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SHORT COMMUNICATION

Molecular characterization of yeast isolated from palm wine in Alakahia, Rivers State, Nigeria

Matthew Onwumah*, Chinemerem Paschal Okoronkwo
and **Enobong Effiong**

Department of Microbiology, University of Port Harcourt Choba, Rivers State, Nigeria

*E-mail address: onwumah01@yahoomail.com

ABSTRACT

Palm wine in Nigeria is considered a beverage. Its usage in local and traditional events pre-dates history of man's existence. Recently bio-mining of this local drink for high throughput microbial strains have not gained much attention to scientist. This study is aimed at characterizing yeasts isolated from freshly tapped palm wine in Alakahia, Rivers State, Nigeria. A total of 4 samples was analysed. Total yeast count was done on glucose yeast agar (GYA) using spread plate technique. The yeast isolates were identified by molecular characterization. DNA sequence analyses was achieved through DNA extraction using CTAB procedure, amplification of regions of rRNA/Internal Transcribed Spacer sequence (ITS) and purification of the PCR products. The amplified region was done using ITS1 and ITS4 which are recommended universal primers for fungi identifications. The total yeast count ranged from 6.5×10^4 to 3.2×10^8 cfu/ml. Gene amplification bands revealed that the molecular weight of the isolates were 500 bp. The BLAST and phylogenetic analysis of F2 and F3 amplicons had 80.04% similarity with *Saccharomyces cerevisiae* strain CS11 with 50% frequency of occurrence while F4 had 100% similarity with *Candida ethanolica* with 25% frequency of occurrence and F1 had 99.8% similarity with *Pichia* sp. *feni 106* with also 25% frequency of occurrence. Statistical analysis using two-way ANOVA showed that the microbial quality of the palm wines is location specific. This investigation further suggests that raffia palm can be harnessed in bioprospecting of high-throughput yeast starter in biotechnological studies.

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***Keywords*:** Palm wine, bioprospecting, biomining, phylogenetic, frequency of occurrence, similarity, High throughput, CTAB procedure, amplicons and primers, *Elaeis guineensis*, *Raphia hookeri*, *Raphia vinifera*, *Phoenix dactylifera*

1. INTRODUCTION

Palm wine is a locally fermented alcoholic beverage produced from the sap of a group of tropical plants belonging to the family; *Palmae* (17; 13). Some popular trees in Nigeria from which palm wine is tapped include *Elaeis guineensis* (palm oil tree), *Raphia hookeri* and *Raphia vinifera* (7). Palm wine is consumed in several parts of the world and it is often referred to as the natural white alcoholic drink. The drink is recognized by different names around the world and known to have a huge suspension of yeasts in fermenting palm sap (Ogueri *et al*., 2016). In Mexico, palm wine is known as 'Tuba'; Ghanaians call it 'Nsafufuo' or 'Doka; Indians call it 'Toddy'; the Chinese call it 'Panamcullo' while in Philistine, it is called 'Lambong. In Nigeria, it is called 'Mmanya' by the Igbos; the Yorubas call it 'Emu' while the Hausas call it 'Bammi (20)

Palm wine is produced and consumed in very large quantities in Nigeria. It is usually served in ceremonies such as traditional weddings and funerals (3). It is also added to herbal remedies and is said to be useful for lactating mothers good for eyesight due to its high yeast content (Ubi *et al*., 2017; Agriculture and Cosumer Protection, 2014). Palm wine yeasts have been found useful in baking, brewing and bio ethanol production (20; 10; 6; 11).

Tapping palm sap is done by puncturing the trunk of a palm tree, inserting a tube in the hole and collecting the palm sap in a container (14). The freshly tapped palm sap has a sugary taste. In a study, the sugars of the sap of *Phoenix dactylifera* comprised of 95.27 % sucrose, 2.51 % glucose and 1.61 % fructose (4). However, it undergoes spontaneous fermentation by yeasts and bacteria to produce a wide range of metabolites including ethanol, lactic acid and acetic acid (17; 19). The drink contains essential nutritionally important components including proteins, sugars, amino acids, and vitamins (11). These nutrients make it a favourable medium for the growth of most microorganisms whose growth in turn alter the physicochemical conditions of the wine, hence, resulting in succession and competition of microorganisms (11).

2. MATERIALS AND METHODS

A total of 4 freshly tapped palm wine samples were collected from 4 different locations in Alakahia, Rivers State, Nigeria. Media and reagents used were of analytical grade.

2. 1. Isolation of Yeast

Yeasts were isolated using the method described by Abosede *et al.*, (1). 1 ml of freshly tapped palm sap was diluted serially in 10 folds to 10^{-6} using sterile peptone water as diluent. Dilutions of 10^{-4}, 10^{-5} and 10^{-6} were cultured in triplicates on glucose yeast agar (GYA) containing 0.1 % lactic acid using spread plate technique. The plates were incubated at 28 °C for 48 hr. Colonies were counted after which the different yeast isolates were purified and stored on GYA (0.1 % lactic acid).

2. 2. Molecular Identification of Yeast

Molecular identification of the isolates was done using the method described by Chikere et al. (5). The DNA of the isolates was extracted using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy growth of the pure culture of yeast isolates was suspended in 200 µl of isotonic buffer into a ZR Bashing Bead Lysis tubes. 750 µl of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for 5 min. The ZR bashing bead lysis tube were centriguged at 10,000 x*g* for 1 min. measure 400 µl of the supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 x*g* for 1 min. measure 1200 µl of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 µl. 800 µl was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 x*g* for 1 min. the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. 200 µl of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000 x*g* for 1 minute followed by the addition of 500 µl of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000 x*g* for 1 min. The Zymo-spin IIC column was transferred to a clean 1.5 µl centrifuge tube. 100 µl of DNA elution buffer was added to the column matrix and centrifuged at 10,000 x*g* for 30 sec to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other downstream reactions.

2. 3. DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer.

2. 4. Internal transcribed space (ITS) amplification

The ITS region of the rRNA genes of the isolates were amplified using the ITS1(TCCGTAGGTGAACCTGCGG) and ITS4(TCCTCCGCTTATTGATATGC) primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 µl for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, $MgCl_2$), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95º for 5 min; denaturation, 95º for 30 sec; anealing, 53º for 30 sec; extension, 72º for 30 sec and final extention, 72º for 5 min. The product was resolved on a 1.5 % agarose gel at 120 V for 15 min and visualized on a UV trans illuminator.

2. 5. Sequencing

Sequencing was done using the Big Dye Terminator kit on on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa.

2. 6. Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to

represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (9).

3. RESULTS

3. 1. Total Yeast Count

The Total yeast count of palm wine samples from the various locations ranged from 6.5 × 10^4 to 3.2 × 10^8 cfu/ml as presented in table 1.

Table 1. Total yeast count of palm wine

Sample Location	Total Yeast count (Cfu/ml)
Alakahia, Obio Akpor	3.2×10^8
UPTH Road, Obio Akpor	2.2×10^6
Choba, Extension	1.0×10^6
Umuchiolu, Ikwerre	6.5×10^4

3. 2. Molecular Characterization

The obtained 16S rRNA-ITS region sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 18S rRNA of the isolate showed a percentage similarity to other species at 99 %. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 18S rRNA of the isolates within the *Candida* sp and revealed a closely relatedness to *Candida ethanolica* than other *Candida* sp. Figure 1 shows the phylogenetic tree of the isolates. Figure 2 shows the agarose electrophoresis of the internal transcribed spacer of the fungal isolates while Table 2 shows the 18S rRNA identification of the palm wine yeast isolates.

Table 2. 18S rRNA identification of the palm wine yeast isolates

Isolate Code	Accession number	Similarity index (%)	Yeast isolates
F4	KM368822.1	100	*Candida ethanolica* voucher UFLA FF 3.4
F2	KU311155.2	80.4	*Saccharomyces cerevisiae* strain CSII
F3	KU311155.1	80.4	*Saccharomyces cerevisiae* strain CSII
F1	KP 223717.1	99.8	*Pichia* sp feni 106

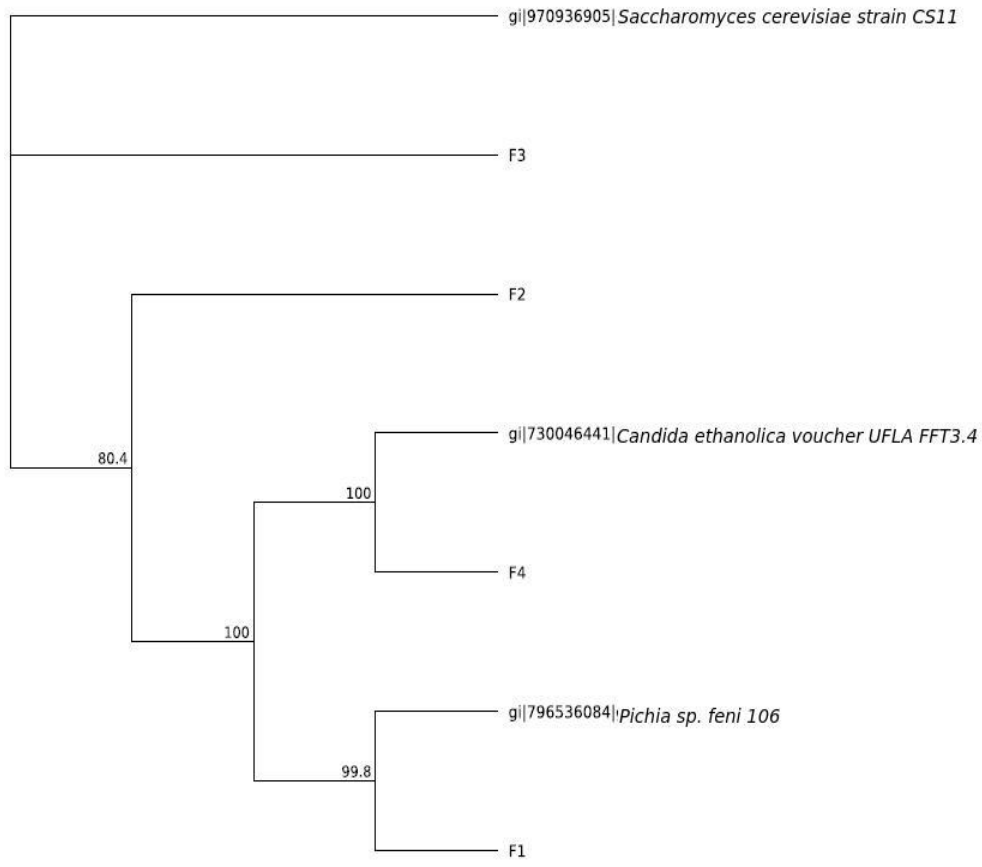

Figure 1. Phylogenetic tree of isolates

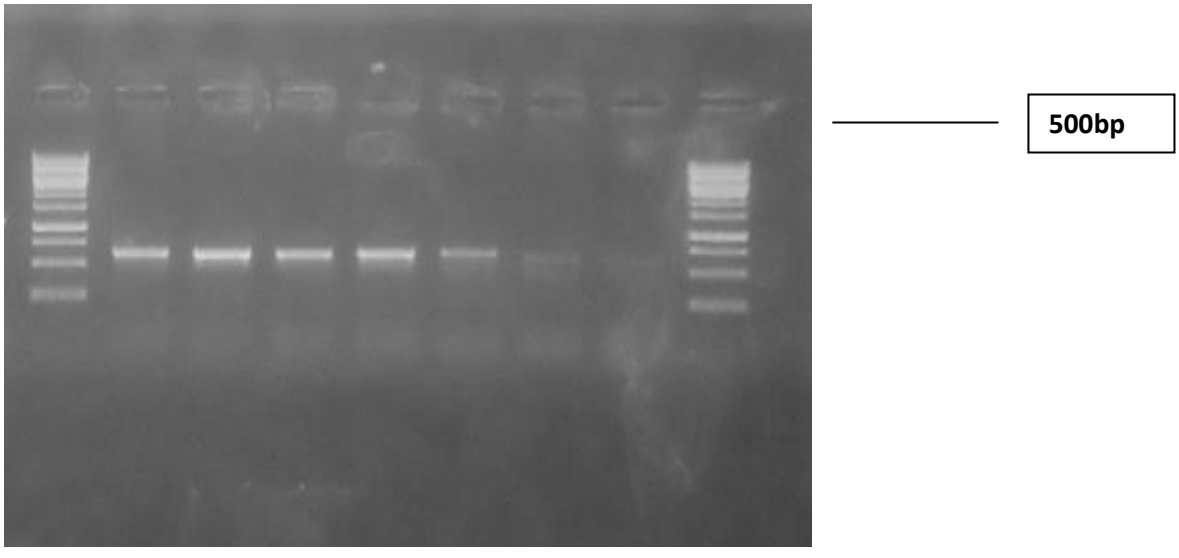

Figure 2. Agarose electrophoresis of the internal transcribed spacer of the fungal isolates

4. DISCUSSION

Palm wine in Nigeria is considered a beverage. Its usage in local and traditional events pre-dates history of man's existence. Recently biomining of this local drink for high throughput microbial strains have not gained much attention to scientist. Areas such biotechnology, pharmaceutical and industrial sectors are yet to explore and harness. Acceptability of palm wine to its consumers is a measure of its proxy to high microbiological and physicochemical preferences, and this primarily affects the organoleptic qualities of the palm wine. These challenges have questioned several scientific discuss on the molecular assessment of indigenous palm wines (12).

The yeast quality of the wines was observed to range from 6.5×10^4 to 3.2×10^8 cfu/ml. This result agree with the finding of Ukwuru and Awah (21) whose study revealed a count of 3.7 to 4.8 $\log_{10}$ cfu/ml of viable yeast. Furthermore, ambient temperature could enhance a rise from 7.6 to 8.9 log10 cfu/ml after 24 h. The lower count in palm wine sourced in Umuchiolu and Choba Extension supports the initial position of Ukwuru and Awah (21), thereby suggesting that the fluxes within the physicochemical and intrinsic parameters could adversely affect the population dynamics of yeast responsible for the aging of wines.

During the study, Isolate F4, had a 100% relatedness with the *Candida ethanolica* UFLA FF 3.4 while isolate F1 had a 99.8% resemblance with *Pichia feni* 106. This agrees with the report of Pretorius (15) whose report had associated wine production as a mutual interaction with the presence of *Pichia* sp in wine production. Furthermore, *Saccharomyces cerevisiae* CS II were reported from palm wines in Alakahia and Choba locations respectively.

This finding agrees with the report of Nwaiwu *et al.* (12) who opined that *S. cerevisiae* is the most popular yeast in the palm wines sold in Nigeria. Again in another study, Some other studies reported that *S. globosus*, *S. carbergensis* were subspecies isolated from the palm wines sold in the South-Eastern states of Nigeria (21).

5. CONCLUSION

Palm sap is a rich substrate for the development of an assortment of organisms including yeasts and microscopic organisms. The proficiency of palm wine yeast in the creation of liquor was assessed and it was observed that *S. cerevisiae* and *C. ethanolica* isolated from raffia palm sap in the Niger Delta was useful in the production of wine with satisfactory quality. It can in this manner be presumed that raffia palm sap fills in as a veritable wellspring of mechanically valuable yeasts. A few types of yeast have been utilized as a part of wine creation.

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