Journal of Advances in Microbiology

20(7): 38-55, 2020; Article no.JAMB.58684
ISSN: 2456-7116

Screening of Yeast Cells for the Production of Wine from Banana and Pineapple Substrates

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Authors' contributions

This work was carried out in collaboration among all authors. Author DNO designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author AA performed the statistical analysis and managed the literature searches. Author JOW managed the analyses of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2020/v20i730264
Editor(s):
(1) Prof. Hung-Jen Liu, National Chung Hsing University, Taiwan.
Reviewers:
(1) Bagiu Iulia-Cristina, "Victor Babes" University of Medicine and Pharmacy, Romania.
(2) Ferymon Mahulette, Pattimura University, Indonesia.
Complete Peer review History: http://www.sdiarticle4.com/review-history/58684

Original Research Article

Received 01 May 2020
Accepted 04 July 2020
Published 04 August 2020

ABSTRACT

Wine, an alcoholic beverage is usually produced from juice of variety of fruits by the fermentative action of microorganisms particularly by yeasts. Several substrates such as pineapple, banana, watermelon, pawpaw and other fruits have been used to produce wine using *Saccharomyces* species. This study was undertaken using non- *Saccharomyces* to produce table wine with pineapple and banana fruits as substrates. Standard microbiological procedures were employed for yeasts cell isolation, sugar (sucrose) fermentation test, pH, ethanol, sucrose and glucose tolerance test were carried out respectively. Alcohol production by the yeasts was screened and the isolates were identified by genomic techniques. Twenty-two (22) yeasts isolated from palm wine (YW), banana (YB) and pineapple (YP) were screened for their ability to ferment sugar and fourteen (14) of the yeast isolates were positive while eight (8) were negative. The fourteen (14) isolates were further screened for their ability to tolerate pH, ethanol, sucrose and glucose. Tolerance tests for these fourteen (14) yeast isolates recorded values between a range of 3.0-5.0, 0-10% v/v, 5-20% w/v and 5-25% w/v for pH, ethanol, sucrose and glucose concentrations respectively. Statistically, there was a significant difference in the interaction effect for pH, ethanol, sucrose and glucose tolerance (OD600 nm) for yeast isolates at p value ≤ 0.0001. Five (5) yeast isolates had high

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tolerance ability to pH, ethanol, sucrose and glucose and were further screened for their ability to produce alcohol. The five (5) yeast isolates were identified as *Meyerozyma guilliermondii* strain 1621, *Pichia guilliermondii* strain PX-PAT, *Meyerozyma caribbica* strain Kw 1S7Y2, *Meyerozyma caribbica* strain Y-27400, *Kodamaea ohmeri* strain ww1-1 and they produced alcohol content of 7.6%, 6.5%, 2.9%, 2.5% and 0.3% respectively. *Meyerozyma guilliermondii* strain 1621 and *Pichia guilliermondii* strain PX-PAT 18 S isolated from palm wine exhibited good characteristics and produced high quantity of alcohol and are suitable for alcohol fermentation of substrates for wine production.

Keywords: Wine production; banana and pineapple substrates; non-saccharomyces species; Meyerozyma guilliermondii; Pichia guilliermondii; yeast tolerance.

1. INTRODUCTION

Wine is a product obtained from the fermentation of grapes and grape wine is the most economically important fruit wine [1]. In the European Union, wine is legally defined as the fermented juice of grapes. Wine can be made from virtually many plant substrates containing 18 to 25% of sugars that can be fermented [2]. Wine making involves the use of yeast to ferment the "must" of a chosen fruit or fruits for a number of days, depending on the objective of the wine maker. The yeast which is the main organism responsible for alcoholic fermentation belongs to the genus, *Saccharomyces*.

Fruits such as banana, cucumber, pineapple and others are used in wine production [3,4,5]. Wine production has been practiced with various fruits such as apple, pear, strawberry, cherries, plum, banana, pineapple, oranges, cucumber, watermelon, guava etc. using species of *Saccharomyces cerevisiae* which converts the sugar in the fruit juices into alcohol and organic acids that later react to form aldehydes, esters and other chemical compounds which also help to preserve the wine [6,7]. The wine produced bears the name of the fruit or fruit mixture used in its production.

Palm wine is the fermented sap of the tropical plant, *Palmae*. It is obtained from the sap of palm trees such as *Elaesi* and *Raphia* species which contain a heavy suspension of living yeasts and bacteria [8]. It is a refreshing alcoholic drink widely produced and consumed in very large quantities in the South-Eastern part of Nigeria, Asia and Southern America [9]. It contains nutritionally important components including amino acids, proteins, vitamins and sugar [8]. These components make this wine a veritable medium for the growth of microorganisms where growth in turn, changes the physicochemical conditions of the wine giving rise to competition and succession of organisms.

Palm wine has varieties of flavours ranging from sweet (unfermented) to sour (fermented) and vinegary. It is produced by a succession of microorganisms (gram- negative bacteria, lactic acid bacteria, acetic acid bacteria and yeasts). Yeasts isolated from palm wine belong to the genera; *Saccharomyces, Pichia, Schizosaccharomyces, Kloeckera, Endomycopsis*, *Saccharomyeoides* and *Candida* which find their way into the wine from a variety of sources like air, rudimentary utensils used for tapping the palm wine, previous brew and the palm trees. Hence, it serves as a source of single cell protein and vitamins [10,6]. Similar reports by other authors have confirmed the efficacy of yeasts isolated from palm wine for wine production [11,12].

Yeasts play a key role in wine production and they can arbitrarily be divided into two categories: *Saccharomyces* and non-*Saccharomyces*. Although many genera and species of yeasts are found in musts, *S. cerevisiae* is the main yeast strain that is commonly reported to be responsible for alcoholic fermentation [13,14]. However, several researchers in their studies have reported the use of mixed cultures of non-*Saccharomyces* (like *Pichia kluyveri, Pichia fermentans Schizosaccharomyces pombe*, *Lachancea thermotolerans*, *Torulaspora delbrueckii*, *Hanseniaspora guilliermondii*) and *S. cerevisiae* in wine production [15,16,17,18,19,20,21,22,23,24,25]. Clemente-Jimenez JM et al, Benito S et al. [25,26] reported *P. fermentans* and *P. guilliermondii* as good strains for must fermentation as mixture with *S. cerevisiae* improves the aroma as well as the characteristics features of the wine. Non-*Saccharomyces* (yeast) genera frequently found in grapes and "must" include; *Hanseniaspora* (*Kloeckera*), *Candida*, *Metschnikowia, Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*,

Rhodotorula, *Zygosaccharomyces*, *Cryptococcus*and *Aureobasidium pullulans* [27,28,29,30,31,32,33].

Recent researches have focused on the role that non- *Saccharomyces* (yeast) play in wine production. The use of controlled mixed cultures of selected *Saccharomyces* and non-*Saccharomyces* strains have advantages over fermentations inoculated with pure cultures of *S. cerevisiae*. The mixed fermentation leads to the production of wines with more desirable characteristics, and starter cultures containing non- *Saccharomyces* (yeast), namely *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Pichia kluyveri* and *Metschnikowia pulcherrima* are available commercially [31].

2. MATERIALS AND METHODS

2.1 Sample Collection

Ripe Queen pineapple (*Ananas spp.*) and Cavendish banana (*Musa spp.*) fruits were purchased from Mile 3 market, Port Harcourt, Rivers State, Nigeria while fresh palm wine from *Elaeis* sp. was obtained from Chokocho community, Etche Local Government Area of Rivers State, Nigeria. The fruits were carried in a sterile plastic container, while sterile plastic bottle was used for collection of fresh palm wine samples in an ice chest cooler at 4°C and transported to the Microbiology Laboratory of Rivers State University for further analyses.

2.2 Microbiological Analyses

All glass wares used were sterilized in an autoclave at 121°C at 15 psi for 15 minutes. Yeast Extract Dextrose Peptone (YEDP) broth was used for isolation and Potato dextrose agar (PDA) was used for the inoculation and identification of the yeast isolates [34].

Yeast Extract Dextrose Peptone (YEDP) broth was prepared with 40 g of peptone water, 10 g of yeast extract and 20 g of dextrose sugar (sucrose) in 1litre of distilled water [35]. The mixture was mixed vigorously and then sterilized by autoclaving at 121°C, 15 psi for 15 minutes. It was allowed to cool before inoculation. Fifty (50) mg/L of tetracycline was added to inhibit bacterial growth.

Potato Dextrose Agar (PDA) medium was used for the isolation of the yeast. This was prepared according to manufacturer's instructions by dispensing 39 g of agar into 1000 ml of distilled water. The mixture was mixed vigorously and then sterilized by autoclaving at 121°C, 15psi for 15 minutes. It was allowed to cool to 45°C and dispensed into petri dishes for solidification and dried in a hot air oven before inoculation. Fifty (50) mg/L of tetracycline was added to the PDA media to inhibit bacterial growth.

2.3 Isolation and Maintenance of the Pure Yeast Cultures

The 48 hours fermented palm wine was agitated in a rotary shaker for thirty minutes for even distribution and an aliquot of 0.1ml of the palm wine was inoculated onto PDA medium in duplicates using the spread plate technique and incubated for 48 hours at 30°C. The colonies that appeared on the plates were further sub-cultured and incubated for another 24 hours at 30°C in order to obtain pure cultures [3].

One hundred (100) grams of the crushed banana and pineapple respectively were added to the broth and incubated for 48 hours at 30°C to enhance microbial growth. An aliquot of 0.1 ml of the YEDP broth containing the banana and pineapple substrates/ juice were inoculated onto Potato Dextrose Agar (PDA) media in duplicates using a glass spreader. The plates were incubated at 30°C for 48 hours.

The colonies that appeared on the plates were further sub-cultured and incubated for another 48 hours at 30°C in order to obtain pure cultures. Twenty-two isolates were obtained and the pure cultures of the isolates were stored in 10% glycerol at 4°C in Bijou bottles.

2.4 Screening of Yeast Isolates

2.4.1 Sugar fermentation test

The twenty- two isolates were tested for their ability to assimilate and ferment sugar. The isolates were inoculated into a test tube containing Yeast extract peptone dextrose (YEPD) broth with an inverted Durham tube. The broth was prepared by adding 15 g of peptone water, 10 g of yeast extract and 20 g of sucrose in 1000 ml of distilled water and sterilized by autoclaving at 121°C Psi for 15 minutes. Ten (10) ml of the broth containing the isolates were incubated for 48 hours at 30°C and the liberation and trapping of gas in the durham's tube indicated the result of each test; the presence of

gas was taken as evidence of fermentative activity and the absence of gas was taken as an evidence of non- fermentative activity [36]. Fourteen out of the twenty-two isolates were positive for the sugar fermentation test and further screened for their ability in wine production. Eight isolates were negative for the sugar fermentation test and were not further screened because their inability to ferment sugar signifies their inability to ferment sugar in wine "must" and produce alcohol.

2.4.2 Ethanol tolerance test

The ability of the fourteen yeast isolates to tolerate different concentrations of ethanol was examined according the procedure described by Kumar RS et al. [37] with some modifications. A loopful of yeast isolate was inoculated into 10 ml of YEDP broth of five different concentrations of ethanol (0%, 5%, 10%, 15%, 20% v/v). The inoculated tubes were incubated at 30°C for 48 hours. The growth of the inoculated yeast isolates was examined and their optical density (OD) was ascertained using a spectrophotometer.

2.4.3 pH tolerance test

The fourteen yeast isolates were examined for their ability to tolerate various pH level using the procedure described by Kumar RS et al. [37] with some modifications. A loopful of yeast isolate was inoculated into 10 ml of YEDP broth of seven different pH (2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 v/v) levels. The inoculated tubes were incubated at 30°C for 48 hours. The growth of the inoculated isolates was examined and their optical density (OD) was ascertained using a spectrophotometer.

2.4.4 Sucrose tolerance test

The ability of the fourteen yeast isolates to tolerate different concentrations of sucrose was examined using the procedure described by Kumar RS et al. [37] with some modifications. A loopful of yeast isolate was inoculated into 10 ml of YEDP broth of seven different concentrations of sucrose (0%, 5%, 10%, 15%, 20%, 25%, 30% w/v). The inoculated tubes were incubated at 30°C for 48 hours. The growth of the inoculated yeast isolates was examined and their optical density (OD) was ascertained using a spectrophotometer.

2.4.5 Glucose tolerance test

The fourteen yeast isolates were examined for their ability to tolerate different concentrations of glucose using the procedure described by Kumar RS et al. [37] with some modifications. A loopful of yeast isolate was inoculated into 10ml of YEDP broth of seven different concentrations of glucose (0%, 5%, 10%, 15%, 20%, 25%, 30% w/v). The inoculated tubes were incubated at 30°C for 48 hours. The growth of the inoculated yeast isolates was examined and their optical density (OD) was ascertained using a spectrophotometer.

2.4.6 Alcohol production test

Five out of the fourteen isolates were positive for the tolerance tests and were further screened for their ability to produce alcohol while the remaining seven isolates were not further screened. A conical flask was cleaned with distilled water, sterilized and allowed to cool. The weight of the cooled dried flask was recorded as W_1. The flask was filled with deionized water; the surface of the flask was cleaned with a cotton wool and weighed as W_2. The flask was emptied and cleaned twice with YEDP broth containing 300 g of sucrose and thereafter the flask was be filled to the brim with the broth containing each isolate. The surface of the flask was cleaned with cotton wool and weighed as W_3. The specific gravity was calculated as:

$$\text{Specific gravity} = \frac{S}{W}$$

Where

S= Weight of the volume of the "broth" (W_3-W_1)

W=Weight of the volume of water (W_2-W_1)

Each of the yeast isolate were inoculated separately in the YEDP broth containing 300 g sucrose and incubated at 30°C for 48 hours. After 48 hours, the specific gravity of the YEDP broth after fermentation was also determined.

The alcohol content was determined using the density and it was calculated according to the method described by Rangana S et al. [38] as:

%ABV= (Original specific gravity – Final specific gravity) X 131.25

Where,

ABV is alcohol by volume.

2.5 Genomic Identification

Molecular identification of the isolates was done using the Polymerase chain Reaction (PCR) to determine the presence of 18 S rRNA. This was done by extracting the DNA, carrying out PCR and sequencing of the amplified DNA (amplicon).

2.6 Extraction (Boiling Method) of the DNA

Extraction was done using a ZR fungal/ bacterial DNA prep extraction kit. A heavy growth of the pure culture of the suspected isolate was suspended in 200 microlitre of isotonic buffer into a ZR Bashing Bead Lysis tubes and 750 microlitre 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 minutes. The ZR bashing bead lysis tubes were centrifuged at 10,000 xg for 1 minute. Four hundred (400) microlitres of supernatant was transferred to a Zymo- Spin IV Filter (orange top) in a collection tube and centrifuged at 700 xg for 1 minute. One thousand two hundred microlitres (1200) of fungal/ bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing it to a final volume of 1600 microlitre. Eight hundred (800) microlitres was then transferred to a Zymo–spin IIC column in a collection tube and centrifuged at 10,000 xg for 1 minute and the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo- spin and spun. Two hundred microlitre (200) of the DNA pre- washed buffer was added to the Zymo- spin IIC in a new collection tube and spun at 10,000 xg for 1 minute followed by the addition of 500 microlitre of fungal/ bacterial DNA wash buffer and centrifuged at 10,000 xg for 1 minute. The Zymo-spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube. 100 microlitre of DNA elution buffer was added to the column matrix and centrifuged at 10,000 xg microlitre for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degrees for other downstream reactions [39].

2.7 Quantification of the Fungal DNA

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two mirolitre of the extracted DNA was loaded onto the lower pedestal and the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button [40].

2.8 Amplification of Internally Transcribed Sequence (ITS) Region

The ITS region of the genes of the isolates was amplified using the ITS4: 5ʹ-TCCTCCGCTTATTGATATGS-3ʹ and ITS5: 5ʹ-GGAAGTAAAAGTCGTAACAAGG-3ʹ, primers on ABI 9700 Applied Bio systems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: X2 Dream taq Master mix (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as a template. The PCR conditions were as follows: Initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles and final extension at 72°C for 5 minutes. The product was resolved on 1% agarose gel at 120 V for 15 minutes and visualized on a blue light transilluminator [39].

2.9 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer. The sequencing was done at a final volume of 10µl, the components included 0.25 ul BigDye terminator v1.1/3.1, 2.25 µl of 5 x BigDye sequencing buffer, 10uM primer, PCR primer and 2-10 ng PCR template per 100 bp. The sequencing condition was as follows: 32 cycles of 96°C for 10 s, 55°C for 5 s and 60°C for 4 minutes [41].

2.10 Phylogenetic Analysis and Restriction Fragment Length Polymorphorism

The obtained sequences were edited using the bioinformatics logarithm Trace edit. Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTIN. These sequences were aligned using Clustal X. the evolutionary history was inferred using the Neighbor- Joining method in MEGA 10.0 [42]. The bootstrap consensus tree inferred from 500 replicates [43] is taken to represent the evolutionary history of the taxa

analyzed. The evolutionary distances were computed using the Jukes- Cantor [44]. Restriction Fragment Length Polymorphism procedure is as follows: Cocktail mix consisting of 10 µL PCR products, 5 µL of 10X NE Buffer, 5 µL of Restriction Enzyme (EcoR1-HF with cat log R3101) and then made up to 50 µL reaction using 30 µL of nuclease free water. The cocktail mix was incubated at 37°C for 1hour after which the enzyme was inactivated at 80°C for 15 mins. The PCR fragments were resolved on 2% agarose gel electrophoresis.

3. RESULTS

Table 1 and Fig. 1 presents the results obtained for isolates subjected to sugar fermentation test after 48 hours. The yeasts were given keys; YB denoting yeasts isolated from banana, YP for yeasts isolated from pineapple and YW, yeasts isolated from palm wine. The isolates from pineapple (YP1, YP2) and palm wine (YW1, YW3, YW4, YW7, YW9) were able to ferment the sugar after 24 hours. The isolates from banana (YB1, YB2, YB4, YB5, YB6), pineapple (YP3, YP4), were able to ferment the sugar after 48 hours while the isolates from banana (YB3), pineapple (YP5, YP6) and palm wine (YW2, YW5, YW8, YW10) didn't have the ability to ferment the sugar at all. The isolates that could not ferment sugar after 24 and 48 hours were not further screened while the yeast isolates that had the ability to ferment sugar after 24 and 48 hours of fermentation were subjected to further screening for their efficacy in wine production.

Figs. 2 and 3 are the results of the tolerance tests for pH used to screen the yeast isolates. The isolates were inoculated into Yeast Extract Dextrose Peptone (YEDP) broth containing various concentrations of pH. The isolates were incubated at 30°C for 48 hours and their optical density (OD) was read using a spectrophotometer. The result of the OD of the yeast isolates were 0.403 – 1.172; 0.53 – 1.394; 1.078 – 1.980; 1.166 – 1.765; 1.187 – 1.722; 1.170 – 1.752 and 1.230 – 1.734 at a pH of 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 respectively. The broken line indicates the line of best fit. The yeast isolates with OD above the broken line were tolerant. The pH values above the line are the level of pH the yeast isolates were able to tolerate. The graph showed a positive slope which indicates that there was an increase in tolerance with an increase in pH value. The isolates were able to tolerate a pH range of 3.0-5.0 with an optimum value at 5.0. Statistically, analysis of variance (anova) test revealed that there was a significance difference between the optical densities of the yeast isolates for pH ($P \leq 0.0001$).

Figs. 4 and 5 are the results of the tolerance tests for ethanol used to screen the yeast isolates. The isolates were inoculated into Yeast Extract Dextrose Peptone (YEDP) broth containing various concentrations of ethanol. The isolates were incubated at 30°C for 48 hours and their optical density (OD) was read using a spectrophotometer. The result of the OD of the yeast isolates were 1.038 – 1.854; 0.909 – 1.771; 0.695 – 1.770; 0.099 – 1.872 and 0.516 – 1.840 for ethanol concentration of 0, 5, 10, 15 and 20% v/v respectively. The broken line indicates the line of best fit. The yeast isolates with OD above the broken line were tolerant. The ethanol concentration above the line is the level of ethanol the yeast isolates were able to tolerate. The graph showed a negative slope which indicates that there was a decrease in ethanol tolerance with an increase in ethanol

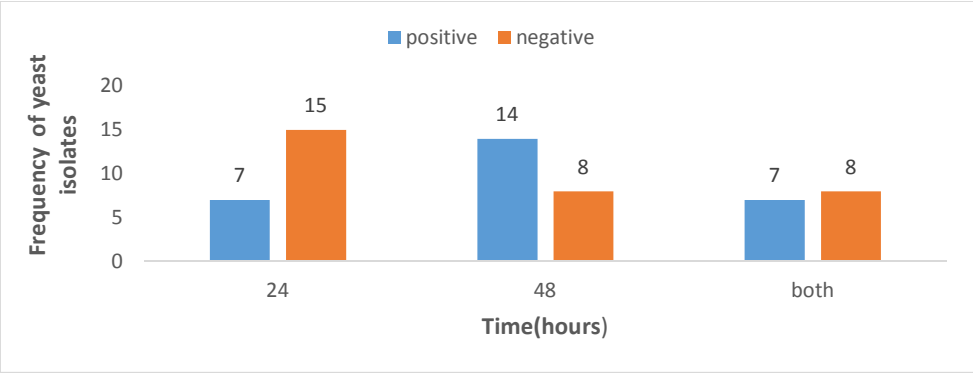

Fig. 1. Sugar fermentation of yeast isolates within 24 and 48 hours
"Both" indicates the frequency of yeast isolates that were positive or negative at 24 and 48 hours

Table 1. Sugar fermentation by yeast isolates at 24 and 48 hours

Isolates	Fermentation at 24	Fermentation at 48
YB1	Negative	Positive
Meyerozyma caribbica (YB2)	Negative	Positive
YB3	Negative	Negative
YB4	Negative	Positive
YB5	Negative	Positive
YB6	Negative	Positive
YP1	Positive	Positive
YP2	Positive	Positive
YP3	Negative	Positive
Kodamaea ohmeri (YP4)	Negative	Positive
YP5	Negative	Negative
YP6	Negative	Negative
Meyerozyma guilliermondii (YW1)	Positive	Positive
YW2	Negative	Negative
Pichia guilliermondii (YW3)	Positive	Positive
YW4	Positive	Positive
YW5	Negative	Negative
YW6	Negative	Negative
YW7	Positive	Positive
YW8	Negative	Negative
Meyerozyma caribbica (YW9)	Positive	Positive
YW10	Negative	Negative

KEY: YB – Yeast isolated from banana;
YP – Yeast isolated from pineapple;
YW– Yeast isolated from palm wine;
Negative – no sugar fermentation;
Positive – sugar fermentation

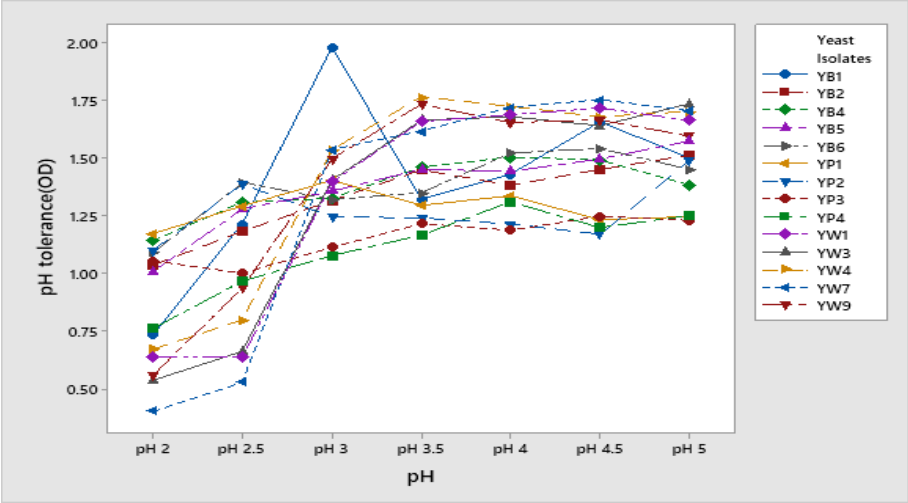

Fig. 2. Interaction effect plot for pH tolerance (OD600 nm) of the yeast isolates

concentration. The yeast isolates were able to tolerate ethanol concentrations of 0-10% with an optimum value at 0% v/v. Statistically, analysis of variance (anova) test revealed that there was a significance difference between the optical densities of the yeast isolates for ethanol ($P \leq 0.0001$).

Figs. 6 and 7 are the results of the tolerance tests for sucrose used to screen the yeast isolates. The isolates were inoculated into Yeast Extract Dextrose Peptone (YEDP) broth containing different concentrations of sucrose. The isolates were incubated at 30°C for 48 hours and their optical density (OD) was read using a

spectrophotometer. The result of the OD of the yeast isolates were 0.476 – 1.587; 1.319 – 1.963; 1.340 – 2.297; 1.000 – 2.297; 1.070 – 2.225; 1.118 – 2.058 and 0.903 –1.988 at sucrose concentrations of 0, 5, 10, 15, 20, 25 and 30 % w/v respectively. The broken line indicates the line of best fit. The yeast isolates with OD above the broken line were tolerant. The sucrose concentrations above the line are the level of sucrose the yeast isolates were able to tolerate. The graph showed a negative slope which indicates that there was a decrease in tolerance with an increase in sucrose concentration. The yeast isolates were able to tolerate sucrose concentrations of 5-20% with an optimum value at 10% w/v. Statistically, analysis of variance (anova) test revealed that there was a significance difference between the optical densities of the yeast isolates for sucrose ($P \leq 0.0001$).

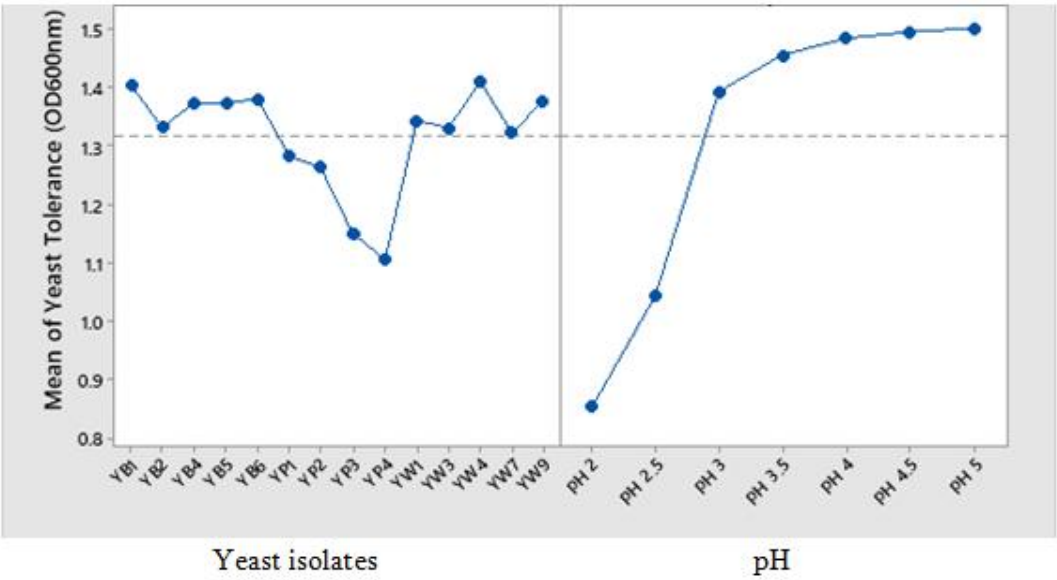

Fig. 3. Main effects plot of yeast isolates pH tolerance (OD600 nm) test

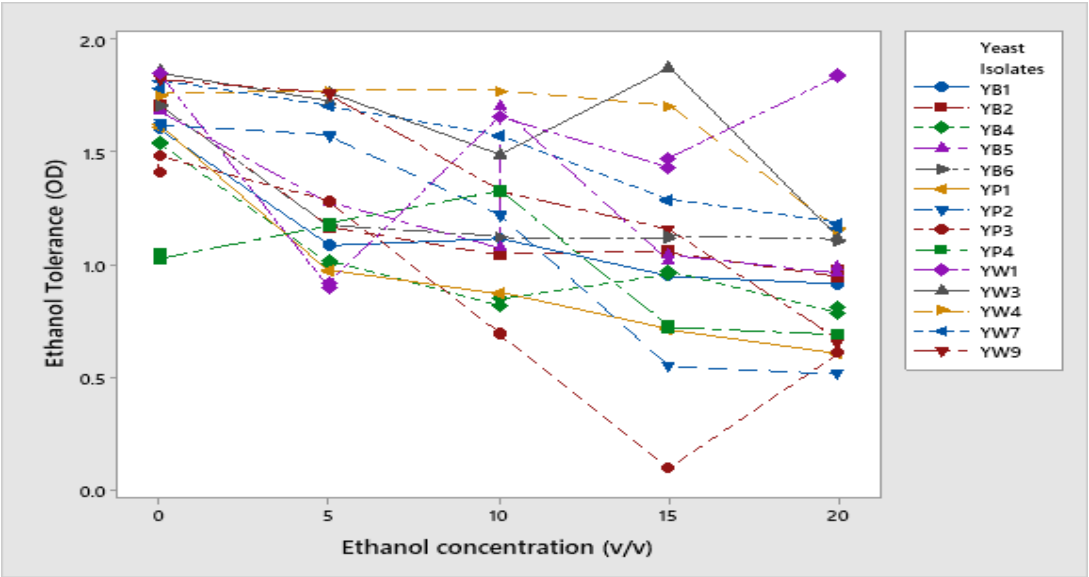

Fig. 4. Interaction effect plot for ethanol tolerance (OD600 nm) of yeast isolates

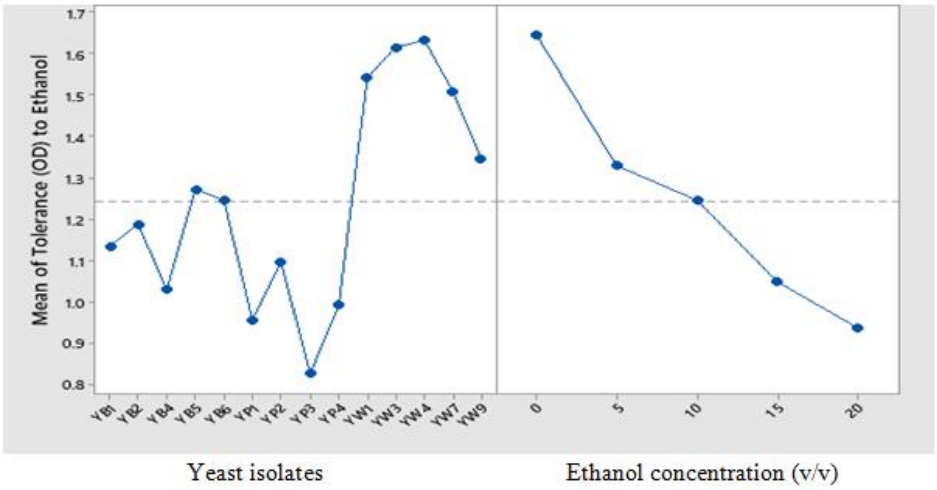

Fig. 5. Main effects plot for ethanol tolerance (OD600 nm) for yeast isolates

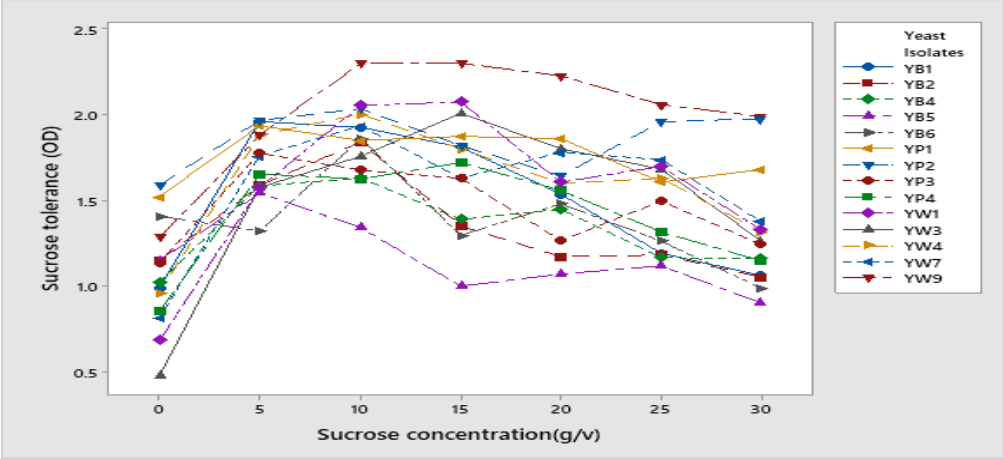

Fig. 6. Interaction effect plot of sucrose tolerance (OD600 nm) of yeast isolates

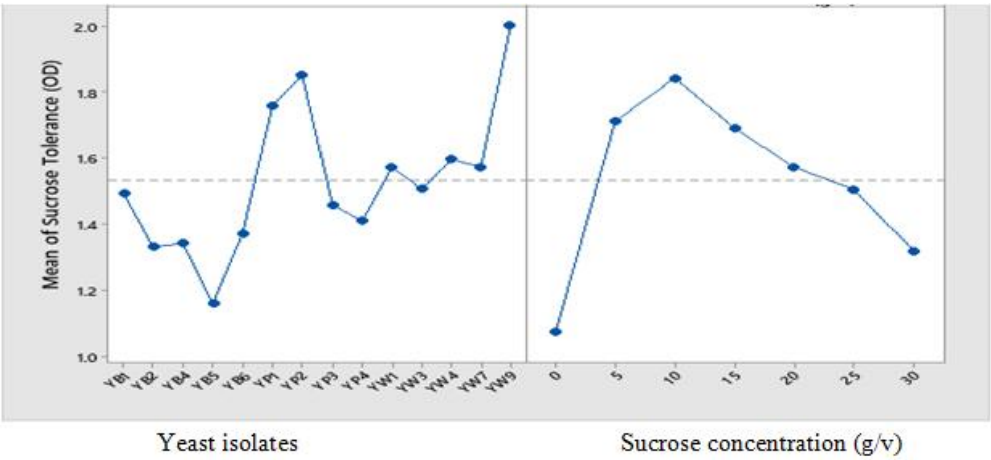

Fig. 7. Main effects plot for sucrose tolerance (OD600 nm) for yeast isolates

Figs. 8 and 9 are the results of the tolerance tests for glucose used to screen the yeast isolates. The isolates were inoculated into Yeast Extract Dextrose Peptone (YEDP) broth containing different concentrations of glucose. The isolates were incubated at 30°C for 48 hours and their optical density (OD) was read using a spectrophotometer. The result of the OD of the yeast isolates were 0.909 – 1.609; 1.389 – 2.210; 1.403 – 2.393; 1.674 – 2.397; 1.394 – 2.365; 1.374 – 2.290 and 1.143 – 2.210 at glucose concentrations of 0, 5, 10, 15, 20, 25 and 30 % w/v respectively. The broken line indicates the line of best fit. The yeast isolates with OD above the broken line were tolerant. The glucose concentrations above the line are the level of glucose the yeast isolates were able to tolerate. The graph showed a negative slope which indicates that there was a decrease in tolerance with an increase in glucose concentration. The yeast isolates were able to tolerate glucose concentrations of 5- 25% with an optimum value at 10% w/v. Statistically, analysis of variance (anova) test revealed that there was a significance difference between the optical densities of the yeast isolates for glucose ($P \leq 0.0001$).

Fig. 10 shows the result of the percentage of alcohol produced by each yeast isolate after 48 hours of fermentation. *M. guilliermondii* strain 1621 produced 7.6% alcohol, *P. guilliermondii* strain PX-PAT 18S produced 6.5% alcohol, *M. caribbica* strain Kw 1S7Y2 18S produced 2.9% alcohol, *M. caribbica* strain Y-27400 18S produced 2.5% alcohol while *K. ohmeri* strain ww1-1 18 S produced 0.3% alcohol. *M. guilliermondii* strain 1621 and *P. guilliermondii* strain PX-PAT 18S produced the highest amount of alcohol of 7.6 and 6.5% respectively and were chosen as the fermenting yeasts for the wine production.

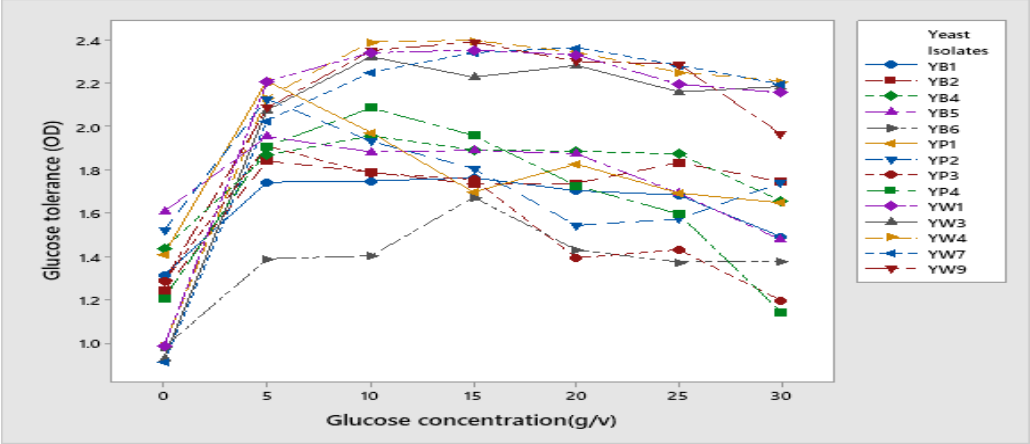

Fig. 8. Interaction effect plot of glucose tolerance (OD600 nm) of yeast isolates

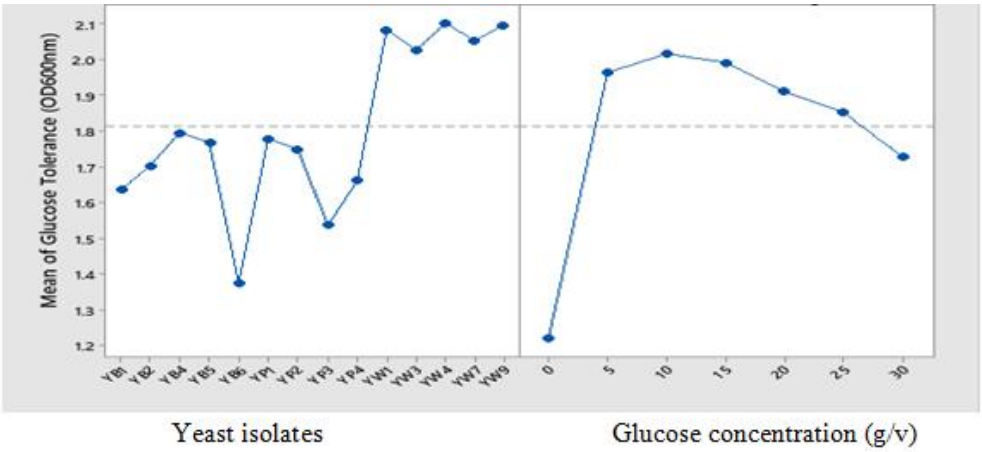

Fig. 9. Main effects plot for sucrose tolerance (OD600 nm) for yeast isolates

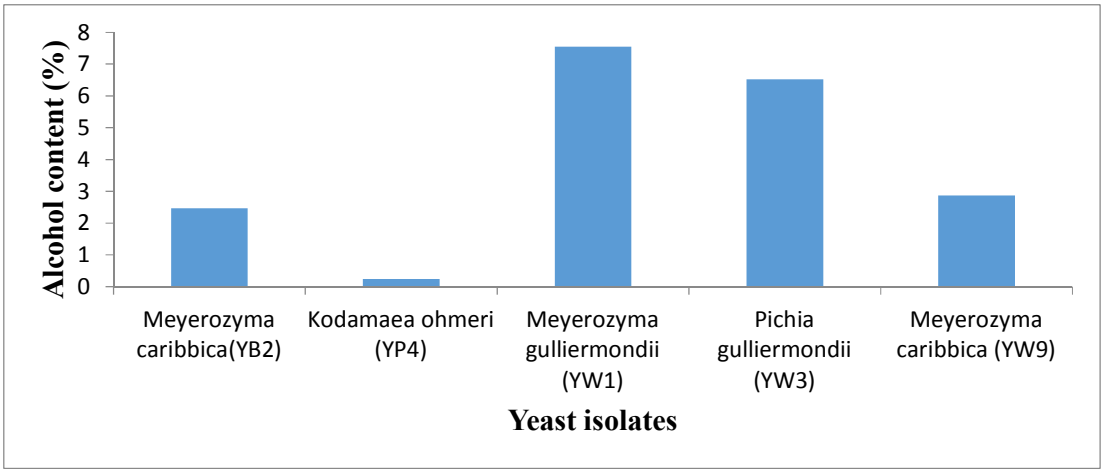

Fig. 10. Alcohol production test of yeast isolates

Table 2. Morphological and microscopic identification of the isolates

Cultural characteristics	Microscopic characteristics	Probable organism
Creamy, circular, raised colonies that are small in size	Oval with budding	Yeast
Creamy, circular, raised colonies that are large in size	Oval with budding	Yeast

Table 2 present the colonial morphology and cell characteristics used to identify the isolates

Plate 1 shows the extracted and amplified internally transcribed sequence (ITS) regions of the yeast isolates after agarose gel electrophoresis. The region is unique amongst fungi. This agarose gel electrophoresis shows that the DNA of the yeasts was actually extracted and amplified. Lanes YW1 to YW9 represents the ITS gene bands of the yeast isolates occurring at 650 base pair (bp) while lane M represents the molecular ladder which is 200bp.

Plate 2 shows RFLP using EcoR1 Restriction Endonuclease. M is a 50 bp molecular weight ladder while lanes YW1 to YW9 indicates the yeast isolates.

Fig. 11 is the phylogenetic tree showing the evolutionary distance between the yeast isolates. The obtained ITS 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 ITS region showed a percentage similarity to other species at 89-96%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of ITS region of the isolate YW1 within the *Meyerozyma sp*. and revealed a closely relatedness to *M. guilliermondii* strain 1621, isolate YB2 also belonged to the *Meyerozyma sp*. and revealed a closely relatedness to *M. caribbica* strain Y-2700 18 S and isolate YW9 also within the *Meyerozyma sp*. revealed a closely relatedness to *M. caribbica* strain Kw 1S7Y2 18 S. Isolate YW3 was within the *Pichia sp.* and revealed a closed relatedness to *P. gulliermondii* strain PX-PAT 18 S while isolate YP4 belonged to the *Kodamaea* sp. and revealed a close relatedness to *K. ohmeri* strain ww1-18S.

Table 3 shows the molecular results of the isolates using the NCBI data base to show the percentage relatedness

4. DISCUSSION

Sugar such as sucrose is one of the main substrates fermented from fruit juice into alcohol (ethanol), carbon dioxide and lactic acid. These constituents contribute to the chemical composition and sensory qualities of wine [45,46] Fourteen (14) out of the twenty-two (22) yeast isolates were able to ferment sugar at 24 and 48 hours of inoculation. This was in accordance with the findings of Antia EU et al. [47] who reported the ability of 20 yeast isolates to ferment sucrose after 24 hours.

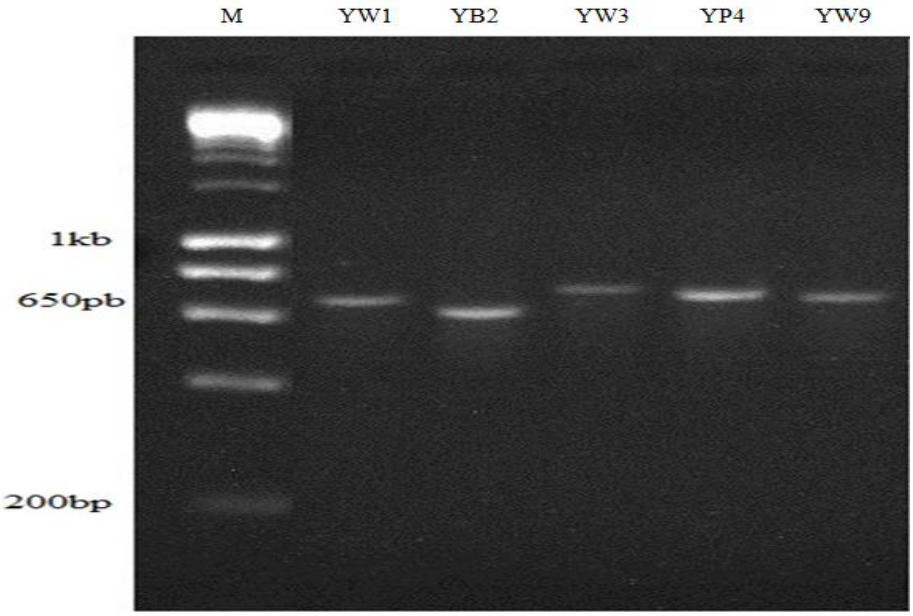

Plate 1. Agarose gel electrophoresis of the ITS region of the yeast isolates
Lanes YW1 to YW9 represents the ITS gene bands of the yeast isolates while lane M represents the molecular ladder

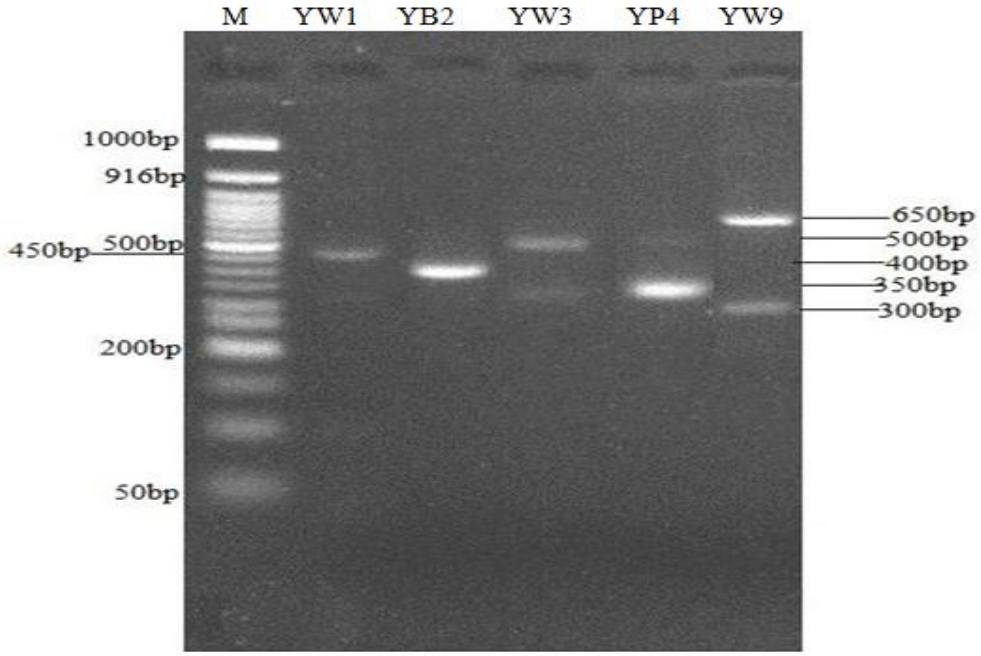

Plate 2. Restriction fragment length polymorphism using EcoR1 restriction endonuclease

According to Fleet GH, [48], pH plays an important role in wine fermentation because it directly affects the stability of wine. Yeasts, lactic acid bacteria and even spoilage bacteria grow well at pH 4.5.The preferred pH for wine production is 3.6. Wine yeasts and some lactic acid bacteria can still thrive in a pH range of 3.3-3.6 while spoilage bacteria and undesirable organisms cannot [45]. The fourteen (14) yeasts isolates were screened for their ability to tolerate

different pH (2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0) and five (5) isolates identified as *M. guilliermondii* strain 1621, *M. caribbica* strain Y-27400 18S, *P. guilliermondii* strain PX-PAT 18S, *K. ohmeri* strain ww1-1 18S and *M. caribbica* strain Kw 1S7Y2 18S were able to tolerate pH range of 3.0 to 4.5. Since the preferred pH for wine production is 3.6, the isolates are suitable for wine production. This is in accordance with the findings of Roukas T, [49], who observed that *S. cerevisiae* was able to tolerate a pH range of 3.5 to 6.5 [50] who reported that yeast isolates were able to tolerate a wide pH range of 2.0 to 4.0.

Ethanol tolerance is a unique property of yeasts that makes it exploitable for industrial applications [51]. The fourteen (14) yeast isolates screened for tolerance to different concentrations of ethanol (0, 5, 10, 15 and 20 % v/v), proved that the yeast isolates were tolerant to ethanol concentrations at 10% v/v. This implies that the yeast strains can remain metabolically active in the fermentation medium, when ethanol builds up to 10%. This result is in accordance with the findings of Nwachukwu IN et al. [52] who reported that *S. globosus* and *S. cerevisiae*, were able to tolerate ethanol concentration at 10 and 11% v/v respectively. Aminu et al. [53] reported that *S. cerevisiae* was able to tolerate ethanol concentration at 8% v/v. This report was in contrast with the findings of Kumar RS et al. [37] and Antia EU et al. [47] who also reported that *S. cerevisiae* was able to tolerate ethanol at 20% v/v.

Table 3. Sequence identification from NCBI BLAST HITS and their percentage relatedness

S/N	Sequence code	NCBI BLAST relative	Accession number	Percentage (%) relatedness
1	YW1	*Meyerozyma guilliermondii* strain 1621	MK418263	91.80%
2	YB2	*Meyerozyma caribbica* strain Y-27400 18S	KJ705036	95.30%
3	YW3	*Pichia guilliermondii* strain PX-PAT 18S	GQ497898	94.00%
4	YP4	*Kodamaea ohmeri* strain ww1-1 18S	EF190229	96.80%
5	YW9	*Meyerozyma caribbica* strain Kw 1S7Y2 18S	KF268353	89.70%

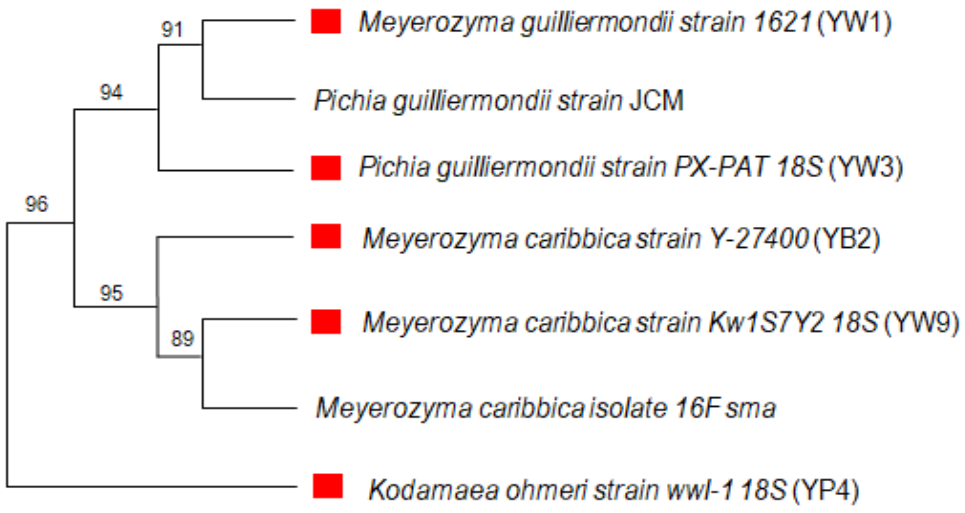

Fig. 11. Phylogenetic tree showing evolutionary distance between the yeast isolates

In wine making, sugar (sucrose or glucose) is added to the must to increase the alcohol content and decrease the acidity of the wine. In this process, the sugar is assimilated by the yeast into ethanol and carbon dioxide [54]. The addition of 18 to 20 w/v of sugar into the substrate enhanced the final concentration of alcohol by 1% after fermentation [55]. The fourteen (14) yeast isolates were also screened for their ability to tolerate different concentrations of sucrose and glucose (0, 5, 10, 15, 20, 25 and 30% w/v). Five (5) of the isolates identified as *Meyerozyma guilliermondii* strain 1621, *Meyerozyma caribbica* strain Y-27400 18 S, *Pichia guilliermondii* strain PX-PAT 18 S, *Kodamaea ohmeri* strain ww1-1 18 S and *Meyerozyma caribbica* strain Kw 1S7Y2 18 S were able to tolerate sucrose of 20% and glucose of 25 w/v. This implies that the yeast strains can remain metabolically active in the fermentation medium containing up to 20% w/v of sucrose and 25% w/v of glucose and utilize these sugars and convert them to alcohol during fermentation. This result was in accordance to the findings of Guimaraes TM et al. [56] who reported the ability of *Saccharomyces cerevisiae* to tolerate sucrose and glucose concentrations of 20 w/v.

Five (5) yeast isolates that had better tolerance were identified by genomic studies as non-*Saccharomyces* species: *M. guilliermondii* strain 1621, *M. caribbica* strain Y-27400 18 S, *P. guilliermondii* strain PX-PAT 18S, *K. ohmeri* strain ww1-1 18 S and *M. caribbica* strain Kw 1S7Y2 18 S. This is in agreement with the findings of some authors who reported the presence of *S. cerevisiae*, *S. globosus* and *H. uvarum* from palm wine [47,52]; *S. cerevisiae*, *S. kluvyeri*, *Debaromyces hansenii* from banana [57]; *S. cerevisiae* from pineapple [58].

Alcohol production is an important parameter to test for the efficiency of yeast strains in wine production and fruit wines contain 6 to 14% alcohol content by volume [59]. Five (5) yeast isolates were screened for their ability to produce alcohol after 48 hours of sugar (sucrose) fermentation. *M. guilliermondii* strain 1621 produced 7.6%, *P. guilliermondii* strain PX-PAT 18S produced 6.5%, *M. caribbica* strain Kw 1S7Y2 18S produced 2.9%, *M. caribbica* strain Y-27400 18 S produced 2.5% while *K. ohmeri* strain ww1-1 18S produced 0.3% alcohol. *Meyerozyma guilliermondii* strain 1621 and *Pichia guilliermondii* strain PX-PAT 18S produced high levels of alcohol of 7.5 and 6.5% respectively which makes them suitable for wine production. This result is similar to the findings of Santoshkumar P et al. [60] who reported that *S. ellipsoideus* produced alcohol level of 8.3%.

The low alcohol production by *M. caribbica* strain Kw 1S7Y2 18S, *M. caribbica* strain Y-27400 18S and *K. ohmeri* strain ww1-1 18 S yeasts may be due to their inability or poor ability to ferment sucrose in the absence of oxygen, so the organisms are unable to carry out fermentation of sugar to alcohol [61].

The ability of *M. guilliermondii* strain 1621 and *P. guilliermondii* strain PX-PAT 18 S to tolerate a pH range of 3.0-5.0, 10% ethanol. 20% sucrose, 25% glucose and produce alcohol at concentrations of 7.6 and 6.5% makes them suitable for wine production from banana and pineapple substrates.

5. CONCLUSION

This study was carried out to screen yeasts isolated from banana, pineapple and palm wine for their efficacy in wine production. Twenty-two (22) isolates were obtained and were screened for their ability to ferment “must” to wine by subjecting them to sugar fermentation test, pH, ethanol, sucrose and glucose tolerance tests. Five (5) isolates identified as *Meyerozyma guilliermondii* strain 1621, *Meyerozyma caribbica* strain Y-27400 18 S, *Pichia guilliermondii* strain PX-PAT 18 S, *Kodamaea ohmeri* strain ww1-1 18S and *Meyerozyma caribbica* strain Kw 1S7Y2 18S were able to tolerate pH range of 3.0 to 4.5, ethanol concentration of 10%, sucrose concentration of 20% and glucose concentration of 25% which are important characteristics for wine production but when the five (5) isolates were subjected to alcohol production test, only two (2) isolates (*M. guilliermondii* strain 1621 and *P. guilliermondii* strain PX-PAT 18 S) were able to produce high alcohol content of 7.5 and 6.5%, respectively. The inability of *M. caribbica* strain Kw 1S7Y2 18 S, *M. caribbica* strain Y-27400 18 S and *K. ohmeri* strain ww1-1 18 S to produce high amount of alcohol may be attributed to inability or poor ability to ferment sucrose in the presence of oxygen.

This study has shown that *Meyerozyma guilliermondii* strain 1621 and *Pichia guilliermondii* strain PX-PAT 18 S are suitable yeast isolates for wine production using banana and pineapple as substrates. However, further studies could be carried out on measures to

increase the alcohol producing ability of *M. caribbica* strain Kw 1S7Y2 18 S, *M. caribbica* strain Y-27400 18 S and *K. ohmeri* strain ww1-1 18S yeasts. Their ability to produce high concentration of alcohol in mixed culture can also be studied.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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