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Research Article
Saccharomyces cerevisiae from Polymerase Chain Reaction (PCR) in Table Wine Using *Tamarindus indica* and *Passiflora edulis* Blends

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Abstract

Background and Objective: Palm wine is an alcoholic beverage produced from the fermentation of sap of different palm species. Palm wine microbiota is mainly yeasts identified as *Saccharomyces cerevisiae* as well as lactic acid bacteria. The objective was to isolate *Saccharomyces cerevisiae* from fresh palm wine and molecularly characterize the isolate using PCR and compare its efficacy with commercial brewer's yeast in fermenting wine formulated from tamarind and passion fruit juice blends. **Materials and Methods:** Fresh palm wine of 12 hrs old was cultured and sub-cultured. Two isolates were obtained and stored as stock for inoculation. The following were carried on the isolates, microscopic and morphological examination, biochemical test, molecular characterization and phylogenic determination. **Results:** Biochemical test of the two yeast isolate sample "L and S" shows that the yeast sample 'L' have higher fermentative capability and was selected as the inoculate for wine production formulated from the blend of tamarind and passion fruit juice. The morphological view of the yeast sample under a microscope shows oval shape single budded cell. Molecularly (using PCR), the DNA of the yeast isolate appeared as a white band of 1.5 agarose gel and the sample was amplified using two pair of primer ITS4/ITS5 at 650 bp of the internal transcribe space (ITS) region. When the sample was blast, it was 99% proven in the gene bank to be *Saccharomyces cerevisiae*. Phylogeny of sample L showed it cluster with other *Saccharomyces cerevisiae* in the phylogenic in the tree. The mould count of the formulated wine from the yeast isolate (*Saccharomyces cerevisiae*) ranged from 4.5×10^5 (sample TPFC) 5.0×10^5 CFU mL^{-1} (sample TFAA). The total viable count ranged from 1.9×10^6 (sample TPFB) to 2.0×10^5 CFU mL^{-1} (sample PTFC). **Conclusion:** *Saccharomyces cerevisiae* from palm wine was identified and molecularly characterized using the real-time polymerase chain reaction (PCR) in place of brewer's yeast that is expensive. The organism could be available and accessible if stored in the gene bank.

Key words: Brewer's yeast, molecular characterization, palm wine isolate, polymerase chain reaction, phylogeny, tamarind, passion fruit juice

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Wine is by common usage defined as a product of the normal alcoholic fermentation of the juice of the sound ripe grape. Nevertheless, any fruit with a good proportion of sugar may be used for wine production. Thus, citrus, banana, apples, pineapples and strawberries among others might all be used to produce wine[1]. Meanwhile, palm wine is an alcoholic beverage created from the sap of various species of palm trees such as the palmyra, date palms and coconut palms. It is known by various names in different regions and is common in various parts of Africa, Asia, the Caribbean and South America[2]. Palm wine is a sweet, milky, effervescent and alcoholic beverage. Palm wine production by small stakeholders and individual farmers may promote conservation as palm trees become a source of regular household income that may be economically worth more than the value of timber sold. Palm wine is known as 'mmanya nkwu' in Nigeria and 'nsafufuo' in Ghana. It is a predominant drink consumed in Ghana, Cameroon and other West African Regions although it is also produced in India. Palm wine in Igbo land comes in two sources: (I) Nkwu (palm tree)-the source of the palm wine is called 'Mmanya nkwu' (ii) 'Ngwo' (raffia palm tree) the source of the palm wine is known as 'Mmanya ngwo. Each of the palm tree categories is regarded in different respects. Each has different occasions depending on of course, how that particular area or village regards it. Palm wine is produced by tapping. The trees are tapped in the same way, by climbing to the required height or the neck of the palm tree and cutting ducts, under which are placed local mugs (calabashes) or plastics gallons. In some places, palm wine is tapped from the fallen tree. It contains nutritionally important components including amino acids, proteins, vitamins and sugar[3]. These make this wine a veritable medium for the growth of a consortium of microorganisms, where growth, in turn, change the physicochemical conditions of the wine giving rise to competition and succession of the organism.

Saccharomyces cerevisiae (commonly known as baker's yeast) is a single-celled eukaryote that is frequently used in scientific research. *Saccharomyces cerevisiae* is an attractive model organism since its genome has been sequenced, its genetics are easily manipulated and it is very easy to maintain in the laboratory. Since many yeast proteins are similar in sequence and function to those found in other organisms, studies performed in yeast could aid in the determination of how a particular gene or protein functions in higher eukaryotes (including humans) according to Jove Science Education Database[4].

Polymerase Chain Reaction (PCR) is a technique that is used to amplify a single or a few copies of a piece of nucleic acid, to generate thousands to millions of copies of a particular nucleic acid. It allows much easier characterization and comparisons of genetic material from different individuals and organisms[5]. Conventional methods for the detection of pathogens and other microorganisms are based on culture methods, but these are time-consuming and laborious and are no longer compatible with the needs of quality control and diagnostic laboratories to provide rapid results[6]. In contrast, PCR is a specific and sensitive alternative that can provide accurate results in about 24 hrs and this thus opens a lot of possibilities for the direct detection of microorganisms in a food product. The targets in the foods are DNA or RNA of pathogens, as spoilage microorganisms, DNA of moulds that can produce mycotoxins, DNA of bacteria that can produce toxins and DNA associated with trace components (e.g. allergens, like nuts) or unwanted components for food authenticity (such as cows' milk in goats' milk cheese).

Tamarind (*Tamarindus indica*) is endemic to tropical Africa, it is a leguminous tree in the family *Fabaceae*. Contrary to its *Indica* classification, the fruit is not of Indian origin. Though the fruit has grown on India's soils for many centuries, tamarinds are native to the tropical regions of Africa-more specifically, Sudan, Cameroon and Nigeria. The fruits, flattish, beanlike, irregularly curved and bulged pods, are borne in great abundance along the new branches and usually vary from 27 in long and from 3/4 to 1, 1/4 inches (2-3.2 cm) in diameter[7]. The tree bears out edible pod-like fruit which is used in almost all cuisines around the globe[8]. In India, tamarind is popular as a gentle laxative. The pulp, which comes from the pods of the tamarind tree, is a gentle laxative that improves the general sluggishness of the bowels. Tamarind is useful in correcting bilious disorders. Tamarind is acidic and excites the bile and other juices in the body. Tamarind is also a blood purifier. Folk medicine uses tamarind leaves for sprains and swelling. The leaves are sometimes used in subacid infusions and a decoction is said to destroy worms in children and is also useful for jaundice and externally as a wash for sore eyes and ulcers. The pulp, leaves and bark also have medical applications. Tamarind pulp concentrate is popular as a flavouring in east Indian and middle eastern cuisine. It is used to season full flavoured foods such as chutney, curry dishes and pickled fish.

Meanwhile, passion fruit (*Passiflora edulis*) is a species of passionflower that produces a fruit about the size of an egg and is used all around the world in culinary and medicinal practices[9]. *Passiflora* is the largest of the Passifloraceae genera, with approximately 530 different species[10]. The genus

is rich in inter-specific and intra-specific variability, a large number of its species being native to Brazil[11]. According to HE *et al.*[12], passion fruit is fat-free, cholesterol-free, sodium-free, low in calories and rich in vitamins and minerals. The pulp and seeds of passion fruit contain the most fibre. The fruit with pulp and seeds contains about 25 g of fibre. Without the pulp and seeds, it contains less than 1 g of fibre. Passion fruit is rich in water-soluble antioxidants, vitamin C or ascorbic acid. This vitamin helps the body gain resistance against infectious agents and pro-inflammatory free radicals. One serving provides 100% of the recommended daily amount. Increased health benefits are found in this fruit because of the combination of iron and vitamin C. Passion fruit also contains the minerals copper, magnesium and phosphorus.

Therefore, the work is aimed to isolate *Saccharomyces cerevisiae* from fresh palm wine and molecularly characterize the isolate using PCR and compare its efficacy with commercial brewer's yeast in fermenting wine formulated from tamarind and passion fruit juice.

MATERIALS AND METHODS

Study area: The study was carried out at Food Microbiology Laboratories, Departments of Food Science and Technology as well as Microbiology, all in the University of Nigeria, Nsukka, Enugu State, Nigeria, from February, 2018 to March, 2021.

Procurement of raw materials: Tamarind (*Tamarindus indica*) fruit was purchased from Maiduguri Monday market in Maiduguri, Borno State, Nigeria. A yellow variety of Passion fruit (*Passiflora edulis*) was obtained from the Department of Crop Science, University of Nigeria, Nsukka. The tamarind and passion fruit were authenticated in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. Freshly tapped palm wine was purchased from Enugu-Ezike market, Nsukka., Enugu state. Brewery's yeast was purchased from the over-head bridge market in Onitsha, Anambra State and the yeast from palm wine was molecularly characterized in the Bioscience Department, International Institutes of Tropical Agriculture (IITA) Ibadan, Oyo State.

Sample preparation: The tamarind fruit and passion fruit were sorted for extraneous foreign materials, spoilt and rotten fruits that would affect the keeping quality of the drink were removed and the good and healthy fruits were washed.

Processing of tamarind pulp into the juice: Tamarind pulp was processed by the method described by Onwuka and Nwokorie[13]. Tamarind juice was processed by weighing 3.5 kg of the sorted and washed fruit, then 8.5 L of water was boiled and mixed with the fruit and left for 5 min to dissolve the fruit pulp. After which the fruits were manually pressed to extract the juice into the hot water. After removal of the seed, the juice was sieved with a muslin cloth to obtain a clearer filtrate and left in stainless steel vat for blending with its counterpart fruit (passion fruit) as shown in Fig. 1.

Processing of passion fruit into juice: Passion fruit was processed into juice by the method described by Onwuka and Nwokorie[13]. The ripened passion fruit was sorted, weighed (14.5 kg), washed and cut with a knife to extract the juice and the seeds which were embedded in the endocarp. The seeds were separated from the juice by passing it through a sieve 2 mm mesh to obtain the juice. A clearer juice was obtained by passing it through a muslin cloth. The total volume of juice obtained was 4.5 and 3 L of distilled water was added to make up the total volume of 7.5 L and left in the stainless vat for blending with tamarind juice (Fig. 2).

Preparation of isolate for inoculation (pitching) into the fruit must for fermentation: Palm wine isolate was pitched into fruit juice using the method described by Nwaiwu *et al.*[14]. The isolate from palm wine was inoculated into sabouraud dextrose agar (SDA) broth (100 mL) and was left in the broth to grow for 72 hrs. After which 6 pieces of centrifuge tubes were sterilized, the grown isolate in the sabouraud dextrose agar (SDA) broth was transferred into each centrifuge tube

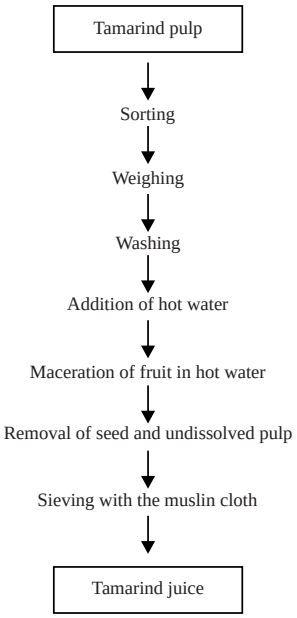

Fig. 1: Modified flow chart of processing method of tamarind pulp into the juice
Onwuka and Nwokorie[13]

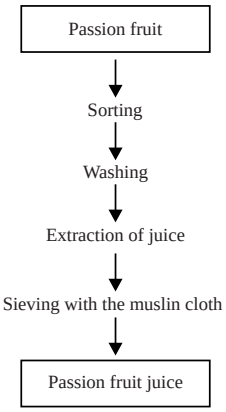

Fig. 2: Modified flow chart processing method of passion fruit pulp into the juice
Onwuka and Nwokorie[13]

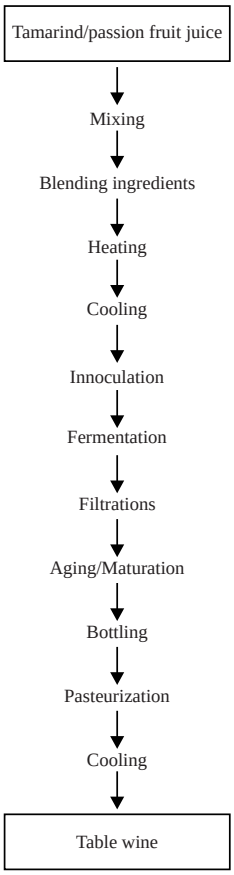

Fig. 3: Modified flow chart for production of tamarind and passion fruit blended wine
Onwuka and Nwokorie[13]

and centrifuge for 10 min at 600 revolutions per minute (making the cells settled at the bottom of the tube), the SDA broth decanted. Sterilized distilled water was poured into the centrifuge tubes for washing of the isolated cells and centrifuged for 10 min, (600 revolution min), the washing procedure was repeated two more times. After cell washing, distilled water was added to the tubes to make it up to 10 mL and thoroughly shaken before inoculating into the fruit must for fermentation.

Production of tamarind and passion fruit blend wine: Tamarind juice (8.5 L) and passion fruit juice (7.5 L) were blended into twelve different ratios of tamarind:passion fruit (1000:0, 900:100, 800:200, 700:300, 600:400, 500:500, 0:1000) mL (Table 1). Apart from the 1000 mL unblended tamarind and passion fruit juice, other blends were divided into two batches, (the first five batches were made to be fermented with an isolate from palm wine while the second five batches were made to be fermented with a commercial brewery's yeast). After which the batches were poured into fermenting vat. Then, 90 g of sugar as a source of carbon, 0.2 g of sodium metabisulphite to prevent the growth of other microbes, 5 g of ammonium sulphate as a source of nitrogen was added to the must and it was pasteurized at 85°C for 15 min, left to cool at room temperature. The isolated cell (10 mL) from a palm which has been washed with distilled water and centrifuged was pitched into six portions of the blends, while the remaining six portions were inoculated with commercial brewer's yeast 50 mL which have been activated the previous day before inoculation. The must be fermented aerobically for 4 days, anaerobically for 8 days and aged for 2 days, it was bottled and pasteurized again for 85°C for 5 min to stop fermentation and cool (Fig. 3).

Microbial analysis on an isolate from palm wine and formulated table wine from tamarind and passion fruit blends

Isolation of yeast from palm wine: Fresh palm wine of 12 hrs old was serially diluted and cultured with sabouraud dextrose agar (SDA) and the culture was incubated for 72 hrs to obtain yeast growth. After which the grown yeast in the petri dish was sub-cultured and stored in the bijou bottle and stored as slants as a stock culture for further uses, as shown in Fig. 4.

Identification of yeast: Identification was done by morphological and biochemical characteristics by the method described by Maicas[15]. The morphological test was done through microscopic examination. The biochemical test was done using a sugar test to carry out the fermentative ability of the isolate on sugars (glucose, galactose, starch, sucrose, lactose, maltose, mannitol, raffinose).

Table 1: Proportion of the formulated must for table wine production from tamarind and passion fruit blend

Samples	Tamarind (mL)	Passion fruit (mL)	Fermenting yeast
TFAA	1000	0	Palm wine isolate
TPFA	900	100	Palm wine isolate
TPFB	800	200	Palm wine isolate
TPFC	700	300	Palm wine isolate
TPFD	600	400	Palm wine isolate
TPFE	500	500	Palm wine isolate
PFAA	0	000	Brewer's yeast
PTFA	900	100	Brewer's yeast
PTFB	800	200	Brewer's yeast
PTFC	700	300	Brewer's yeast
PTFD	600	400	Brewer's yeast
PTFE	500	500	Brewer's yeast
GRFW	0	0	-

TFAA: 1000 mL tamarind fruit juice fermented with Isolate from palm wine, TPFA: 900 mL tamarind+100 mL passion fruit fermented with isolate from palm wine, TPFB: 800 mL tamarind+200 mL pasion fruit fermented with isolate from palm wine, T PFC:700 mL tamarind+300 mL passion fruit fermented with isolate from palm wine, TPFD: 600 mL tamarind+400 mL passion fruit fermented with isolate from palm wine, TPFE: 500 mL tamarind+500 mL passion fruit fermented with isolate from palm wine, PFAA: 1000 mL passion fruit fermented with commercial brewer's yeast, PTFA: 900 mL tamarind+100 mL passion fruit fermented with brewer's yeast, PTFB: 800 mL tamarind+200 mL passion fruit fermented with brewer's yeast, PTFC: 700 mL tamarind+300 mL passion fruit fermented with brewer's yeast, PTFD: 600 mL tamarind +400 mL passion fruit fermented with brewer's yeast, PTFE: 500 mL tamarind+500 mL passion fruit fermented with brewer's yeast and GRFW: Commercial grape fruit wine (control)

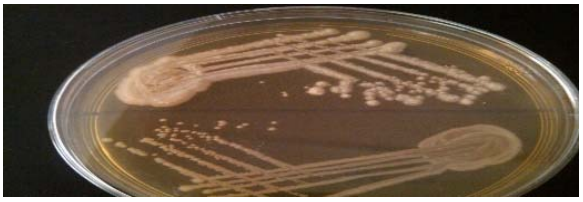

Fig. 4: Microbial culture of palm wine isolates after 72 hrs of incubation

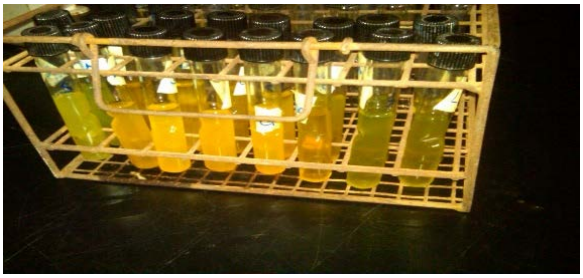

Fig. 5: Fermentation of eight different sugars with yeast isolate from palm wine

Procedure for microscopic examination: A drop of fresh saline was placed on one end of a clean dry grease-free microscopic slide and a drop of lactophenol blue on the other end. Using a wire loop, a small amount of fresh specimen (palm wine isolate from incubated petri dish) was mixed with each drop and was overlaid with a coverslip. The preparations were examined under a light binocular microscope using ×10 and ×40 objectives with the condenser iris closed sufficiently to give a good contrast. The preparation was examined especially for yeast cells.

Procedure for sugar test: Two millilitres of bromothymol blue, 5 mL of basal medium was added to eight different test tube of 12×150 mm size each. Then, 2% sugars (glucose, galactose, starch, sucrose, lactose, maltose, mannitol, raffinose) each were added to the eight different test tubes containing the basal medium and bromothymol blue then Durham tube was inserted inside the test tube by inversion. The contents were sterilized in the autoclave at 121°C for 15 min and left to cool, before inoculating the palm wine isolate using a sterile wire loop. After 24 hrs, the inoculated tubes were frequently inspected at the interval for the accumulation of gas in the 'insert' and colour change from light yellow to deep yellow, if the sugar were fermented by the isolate, the reaction was monitored for 5 days (Fig. 5). The results were scored depending on the time taken to fill the 'insert' with gas and the amount of accumulation. If the 'insert' was more than one-third filled with gas, it was rated strongly positive. If the 'insert' were less than one-third filled with gas, it is rated weakly positive. If no gas in the 'insert, is rated negative and if there is a deep colour change from light yellow to deep yellow in the whole solution, it was rated positive but if no colour change was observed since after the day it was inoculated, it was rated negatively.

Molecular characterization of an isolate from palm wine

Procedure for deoxyribonucleic acid (DNA) extraction of Isolate: The DNA extraction was done using the method of fungal, bacterial DNA extraction kit documented by Zymo Research (ZR) Company.

Table 2: PCR cocktail mix

DNA isolate was subjected to the following cocktail mix	
Component	Quantity
10×PCR buffer	2.0
25 mm $MgCl_2$	1.0
5 pmol forward primer	1.0
5 pmol reverse primer	1.0
Dmso	1.0
2.5 mm dntps	2.0
taq 5 μ μL^{-1}	0.1
10 ng μL^{-1} DNA	3.0
H_2O	13.4
	25 μL

Primers used for the sequencing of sample 'L' are universal primer, ITS4 (-TCCTCCGCTTATTGATATGS-), ITS5 (-GGAAGTAAAAGTCGTAACAAGG-)

One hundred milligrammes (100 mg) by (wet weight) fungal cells that had been resuspended in up to 200 μL of water or isotonic buffer (e.g., phosphate buffer saline, PBS) or up to 200 mg of tissue to a ZR Bashing™ Lysis Tube. Then, 750 uL Lysis Solution was added to the tube. A secured bead fitted with a 2 mL tube holder was assembled and processed at maximum speed for >5 min. The ZR Bashing Bead™ Lysis Tube was centrifuged in a microcentrifuge at >10,000×g for 1 min. Four hundred microlitre (400 μL) supernatant was transferred to a Zymo-Spin™ IV Spin Filter (orange top) in a collection tube and centrifuged at 7,000×g for 1 min (Note: Snap off the base of the Zymo-Spin™ Spin filter before use). After which 1,200 μL of Fungal DNA Binding Buffer was added to the filtrate in the collection tube. Eight hundred microlitres (800 μL) of the mixture from 1,200 μL of fungal DNA was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10,000×g for 1 min (Note: The Zymo-Spin™ IIC Column has a maximum of 800 μL). The flow-through was discarded from the collection tube and centrifuged again. Two hundred microlitres (200 μL) DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in the new Collection Tube and centrifuged at 10,000×g for 1 min. Five hundred microlitres (500 μL) of the Fungal DNA wash buffer was added to the Zymo-Spin™ IIC column and centrifuge at 10,000×g for 1 min, the Zymo-Spin™ IIC Column was transferred to a clean 1.5 mL microcentrifuge tube and 100 μL was added. Thirty-five microlitres (35 μL minimum) DNA Elution Buffer was direct to the column matrix and centrifuged at 10,000×g for 30 sec to elute the DNA. After the elution, the DNA was now suitable for PCR.

PCR protocol for DNA amplification and visualization of sample 'L' (unidentified yeast cell): The PCR protocol was performed using Bioline PCR kit (Table 2) as described, documented and instructed by the manufacturer. In each PCR, 10 ng of DNA of the tested strain, 5 pmol forward and 5 pmol reverse primers were used. The DNA of the studied strain is amplified using universal primers internal transcribed spaced (ITS) at a region of 4 and 5 - ITS4 (- TCCTCCGCTTATTGATATGS-) and ITS5 (-GGAAGTAAAAGTCGTAACAAGG-). The operating condition for the PCR-Thermo scientific cycler (Gene amp 9700 PCR system, Applied biosystem. the UK) include, 1 cycle initial denaturation temperature of 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, extension temperature at 72°C for 45 sec each at 36 cycles, final extension at 72°C for 7 min and holding temperature at 10°C to infinity (α). The resulting product was loaded and visualized on 1.5% agarose gel stained with ethidium bromide solution. The ladder used was 1kb plus. The expected base pair of the amplicon was around 650 bp.

Method of phylogenic determination: The evolutionary history was inferred by using the Maximum Likelihood method based on the model Carlos *et al.*[16]. The tree with the highest log likelihood (-2027.2392) was shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 854 positions in the final dataset. Evolutionary analyses were conducted using the MEGA7 (2016) version.

Determination of total viable count: The total viable count was determined using the pour plate method described by Karamoko *et al.*[17]. Then, 23 g of nutrient agar (NA) was dissolved in 1000 mL of distilled sterile water. The samples were serially diluted and 1 mL of the appropriate dilution was used to inoculate the plate. The cultured plate was then incubated at 37°C for 18-24 hrs and the GallenKamp colony was counted as a colony-forming unit.

Determination of mould count: Plating was done on potatoes dextrose agar using the pour plate method as described by Karamoko *et al.*[17]. Then, 39 g of potatoes dextrose agar (PDA) was dissolved in 1000 mL of distilled sterile water. Serial dilution was carried out using 1.0 mL of the formulated wine sample to 9.0 mL of water to reduce the microbial load. After

dilution, 1 mL was then plated out into triplicate of the petri dish, after which the potatoes dextrose agar was poured out and swirled gently and allowed to solidify. They were then incubated at room temperature of 28°C for 3 days and counted as colony-forming units.

Data analysis and experimental design: The experimental design was carried out using Completely Randomized Designed (CRD). The mean and standard deviation were calculated using One-way Analysis of Variance (ANOVA) using Computer Software for Solving Solution (SPSS) version 21. Means were separated using Duncan multiple range test. Significance was accepted at $p<0.05$ according to Bécue-Bertaut[18].

RESULTS AND DISCUSSION

Biochemical characterization of an isolate from palm wine: Table 3 shows the sugar fermentative capability of two yeast isolates from palm wine. It was observed from the result that both isolates 'L' and 'S' were able to utilize the sugars (starch, sucrose, raffinose, glucose, galactose, mannitol, lactose), thereby producing carbon (IV) oxide gas, which was obvious in the Durham tube and colour change from light yellow to deep yellow after 24 hrs of inoculation, except for isolate 'L' which produce gas (carbon dioxide) when fermented with lactose sugar, but isolate 'S' could not. Therefore, isolate 'L' was used for molecular characterization and fermentation of the formulated table wine from the blend of tamarind and passion fruit. The result of the sugars (sucrose, raffinose, glucose, mannitol, galactose and lactose) corresponds to the sugar test result reported by Olowonibi[19] who carried out research work on isolation and characterization of palm wine strain of *Saccharomyces cerevisiae* potentially useful as bakery yeast. Which confirm the probable organism to be *Saccharomyces cerevisiae*.

Morphology of the yeast cell under microscopic view: Figure 6a-b shows the microscopic view of the palm wine isolate after 72 hrs (3 days) of culture. The yeast cells were seen as oval-shaped single or budded cells as reported by Maicas[15].

Molecular characterization of yeast from palm wine

Genomic DNA extract from sample L (unidentified yeast cell): Genomic DNA was successfully extracted from sample L. The genomic DNA appeared as a thick white band (Fig. 7) on agarose gel after capturing under ultraviolet (UV) light using the Gel Documentation System.

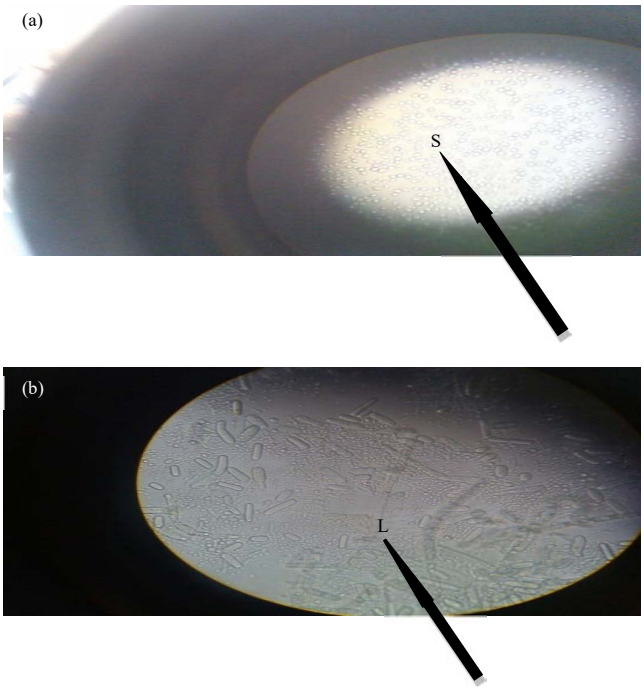

Fig. 6(a-b): Unidentified yeast cell, (a) Yeast cell labelled 'S' and (b) Yeast cell labelled 'L'
S: Unidentified yeast cell and L: Unidentified yeast cell

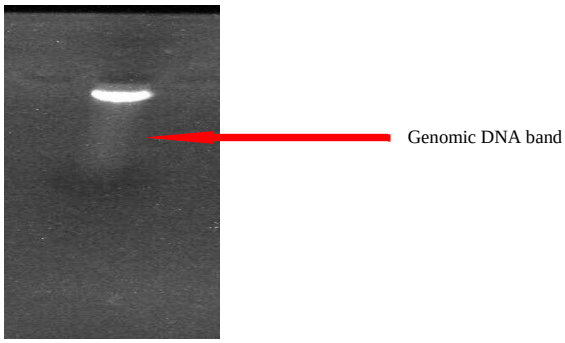

Fig. 7: Genomic DNA band of sample L on 1.5% agarose gel

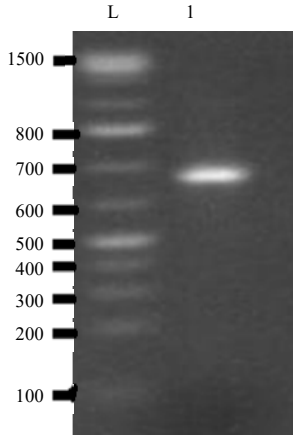

Fig. 8: Amplified ITS region of sample L
L: 100 bp DNA Ladder, 1: Sample L

Amplification of internal transcribed spaced (ITS) region of Sample L: The two primers pair ITS4/ITS5 and the PCR thermal conditions successfully amplified approximately 650 bp of the ITS region of sample L. The amplified fragment of the gene occurs between the 600 and 700 bp of 100 bp DNA Ladder (Fig. 8).

Sequence and phylogeny of sample L (unidentified yeast cells): The amplified gene (ITS region) of sample L was sequenced successfully both in the forward and reverse directions. The raw sequences of sample L are indicated thus: Sample L f = forward sequence of sample L and sample L r = reverse sequence of sample L (Fig. 9). The horizontal rectangles represent where the forward and reverse sequences matched each other. The extremes where the sequences did not match are unreliable and were trimmed off before phylogenetic analysis. A BLAST search of the raw sequence of sample L in GenBank had a score of 99% with *Saccharomyces cerevisiae* while a BLAST search of the edited sequence of sample L in GenBank have a score of 100% with *Saccharomyces cerevisiae* thus confirming sample L as *Saccharomyces cerevisiae*.

However, genes are sequenced in forward and reverse directions for greater accuracy of the sequence data generated. Where the forward sequence misreads the bases, the reverse supplies it and vice versa.

Phylogenetic tree of sample L (*Saccharomyces cerevisiae*): Phylogenetic analysis clustered sample L with *Saccharomyces cerevisiae* from GenBank (Fig. 10). This confirms sample L to be *Saccharomyces cerevisiae*. Other species of *Saccharomyces cerevisiae* from GenBank, the GenBank accession numbers and the country they were isolated are indicated on the tree for instance *Saccharomyces cerevisiae* AM262827 Austria. These are the sequences used to match and confirm the identity of sample L to be *Saccharomyces cerevisiae*. *Aspergillus niger* from Canada was downloaded from GenBank and used as the root of the tree.

Comparing the result obtained through the phylogenic tree analysis on the sample L (*Saccharomyces cerevisiae*) with the phylogenic tree analysis research work by Nwaiwu and Itumoh[20] on molecular phylogeny of yeasts from palm wine and enological potentials of the drink. According to Nwaiwu and Itumoh[20], it was observed that the yeast (*Saccharomyces cerevisiae*) isolated from palm wine in Nigeria with accession number starting with alphabet HG, yeast (*Saccharomyces cerevisiae*) isolated from palm wine in Burkina Faso with accession number starting with alphabet HE and yeast (*Saccharomyces cerevisiae*) isolated from palm wine in Mexico with accession number starting with alphabet KF were observed to all clustered in the same bootstrap 39, 52, 56 and 87 of the phylogenic tree. Indicating the Intraspecies similarities of the organism, it was also obvious in the phylogenetic tree obtained for sample L in the phylogenic tree (Fig. 11).

Mould and total viable counts of the formulated table wine from the blend of tamarind and passion fruit blend: Table 4 shows the microbial count of the formulated wine from the blends of tamarind and passion fruits which were fermented for two weeks. After four weeks of production, bacterial growth was observed after analysis, the total bacterial growth ranges from 1.9×10^6 (sample TPFB) to 2.0×10^5 CFU mL^{-1} (sample PTFC).

The total mould count in the formulated table wine from tamarind and passion fruit blends was observed to range from 4.5×10^5 (sample TPFC) and 5.0×10^4 CFU mL^{-1} (sample TFAA). It was observed that some of the microorganisms were yeasts, which could be as a result of the yeast that was used in fermentation that reactivated after some weeks of storage.

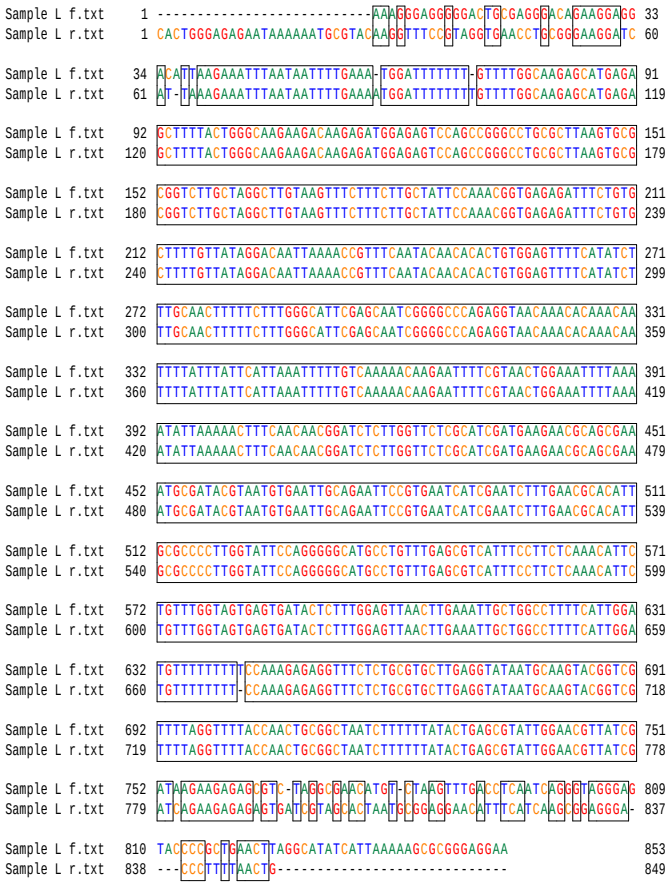

Fig. 9: Aligned forward and reverse raw sequences of sample L

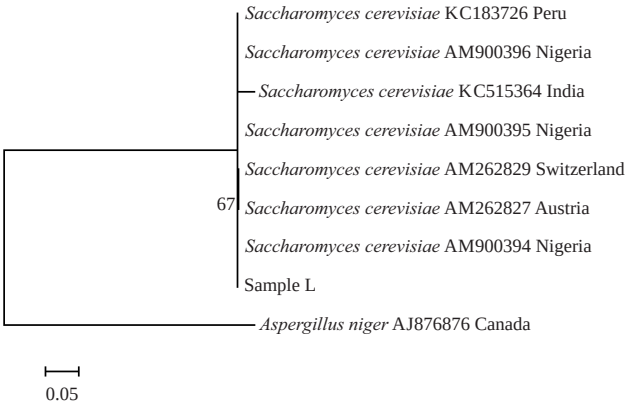

Fig. 10: Phylogenetic tree of sample L based on 650 bp ITS4 and ITS5 region

Table 3: Biochemical characterization of an isolate from palm wine

	Colour change gas							
Yeast cell	Starch	Sucrose	Raffinose	Glucose	Galactose	Mannitol	Maltose	Lactose probable organism
L	+ - wk	+ - str	+ - str	+ - str	+ - str	- -	- -	+ - *Saccharomyces cerevisiae*
S	+ +	+ + str	+ + str	+ + str	+ + str	- -	- - wk	- - *Saccharomyces cerevisiae*

+: Positive, -: Negative, str: Strong gas production (carbon (IV) oxide), wk: Weak gas production, L: Unidentified yeast cell (isolate from palm wine), S: Unidentified yeast cell (isolate from palm wine)

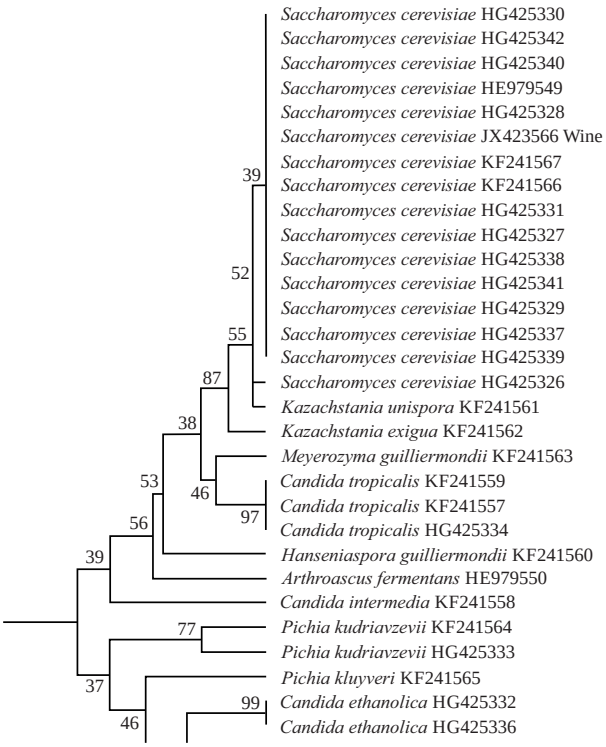

Fig. 11: Phylogenic tree of yeast isolated from palm wine in three different countries
Nwaiwu and Itumoh[20]

Table 4: Microbial count (CFU mL^{-1}) of formulated table wine from tamarind and passion fruit blends

Samples	Mould count (CFU mL^{-1})	Total viable count (CFU mL^{-1})
TFAA	5.0×10^4	5.00×10^5
TPFA	8.0×10^4	3.2×10^5
TPFB	2.4×10^5	1.9×10^6
TPFC	45×10^5	5.8×10^5
TPFD	3.1×10^5	9.0×10^5
TPFE	2.8×10^5	4.6×10^5
PFAA	3.0×10^5	5.2×10^5
PTFA	2.7×10^5	4.6×10^5
PTFB	2.0×10^5	4.1×10^5
PTFC	1.6×10^5	2.0×10^5
PTFD	1.3×10^5	3.1×10^5
PTFE	1.6×10^5	2.6×10^5
GRFW	2.3×10^5	4.0×10^5

Values are means of triplicate determinations, TFAA: 1000 mL tamarind fruit juice fermented with Isolate from palm wine, TPFA: 900 mL tamarind+100 mL passion fruit fermented with isolate from palm wine, TPFB: 800 mL tamarind+200 mL pasion fruit fermented with isolate from palm wine, TPFC: 700 mL tamarind+300 mL passion fruit fermented with isolate from palm wine, TPFD: 600 mL tamarind+400 mL passion fruit fermented with isolate from palm wine, TPFE: 500 mL tamarind+500 mL passion fruit fermented with isolate from palm wine, PFAA: 1000 mL passion fruit fermented with commercial brewer's yeast, PTFA: 900 mL tamarind+100 mL passion fruit fermented with brewer's yeast, PTFB: 800 mL tamarind+200 mL passion fruit fermented with brewer's yeast, PTFC: 700 mL tamarind+300 mL passion fruit fermented with brewer's yeast, PTFD: 600 mL tamarind +400 mL passion fruit fermented with brewer's yeast, PTFE: 500 mL tamarind+500 mL passion fruit fermented with brewer's yeast and GRFW: Commercial grape fruit wine (control)

The mould count of the formulated table wine from the blends of tamarind and passion fruits is found to be higher than that of Anvoh *et al.*[21], whose research work on the comparison of biochemical changes during alcoholic fermentation of cocoa juice conducted by spontaneous and induced processes for the production of ethanol. Yeast and mould count averaged 5.2×10^4 CFU mL^{-1} in cocoa juice, 1.61×10^4 CFU mL^{-1} on the cocoa shield and 1.3×10^2 CFU mL^{-1} on banana leaves. Aerobic mesophilic germs were encountered at a concentration of 2.5×10^3 CFU mL^{-1} on banana leaves, 8.1×10^4 CFU mL^{-1} in cocoa juice and 1.2×10^4 CFU mL^{-1} on the cocoa shield.

CONCLUSION

From the study, it can be deduced that the two microorganisms isolated from palm when subjected to biochemical test shows the potentiality of *Saccharomyces cerevisiae* and when 'sample L' was molecularly characterized using PCR proved 99% *Saccharomyces cerevisiae* when a blast in the Genbank. The phylogenic determination shows a cluster of 'sample' with other *Saccharomyces cerevisiae* in the tree. The mould count result of the formulated wine from tamarind and passion fruit juice ranged from 4.5×10^5 (sample TPFC) and 5.0×10^5CFU mL^{-1} (sample TFAA) while the total viable count ranged from 1.9×10^6-2.0×10^5 CFU mL^{-1} (sample PTFC).

SIGNIFICANCE STATEMENT

The efficacy of *Saccharomyces cerevisiae* as a starter culture for wine production is very essential. The comparison of yeast isolated from palm wine and characterized using PCR with commercial brewery's yeast used in the fermentation of wine formulated from tamarind and passion fruit juice blend shows similarity in alcohol production. This study discovers the possible synergistic effect of vitamin E, calcium and vitamin D combination that can be beneficial for osteoporosis-induced ovariectomized rats. This study will help the researcher to uncover the critical area of postmenopausal bone loss that many researchers were not able to explore. Thus, a new theory on these micronutrients combination and possibly other combinations, may be arrived at.

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