International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2019): 7.583

Isolation and Identification of Yeast Cells from Palm Wine in Ado Ekiti Southwest Nigeria

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Abstract: ***Investigations were carried out on yeast cells isolated from palm wine samples obtained from Ado Ekiti in southwestern Nigeria. The isolates were characterized for certain attributes that enhanced ethanol production. Isolations were made from palm wines that were stored for 10 days [240hours]. The investigations were performed on the following attributes, ethanol tolerance and sedimentation rates. The isolates include – strains of Saccharomyces cerevisiae, strain of S .globosus and strains of Hanseniaspora uvarum. Fermentation of carbohydrates [sugars] was carried out. Results of the ethanol tolerance revealed a range of 10-20 % [v/v], ethanol tolerance for the isolates. The sedimentation rates varied from 50.5 to 92.1%.***

Keywords: Isolation, Characterization, Yeast, Palm wine, Ado Ekiti.

1. Introduction

The practice of isolating and evaluating local microbial strains for defined or potential commercial attributes is a common practice in the industrial microbiology and biotechnology (Olowonibi, 2017).

For isolating organisms, diverse environments such as water, soil, foods [including palm wine] are usually screened by using appropriate techniques. *Saccharomyces cerevisiae* is yeast [unicellular fungus] (Okerentugba *et al.* 2016, Yu *et al*., 2018).
The organism is usually isolated from sugary foods and beverages such as palm wine.

Saccharomyces cerevisiae is common yeast of economic importance in food and beverage industries (Umeh, 2016, Yu *et al*., 2018).

Palm wine is a milky alcoholic beverage produced from palm wine. Palm wine contain diverse microbial population including bacteria, yeasts or moulds. Different species of yeast exist in palm wine. Yeast population among other organisms have been found to vary in palm wine depending on the source of the palm wine. The organism has fermentative and oxidative capabilities. Thus, it can convert the sugar content of the palm wine into alcohol [ethanol] and carbon dioxide under anaerobic conditions (Nwachukwu *et al*. 2006).

The metabolic activities of the yeast in the palm wine normally create the physiochemical condition of the palm wine. Bechem *et al.* (2007) studied the physiological characteristics of some palm wine yeast isolates and some of these isolates showed to high concentration of sucrose and ethanol. Similarly, Chilaka *et al.* (2010) evaluated the efficiency of yeasts isolated from palm wine in diverse fruit wine production and concluded that acceptable wine could be produced from fruits using yeasts isolated from palm wine. Palm wine yeast have been found to possess good sedimentation properties for high product recovery (Ukwuru and Awah, 2013).

A milky juice containing initially well over 13% sucrose when it is collected fresh in the container from the palm tree, immediately after leaving the palm tree, yeast spores especially those of *Saccharomyces cervisae* infect the juice and start to ferment the fermentable sugars (Obahiagbon and Osagie, 2007).

Palm wine is predominantly fermented by yeast (Amoa-Awua *et al.* 2007; Ogunremi *et al.* 2017). Successful fermentations to produce ethanol using yeast requires tolerance to high concentrations of both glucose and ethanol. These characteristics are important because of high gravity fermentation, which are common in the ethanol industry, give rise to high sugar concentration at the beginning of the process and high ethanol concentration at end of the process (Tsegay, 2016, Yu *et al*., 2018).

Saccharomyces cerevisiae is an important microorganism in bio-industries and its tolerance to ethanol is one of the characteristics to decide if it can be used for bio-fermentation resources (Tsegay, 2016, Yu *et al.*, 2018).

2. Materials and Methods

Sources and collection of palm wine samples:
Fresh palm wine samples obtained from oil palm trees (*Elaesnsis guineensis*) were purchased from palm wine tapers within the period of 120 seconds of the taping in sterile containers firmly covered and brought to the food microbiology laboratory of the Federal Polytechnic, Ado Ekiti Ekiti state for analysis.

Isolation and preliminary identification of yeast:
About five {5} different samples of palm wine were purchased for analysis. These samples were labeled according to the areas where they were purchased. The areas are Irona, Ijigbo, Ajilosun, Erinfun and Odo-ado all in Ado Ekiti, Ekiti state. The palm wine samples were stored for 10 days, after which each sample was shaken and inoculated on potato dextrose agar {YPDA}that has been mixed with antibiotic chloramphenicol {0.05mg/l} to prevent the growth of bacteria (Nwachukwu, 2001).

The plates that were inoculated with palm wine samples were incubated at 27 ±5^{o}C for 48 hours. The isolates that were confirmed to be yeasts by microscopy, were purified by streaking on YPDA and the pure isolates were stored on slopes of YPDA. Yeast isolates were identified by standard morphological and physiological methods (Nwachukwu *et al.* 2006).

Table 1: Sources and codes for palm wine samples

Sample code	Type of wine	Source
Rw	Raffia wine	Irona
Rw	Raffia wine	Ijigbo
Pw	Palm wine	Ajilosun
Pw	Palm wine	Erinfun
Pw	Palm wine	Odo-ado

Microscopic Examination of Isolates
The isolates were observed microscopically for the following feature colony elevation, colour and other unique features. For microscopy, thin smear was prepared in accordance to Olowonibi (2017, Yu *et al*., 2018).

Growth in liquid media:
Yeast isolates were cultured in liquid medium {yeast potato dextrose broth} the medium was autoclaved at 121^{o}C for 15mins. and cooled . 15ml portion of the medium was inoculated with 48 hrs. Old yeast strain and incubated for 72 hrs. at 30 ^{o}C. The culture was examined visually for growth on the surface of YPDB. According to Kurtzman *et al.* (2011) the morphological changes in the cultured yeast {*Saccharomyces cerevisiae*} leads to filament fermentation under unfavorable fermentation conditions, this form scum formation.

Glucose fermentation test
The isolates were tested for their ability to assimilate and to ferment glucose**.** The isolates were inoculated into a test tube containing an inverted durham tube and peptone water containing glucose and a drop of indicator {Andrade}. The test tube was incubated for 24hrs, changes in colour {pink – yellow} or otherwise, as well as production and trapping of gas in the durham tube indicates the results of positive glucose fermentation (Nwachukwu *et al.* 2006, Yu *et al.*, 2018).

Determination of pH and titratable acidity {TA} of palm wine:
The pH of palm wine sample was determined at 24hrs. interval over a period of 72hrs.by using a pH meter. The titratable acidity {TA} was determined in triplicates at 24hourly interval over a period of 72 hours . It was determined measuring 10ml of palm wine sample into a conical flask and titrate against 1.0m NaOH using phenolphthalein as indicator. The appearance of pink colour indicated the end point. Readings were taken in triplicate. Titratable acidity was calculated according to AOAC (2007).

$$\text{Titratable acidity } \{g/100ml\} = \frac{\text{Volume of NaOH}}{\text{Volume of sample}} \times 10$$

Enumeration and isolation of yeast from palm wine:
1 ml of palm wine sample was diluted serially in 9ml of sterile distilled water. 1ml of the diluted sample was dispensed into 2 petri dishes each that contain yeast peptone dextrose agar {YPDA} that was supplemented with 0.1mg/ml of chloramphenicol to inhibit the growth of bacteria. The plates were incubated at 30^{o}C for 48 hours. After 48hours, the plates were examined for the development of colonies, and these colonies were counted and recorded as colony forming units{cfu/ml}of the sample (Osho, 2005).

Distinct colonies of yeast from each plate were selected at random and purified by streaking on fresh yeast peptone dextrose agar that has no chloramphenicol. Pure culture of each yeast isolate was preserved in the {YPDA}slant at 5^{o}C and renewed every 2 weeks (Kurtzman *et al*. 2011).

Characterization and identification of yeast isolates
Pure cultures of yeast strains were characterized morphologically and biochemically. Six colonies of fresh cultures of yeast isolates were observed for texture, colur, surface, elevation and margin. Cellular morphology was determined by taking a portion of the yeast colony into a drop of lacto-phenol cotton blue on a clean glass slide. The slide was examined under the microscope using X40 objective (Kurtzman *et al.* 2011).

Biochemical tests were performed to determine acid production from glucose, hydrolysis of urea and sugar fermentation as described by Kurtzman *et al*. (2011).

The sugars that were used in this work include glucose, sucrose, maltose and lactose. The isolated yeast strains were identified with reference to Boboye *et al*. (2008) and Ogunremi *et al.* (2017).

Isolation and identification of yeasts was by the use of standard morphological and physiological tests and identification keys described by Nwachukwu *et al*. (2011).

Incubation was at 28 ^{o}C under aerobic and anaerobic conditions. The morphological and cultural characteristics of the yeasts were studied after isolation on glucose yeast agar {GYA} and yeast malt agar {YMA). These tests included morphology, surface characteristics, presence of pseudohyphae, ascospore formation and vegetative reproduction. Fermentation tests included sugars stated by Boboye *et al*. (2008) and Ogunremi *et al.* (2017).

Physiological characterization

Fermentation of carbohydrates {sugars}
Yeast fermentation broth base with durham tube was used for testing of yeasts for carbohydrate fermentation. Yeast

fermentation broth media were used for identification of yeasts based on fermentation specific carbohydrates of fermentation pattern. The carbohydrate used were glucose {dextrose}, arabinose, maltose, sucrose, lactose, fructose, and xylose. Yeast fermentation broth was modification of media for the determination of carbohydrate fermentation by yeasts. For fermented carbohydrate by yeasts, the colour of the medium changed from red to yellow due to the formation of acids and gas produced.

Testing of selected strains for carbohydrate fermentation, the ability to ferment seven different carbohydrates was examined an-aerobically, was assessed by looking for the formation of gas {CO_2 } in the durham tube and colour change of the fermentation media.

Isolation of ethanol tolerant yeasts

The 10 days old palm wine samples were centrifuged in sterile centrifuge tubes for 5minutes at low speed, one ml of the serially diluted sediment is inoculated by streaking on plates of glucose yeast agar {GYA}. These were supplemented with chloramphenicol {0.05mg/l} Nwachukwu (2001, Yu *et al*., 2018). The yeast colonies that developed are isolated and purified by further streaking on GYA.

Yeast Identification

Isolation and identification of yeasts was by the use of standard morphological and physiological tests and identification keys described by Nwachukwu *et al* (2011). Incubation was at 28 ^{o}C under aerobic and anaerobic conditions. The morphological and cultural characteristics of the yeasts were studied after isolation on glucose yeast agar {GYA} and yeast malt agar {YMA}. These tests included morphology, surface characteristics, presence of pseudohyphae, ascospore formation and vegetative reproduction. Fermentative tests included sugars. Other test include nitrate assimilation, growth in 10% NaCl + 50% glucose in yeast extract, growth at 37 ^{o}c and growth in 50% w/w glucose yeast extract.

Sedimentation Rate Determination

Cultures of the yeast grown on malt yeast extract glucose peptone medium for 24 hours were used for the experiment. The yeasts were harvested by high centrifugation at 1600 rpm. For 10 minutes and used to prepare a standard cell suspension of 1.5 x 10^8 cells/ml. in 0.9% NaCl [by aid of Neubuer counting chambers] solution. These were then used for measuring reduction/decrease in optical density reading over a period of 2 hours at 650 mm using a corning colorimeter, the sedimentation rate is expressed by the formula. The sedimentation properties of the ethanol tolerant yeasts were compared with a brewing strain (Nwachukwu *et al*., 2011).

$$\text{Sedimentation} = \frac{\text{Total drop in reading x 100\%}}{\text{Colorimeter reading at 0 hour}}$$

Temperature Tolerance Test

The isolates were plated out in Yeast Peptone Agar [YPA] and Yeast peptone broth, incubated at 25, 30, 37 and 45 ^{o}C respectively for 48 hours. After the incubation, the growth of each isolate was studied using serial dilution on Yeast Extract Agar [YEA].

Fluctuation Test

The isolates were inoculated in 10 ml of liquid yeast peptone and incubated at 30 ^{o}C for 72 hours. After incubation, the tubes were agitated for visualization of fluctuation.

Hydrogen Sulfide Production

The isolates were plated out in Yeast Peptone Agar and incubated at 30 ^{o}C for 10 days (Thais M. Guimaraes *et al*., 2006, Ukwuru and Awah, 2013).

3. Results and Discussion

The yeast strains that were isolated from five palm wine samples and five raphia wine samples include *Saccharomyces cerevisiae* , *Saccharomyces globosus* and *Hansenula uvarum*

Table 1 shows palm wine samples, there codes and sources, it is generally noted that palm wine is gotten from both Raffia and Palm trees.

Table 2: This Table shows yeast isolates, sources, Name, sedimentation rate and ethanol tolerance

Time Sample	Source	Name	Sedimentation rate	Ethanol tolerance (% v/v ethanol)
Ry1	Raffia	*S. cerevisiae*	81.2	15.0
Ry2	Raffia	*S. cerevisiae*	74.5	12.0
Ry3	Raffia	*S. cerevisiae*	82.5	12.0
Ry4	Raffia	*S. cerevisiae*	57.5	10.0
Ry5	Raffia	*S. globosus*	56.5	12.0
Py1	Oil palm	*S. cerevisiae*	83.6	17.0
Py2	Oil palm	*S. cerevisiae*	82.0	16.0
Py3	Oil palm	*H. uvarum*	60.2	12.0
Py4	Oil palm	*S. cerevisiae*	90.0	15.0
Py5	Oil palm	*S. globosus*	64.5	10.0
S. carlsbergensis		75.0	-	

Ethanol tolerance yeasts were isolated from the palm wine and raffia wine samples after 240 hours. These include *Saccharomyces cerevisiae*, *Saccharomyces globosus* and *H. uvarum*. The frequencies of isolation were 64%, 24% and 12% respectively for *S. cerevisiae*, *S. globosus* and *H. uvarum*.

The yeast isolates (*S. cerevisiae*) display the highest flocculation/sedimentation rate (90.0). The lowest sedimentation rate observed was for the yeast R_5 (*S.globosus*: 56.5). The sedimentation rate of the brewing strain of *Saccharomyces carlsbergensis* was 75.0% after 24 hours. No significant difference was observed in sedimentation rates recorded for raffia and palm wine isolates using the ANOVA P=0.05 (Nwachukwu and Egbulonu 2000).

Table 3: Morphological Characteristics of yeast cells

Isolates	Surface	Margin	Colony Size (mm)/color	Shape	Vegetative Reproduction	Probable Isolates
R1	Smooth	Entire	0.5 cream	Spherical	Budding	*S. cerevisiae*
R2	Smooth	Entire	0.5 cream	Spherical	Budding	*S. cerevisiae*
R3	Smooth	Entire	0.5 cream	Spherical	Budding	*S. cerevisiae*
R4	Smooth	Entire	0.5 cream	Spherical	Budding	*S. cerevisiae*
R5	Smooth	Entire	0.5 cream	Spherical	Budding	*S. globosus*
P1	Smooth	Entire	0.3 cream	Elipsoidal	Budding	*S. cerevisiae*
P2	Smooth	Entire	0.3 cream	Elipsoidal	Budding	*S. cerevisiae*
P3	Smooth	Entire	0.3 cream	Elipsoidal	Budding	*H. uvarum*
P4	Smooth	Entire	0.3 cream	Elipsoidal	Budding	*S. cerevisiae*
P5	Smooth	Entire	0.3 cream	Elipsoidal	Budding	*S. globosus*

Yeast colonies on the Yeast Extract Agar were observed for Surface, Margin, size(mm)/color, Shape, Relative Reproduction and Probable Isolates. These observations were carried out using high powered microscope of objective lens (x100).

Table 4: Carbohydrates Fermentation By Yeast Isolates

Carbon Source	Ry1	Ry2	Ry3	Ry4	Ry5	Py1	Py2	Py3	Py4	Py5
Glucose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-	-	-	-
Raffinose	+	+	+	+	+	+	+	+	+	+

It was observed that all the yeast isolates have the same pattern of carbohydrate fermentation.

Table 5: Total Plate Count of Yeast (cfu/ml) in Palm Wine Samples carried out at different times viz; 0, 6, 12, 18 and 24 hours respectively

Time (h) Sample	0	6	12	18	24
Ry1	2.0	4.5	6.2	7.8	8.2
Ry2	3.6	5.3	5.7	6.5	7.6
Ry3	4.0	4.7	5.5	6.3	7.4
Ry4	5.0	5.7	6.1	6.8	7.9
Ry5	5.2	5.8	6.5	7.0	7.7
Py1	4.5	4.8	5.1	5.7	6.6
Py2	5.4	5.6	6.4	6.9	7.3
Py3	5.8	6.0	6.7	7.3	7.5
Py4	3.9	4.4	4.8	5.6	6.3
Py5	6.4	7.0	7.8	8.2	8.6

Table 6: PH and Acidity of palm wine samples with regard to Time

Time Sample	0	6	12	18	24
Ry1	4.5	4.3	4.2	4.0	3.8
Ry2	4.8	4.6	4.4	4.2	4.1
Ry3	5.1	4.8	4.5	4.3	4.1
Ry4	5.3	5.0	4.9	4.6	4.4
Ry5	5.4	5.2	5.1	4.8	4.5
Py1	5.7	5.6	5.3	5.2	5.0
Py2	5.8	5.7	5.5	5.3	5.1
Py3	5.9	5.8	5.6	5.4	5.2
Py4	6.1	6.0	5.8	5.7	5.4
Py5	4.3	4.1	3.8	3.6	3.5

This table shows level of acidity of the palm wine samples based on temperature of storage. The PH reduces as the time increases, that is PH at 0 time is greater than PH at time of 6 hours later and PH of 6 hours is greater than 12 hours and PH of 12 is greater than 18 hours and PH of 18 hours is greater than 24 hours.

4. Conclusion

The properties of yeast isolates from oil palm wine and raffia palm wine were studied and examined in this research. The yeast isolates include *Saccharomyces cerevisiae*, *Saccharomyces globosus* and *Hansenula uvarum.* These yeast cells were viable and exhibit high rate of sedimentation and ethanol tolerance. These yeasts were isolated from both oil palm wine and raffia palm wine, but it was observed that Saccharomyces produced high level alcohol in palm wine.

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