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Molecular Characterization of Successive Yeasts Strains and their Optimal Invertase Producing Conditions in Fresh Palm Wine (*Raphia hookeri*) obtained from Lagos, Nigeria

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ABSTRACT: *Saccharomyces cerevisiae* is known to produce invertase. However, molecular characterization of successive yeast strains and their optimal invertase producing conditions in Nigerian *Raphia hookeri* has not been documented. In this study, molecular characterization of successive yeast strains and their optimal invertase producing conditions in fresh palm wine (*Raphia hookeri*) obtained from Lagos, Nigeria was evaluated. To achieve this, sequencing of 18S rDNA was carried out following DNA extraction from the yeast isolates. Invertase production within 5-day period was also monitored at different conditions for the yeast strains. The physicochemical parameters investigated include pH, optical density, titratable acidity, sugar concentration and alcohol content. Out of the 21isolates obtained, 18 were identified by 18S rDNA to be *S. cerevisiae*, while 3 were *Penicillium chrysogenum*, *Blumeria graminis* sp. *tritici* and *Fusarium culmorum*. The fungal population density decreased from 1.01 x 10^7 to 0.9 x 10^5CFU/mL. The pH and sugar concentration decreased with increase in age of the wine ranging from 6.05-3.33 and 3.70-1.10mg/100mL respectively. The optical density, titratable acidity and alcohol content increased with time and ranged from 0.454-0.904, 0.266-0.412 % and 0.920-5.640 % respectively. All isolates produced invertase optimally at pH 6, 30°C after 48 h incubation with *S. cerevisiae* CP006433.1 having the highest production (1.745U/mL).The results highlight the effect of fermentation conditions on the survival and invertase production of yeast strains in Nigerian palm wine.

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Palm wine is the collective name for a group of alcoholic beverages produced by the natural fermentation of the sap obtained from various tropical plants of the Palmae family and consumed in different regions of the world (Okafor, 1978). It contains nutritionally important components including amino acids, proteins, vitamins and sugars (Okafor, 1987). These constituents of palm wine make it a veritable medium for the growth of a consortium of microorganisms, whose growth in turn, alter the physicochemical conditions of the wine, promoting competition and successions of organisms. Santiago-Urbina et al. (2013) reported the production of several metabolites including ethanol, lactic acid and acetic acid from spontaneous fermentation of the sap of the palm trees, which is originally sweet and serves as a rich substrate for the growth of various types of yeasts and bacteria. Thus, palm sap fermentation has been reported to be alcoholic, lactic and acetic (Santiago-Urbina et al., 2013). Yeasts, lactic acid bacteria and

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acetic acid bacteria are considered to play the most important role in the palm wine production. Yeasts are eukaryotic microorganisms, classified in the kingdom fungi, with about 1500 species currently identified and described. They are estimated to be 1% of all fungal species (Kurzman and Piskur, 2006) and measures up to 3-4 µm in diameter. Some can reach over 40 µm depending on the species (Walker et al., 2002). Most young yeast colonies are moist, somewhat slimy in appearance and may also appear mealy. However, some yeast colonies are whitish, cream-coloured or pink and change slightly with age, while others become dry and wrinkled. Yeasts are oxidative, fermentative or both. Oxidative yeast (film yeasts) may grow as a film or scum on the surface of a liquid medium whereas fermentative yeasts grow throughout the liquid (Okerentugba et al., 2016). They are chemo-organotrophs, hence require enriched incubation for growth (Baker et al., 2019). They could be aerobes, facultative anaerobes but never strict or obligate anaerobes (He and Chen, 2005). Yeasts are widely dispersed in the environment. They grow best in neutral or slightly acidic pH environments. Yeasts occur in palm wine as indigenous micro flora and are mainly from the genus *Saccharomyces, Schizosaccharomyces, Pichia, Candida, Kleockera, Hansenula, Endomycopsis and Saccharomycoides* (Tuntiwongwanich and Leenanon, 2009). *Saccharomyces cerevisiae* has been reported to produce invertase (Ikram-Ul-Haq, Ali, 2007). However, molecular identification and optimal producing conditions in Nigerian *Raphia hookeri* is yet to be reported. The objective of this study is molecular characterization of successive yeast strains and their optimal invertase producing conditions in fresh palm wine (*Raphia hookeri*) obtained from Lagos, Nigeria.

MATERIALS AND METHODS

Sample Collection: Freshly tapped palm wine (*Raphia hookeri*) was collected from local palm wine tappers at Isolo metropolis, Lagos State, Nigeria. The palm wine sample was collected directly from the palm tree using pre-sterilized, labeled 100 mL capacity sample bottles with perforated screw caps. The samples were transported to the laboratory in an ice containing cooler pack for analysis within an hour of collection to slow down fermentation rate.

Microbial isolation and succession in palm wine: One ml of the palm wine was taken aseptically and serially diluted 5-fold. Aliquots (0.1mL) from 10^{-2} and 10^{-5} dilutions were plated out in duplicates on Rose-bengal chloramphenicol agar base (CM0549; Oxoid) using spread plate method. The inoculated plates were incubated at 30^{o}C for 72hrs. Pure cultures were obtained from developed distinct colonies and stored on agar slants for characterization. This procedure was repeated every 24hrs for 5 days to determine yeast strain succession.

Molecular identification of isolates: Pure cultures of the strains maintained on potato dextrose broth (PDB) in McCartney bottles were identified at the Bioscience Centre, International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria. The identification was done by employing deoxyribonucleic acid (DNA) extraction and sequencing. Species confirmation was carried out by subjecting sequences obtained to a basic local alignment search tool (BLAST) activity on the European Molecular biology Laboratory-European Bioinformatics Institute (EMBL-EBI) database (http://www.ebi.ac.uk/tools/sss/ncbiblast/nucleotide.html), after which identification was carried out based on sequences of closest relatives.

Extraction of genomic DNA: The DNA extraction was carried out using Zymo research extraction kit. Cells were harvested from an overnight culture plate and re-suspended in up to 200 µl of isotonic buffer (PBS) in a ZR Bashing ™ Lysis Tube. 750µl Lysis Solution (EDTA) was added to the tube and secured in a bead fitted with 2 mL tube holder assembly and processed at maximum speed 3000 x g for > 5 mins. The ZR Bashing Bead ™ Lysis Tube was centrifuged in a micro-centrifuge at > 10,000 x g for 1 min. Then, 400 µl supernatant was transferred unto a Zymo-Spin ™ IV Spin Filter (orange top) in a collection tube and centrifuged at 7,000 x g for 1 min. The base of the Zymo-Spin TM Spin filter snapped off prior to use. Subsequently, 1,200 µl of fungal DNA binding buffer was added to the filtrate in the collection tube. Then, 800 µl of the mixture was transferred to an 800 µl capacity Zymo-Spin ™ IIC column in a collection tube and centrifuged at 10,000 x g for 1 min. The flow through from the collection tube was discarded and centrifugation in Zymo-Spin ™ IIC column was repeated. DNA pre-wash buffer (200 µl) was added to the Zymo-Spin ™ IIC column in new collection tube and centrifuged at 10,000 x g for 1 min. Fungal DNA wash buffer (500 µl) was added to the Zymo-Spin ™ IIC Column and centrifuged at 10,000 x g for 1 min. Zymo-Spin ™ IIC Column was transferred to a clean 1.5 mL micro-centrifuge tube and 100µl DNA elution buffer was added directly to the column matrix. Centrifugation was done at 10,000 x g for 30 seconds to elute the DNA.

Gel Electrophoresis: Agarose gel electrophoresis was used to determine the quality and integrity of the DNA by size fractionation on 1.5 % agarose gels. Agarose gels (biotechnology grade) were prepared by

dissolving and boiling 1.5 g agarose in 100 mL 0.5 X sodium borate buffer solution. The gels were allowed to cool down to about 45°C and 10 µl of 5 mg/mL ethidium bromide was added, mixed together before pouring it into an electrophoresis chamber set with the combs inserted. After the gel has solidified, 3 µl of the DNA with 5 µl sterile distilled water and 2 µl of 6X loading dye was mixed together and loaded in the well created. Electrophoresis was done using the Consort EV231 electrophoresis machine at 80V for 2 hours. The integrity of the DNA was visualized and photographed on UV light source by the UV transillumination.

Polymerase chain reaction (PCR): Amplification of the 18S rDNA genes by polymerase chain reaction was carried out as described by Okerentugba *et al*. (2016) using the MJ research thermal cycler (PTC-200). The amplicon was further purified prior to sequencing using 2 M sodium acetate wash technique. The primer used for the reaction was ITS 4 and ITS 5. PCR reaction was performed with a 25 µl (final volume) of the PCR cocktail mixture: 10 × PCR buffer (2.5 µl), 1.0 µl 25 mM $MgCl_2$, 1.0 µl 5 pMol forward primer. 1.0 µl 5 pMol reverse primer, 1.0 µl DMSO. 2.0 µl 2.5 Mm dNTPs, 0.1 µl Taq 5 u/ul, 3.0 µl 10 ng/µl DNA, 13.4 µl H_2O. The protocol used consisted of an initial denaturation step (94^0C for 5 min), followed by 36 cycles of denaturation at 94^0C for 30secs, primer annealing at 54^0C for 30 secs and elongation at 72^0C for 45 secs. This was followed by an additional 7 min cycle at 72^0C to finalize the reaction.The amplicons from the reaction above was loaded on 1.5% agarose gel and the ladder used was hyper ladder 1 from Bioloine. The gel was stained with ethidium bromide and the bands visualized under UV illumination. Sequencing was carried out using the applied biosystem; ABI 3130X1 model. The isolates' genes were sequenced using the ITS 4 and ITS 5 primer. The primer sequences are: ITS 4: TCCTCCGCTTATTGATATGS and ITS 5: GGAAGTAAAAGTCGTAACAAGG.

Identification of 18S rDNA sequences and phylogenetic assignment: The 18 S rDNA sequences were used to determine taxonomic assignments by comparing them with the nucleotide database at GenBank using the basic local alignment search tool (BLAST) program. The 18S rDNA sequences were subsequently aligned with representative 18S rDNA sequences from GenBank corresponding to organisms previously identified in the BLAST analysis.

Physicochemical analysis: Determination of pH and titrable acidity: The pH of palm wine samples was determined directly using a pH meter (Thermometer Electron Corporation, Orion 4 star (pH ISE portable) after calibration with standard buffers. For determination of titrable acidity, 10 mL of sample was made up to 200 mL with distilled water and 80 mL was titrated against 0·1 M NaOH using 1% phenolphthalein as indicator(Kirk and Sawyer, 1991).

Determination of sugar concentration: The Anthrone method was used. Anthrone reagent was prepared by dissolving 200mg Anthrone reagent in 100mL H_2SO_4. Stock standard was prepared by diluting 100mL of palm wine sample with 100mL distilled water while working standard was prepared by diluting 10mL of stock standard solution with 100mL distilled water. A blank was prepared with 1mL of distilled water and 4mL of Anthrone reagent. Subsequently, 0.2 to 1mL of working standard solution was prepared in five test tubes, water was added to bring the volume to 1mL. In each test tube, 4mL of Anthrone reagent was added, the contents were mixed as well and the test tubes were placed in water bath for 10mins, then cooled to room temperature. The optical density was measured with spectrophotometer (Thermo Electron Corporation, Soectron 20D+) at 620 nm (Santos et al., 2013)

Determination of alcohol concentration from specific gravity: The percentage of alcohol by volume from specific gravity was determined according to AOAC (1990). Palm wine (100mL) was diluted with 50 mL of distilled water. After collecting the distillate, its relative specific gravity was determined by dividing the weight of 25 mL of the distillate by the weight of an equal volume of water using a 25 mL specific gravity bottle and comparing with a reference table. All experiments were carried out in duplicates.

Invertase Production: Preparation of inoculum: Colonies of each yeast isolate from slants were standardized to ~1.5×10^8 (Mac Farland standard) and transferred into 25mL of sucrose yeast extract peptone medium in a flask containing (g/liter) sucrose 30.0, peptone 5.0 and yeast extract 3.0 at pH 6. The flasks were incubated at 30°C at 150rpm for 24hr (Chauhan et al., 2016). All experiments were carried out in duplicates.

Submerged Fermentation: Sucrose yeast extract (SYE) peptone medium was used for invertase production under submerged fermentation condition. Erlenmeyer flasks (250mL) containing 100mL of the SYE medium was autoclaved at 121°C and 15lbs pressure for 15mins and cooled at room temperature. The sterilized fermentation flask was inoculated with 5mL of 24hrs old culture previously prepared in the 25mL sucrose yeast extract peptone medium of McFarland standard (~1.5×10^8) and incubated at

30ºC, 150rpm for 48hrs. The samples were collected and centrifuged at 5000 rpm for 20mins. The cell-free supernatant was used as the source of crude invertase enzyme (Chauhan et al., 2016).

Enzyme Assay: Invertase activity was determined as described by (Chauhan et al., 2016) with slight modification by incubating 1mL of enzyme solution with 2mL of sucrose in 0.1M acetate buffer (pH 5.0) and incubated at 55°C for 10mins. To terminate the reaction, 1mL of the dinitrosalicylic acid reagent (DNSA) was added and heated for 10mins in boiling water bath. Finally, the absorbance was read at 540nm in spectrophotometer (Miller, 1959). One unit of enzyme activity (IU) is defined as the amount of enzyme which liberates 1 micro mole of glucose/minute/ml under standard assay condition.

Optimization studies: Effects of parameters on invertase production: The optimization study for the highest invertase producing organism (*S. cerevisiae* CP006433.1) at different parameters viz pH, incubation time and temperature was carried out.

Effect of time on invertase production: Sucrose Yeast Extract Peptone broth medium containing (g/liter) sucrose 30.0, peptone 5.0 and yeast extract 3.0 was inoculated with 5% inoculum of the selected yeast strain. The broth was incubated at different times (48hrs, 72hrs, 96hrs, 120hrs and 144hrs) at 30°C and pH 6 under shaking conditions at 150rpm. The samples were withdrawn at 24hrs time interval, centrifuged at 5000 rpm for 20mins and supernatant was used as crude enzyme source.

Effect of pH and temperature on invertase production: The pH of fermentation medium was adjusted in the range of 6, 7 and 9 using 1N NaOH and 1N HCL. Then, each flask was inoculated with 5% of the inoculum and incubated at 30°C, 37ºC and 45ºC for 48hrs under shaking conditions at 150rpm. The samples were collected after 48hrs of incubation and centrifuged at 5000 rpm for 20 min. The cell-free supernatant was used as the source of crude invertase enzyme.

Statistical Analysis: To verify these results statistically, the Kruskal-Wallis rank sum test was employed to test the null hypothesis that invertase production is equal at different parameter conditions. It is the non-parametric analogue to the F-test used in analysis of variance. While analysis of variance tests depend on the assumption that all populations under comparison are normally distributed, the Kruskal-Wallis test places no such restriction, so we assume non-normality and that the observations are independent within each sample (Obidi et al., 2018).

RESULTS AND DISCUSSION

Cultural and morphological characteristics: The yeasts isolated from the fresh palm wine samples showed similar colonial morphologies ranging from small to large spherical colonies which are opaque, flat and with entire margins. Microscopic investigation showed cells were small and ellipsoidal in shape.

Mean Population Density of Successive Species in Palm Wine: Generally, the population density of isolates from the palm wine samples was highest (1.01×10^7 CFU/mL) at 0 hr. It further decreased to 2.65 x 10^6 at 24 hrs. The count rose slightly to 4.1 x 10^6 and 4.7 x 10^6 at 48 and 72 hrs respectively which could be the effect of an adaptation or Lag phase. The lowest population density (2.4 x 10^6 CFU/mL) was observed at 96 hrs (Fig. 1).

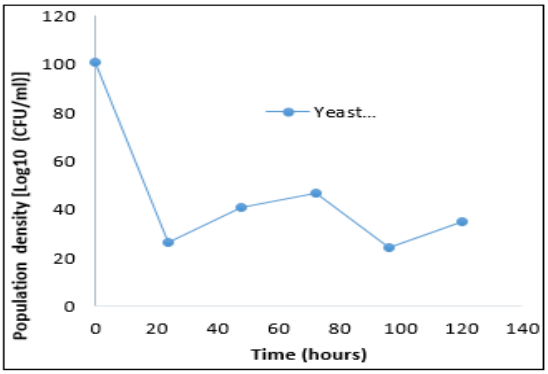

Fig. 1: Mean population density of isolates from palm wine samples with time.

Isolates QW1 (*S. cerevisiae* CP006433.1) had the highest population density which was observed at 0 hr of fermentation with a value of 1.01×10^7 CFU/mL. Its population reduced with age of the palm wine from $2.0\text{x}10^6$ at 24hrs to $1.8\text{x}10^6$ CFU/mL at 72 hrs and subsequently to a very low value of $0.9\text{x}10^5$ CFU/mL at 120 hrs. QW2 and QW3 (though suspected to be contaminants) were both observed only once at 24 hrs and had the lowest population count of $3.5\text{x}10^5$ and $3.0\text{x}10^5$ respectively. QW4 and QW5 appeared at age 48 hrs with values of 1.2 x 10^6 and 1.1 x 10^6 CFU/mL respectively while QW6 appeared at age 72 hours with value of 2.9 x 10^6. These three strains were also observed once throughout the days of isolation. Isolates QW7 appeared twice throughout the hours of fermentation. It appeared at 96 and 120 hrs with values 2.0 x 10^6 and 2.6 x 10^6 CFU/mL respectively. Its population increased with increase in the age of the palm wine. QW8 appeared only at 96 hrs at a low population of 4.0 x 10^5(Table 1)

Table 1: Molecular identification and total population density based on yeast occurrence.

Days	Codes	Identification	Ascension No	Population density
At 0hr	QW1	*Saccharomyces cerevisiae*	CP006433.1	01×10^7
		Saccharomyces cerevisiae	CP006433.1	
		Saccharomyces cerevisiae	CP006433.1	
		Saccharomyces cerevisiae	CP006433.1	
At 24hrs	QW1	*Saccharomyces cerevisiae*	CP006433.1	2.0×10^6
		Saccharomyces cerevisiae	CP006433.1	
		Saccharomyces cerevisiae	CP006433.1	
	QW2	*Blumeragraminis* sp *Tritici*	FR779027	$3.5x10^5$
	QW3	*Fusariumvculmorum*	LT598661	$3.0x10^5$
At 48hrs	QW4	*Saccharomyces cerevisiae*	KY109259.1	1.2×10^6
	QW5	*Saccharomyces cerevisiae*	KY105076.1	1.1×10^6
	QW1	*Saccharomyces cerevisiae*	CP006433.1	1.8×10^6
At 72hrs	QW1	*Saccharomyces cerevisiae*	CP006433.1	1.8×10^6
		Saccharomyces cerevisiae	CP006433.1	
		Saccharomyces cerevisiae	CP006433.1	
	QW6	*Saccharomyces cerevisiae*	U09327.1	2.9×10^6
At 96hrs	QW7	*Saccharomyces cerevisiae*	KY109257.1	2.0×10^6
	QW8	*Penicilliumchrysogenum*	AM920428	4.0×10^5
At 120hrs	QW7	*Saccharomyces cerevisiae*	KY109257.1	2.6×10^6
	QW1	*Saccharomyces cerevisiae*	CP006433.1	0.9×10^5

Molecular identification and succession of yeast in palm wine: The molecular characterization of yeast isolates from the fermenting palm wine showed narrow yeast diversity as only one yeast species (*Saccharomyces cerevisiae*) was isolated. Out of a total of 21isolates obtained in the study, eighteen were different strains of *Saccharomyces cerevisiae* which occurred at different hours during the fermentation period. Twelve of the isolates (QW1) were *Saccharomyces cerevisiae* CP006433.1 and occurred at 0, 24, 48, 72 and 120 hrs (Table 1). Three of the isolates (QW7) were *Saccharomyces cerevisiae* KY109257.1 and appeared at 96 and 120 hrs of fermentation. QW7 was absent on the first four isolation periods, this could be due to its inability to thrive under the physicochemical conditions at these periods. This isolate therefore, showed high tolerance to the conditions of the palm wine at the ages when there was increase in the acidity and alcohol content of the palm wine. (Table 1).*Saccharomyces cerevisiae* KY109259.1 (QW4), *Saccharomyces cerevisiae* KY105076.1 (QW5) *and Saccharomyces cerevisiae* U09327.1 (QW6), appeared just once at 48, 48 and 72 hrs of fermentation respectively. QW2, QW3 and QW8's sequences were incomplete after molecular identification. The BLAST result showed *Blumer agraminis* sp *tritci* FR779027, *Fusarium culmorum*LT598661 and *Penicillium chrysogenum* AM920428. This probably occurred as a result of contamination during the molecular analysis.

Physicochemical parameters of palm wine: All the physiochemical parameters tested which included pH, alcohol concentration, titratable acidity, sugar concentration and optical density varied with respect to time as shown in Table 2.

Table 2. Physicochemical Analysis of Palm Wine at Different Time Intervals

Parameters	0hr	24hrs	48hrs	72hrs	96hrs	120hrs
pH	6.05	3.57	3.41	3.68	3.43	3.33
Alcohol content (%)	0.920	2.100	2.890	3.940	4.860	5.640
Optical Density	0.454	0.622	0.640	0.772	0.847	0.904
Titratable acidity (%)	0.266	0.345	0.360	0.389	0.399	0.412
Sugar Conc. (mg/100mL)	3.70	3.40	3.17	2.55	1.50	1.10

The pH decreased with increase in age of the palm wine. The results of pH confirmed the importance of other microorganisms coexisting with yeast such as lactic acid and acetic acid bacteria in the fermentation of palm wine. From near neutral value of 6.05 at 0 hour, the pH dropped to values between pH 3.57 and 3.41 at 24 and 48 hrs respectively. An increase to 3.68 at 72 hrs and subsequent decrease to 3.43 and 3.33 at 96 and 120 hrs was observed respectively (Figure 2). The concentration of alcohol in palm wine sample at zero hr of tapping was found to be low (0.920 %,) and might have occurred between the time of tapping and laboratory. At 24 and 48 hrs, the alcohol concentration increased steadily to 2.100 % and 2.890 % respectively. The highest concentration of alcohol was 5.640 %, observed at 120 hrs (Figure 3**)**. The level of titratable acidity also increased with increase in age of the sample. The wine showed higher value of 0.412 % at age 120 hrs and a lowest value of 0.266 % at zero hr (Figure 4).

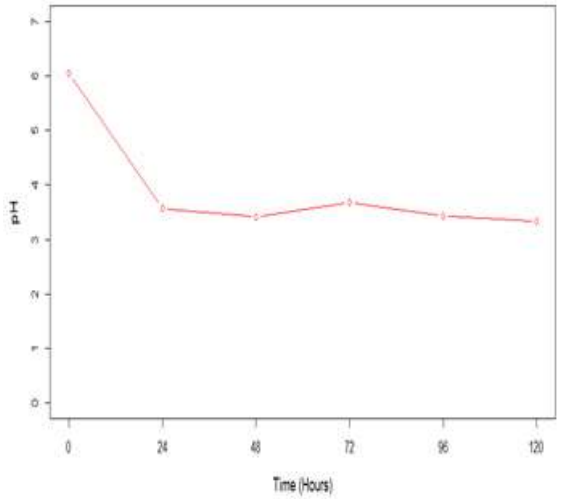

Fig. 2: Mean pH of the fermenting palm wine with time.

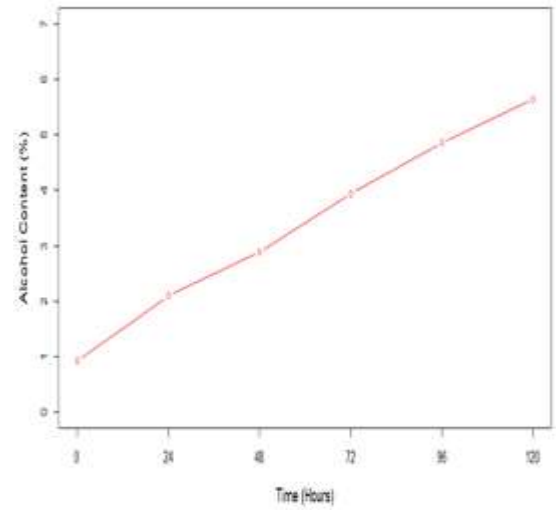

Fig. 3: Mean changes in alcohol levels of palm wine sample with time (hours).

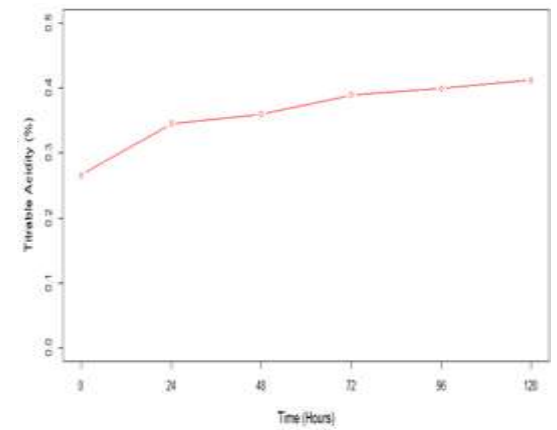

Fig. 4: Mean changes in titratable acidity (parts per million) of palm wine sample with time (hours).

Yeast growth in the sample was authenticated by the steady increase in the optical density in relation to age of the palm wine (Figure 5). On the contrary, sugar concentration decreased with increase in age of the palm wine. At 0 hr, higher value of 3.70 mg/100mL was observed and a slight decrease to 3.40 mg/100mL after 24 hrs. There was sharp decrease at 72 hrs with value 2.55 mg/100mL and at 120 hrs, a lowest value of 1.10 mg/100mL was observed (Figure 6).

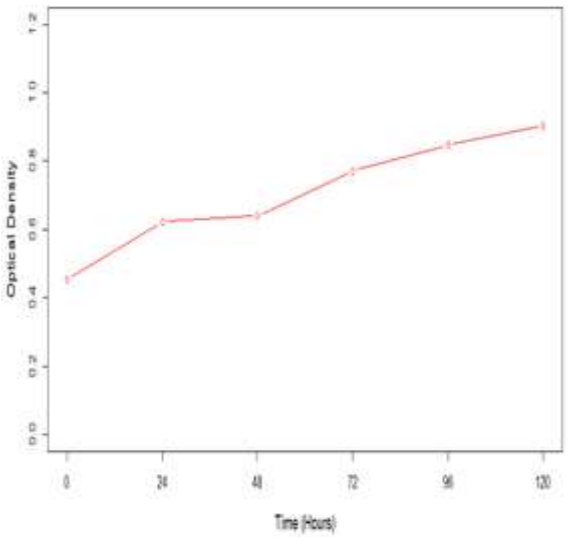

Fig. 5: Mean changes in optical density of palm wine sample with time (hours).

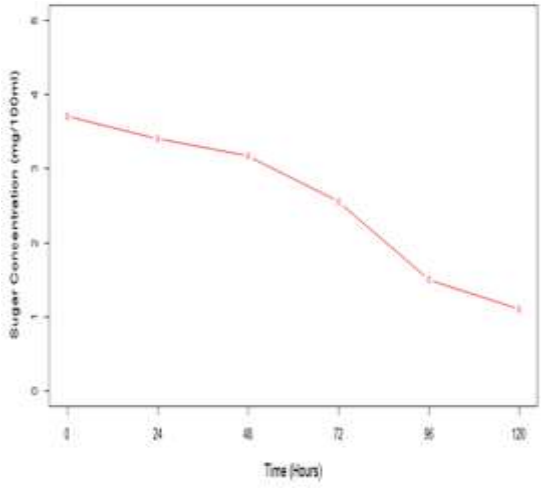

Fig. 6**:** Mean changes in sugar concentration (mg/100mL) of palm wine sample with time (hrs).

Screening of palm wine isolates for invertase production: Twenty one isolates obtained from palm wine samples were screened for their ability to produce invertase. Generally, all the twenty one isolates tested positive to the enzyme assay at 30^0C, pH 6 and at 48hrs incubation time with different levels of activity (Fig. 7).

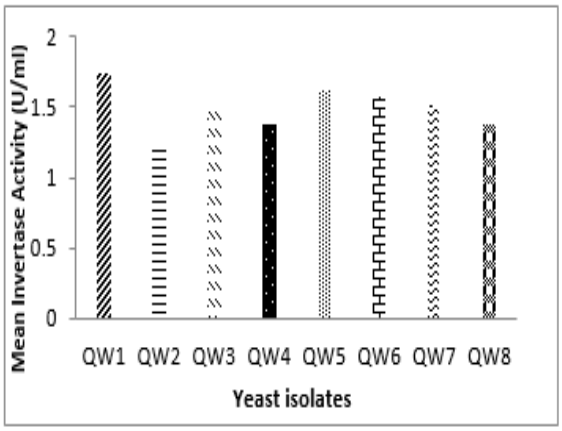

Fig. 7. Mean invertase activity (U/ml) of individual isolates at 48hrs and pH 6

The invertase activity ranged from 1.205U/mL to 1.745U/mL. However, of all the twenty one isolates, isolate QW1 (*S. cerevisiae* CP006433.1) had the highest activity at 1.745U/mL after 48hrs at pH6 and was subsequently selected for optimization studies. A Line plot of invertase production with respect to parameters measured is shown in Figure 8. It reveals the relationships between invertase production and each parameter measured.

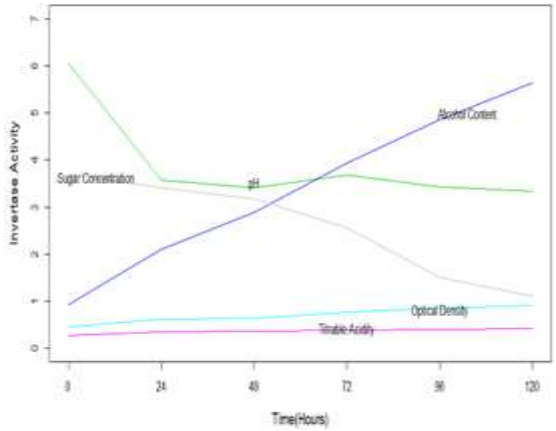

Fig. 8. Line plot of invertase production with respect to parameters measured

Effect of time course on invertase production by S. cerevisiae CP006433.1: The effect of fermentation time was tested on the selected *S. cerevisiae* CP006433.1 for a period of 144hrs (6 days). It was observed that invertase activity reduced with an increase in fermentation time. At 48hrs of fermentation, invertase activity was 2.23U/mL which reduced drastically to 1.56U/mL and 1.08U/mL at 77 and 96hrs respectively. After the 96th hr, the activity reduced steadily to a final value of 0.75U/mL at 144 hrs. Therefore, the maximum amount of invertase production for *S. cerevisiae* CP006433.1was observed at 48hrs of incubation (Fig. 9).

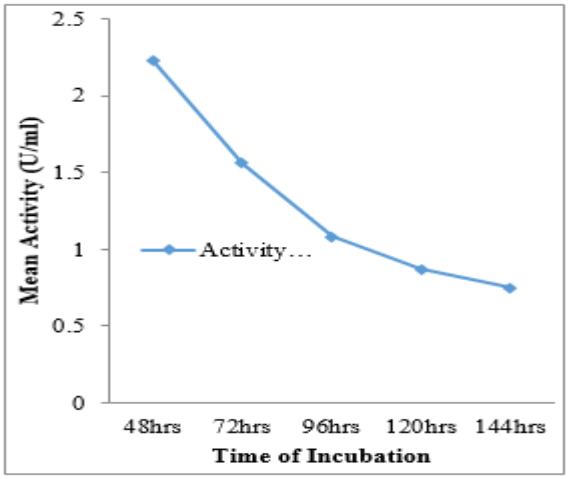

Fig 9: Effects of time course on invertase production by *S. cerevisiae* CP006433.1

Effect of pH and temperature on invertase production by S. cerevisiae CP006433.1: In order to determine optimum pH for invertase production, invertase production was assessed at different pH values (6.0, 7.0, and 9.0). Optimal invertase production (1.13U/mL to 1.90U/mL) was observed at pH 6.0 while minimum invertase production (0.62U/mL to 1.50U/mL) was at pH 9.0 (Fig.10). Figure 10 also shows the effect of incubation temperature on invertase production. It was observed that maximum invertase production (1.50U/mL to 1.90U/mL) occurred at 30^{0}C. On the other hand, minimum production (0.62U/mL to 1.13U/mL) was at 45^{0}C. At 37^{0}C, invertase activity ranged from 0.93U/mL to 1.76U/mL. Therefore, maximum production was achieved at 30^{0}C at pH 6.

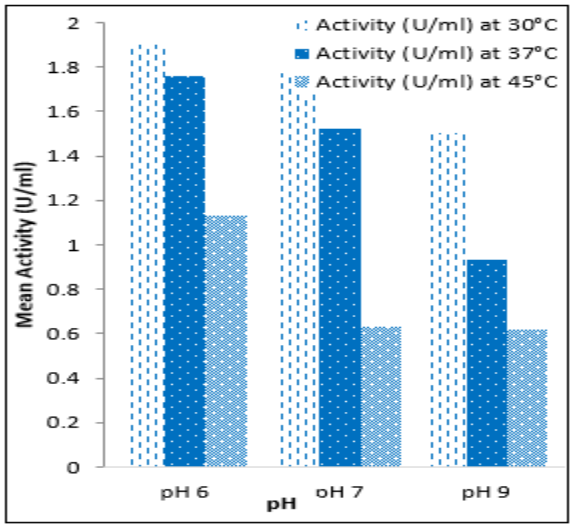

Fig 10: Effects of pHinvertase activity by *S. cerevisiae* CP006433.1

Statistical Analysis: Kruskal-Wallis H test showed that there was a statistically significant difference in invertase production at the different time levels. Kruskal-Wallis chi-squared = 23.411, degrees of freedom = 4, p-value = 0.0001048.Since p-value is less than 0.05 significant level, the test is significant and we reject H_0 which confirms that invertase production at various time levels is not the same for different parameters. A significant Kruskal–Wallis test indicates that at least one sample stochastically dominates other samples. The stochastically dominant distribution appears to be pH from the Boxplot in Figure 11 whose median value is higher than other parameters.

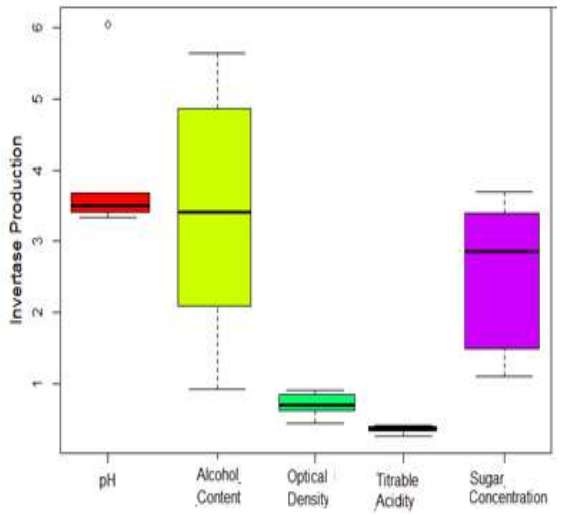

Fig. 11. Box plot showing a Non-Parametric Test of invertase production against culture conditions

The high carbon dioxide environment among other conditions during palm wine fermentation favours the proliferation of *Saccharomyces* (Stringini et al., 2009) and that probably contributed to *S. cerevisiae* being the dominant yeast isolated. Although Santiago-Urbina *et al*. (2015) failed to find any *S. cerevisiae* strain in any of three palm saps analyzed in Mexico, the dominance of *S. cerevisiae* among yeast species in palm wine is widely reported in other locations (Amoa-Awua et al., 2007). Although**,** microbial population reduced to 0.9 x 10^5 at 120 h from the initial 1.01 x 10^7 at 0 hr of isolation, the persistence of *Saccharomyces* throughout the fermentation period is an indication of their acid and alcohol tolerance as also reported by Nwachukwu *et al.*(2006). This characteristic make *S. cerevisiae* remain the primary choice for fermentation (Chandra and Panchal, 2003). The results of physicochemical parameter determinations points to the enormous activities of the microorganisms which also contribute to yeast succession and the nutritional quality of palm wine (Okafor, 1972; Amoa-Awua et al., 2007). The rapid decline in sugar concentration in the palm wine as fermentation progresses is an indication of its preference for metabolism by the yeast strains (Priest and Campbell, 1996). The steady increase in the level of titratable acidity could be attributed to the formation of more organic acids by the fermenting organisms with time. This explanation is supported by the reduction in pH of the palm wine sample with time as corroborated by Okafor, (1972); Oyagbade et al., 2004). In the same vein, the increase in alcohol level of the palm wine with time to a peak at 120 hrs suggests that carbohydrate present in the palm wine is being used up by the microorganisms which convert it to alcohol (Nwachukwu et al., 2006). Since the synergy between some of the acids such as acetic acid and ethanol has been established (Nwachukwu et al., 2006), it is possible that their presence in palm wine would result to toxic effect on the microflora in the palm wine. Consequently, the medium would be increasingly selective for only organisms that can resist this toxic effect. The yeast strains isolated at 120 hrs (*Saccharomyces cerevisiae* CP006433.1 and *Saccharomyces cerevisiae* KY109257.1) clearly showed tolerance to this effect at low pH, high titratable acidity and alcohol concentration. The isolation of *Saccharomyces cerevisiae* at 120 hrs of fermentation further corroborated their high tolerance for ethanol as reported by Nwachukwu *et al.* (2006).

Saccharomyces cerevisiae CP006433.1 has been confirmed in the present work as the dominant yeast strain responsible for the fermentation of palm wine tapped from the palm of *Raphia hookeri*. In the palm wine samples, the only yeast species isolated were strains of *S. cerevisiae* and *S. cerevisiae* CP006433.1 appear to completely dominate the fermentation of palm wine sample amongst others. This is in agreement with other studies carried out in Ghana (Brown, 1994). Brown (1994) used restriction fragment length polymorphism to identify the yeast isolates from the market samples of palm wine from several towns in southern Ghana and reported the presence of only *S. cerevisiae*. In the palm wine samples tapped from the upright trees in other countries, other yeast species have been reported to be important in the fermentation of palm wine in addition to *S. cerevisiae*. In Nigeria, many studies have reported the occurrence of yeast species in palm wine. Owuana and Saunders (1990) isolated both *S. cerevisiae* and *Kloeckera apiculata* from the palm wine, whilst Ezeronye and Okerentugba (2000) reported the presence of *S. cerevisiae*. Enwefa *et al*. (1992), however, reported the presence of several genera of yeasts including *Saccharomyces, Candida, Endomycopsis, Hansenula, Kloeckera, Pichia,*

Saccharomycoides and *Schizosacchromyces* in palm wine tapped from oil and raffia palms. Earlier studies had reported *S. cerevisae* and *Schizosacchoromyces pombe* as the dominant yeasts in palm wine (Okafor, 1972; Faparusi, 1973), whilst Fahwehinmi (1981) also reported the presence of *Saccharomyces chevalieri* and *Pichia membrabefaciens*. Recently, Boboye *et al*. (2008) reported the isolation of *Schizosaccharo myces pombe*, *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Geotrichum lactis* and *Zygosaccharo mycesrouxii* from a freshly tapped palm wine obtained from Akure, Nigeria. In the present study, we have gone a step further to reveal the molecular identification of such yeasts present in traditional Nigerian palm wine in order to understand their phylogenetic relatedness.

The production of invertase has been previously reported in several organisms such as such as *Neurospora crassa*, *Candida utilis*, *Fusarium oxy sporium*, *Phytophthora meganosperma*, *Aspergillus niger*, *Saccharomyces cerevisiae*, *Schizosaccharo myces pombe*, and *Schwanniomyces occidentalis* (Silveira et al., 2000). However, *S. cerevisae* was observed in the present study as a major invertase producer. This is in line with the findings of Ikram-ul-Haq et al., 2005) who reported *S. cerevisae* as the highest producing isolate amongst other tested isolates. It is noteworthy that possible differences in the chemical composition of palm wine from different environments impacts the *S. cerevisiae* diversity, succession, invertase production and prevalence in the yeast biota of palm wine. Maximum invertase production was achieved at pH 6 at 30ºC after 48hrs of incubation, indicating that invertase production was more pronounced at lower pH and was less produced at higher pH. These results are similar to the work of UlHaq Ali et al. (2005) who specified that the maximum production of invertase was obtained at pH 6 and suggested that less enzyme production at higher developed pH was due to blocked enzyme secretion from the yeast cells. Similarly, Uma et al. (2012) reported maximum invertase activity at pH 6.0 by *Cladosporium cladosporioides*. Additionally, Shankar et al., (2013) studied the effect of different pH on invertase production after 48hrs of incubation period at 30°C, it was observed that the maximum amount of invertase production was at pH 6.0 (0.35 ± 0.005) and minimum invertase production was recorded at pH 8.0.

Conclusion: This study contributes to a better understanding of the yeast community structure and effect of fermentation on survival of yeast strains and their invertase production in palm wine. *S. cerevisiae* emerged the only yeast isolated throughout the days of fermentation *S. cerevisiae* CP006433.1 and *S. cerevisiae* KY109257.1 could be important strains as starter cultures in the fermentation of African indigenous foods and beverages and also in ethanol producing industries in Nigeria. Invertase production by *S. cerevisiae* CP006433.1 proved optimum at 30ºC and pH 6 after 48 hrs of incubation.

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