to grow and carry out fermentation as impaired yeast. The impaired yeast growth (Ivorra *et al*., 1999) during the fermentation usually does not grow in optimal conditions and continuously exposed to several stress especially to osmotic and ethanol stress (Querol *et al*., 2003). All the thirteen yeast strains including commercial yeast showed the necessary characteristics suitable for bread making as indicated in Table 2. Our findings were in agreement with that of Pataro *et al*. (2000) who reported that most of *S. cerevisiae* strains isolated from conventional

fermentation processes were physiologically adapted to extreme conditions. In this case the strains were able to grow on medium (YP) containing 20% (w/v) glucose and 8% (v/v) ethanol and incubated at 37°C. Since the yeast cells in bread making produce ethanol as secondary metabolites the ethanol stress were conducted to observe its tolerance to ethanol.

The primary role of baker's yeast in dough development, fermentative capacity using carbon source simultaneously with the production of carbon dioxide is important parameter for bread making (Benitez *et al*., 1996). All yeast strains tested were identified as *S. cerevisiae* due to its ability to ferment sucrose, maltose, fructose, glucose, galactose and raffinose but not on lactose (Thais *et al*., 2006). Table 3 shows the fermentative ability of thirteen *S. cerevisiae* strains including commercial strain using 7 different carbon sources. *S. cerevisiae* strains of BY2, BY3, FY9, SY1 and SY10 showed their ability to change the broth medium color from green to yellow (for sucrose, maltose, fructose, glucose and galactose) within 2-5 hour fermentation. But other strains took more than 24 hours incubation time to produce the similar results. This indicated that they went through acidic fermentation and was able to initiate the fermentation process faster than the commercial strain. The commercial *S. cerevisiae* strain was acidic fermenting yeast and the color changes took 18 hours of fermentation. In contrast, for carbon source of raffinose *S. cerevisiae* strains BY6, FY4, and SY6 showed the changes of color from green to blue, which indicated an alkaline fermentation. All yeast strains including commercial strain did not ferment lactose. Tarek (2001) reported the *S. cerevisiae* cells which were unable to ferment lactose were actually due to lack of lactase or β-galactosidase system. It was also showed that all yeast strains which utilized respective sugars also produced carbon dioxide (Table 3). The carbon dioxide released during dough fermentation process is prominent as a leavening agent of dough (Thais *et al*., 2006).

CONCLUSIONS

In conclusion, local beverages (burukutu and fura) and fruit (shaddock) could be sources of novel *Saccharomyces cerevisiae* which can be potentially used as leavening agent for bread and other confectionary makings in our food industries.

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