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Original article

Determination of fermentative properties of yeast strains isolated from spoilt fruits and beverages

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ABSTRACT

In this study, seven yeast isolates were obtained altogether from both spoilt fruit and brukutu alcoholic drink using the spread plate method. Yeast strains were isolated from burukutu alcoholic drink, spoilt pineapple fruit and spoilt watermelon fruit, confirmed using API Test kit and subjected to flocculation, ethanol tolerance, hydrogen sulphide, temperature tolerance test and stress tolerance test to determine their fermentative potentials. In all 7 species *Candida sphaerica*, *Pichiaspp*, *Candida guilliermondii*, *Candidafamata* *Candida pelliculosa*, *Cryptococcus humicola* and *Kloeckeraapiculata* were isolated. *Candida sphaerica*, *Candida famata* and *Kloeckeraapiculata* displayed the highest flocculation ability while only *Candida famata* and *Candida pelliculosa* survived 15% ethanol concentration. Hydrogen sulphide test showed that only *Candidafamata* and *Candida pelliculosa* can survive the presence of hydrogen sulphide. All yeast isolated grew very well at 25°C except *Pichia species* while only *Candida pelliculosa* grew at 45°C. All the seven yeast isolated survived the first and second stage of stress while only *Candida pelliculosa* survived the last stage.

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1. Introduction

Yeasts are simple eukaryotic and unicellular organisms classified in the kingdom Fungi with over 1,500 species known (Kurtzman et al., 1998). In wine and beer making, yeast is the catabolite that converts the simple sugars into ethanol and carbondioxide. The most common species used are *Saccharomyces* species. It has been known for a long time that freshly crushed grape juice harbours a diversity of yeast species, principally within the genera *Hanseniaspora* (anamorph *Kloeckera*), *Pichia*, *Candida*, *Metschnikowia*, *Kluyveromyces* and *Saccharomyces*. Occasionally, species in other genera such as *Zygosaccharomyces*, *Saccharomyces*, *Torulasporea*, *Dekkera* and *Schizosaccharomyces* may be present (Graham et al., 2008). It is also well known that many of these non-*Saccharomyces* species (especially species of *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia*) can initiate spontaneous alcoholic fermentation of the juice (Graham et al., 2008).

According to FAO (1998) fermentation is one of the most ancient and most important food processing technologies which had been neglected by scientists and policy makers especially in traditional fermented products from developing countries. Fermentation is a relatively efficient, low energy preservation process which increases the shelf-life and decreases the need for refrigeration or other form of food preservation technology. It is therefore a highly appropriate technique for use in developing countries and remote areas where access to sophisticated equipment is limited. Fermented fruits wines are popular throughout the world, and in some regions it makes a significant contribution to the diet of millions of individuals.

Individual yeast strains that carry out fermentation possess different physiological traits. There are several traits that are highly desired in wine strains of *Saccharomyces* (Jolly et al., 2003). The most important characteristic is that the strain be able to complete the fermentation, leaving little to no residual sugar. It is also critical that the strain display a reasonable rate of fermentation. A slow rate of fermentation becomes difficult to distinguish from problem fermentation. If the rate is too fast, the fermentation may reach too high of a temperature due to the rate of heat release from metabolism. Rapid fermentations may also lead to increased loss of volatile components (Jolly et al., 2003).

In Nigeria, there are several fermentative industries which depend on imported strains and with the cost of importation there is need to seek for indigenous strain of fermentative yeasts which can be a good substitute and most of them have not been well screened. The work is therefore aimed at isolation and characterization of yeast species from fruits and alcoholic beverages and screening them for their fermentative properties.

2. Materials and methods

2.1. Sample collection and preparation

Spoilt watermelon (*Citrulus vulgaris*) and spoilt pineapple (*Ananascomosus*) fruits were collected from Samaru market in Zaria metropolis in Kaduna State from which the yeasts used in fermentation were isolated. Burukutu alcoholic drink was also purchased in Zaria metropolis in Kaduna State, Nigeria. It was collected aseptically using a clean bowl and transferred aseptically into sterile plastic containers that had already been autoclaved in the laboratory with fitted seal. The containers were then well sealed, labelled and transferred to the laboratory in sterile polythene bags.

All glass wares were washed with detergent, rinsed severally in clean water, and sterilized in hot air oven at 180°C for isolation, identification and growing of the yeast cells. The plastic containers, stainless steel trays, kitchen knife and the warring blender that were also used in the research were thoroughly cleaned while the work bench were swabbed with cotton wool soaked in absolute ethanol. Spoilt pineapple and watermelon fruits used were washed with potassium metabisulphite solution and rinsed aseptically then they were peeled and sliced aseptically with sterile knife.

2.2. Isolation of yeast strains

One each of spoilt pineapple and watermelon fruits samples was taken and 10g of aseptically cut fleshy part of the spoilt fruit sample was smashed and mixed in sterilized nine milliliters peptone water then a serial dilution was made up to 10^{-5} , 0.1ml of the last dilution was cultured by spread plate method on Malt yeast extract agar also 1ml of burukutu alcoholic drink was taken and mixed with sterilized nine milliliters peptone water then a serial of dilution was also made up to 10^{-5} , 0.1 ml of the last dilution was then cultured by spread plate method on Malt

yeast extract agar. The inoculated plates were incubated for 48h at 30°C (Panneerselvam et al., 2011). Typical yeast colonies were subcultured on malt yeast extract agar plates and incubated at 30°C for 48h to check its purity. Purified cultures were maintained on malt yeast extract agar slants and kept at -4°C for subsequent identification.

2.3. Characterization of yeast strains

Pure culture of the yeast isolates were characterized using cultural, morphological, biochemical test and confirmed using API test kit known as API 20 C identification kit.

2.3.1. Cultural characterization of yeast isolates

The cultural properties of the yeast isolates were determined using the scheme of Kurtzman et al. (2011). Cultural characteristics such as texture (whether mucoid, fluid or viscous, butyrous, friable or membranous), colour, surface appearance (whether glistening or dull, smooth, rough, sectorized, folded, ridged or hirsute), elevation (whether flat, depressed in the centre, raised and dome-like or conical) and margin (whether the edge of the colony is entire, undulating, lobed, erose or fringed with hyphae or pseudohyphae) were observed.

2.3.2. Morphological characterization of yeast isolates

A single colony of yeast isolate was mixed in a drop of distilled water placed on glass slide and smeared and allowed to dry off. The smear was then stained using diluted methylene blue dye, air dried and observed under light microscope at 10x, 40x and 100x objective with oil immersion (Noroul et al., 2013). The shape and size of the vegetative cells were observed such as globose, subglobose, ellipsoidal, ovoidal, cylindrical, elongated, apiculate, lunate and triangular also the mode of conidia formation, position and site of bud formation were observed.

2.3.3. Biochemical characterization of yeast isolates

2.3.3.1. Carbon assimilation test

In this test, the fermentative ability media was prepared and the test was conducted as described by Atlas and Parks (1997). Before yeast cells grew in the 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, maltose, lactose, galactose and raffinose were filter sterilized, separately. One colony of each organism was transferred into 10ml of YPD(0.1g yeast extract, 0.2g peptone and 0.2g dextrose) and incubated for 24hrs after which 1ml was transferred into 200ml YPD(2g yeast extract, 4g peptone and 4g dextrose) and allowed to grow at 30°C for 3-4hrs on a shaker. It was then centrifuged at 5000 rpm for 5min with a G force of 2200. The supernatant was decanted and the pellet formed was resuspended in 30ml of distilled water. Aliquot of 9ml of the Yeast Fermentation Broth medium was dispensed into test tubes containing Durham tubes. The tubes were plugged in cotton wool and sterilized at 121°C for 15minutes after cooling, 2ml of filtered sterilized solution of sugar was added into different test tubes after which 1ml of the yeast suspension of the washed yeast cells was also added and incubated at 30°C for 24-48h. The yeast cells were then examined for their fermentative ability using different carbon source. The Durham tubes were placed in the media to trap the carbon dioxide released.

2.3.3.2. Analytical profile index confirmation of yeast isolates using API 20C test kit

This test kit consists of a single-use disposable plastic strip with 32 wells containing substrates for 29 assimilation tests (carbohydrates, organic acids, and amino acids), one susceptibility test (cycloheximide), one colorimetric test (esculin), and a negative control. The yeast identification procedures were conducted in accordance with the manufacturer's instructions.

2.4. Screening and selection of yeast strain with the best fermentative properties

2.4.1. Stress exclusion test

Stress exclusion test was conducted as described by Thais et al. (2006). The continuously growth of stress exclusion test was carried out for 15 days incubation onto different media. The ability to grow under different stress conditions was observed by growing yeast isolates on Yeast Peptone Glucose (YPG) (1 g/ml yeast extract, 1 g/ml peptone, 2 g/ml glucose and 2 g/ml agar) medium and incubated at 30°C for 3 days. A single colony was then transferred and continuously grown on YPG medium and incubated at 37°C for another 3 days, before further subculture of the isolated yeast colony on YPG medium containing 8% (v/v) ethanol and incubated at 30°C for 3

days. A single isolated colony on YPG with 8% ethanol was further subcultured on YPG supplemented with 20% (w/v) glucose and incubated under the same conditions as above. Finally the yeast cells were transferred to YP (1 g/ml yeast extract, 1 g/ml peptone) medium supplemented with 2% (w/v) sucrose and 8% (v/v) ethanol and incubated under the same conditions as above.

2.4.2. Ethanol tolerance test

The ability of the isolated yeast strains to grow in higher ethanol concentrations medium was tested by growing them in yeast peptone glucose broth containing 3 different concentration of ethanol, 10% , 13% and 15% (v/v), respectively and incubated at 30°C for 48 h (Thais et al., 2006).

2.4.3. Temperature tolerance test

The ability of the yeast to grow at higher temperatures was determined by plating the yeast isolates onto yeast peptone glucose agar medium and incubated at 3 different temperatures i.e. 25°C, 37°C and 45°C for 48h (Thais et al., 2006).

2.4.4. Hydrogen sulfide production test

The ability of the yeast to produce hydrogen sulphide (H₂S) was examined by growing the yeast isolates on lead acetate medium (4 g/ml glucose, 0.5 g/ml yeast extract, 0.3 g/ml peptone, 0.02 g/ml ammonium sulfate, 0.1 g/ml lead acetate and 2 g/ml agar) and incubated 30°C for 10 days (Ono et al., 1991).

2.4.5. Flocculation test

In this test, isolates were inoculated in 10 ml of yeast peptone glucose broth and incubated at 30°C for 3 days. After incubation, tubes were agitated to observe the flocculation formed (Thais et al., 2006).

3. Results and discussion

3.1. Isolation and identification of yeast strains from spoilt fruits and alcoholic beverages

In this study, a total of three sample types which are burukutu alcoholic drink, spoilt pineapple fruit and spoilt watermelon fruit were opted for the isolation of yeast other than *Saccharomyces*, of which seven (7), yeasts were obtained as shown in Table 3.1 below.

Table 3.1
Sources of yeasts isolated, characterized and identified.

Sample type	No. of yeasts		Codes used for each yeast
	isolated	Specific yeasts isolated	
Burukutu drink	2	<i>Candida sphaerica</i>	BKT 1
		<i>Pichiaspp</i>	BKT 2
Spoilt pineapple	2	<i>Candida pelliculosa</i>	SPP 5
		<i>Cryptococcus humicola</i>	SPP 6
Spoilt watermelon	3	<i>Candida guilliermondii</i>	SWW 3
		<i>Candida famata</i>	SWW 4
		<i>Kloeckeraapiculata</i>	SWW 7

The isolates obtained were further subcultured on malt yeast extract agar and the colonies formed varied in colour from white to cream, they had smooth surface appearance, entire margin and raised colonies except for *Candida famata* that had rough, whitish colony with undulating margin and convex elevation as shown in Table 3.2 below. The yeasts isolated were confirmed using API Test kit for the fermentation of the various sugars as shown in Table 3.3.

3.2. Screening and selection of yeast strain with the best fermentative property

All the seven yeast isolate obtained, characterized and identified were screened by carrying out different test on the individual yeast to select the yeast with the best fermentative property for fermentation. Test carried out

include flocculation test, ethanol tolerance test, hydrogen sulphide test, temperature tolerance test and stress tolerance test as presented in Table 4.4 and 4.5 below. In the stress tolerance test all the organisms survived up to the third stage of stress tolerance but only *Candida pelliculosa* survived the fourth stage of stress tolerance test.

Table 3.2.
Cultural, microscopic and fermentative characteristics of yeasts isolates.

Isolates codes	Growth on MYEPG	Microscopy using Methylene blue	Sugar fermentation							Inference
			Glu	Suc	Fru	Mal	Lac	Gla	Raf	
BKT 1	The surface appearance is smooth, creamy in color, its elevation is raised and has an entire margin	Spherical cell without budding and pseudohyphae is absent	+	+	-	-	+	+	-	<i>Candida sphaerica</i>
BKT 2	Mucoid, whitish, smooth colonies with entire margin and elevation is raised	Small elongated budding yeast cells, pseudohyphae is present	+G	-	+	-	-	-	-	<i>Pichia specie</i>
SWW 3	Creamy, mucoid, smooth, with entire margin that is conical	Spherical budding cells. Pseudohyphae is present and branched	+G	+G	+	+	-	±	+	<i>Candida guilliermondii</i>
SWW 4	Rough, white, with undulating margin. It has a convex elevation and the cells are spherical	Ovoid budding cell, no pseudo hyphae is present	±G	±G	+	±G	-	±G	+	<i>Candida famata</i>
SPP 5	Smooth, mucoid, creamy colony with entire margin, the cells are raised	Ovoid budding yeast cells, pseudohyphae is present	+G	+G	+	+	-	±	+	<i>Candida pelliculosa</i>
SPP 6	Smooth, mucoid, creamy colony with entire margin and raised cells	Ovoid budding cells. Pseudohyphae is present	+	+	+	+	-	+	±	<i>Cryptococcus humicola</i>
SWW7	White, smooth, mucoid colony that is slightly raised at the centre	Spherical budding cells pseudohyphae is absent	+G	±	+	-	-	+	+	<i>Kloeckeraapiculata</i>

MYEPG: Malt yeast extract peptone glucose medium; Glu: Glucose; Suc: Sucrose; Fru: Fructose; Mal: Maltose; Lac: Lactose; Gla: Galactose; Raf: Raffinose; +: Positive; -: Negative; G: Gas; ±: Weak.

Table 3.3
API confirmatory test for yeast isolates.

Sugars	BKT 1	BKT 2	SWW 3	SWW 4	SPP 5	SPP 6	SWW 7
Control	-	-	-	-	-	-	-
D-glucose	+	+	+	+	+	+	+
Glycerol	-	-	-	+	+	+	-
Calcium 2-keto-gluconate	-	+	+	+	-	+	+
L-arabinose	-	+	-	-	-	+	-
D-xylose	+	+	-	-	+	+	-
Adonitol	-	-	+	+	-	+	-
Xylitol	-	-	+	-	+	+	-
D-galactose	+	+	+	+	-	+	-
Inositol	-	-	-	+	-	+	-
D-sorbitol	+	-	+	+	+	+	-
Methyl-Ad-glucopyranoside	-	+	+	-	+	+	-
N-Acetyl-Glucosamine	-	+	+	+	-	+	-
D-cellobiose	-	-	+	+	+	+	-
D-lactose	-	-	+	+	-	+	-
D-maltose	+	+	+	+	+	+	-
D-saccharose (sucrose)	+	+	+	+	+	+	-
D-trehalose	+	+	+	+	+	+	-
D-melezitose	-	-	+	+	+	+	-
D-raffinose	+	-	+	+	+	+	-
Inference	<i>Candida sphaerica</i>	<i>Pichiaspp</i>	<i>Candida guilliermondii</i>	<i>Candida famata</i>	<i>Candida pelliculosa</i>	<i>Cryptococcus humicola</i>	<i>Kloeckeraapiculata</i>

+: Positive; -: Negative.

Table 3.4

Screening and selection of yeast strains for best fermentative properties.

Test	BKT 1	BKT 2	SWW 3	SWW 4	SPP 5	SPP 6	SWW 7
Flocculation test	+++	+	++	+++	++	+	+++
Ethanol 15%	-	-	-	+	+	-	-
Ethanol 13%	+	+	+	+	+	-	+
Ethanol 10%	+	+	++	++	++	-	++
Hydrogen sulphide test	-	-	-	+	+	-	-
Temp. 25°C	++	+	++	++	++	++	+++
Temp. 30°C	+	+	++	+	++	+	++
Temp. 37°C	+	+	++	+	++	+	+
Temp. 45°C	-	-	-	-	+++	-	-
Inference	<i>Candida sphaerica</i>	<i>Pichiaspp</i>	<i>Candida guilliermondii</i>	<i>Candida famata</i>	<i>Candida pelliculosa</i>	<i>Crypococcus humicola</i>	<i>Kloeckeraapiculata</i>

-: Not turbid/ no growth; +: It is turbid but no flocculant was formed/Growth is observed; ++: Flocculant are formed/Moderate growth; +++: Flocculant are densely formed/ Highly populated.

Table 3.5

Stress tolerance test of isolated yeasts.

Test	BKT 1	BKT 2	SWW 3	SWW 4	SPP 5	SPP 6	SWW 7
YPG at 30°C for 3 days	+++	++	+++	+++	+++	+++	+++
Further subcultured at 37°C for 3 days	+++	+++	+++	+++	+++	+++	+++
Further subcultured on YPG+8% ethanol at 30°C for 3 days	+	+	+	+	++	+	+
Further subcultured on YPG+20% glucose	-	-	-	-	+	-	-
Inference	<i>Candida sphaerica</i>	<i>Pichiaspp</i>	<i>Candida guilliermondii</i>	<i>Candida famata</i>	<i>Candida pelliculosa</i>	<i>Crypococcus humicola</i>	<i>Kloeckeraapiculata</i>

-: Not turbid/ no growth; +: Growth is observed; ++: Moderate growth; +++: Highly populated; YPG: yeast peptone glucose medium.

From the result obtained in table 3.1 above two (2) yeasts were isolated from burukutu drink, two (2), from spoilt pineapple and three (3) yeasts from spoilt watermelon. This was due to the richness in the nutritional composition of the fruits and alcoholic drink. This agreed with the work of Onuorah et al. (2013) who isolated fungi such as *Candida specie*, *Pichia specie*, *Fusarium specie*, *Rhizopus specie* and *Aspergillus specie* from spoilt pineapple fruit and stated that high moisture content of pineapple fruit is a limiting factor in their preservation. Effiuvwevwere and Oyelade et al. (2000) also reported that *Aspergillus specie* and *Candida specie* are responsible for the rotting of pineapple fruit. Tournas et al. (2006) isolated *Pichia specie*, *Candida specie*, and *Rhodotorula specie* from pineapple and watermelon fruits. Also, during the study, *Candida specie* was predominant this was due to nutritional variability of the specie and their ability to tolerate presence of high alcohol. This agreed with the work of Kindu et al. (2015) who stated that *Candida specie* occurred more in spoilt fruits due to their ability to tolerate acidic environments. In Table 3.2 above, all the yeast smeared and observed appeared spherical to ovoid in shape in presence of methylene blue stain this is because the actual shape of yeasts are spherical to ovoid in

shape and methylene blue stain only provides a platform to see them more clearly as cells that are non-viable are stained dark blue while Cells that are viable remain clear of the stain. This agrees with the work of Alaa et al. (2015) who stated that methylene blue stains the yeast cell darker and makes them visible to the eyes. Moreover, all the yeasts isolated were able to ferment glucose with gas production except for *Candidasphaerica* and *Cryptococcus humicola*. The yeasts isolated were confirmed using API Test kit for the fermentation of the various sugars as shown in Table 3.3 above.

In this research, during the screening of the yeast to identify the one with the best fermentative property, it was observed that *Candida sphaerica*, *Candida famata* and *Kloeckeraapiculata* had the best flocculation ability. This agreed with the work of Kocher et al. (2011). In the ethanol test, only *Candida famata* and *Candida pelliculosa* were able to grow in presence of 15% ethanol concentration at normal temperature, while all the yeasts isolated grew in presence of 13% ethanol at normal temperature except *Cryptococcus humicola* which could not even grow at 10% ethanol. This work agrees with the work of Ludmila et al. (2012) who stated that *Cryptococcus humicola* cells grown on glucose and ethanol died at 0.05mg/ml and 0.2mg/ml cellobiose lipid, respectively. *Candida guilliermondii*, *Candida famata*, *Candida pelliculosa* and *Kloeckeraapiculata* grew well in presence of 10% ethanol. This agrees with the work of Kindu et al. (2015) who stated that *Candida* occurred more in spoiled fruits due to their ability to tolerate presence of acidic environment also Bilbao et al. (1997) stated that the growth of *Kloeckeraapiculata* was enhanced at lower temperature in higher concentration of ethanol since high concentration of alcohol is reported to be toxic to yeast by inhibiting the cell growth due to the destruction of the cell membrane of the yeast.

In the stress tolerance test as seen in Table 3.5 all the organisms' survived up to the third stage of stress tolerance but only *Candida pelliculosa* survived the fourth stage of stress tolerance test. This shows that strain survival of yeast under stressful condition provides useful information on their ability to grow and carry out fermentation as impaired yeast. This is because yeast growth during fermentation usually does not occur in optimal condition and are continuously exposed to several stress especially osmotic and ethanol stress. But since all isolates could survive to the third stage of stress tolerance test, this indicates that they can survive most of the conditions they will be confronted with in the fermentation vessel. This agrees with the work of Lee et al. (2011) who stated that *Candida specie* and *Pichia specie* could survive alcohol and osmotic stress and are alcohol tolerant yeast. However, from the results obtained above, *Candida pelliculosah* as proven itself in all the test carried out to be excellent.

4. Conclusion

It can be concluded from the strength of this research that *Candida pelliculosah* has higher tolerance due to high ethanol concentration, high temperature, hydrogen sulphide and stress tolerance and is thus the organism with the best fermentative properties.

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