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Stress Tolerance of Locally Sourced Wild Non-Saccharomyces Suitable for Vinification

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ABSTRACT

This study illustrates the stress tolerance levels of locally sourced yeast for vinification. *Candida tropicalis*$^{(H4)}$ and *Clavispora lusitaniae*$^{(H7)}$ isolated from honey; *Candida tropicalis*$^{(B10)}$ and *Candida tropicalis*$^{(B7)}$ isolated from banana using standard mycological methods and *Candida tropicalis*$^{(CY)}$, a commercial wine yeast, were subjected stress tolerance at various percentages of ethanol (v/v), glucose, sucrose, fructose, metabisulphite (w/v) and pH. The decreasing order of the optimal stress tolerance of yeasts for ethanol was H4 (0.56 OD, 25%) > B7 (0.56 OD, 20%) > B10 (0.58 OD, 20%) and H7 (0.50 OD, 15%) > CY (1.07 OD, 10%); Glucose tolerance, B10 (1.24 OD, 30%) > B7 (1.07 OD, 30%) > CY (1.01 OD, 30%) > H4 (1.07 OD, 25%) > H7 (1.06 OD, 20%); Sucrose tolerance, H4 (1.46 OD, 30%) > B10 (1.23 OD, 30%) > CY (1.17 OD, 30%) > H7 (1.08 OD, 30%) > B7 (1.04 OD, 25%); fructose tolerance, CY (1.636 OD, 20 %) > B10 (1.648 OD, 20 %) > B7 (1.089 OD, 5 %) > H4 (1.362 OD, 10 %) > H7 (1.124 OD, 5%) > B7 (1.089 OD, 5 %); pH tolerance, B7 and B10 (1.22) > H7 (1.21) > CY (1.12) > H4 (0.73) (OD) at pH 2. Metabisulphite tolerance, H4 (0.68 OD, 0.3g/100ml) > B10 (0.63 OD, 0.3g/100ml) > B7 (0.52 OD, 0.3g/100ml) > CY (0.68 OD, 0.25g/100ml) > H7 (0.56 OD, 0.25g/100ml). The wild non-Saccharomyces isolates possess stress tolerance ability.

Keywords: Stress Tolerance, Banana, Honey, Vinification, Non-Saccharomyces.

Introduction

Nigeria is a country located in tropical region, rich with abundance of fruits (Ewekeye *et al.*, 2013). Conversion of fruit juice into wine will serve as a nutrient supplement for seasonal fruits throughout the year (Varela, 2016; Kavitha & Kannahi, 2018). Postharvest spoilage of tropical fruits has been reported by several researchers (Amadi *et al.*, 2014; Esteve-Zarzoso *et al.*, 2013). Fermentation is the cheapest and energy efficient process of preserving perishable raw materials like pawpaw, mango, pineapple, banana, lemon and watermelon (Okafor *et al*., 2018).

Over the last few decades, the control of microorganisms using biotechnological approaches has increased in the field of winemaking in monitoring and controlling of undesired yeasts (Ciani & Comitini, 2011); with the knowledge that winemaking is a complex process requiring microorganisms that possess interesting biotechnological properties.

Therefore, it is worthy of acceptation that search for yeast with oenology potentials is necessary: screening and strain selection of non-Saccharomyces yeasts possessing desirable characteristics (Mateo & Maicas, 2016), that could improve wine quality, stability and food safety of wine (Suárez-Lepe and Morata, 2012). Recent studies on winemaking are recommending mixed fermentation (combining *Saccharomyces cerevisiae* and non-Saccharomyces yeasts cells for fermentation) as a practical way to improve complexity and a particular characteristic of wine (Ciani *et al.*, 2009), as non-*Saccharomyces* yeasts persevere at various stages of wine fermentations with *Saccharomyces cerevisiae* as co-fermenter or pure starter culture (Comitini *et al.*, 2011).

The biotechnological characteristics of wine yeast include sugar fermentation, stress tolerance (Viana *et al.*, 2014), flocculation (Soares, 2011; Stewart, 2018), low hydrogen sulphide production (Mendes-Ferreira *et al*., 2002), alcohol production and organic acid production (Nandy & Srivastava, 2018).

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Industrial yeasts should be proficient to tolerate numerous stresses and acclimatise to adverse environmental factors to avoid significant viability loss as alcohol common to fermented beverages is the consequence of yeast metabolic activity on substrates. The autochthonous (wild *Saccharomyces* and non-*Saccharomyces*) yeasts are responsible for spontaneous fermentation (Varela, 2016), which are not employed in winemaking due to off-flavours (Benito *et al.*, 2016).

Acetic acid, ethyl acetate, acetaldehyde and high concentration of acetoin, limited fermentation potential and low Sulphur dioxide (SO_2) resistance (Benedictis *et al.*, 2011) while multi-tolerance and similar or even better fermentation efficiency of non-*Saccharomyces* when compared to *Saccharomyces cerevisiae* in the presence of certain stressful conditions (Mukherjee *et al.*, 2017a) had been reported. This implies that there is need for biotechnological evaluation of locally sourced yeasts for co-fermentation or pure culture with desired results.

Materials and Methods

Source of Wild Yeast and Commercial Wine Yeast

Wholesome ripe banana fruit and Honey were purchased from Oro-Ekpo junction market in Port Harcourt metropolis and Ekpoma in Edo State, respectively, while commercial wine yeast "Muntons GV1 Gervin Universal Wine Yeast" was imported from United States of America.

Isolation of yeast from banana fruit and honey

An overripe banana fruit (20.0 g) and honey (20.0 ml) were introduced into separate 200 ml sterile peptone water and incubated at 30°C for 24-72 hours. Aliquot (0.1ml) were plated on Yeast Extract Peptone Glucose (YEPG) agar supplemented with 0.003g/ml erythromycin to prevent bacteria growth and incubated at 30oC for 24 h.

The isolated yeast colonies were sub-cultured using YEPG agar to obtain pure culture (Guimaraes et al., 2006). The isolates were streaked on YEPG agar slants and maintained at 4°C (Nnodim *et al*., 2021).

Yeast starter culture resuscitation

The yeast starter culture was maintained at 4 ºC in refrigerator prior to its use. One (1) gram of commercial wine yeast cells were transferred into test tube holding 10 ml YEPG (containing 5 g/l yeast extract, 10 g/l peptone, 10 g/l glucose, 20 g/l agar and pH 5.6) broth and incubated with agitation (120 rpm) at 30 ºC for 24 h. Subsequently, 0.1 ml of the test tube content was transferred to YEPG agar supplemented with 0.003g/ml erythromycin and incubated at 30 ºC for 24 h (Nissen *et al.*, 2003).

Molecular identification of yeast isolates

DNA extraction, PCR amplification of the fungi 18S rRNA PCR gene and gel electrophoresis of the screened isolates were carried out at Bioinformatics services, Ibadan. The PCR product was sent to the International Institute of Tropical Agriculture (IITA), Ibadan for sequencing the 18S rRNA. Yeast DNA was extracted using Zr Fungal DNA Miniprep (Manufactured by Zymo research, cat number: D6005). Two (2) millilitres of pure culture of fungal cells broth and 750µl Lysis Solution was added to ZR BashingTM Lysis Tube, secured in a bead fitted with 2 ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR Bashing BeadTM Lysis Tube was centrifuge in a microcentirifuge at 10,000 rpm x g for 1 minute. Four hundred microliter (400 µl) of supernatant was transferred to a Zymo-SpinTM IV Spin Filter (orange top) in a collection tube and centrifuge at 7000 rpm x g for 1 minute. Fungal/Bacterial DNA Binding Buffer (1200 µl) was added to the filtrate in the collection tube with a volumetric capacity of 1600 ul. Transfer 800 µl of the mixture to a Zymo-SpinTM IIC Column in a collection tube and centrifuge at 10,000 rpm x g for 1 minute, then discard the flow through from the collection tube, the remaining volume was transferred to the same Zymo-SpinTMIIC and centrifuged at 10,000 rpm x g for 1 minute. Two hundred microliters (200 µl) DNA Pre-Wash Buffer was added to the Zymo-Spin TM IIC Column in new collection tube and centrifuge at 10,000 rpm x g for 1 minute and then, 500 µl Fungal DNA Wash Buffer was added to the Zymo-SpinTM IIC Column and centrifuge at 10,000 x g for 1 minute. The Zymo-SpinTM IIC Column was transfer to a clean 1.5 µl microcentrifuge tube and 100µl (35 µl minimum) DNA Elution Buffer was added directly to the column matrix, Centrifuge at

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10,000 rpm x g for 30 seconds to elute the DNA. The extracted DNA was then stored at -200°C for other downstream reactions. The concentration and purity of the extracted genomic DNA of the fungal isolates were estimated using a Nanodrop 1000 spectrophotometer. The absorbance was taken at 260 nm and 280 nm for each sample and the ratio of absorbance at 260 nm and 280 nm were used to assess the purity of the DNA. A ratio of -1.8 is generally accepted as "pure" for DNA while a ratio of -2.0 is generally accepted as "pure" for RNA. The primer sequences ITS4 (5´-TCCTCCGCTTATTGATATGS -3´) and ITS5 (5´-GGAAGTAAAAGTCGTAACAAGG-3´) were employed for PCR amplification of fungi 18S rRNA gene for fungi Characterization (Guimarães et al., 2006). The PCR mix, made up of 12.5µL of Taq 2X Master Mix from New England Biolabs (M0270), 1µL each of 10µM forward and reverse primer was with 2µL of DNA template and then made up with sterile 8.5µL Nuclease free water.

The sequencing machine used was 3130XL genetic analyser from Applied Biosystems while the PCR thermal cycler used was GeneAmp PCR system 9700. The PCR cycling parameters were: Initial denaturation at 94˚C for 5mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 55˚C for 30secs and elongation at 72˚C for 45sec. Followed by a final elongation step at 72˚C for 7 minutes and hold temperature at 10˚C. After the PCR reaction, 5 microliters (5 µL) of the amplified products were separated on a 1% agarose gel. Six hundred base pair (600 bp) DNA ladder was used as DNA molecular weight marker. Electrophoresis was done at 120 V for 20 min. and the gel was visualised using UV transilluminator to determine the size of the DNA of the isolates.

The Sanger method and 3130XL genetic analyser from Applied Biosystems were used to sequence the amplified 18S products. The sequence generated by the sequencer was visualised using Bioformatic Algorithms such as Chromoslite for base calling. MEGA12 software was used for sequence editing before performing a Basic Local Alignment Search Tool (BLAST) using NCBI (National Centre for Biotechnology Information) database (https://blast.ncbi.nim.nih.gov/Blast.cgi). Similar sequences were downloaded and aligned with MUSCLE in MEGA 12 software (Guimarães et al., 2006; Maicas *et al.*, 2016; Nnodim *et al.*, 2021).

Determination of Stress tolerance

The stress tolerance of yeast isolates was quantified based on the broth turbidity measured in OD. As increase or decrease in OD measured at 600 nanometer wavelengths (OD_{600nm}) across the concentrations signifies tolerance or intolerance to ethanol, sucrose, glucose, pH or metabisulphite: low OD, low tolerance and higher OD, higher tolerance. For the main effect and interaction plot, concentrations (%) with points above the reference line (broken red line) are considered tolerated by the yeast isolates, while points below the reference line are concentrations not tolerated by yeasts isolates. Yeast isolates with points above the reference line (broken line) are regarded as tolerant yeasts; below the reference line are intolerant yeasts.

Ethanol tolerance test

Ethanol tolerance was carried out according to Alabere *et al.* (2020) with modification. Ten millilitres of sterile YEPG medium supplemented with 0 %, 5 %, 10 %, 15 %, 20 %, 25% and 30 % (v/v) of 99.7 % ethanol, then inoculated with yeast isolates cell suspension and incubated at 30 °C for 48 hours. The cell density was measured with spectrophotometer (OD_{600nm}) to estimate the actual optical density (final optical density - initial optical density) of each suspended yeast cells and read off on spectrophotometer (OD_{600nm}) against the medium as the blank. Growth was measured by spectrophotometer (OD600nm) and ethanol threshold for tolerance was set at 0.500 OD600nm.

Sugar tolerance test

The yeast isolates were screened for sugar (glucose and sucrose) tolerance according to Lee *et al.* (2011) with required modification. Ten millilitres of sterile YEPG medium supplemented with 0 %, 5 %, 10 %, 15 %, 20 %, 25 % and 30 % (g/v) of glucose and sucrose respectively, autoclave at 121 °C, 15 Psi for 15 minutes and cooled, then inoculated with yeast isolates cell suspension (0.1) and incubated at 30 °C for 48 hours. The cell density was measured with spectrophotometer (OD_{600nm}) to estimate the actual optical density (final optical density - initial optical density) of each suspended yeast cell by spectrophotometer (OD_{600nm}) against the medium as the blank and sugar threshold for tolerance was set at 1.000 OD_{600nm}.

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pH tolerance test

pH tolerance of yeast isolates was carried out as per the procedure of Alabere *et al.* (2020) with slight modification. 10.0 ml YEPG broth pH was adjusted to pH 2, 2.5, 3, 3.5, 4, 4.5 and 5 respectively using H_2SO_4, afterward autoclaved (at 121ºC, 15 Psi for 15 minutes) and cooled. Each test tube contains YEPG media with different pH and blank media were then inoculated with yeast cell suspension (0.10 ml) that is 48 hours old and incubate at 30 °C for 48h. After 48h, growth was measured by spectrophotometer (OD_{600nm}) and pH threshold for tolerance was set at 0.500 OD_{600nm}.

Sulphite tolerance test

To determine sulphite (metabisulphite) tolerance of yeast isolates, the method of Nardi *et al*. (2010) was adopted with slight modification. Ten (10) millilitre sterile peptone water broth was inoculated with yeast isolates from a 48 h YEPG broth culture at a final OD_{600} of 0.05 in micro-aerobic conditions (flasks fitted with stoppers to maintain anaerobic condition). 0.1 ml of broth culture was inoculated into 0, 0.10, 0. 15, 0.20, 0.25 and 0.30 g/100ml of metabisulphite prepared (in peptone water broth, pH 6.0) and incubated at 30 °C for 48 hours. Growth was measured by a spectrophotometer (OD_{600nm}) and a sulphite threshold for tolerance was set at 0.500 OD_{600nm}.

Statistical analysis

Analysis of variance (ANOVA) was applied to the experimental data using Minitab version 19.1. The significant differences were determined by means of the Tukey test, and the results were considered significant if the associated P values are below 0.05. Data were presented in tables and graphs (Comitini *et al.*, 2011).

Results

The Gel electrophoresis (DNA finger printing) of the yeast isolates B7 and B10 isolated from banana fruit; H4 and H7 isolated from wild honey and commercial yeast CY a starter culture (from Muntons Plc, Stowmarket, United Kingdom) screened for stress tolerance characteristic that is indispensable in vinification process is shown in Plate 1.

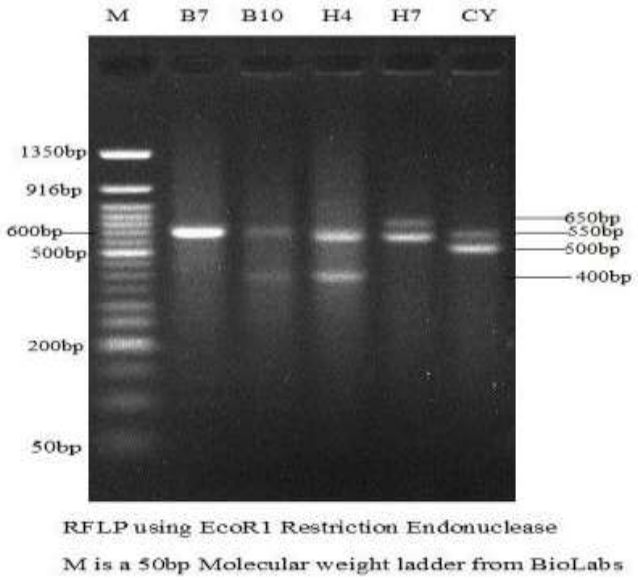

Plate 1: DNA fingerprinting yeast isolates from wild and commercial wine yeast. Molecular Ladder (M); *Candida tropicalis* (B7); *Candida tropicalis* (B10); *Candida tropicalis* (CY); *Candida tropicalis* (H4); *Clavispora lusitaniae* (H7).

The yeasts isolates cells showed a common gene fragment of 550bp molecular weight. The isolates were identified as *Candida tropicalis* Pe1, *Candida tropicalis* WC65-1, *Candida tropicalis* WC57, *Clavispora lusitaniae* WM03.178 and *Candida tropicalis* zhuan4 respectively. Sequence Identification from NCBI BLASTN Hits and Percentage Relatedness and Accession numbers of the yeast isolates are presented in Table 1. The evolutionary distance of five yeast isolates as inferred from their nucleotide sequence is presented in the Phylogenetic tree in Figure 1. The unrooted optimal phylogram grouped the yeast isolates into two (2) clades. The first clade consists of *Candida tropicalis*^{B7}, *Candida tropicalis*^{H7,} *Candida tropicalis*^{CY} being a paraphyletic taxon, and *Clavispora lusitaniae*^{H7} and *Candida tropicalis*^{B7} are 100 % similar in nucleotide sequence, while the second clade consists of *Candida tropicalis*^{B10} and *Candida tropicalis*^{H7} with 100 % similarity in nucleotide sequence. The rate of mutation per nucleotide sequence site is 0.003 and 0.346 for the first and second clade respectively. Within the first clade, 0.104 and 0.275 mutation per sequence site of the ancestor (node 7) of *Candida tropicalis*^{B7} *and Clavispora lusitaniae*^{H7}, and *Candida tropicalis*^{CY},

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respectively, while *Candida tropicalis*^{B7} and *Clavispora lusitaniae*^{H7} had mutation rates of 0.246 and 0.154, respectively. *Candida tropicalis*^{H4} and *Candida tropicalis*^{B10} have 0.005 and 0.007 mutations per sequence site respectively in the second clade.

The result of the main effect of Ethanol stress tolerance of yeast isolates is presented in Figure 2. Ethanol concentration of 0 – 20 (% v/v) are considered tolerable by the yeast isolates and 25 – 30 (% v/v) are considered inhibiting ethanol concentrations by the yeast isolates. Ethanol stress tolerance (OD_{600nm}) of the yeast isolates decreased with increase in ethanol concentration, showing a negative correlation. The OD values of ethanol concentrations (0 – 30%) for yeast isolates range from: *Candida tropicalis*^{B10} (1.418±0.03 – 0.268±0.02), *Candida tropicalis*^{B7} (1.604±0.13 – 0.422), *Candida tropicalis* ^{CY} (1.424±0.03 – 0.307±0.02), *Candida tropicalis*^{H4} (1.489±0.02 – 0.493±0.01) and *Clavispora lusitaniae*^{H7} (1.389±0.02 – 0.396±0.01): *Candida tropicalis*^{H4} and *Candida tropicalis*^{B10} had the highest and lowest OD values across ethanol concentrations (Figure 3). There was a decrease in the tolerance of yeast isolates with an increase in ethanol concentration, *Candida tropicalis*^{B7} and *Candida tropicalis*^{H4} tolerating 25 % (v/v), *Candida tropicalis*^{B10} and *Clavispora lusitaniae*^{H7} tolerating 15 % (v/v) and *Candida tropicalis* ^{CY} tolerating 10 % (v/v) ethanol concentration. Statistically, there is a significant difference in ethanol tolerance among the yeast isolates at p-value ≤ 0.0001.

Table 1: Sequence Identification from NCBI BLASTN Hits and Percentage Relatedness

Isolate code	NCBI BLASTN relative	Accession number	E Value	% Relatedness
B7	*Candida tropicalis* Pe 1	MK752669	2.50E-179	93.10
B10	*Candida tropicalis* WC65-1	EF190223	0	95.00
CY	*Candida tropicalis* zhuan4	EF192229	0	92.10
H4	*Candida tropicalis* WC57	EF198007	0	94.80
H7	*Clavispora lusitaniae* WM03	KF268353	8.52E-35	77.60

Keys: B = yeast isolated from banana; H = yeast isolated from honey and CY = commercial wine yeast.

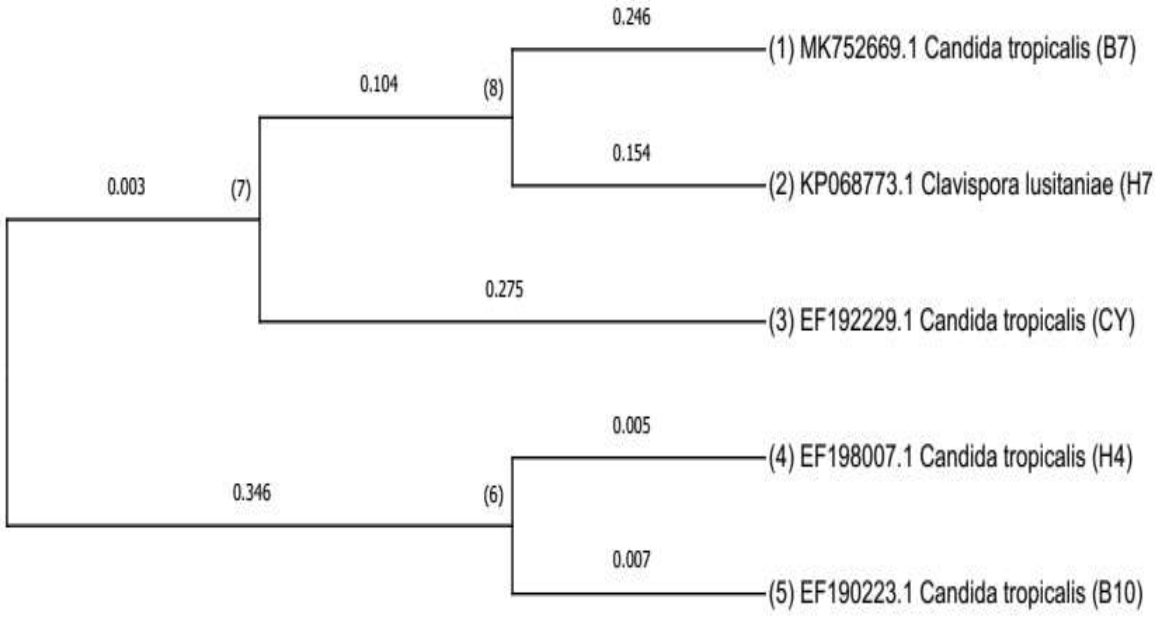

Fig. 1: Evolutionary distance of five yeast isolates as inferred from their nucleotide sequence

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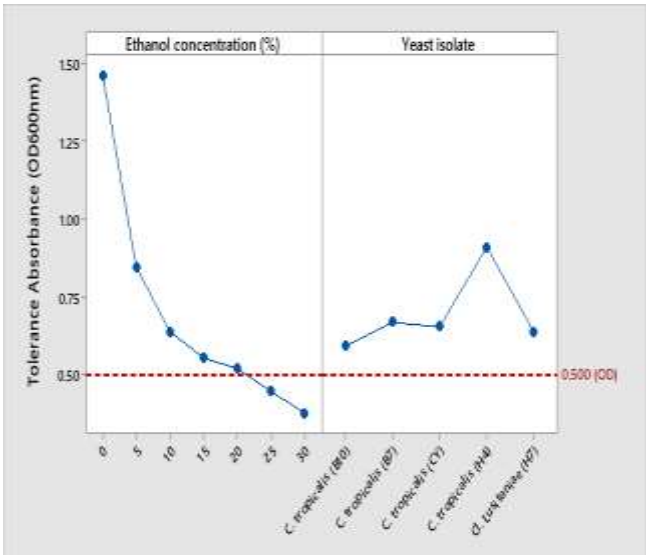

Fig. 2: Main effect plot of ethanol stress tolerance of wild yeast isolates and commercial wine yeast

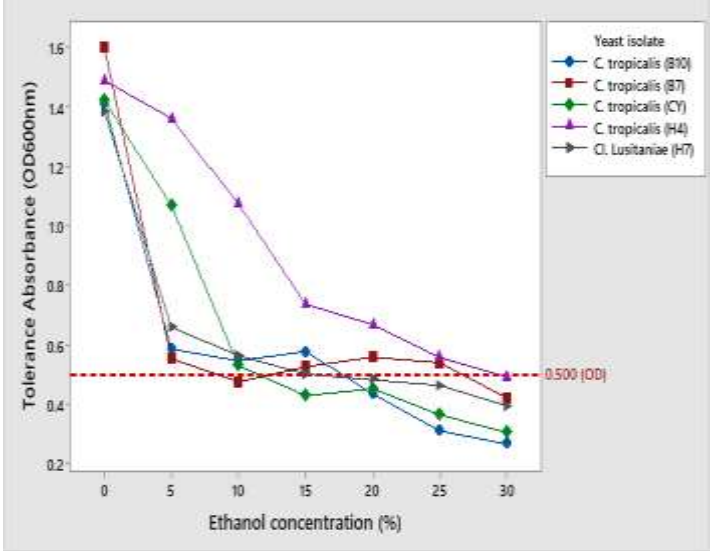

Fig. 3: Interaction plot showing ethanol stress tolerance of wild yeast isolates and commercial wine yeast

The result of the main effect of Glucose stress tolerance of yeast isolates is presented in Figure 4. The glucose stress tolerance with respect to glucose concentration (%) impact and yeast isolates responses are shown respectively. The concentrations of glucose (5 – 25 %[v/v]) are tolerated by the yeast isolates, thus can be referred to as glucose tolerant yeasts. Glucose stress tolerance (at OD_{600nm}) decreased with increase in glucose concentration, with optimum tolerance at 5 % Glucose for *Candida tropicalis*^{B10} (1.323±0.02), *Candida tropicalis* ^{CY} (1.424±0.01), *Candida tropicalis*^{H4} (1.761±0.01), *Clavispora lusitaniae*^{H7} (1.460±0.06) and optimum tolerance at 15 % for *Candida tropicalis*^{B7} (1.614±0.04). There is a significant difference in glucose tolerance among the yeast isolates at p-value ≤ 0.0001. The interaction plot (Figure 5) reveals a decline in the stress tolerance ability of the yeast isolates with an increase in glucose concentration (%). The yeast isolates OD values for glucose tolerance recorded the following *Candida tropicalis*^{B7} (0.542 - 1.614), *Candida tropicalis*^{CY} (0.817 - 1.424), *Candida tropicalis*^{H4} (0.548 - 1.761), *Candida tropicalis*^{B10} (0.811 - 1.345) and *Clavispora lusitaniae*^{H7} (0.586 – 1.460 OD) and the highest glucose concentration tolerance of the yeast isolates are as follows: *Candida tropicalis*^{B7} (1.073 OD, 30 %) > *Candida tropicalis*^{CY} (1.205 OD, 25 %) > *Candida tropicalis*^{H4} (1.068 OD, 25 %) > *Candida tropicalis*^{B10} (1.073 OD, 20 %) > *Clavispora lusitaniae*^{H7} (1.057 OD, 20 %). Yeast isolates showed an increase in glucose concentration, with significant difference at p-value = 0.001. The result of the main effect of fructose stress tolerance of yeast isolates is presented in Figure 6. The points below or above the red broken line (1.000 OD) indicate intolerance or tolerance. The main effect plot shows that fructose concentrations 5 – 20 (%) were tolerated and 25 – 30 (%) were not tolerated, the trend of fructose concentration tolerated are as follows: 15 % (1.079 OD) < 5 % (1.187 OD) < 10 % (1.151 OD) < 20 % (1.226 OD). There is a significant difference in stress due to fructose concentrations at a p-value ≤ 0.001. *Candida tropicalis*^{B10} (1.280 OD) > *Candida tropicalis*^{CY} (1.076 OD) > *Candida tropicalis*^{H4} (1.026 OD) >*Candida tropicalis*^{B7} (0.928 OD) are fructose tolerant, while *Clavispora lusitaniae*^{H7} (0.882 OD) is fructose intolerant. Statistically, there is a significant difference in yeast isolates fructose tolerance ability at p-value ≤ 0.001. The interaction effect plot of fructose tolerance by the yeast isolates shown in Figure 7. The ranking of the optimal fructose tolerance of yeast isolates are as follows: *Candida tropicalis*^{CY} (1.636 OD, 20 %) > *Candida tropicalis*^{B10} (1.648 OD, 20 %) > *Candida tropicalis*^{H4} (1.362 OD, 10 %) > *Clavispora lusitaniae*^{H7} (1.124 OD, 5%) > *Candida tropicalis*^{B7} (1.089 OD, 5 %). Only *Candida tropicalis*^{B10}, *Candida tropicalis*^{CY} and *Candida tropicalis*^{B7} were able to tolerate up to 20 % of fructose. There is a significant difference in the fructose tolerance of yeast isolates and fructose concentrations interaction at p-value ≤ 0.001.

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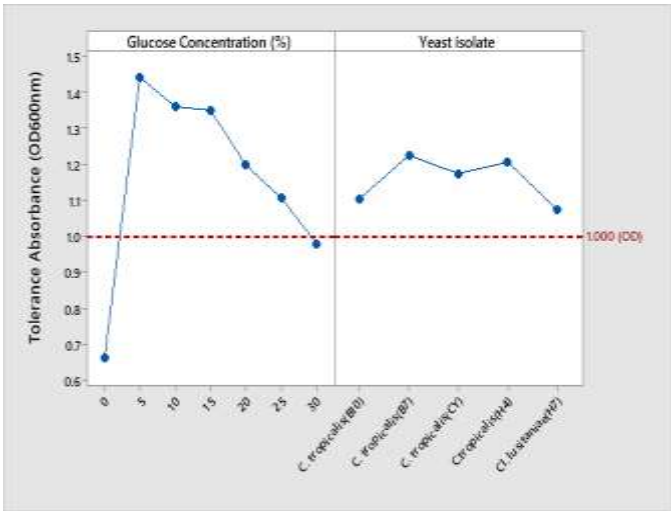

Fig. 4: Main effect plot showing glucose stress tolerance of wild yeast isolates and commercial wine yeast

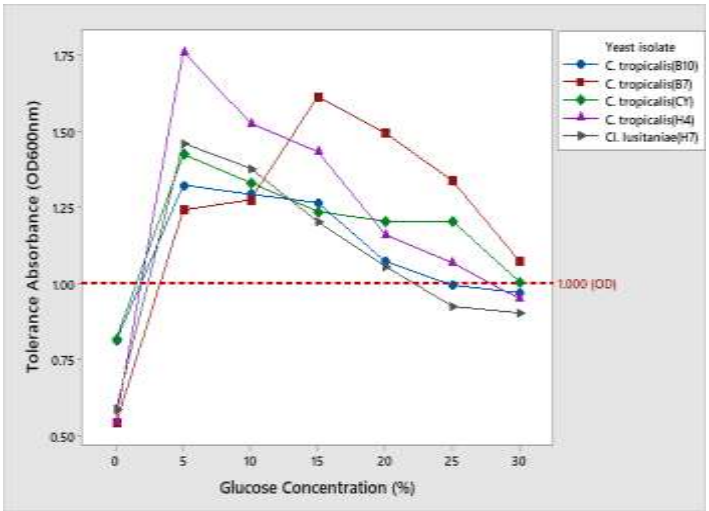

Fig. 5: Interaction effect showing Glucose stress tolerance of wild yeast isolates and a commercial wine yeast

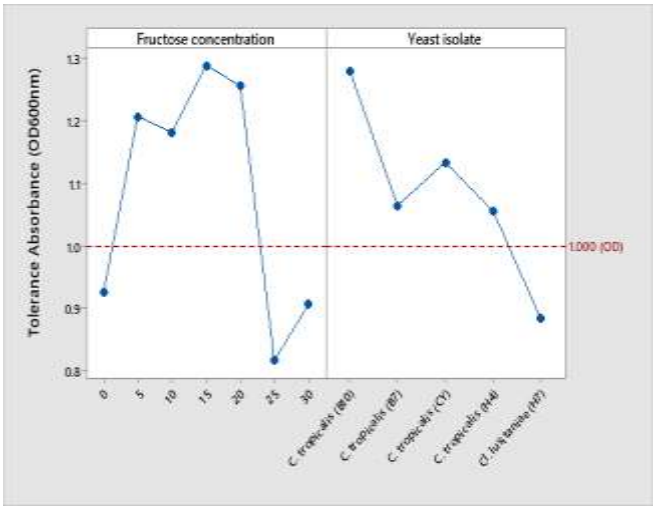

Fig. 6: Main effect plot showing fructose stress tolerance of wild yeast isolates and commercial wine yeast

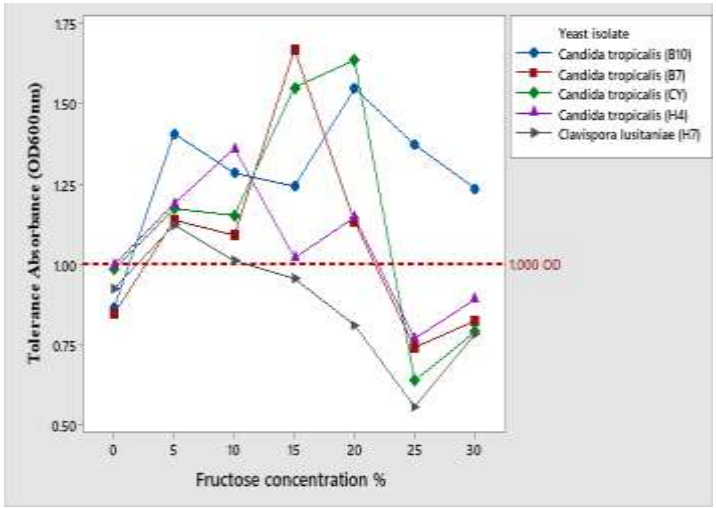

Fig. 7: Interaction effect showing Fructose stress tolerance of wild yeast isolates and a commercial wine yeast

The result of the main effect of sucrose stress tolerance of yeast isolates is presented in Figure 8, which illustrates the sucrose stress tolerance (OD_{600nm}) trend (main effect plot) of yeast isolates and sucrose concentration. Sucrose concentration was above the reference line marked at OD value 1.000, with 15% (g/v) as the optimum sucrose stress tolerance concentration; while *Candida tropicalis*^{H4} and *Candida tropicalis*^{B7} had the highest and lowest sucrose tolerance, respectively.

The OD value of sucrose stress tolerance is above 1.000 for each yeast isolates (*Candida tropicalis*^{B10} (1.335, 10%); *Candida tropicalis*^{B7} (1.539, 15%); *Candida tropicalis* ^{CY} (1.622, 15%); *Candida tropicalis*^{H4} (1.517, 5%, and 1,357, 15%) and *Clavispora lusitaniae*^{H7} (1.365, 5%), respectively, with *Candida tropicalis*^{CY} as the most sucrose tolerant yeast (Figure 9). Yeast isolates, Sucrose concentration and yeast isolates* Sucrose concentration shows a p-value ≤ 0.0001.

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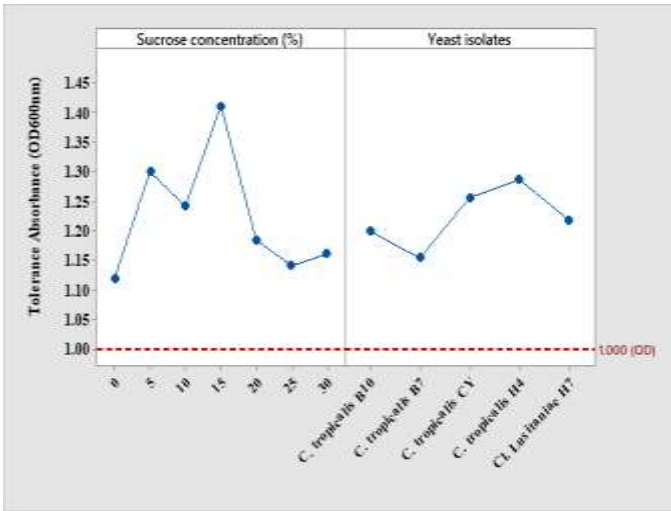

Fig. 8: Main effect plot showing Sucrose stress tolerance of wild yeast isolates and a commercial wine yeast

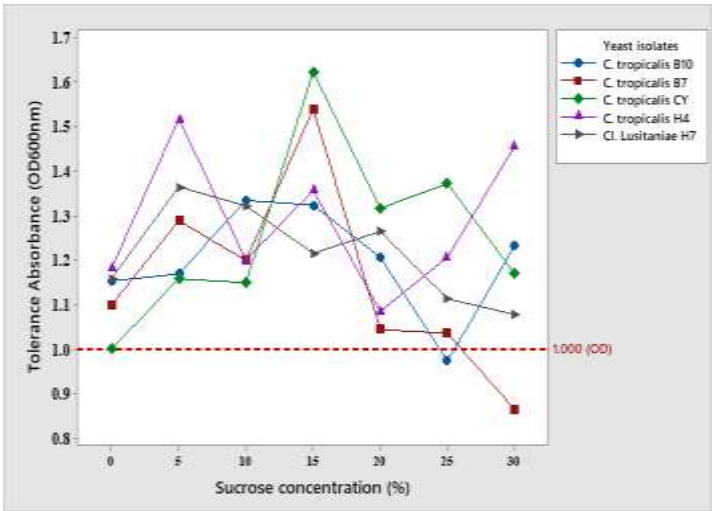

Fig. 9: Interaction effect showing Sucrose stress tolerance of wild yeast isolates and a commercial wine yeast

The result of the main effect of metabisulphite stress tolerance of yeast isolates is presented in Figure 10**.** The main effect plot of metabisulphite concentration stress and yeast isolates tolerance are shown, respectively. Increase metabisulphite concentrations (g/100 ml) and bring about increased metabisulphite stress (decrease in OD value 0 % (1.264 OD), 10 % (0.755 OD), 15 % (0.710 OD), 20 % (0.686 OD), 25 % (0.550 OD) and 30 % (0.457 OD)); and metabisulphite concentration with OD values ≥ 0.500 OD (reference red line) are considered to tolerate metabisulphite concentration. The yeast isolates above the reference line were considered metabisulphite tolerant: Yeast isolates *Candida tropicalis*^{H4} (0.852 OD) expressed the highest tolerance ability, while *Candida tropicalis*^{CY} (0.676 OD) expressed the least tolerance ability to metabisulphite. The interaction plot (Figure 11) showing a linear decline due to the inhibitory effect of metabisulphite concentrations (g/100 ml) on the yeast isolates, and metabisulphite concentration and yeast isolate interaction with OD values above the reference line (≥ 0.500) is considered tolerated metabisulphite concentration by a specific yeast.

The OD values reported are as follows: *Candida tropicalis*^{B10} (1.358 - 0.545 OD), *Candida tropicalis*^{B7} (1.365 - 0.465 OD), *Candida tropicalis*^{CY} (1.300 - 0.400 OD), *Candida tropicalis*^{H4} (1.200 - 0.475 OD) and *Clavispora lusitaniae*^{H7} (1.103 - 0.400 OD) from 0 - 0.30 (g/100ml), respectively. The highest tolerated metabisulphite concentrations stress by yeast isolates are as follows: *Candida tropicalis*^{B10} (0.545 OD, 0.30 g/100ml) > *Candida tropicalis*^{H4} (0.679 OD, 0.25 g/100ml) > *Candida tropicalis*^{B7} (0.519 OD, 0.25 g/100ml) > *Candida tropicalis*^{CY} (0.675 OD, 0.20 g/100ml) > *Clavispora lusitaniae*^{H7} (0.559 OD, 0.20 g/100ml). Statistically, there is a significant difference in the interaction between metabisulphite tolerance of the yeast isolates and metabisulphite concentrations (g/ 100ml) at p-value < 0.001.

The result of the main effect of pH stress tolerance of yeast isolates is presented in Figure 12. The inhibitory property of pH (measured in OD) declined with increase in pH value and the points above the broken red line (> 1.000 OD) is considered as tolerated pH point - 2.0 (0.350 OD), 2.5 (1.099 OD), 3.0 (1.425 OD), 3.5 (1.523 OD), 4.0 (1.529 OD), 4.5 (1.458 OD), and 5.0 (1.450 OD), with pH 3.5 (1.523 OD) and 4.0 (1.529 OD) as the optimal pH values. The yeast isolates tolerate the different pH ranges. The interaction plot shows the relationship between the various pH values and yeast isolates tolerance represented in Figure 13., and OD values ≥ 1.000 are considered pH tolerant. The OD values recorded for each pH by the yeast isolates are as follows, *Candida tropicalis*^{B10} (0.428 - 1.572 OD), *Candida tropicalis*^{B7} (0.333 - 1.636 OD), *Candida tropicalis*^{CY} (0.346 - 1.562 OD), *Candida tropicalis*^{H4} (0.330 - 1.506 OD) and *Clavispora lusitaniae*^{H7} (0.314 - 1.577 OD). The yeast isolates had OD values ≥ 1.000 at a pH of 2.5 – 5, except *Clavispora lusitaniae*^{H7} which could tolerate pH values of 3.0 – 5.0. Statistically, there is a significant difference in the main effect and interaction of pH tolerance ability of the yeast isolates and pH values at p-value < 0.001, respectively.

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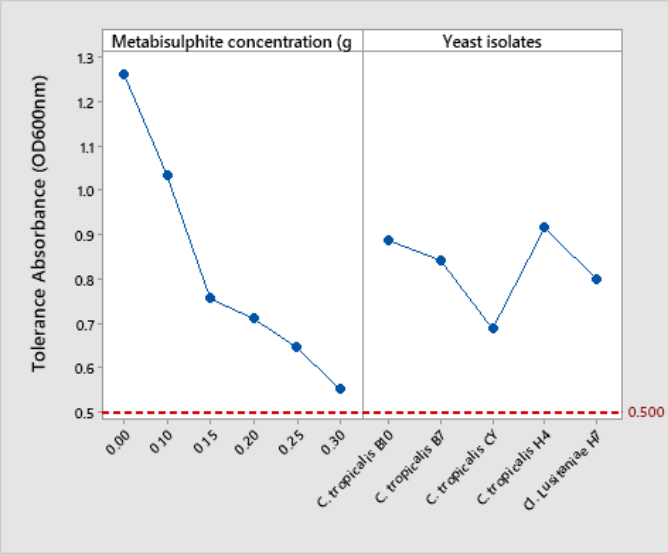

Fig. 10: Main effect of metabisulphite stress tolerance of wild yeast isolates and a commercial wine yeast

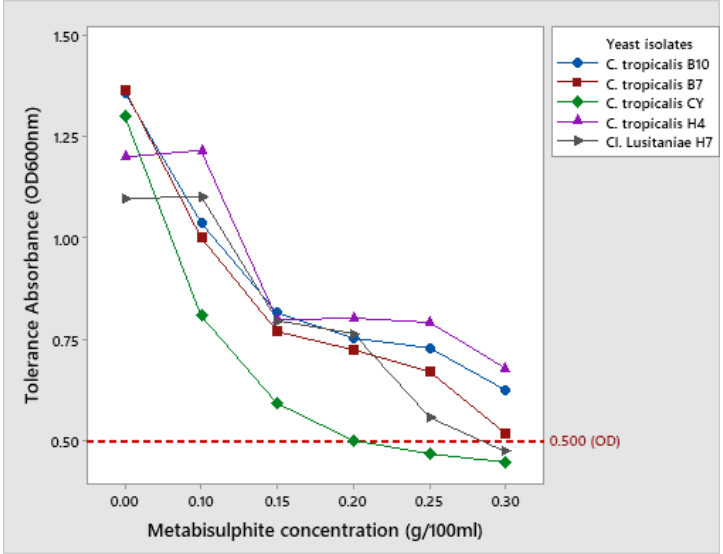

Fig. 11: Interaction effect of metabisulphite stress tolerance of wild yeast isolates and commercial wine yeast

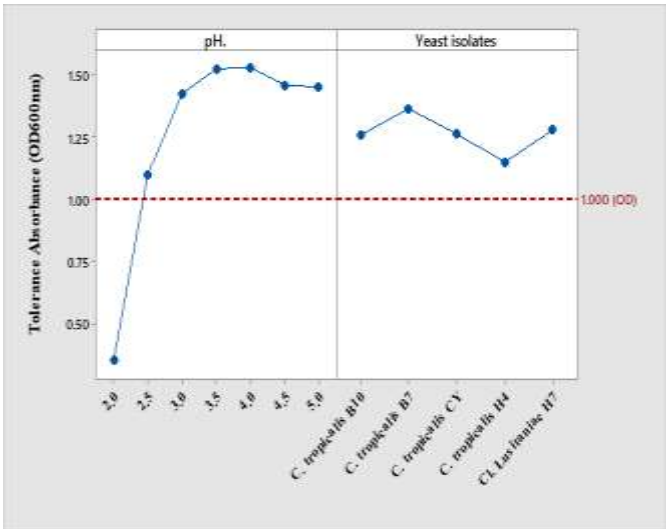

Fig. 12: Main effect plot showing pH stress tolerance of wild yeast isolates and a commercial wine yeast

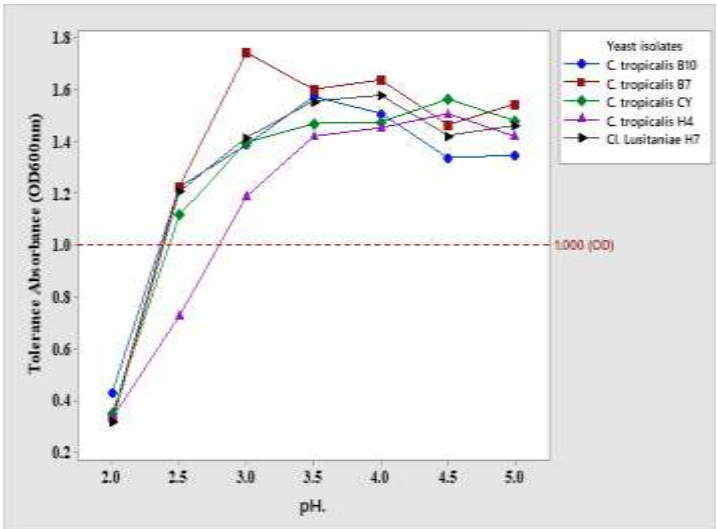

Fig. 13: Interaction effect showing pH stress tolerance of wild yeast isolates and a commercial wine yeast

The result of the cluster variables analysis of the similarity in stress impact of fructose, glucose, sucrose, ethanol, metabisulphite and pH is presented in Figure 14. Understand that variables in the group have similar characteristics. The dendrogram grouped the effect of the treatments into 2 major clusters (cluster 1[blue] and 2 [red]) with inhibition similarity of 6.51 %. Cluster 1, ethanol and metabisulphite variables possess 90.17 % similarity; Cluster 2, fructose-sucrose and glucose-pH nodes have similarity of 51.33 %. Fructose and sucrose variables have similarity of 60.73 %, while that of glucose and pH was76.86 %.

The similarity in yeast isolates tolerance to the inhibitory effect of sugars, metabisulphite, pH, and ethanol is shown on the dendrogram (Figure 15). The response of yeast isolates to stress was grouped into 2 clusters (cluster 1[blue] and 2 [red]) with a tolerance similarity of 85.27 %. In cluster 1, *Candida tropicalis*^{B7}, *Candida tropicalis*^{CY} and *Candida tropicalis*^{B10} variables showed 91.94 % similarity, while *Candida tropicalis*^{B7} and *Candida tropicalis*^{CY} have 93.73 % similarity. Cluster 2, *Candida tropicalis*^{H4} and *Clavispora lusitaniae*^{H7} showed 90.46 % similarity.

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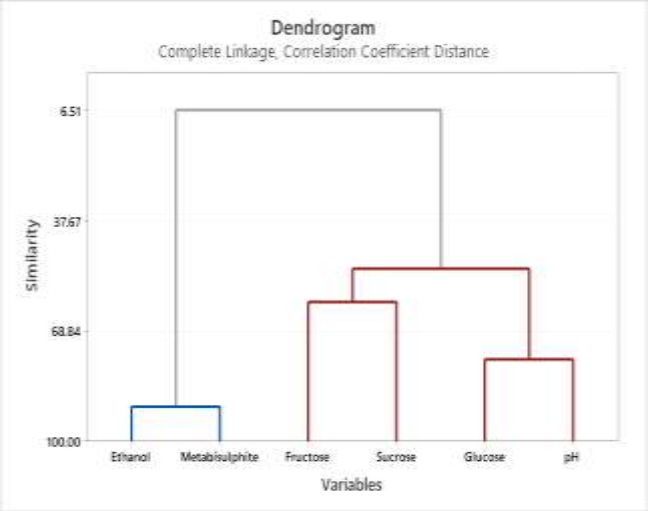

Fig. 14: Cluster variable analysis showing relative similarity in stress impact of samples

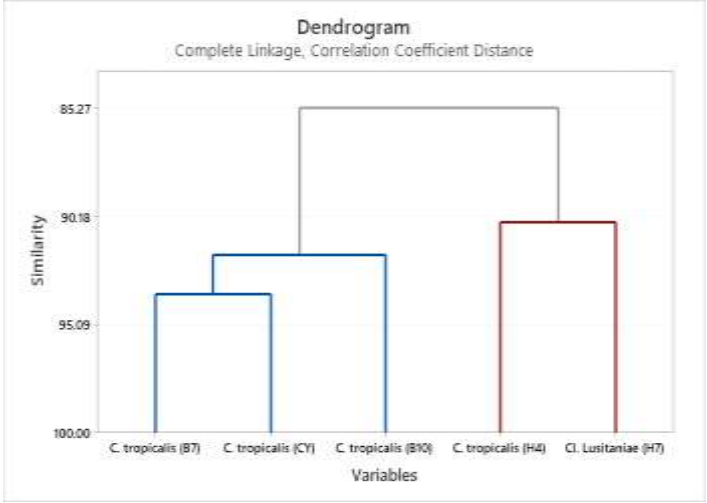

Fig. 15: Cluster variable analysis showing relative similarity in tolerance of yeast isolate

Discussion

The isolation of *Candida tropicalis* strains and *Clavispora lusitaniae* from banana fruit and honey implicated every sugar-rich source as yeast sustaining and populated environment. This finding agrees with the report of (Mateo & Maicas, 2016) and (Mukherjee *et al.*, 2017a) that non-Saccharomyces yeasts are ubiquitous in all sorts of niches, such as fruits surface. Flowers of plants, fruits pulp and honey were reported as a common and sufficient environment for yeast, while honeybees are agents for yeast dissemination (Siavoshi *et al.*, 2018). Yeast isolates were isolated from fermented fruits, vegetables (banana, cabbage, grapes, lime, and mango), pudding, bee honey, toddy, fermented fish (Chandimala *et al.*, 2022), palm wine and date fruits (Bose *et al.*, 2018). Comitini *et al.* (2011) and (Gil *et al.*, 2008) affirmed that non-*Saccharomyces* yeasts of the genera *Candida*, *Kluyveromyces*, *Metschnikowia* and *Torulaspora* are rich sink of untapped biodiversity for vinification due its beneficial fermentation characteristics and there were no significant differences among the genera *Candida*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces* in metabolite production. The presence of *Clavispora lusitaniae* has been reported in fermented cotton seed, rice beverage (Ramos *et al.*, 2011), Tagus estuary (de Almeida, 2005), Domiati cheese, kariesh cheese, and Matared cream (El-Sharoud *et al*., 2009). It has been widely considered the second most virulent Candida species, as a biofilm former, produces several virulent factors, adhesion to buccal epithelial and endothelial cells; secrete lytic enzymes (proteinases, phospholipases, and hemolysins), bud-to-hyphae transition and phenotypic switching. Currently, *Candida tropicalis* has emerged as a biotechnologically important Candida species (Zuza-Alves *et al.*, 2017).

Ethanol demonstrated a high degree of inhibition of yeast isolate cells with increase in concentration, especially against the commercial wine yeast than the wild yeast isolates, which reveals the inhibitory effect of ethanol on yeast isolates. This finding correlates with the report that non-*Saccharomyces* yeasts are ethanol tolerant (Mateo & Maicas, 2016; Mukherjee *et al.*, 2017b) like *Saccharomyces cerevisiae* (Archana & Ravi, 2015). But disagree with (Suárez-Lepe and Morata, 2012) and (Arellano-plaza & Gschaedler, 2013) that non-Saccharomyces yeasts have low ethanol tolerance. The ethanol tolerance is due to the membrane lipid composition and fluidity (Ishmayana *et al.*, 2017; Vazquez *et al.*, 2003), vacuole function maintenance, protein turnover and ion homoeostasis (Nandy & Srivastava, 2018), and the cell wall as genes (*ETR1*, *GPD1*, *DAK1*, *PCT1*, *OPI3*, *MCR1*, *FAA1*, *GRE2*) regulating fatty acid, lipid, and isoprenoid metabolism are expressed under ethanol stress (Yang *et al.*, 2012). But increasing ethanol concentration resulting from fermentation leads to growth inhibition, cell death (Archana *et al*., 2015) or adaptation (Biazi *et al.*, 2022; Teixeira *et al.*, 2012). Thus, non-Saccharomyces yeasts have potential for industrial application in ethanol production (Arellano-plaza & Gschaedler, 2013).

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The study also agrees with the report that non-Saccharomyces can tolerate up to 9 - 10 (% v/v) ethanol, its toxic effects on yeast cells involve loss of cell viability and inhibition of yeast growth and various transport systems (de la Torre-gonzález *et al.*, 2016). Non-Saccharomyces fermentative species inability to survive the increasing concentrations of ethanol greater than 6% v/v (Pina *et al.*, 2004) is not disagrees with my finding of non-Saccharomyces tolerating 20 (% v/v) ethanol; as 5% (v/v) ethanol supplement during molasses fermentation yield maximum ethanol concentration of 25.7% and 42.9% higher than the wild-type (Thammasittirong *et al.*, 2013), demonstrating ethanol stress tolerance of non-Saccharomyces species (Thontowi, 2017).

The ability of wine yeasts to tolerate and adapt to the harsh environment of the must during fermentation is of great concern to oenologists: it is a challenge which had resulted in sick or slow fermentation (Bauer and Pretorius, 2000). This current study investigated the tolerance of wild yeasts isolated from banana and honey comparing them to the sugar tolerance of commercial wine yeasts (that serve as a reference). The ability to withstand harsh osmotic environment is required of yeast for wine production. The findings from this study showed that fructose had more inhibitory effect than sucrose and glucose, and the wild yeast isolates had optimum tolerance at different concentrations yet outclassed the commercial wine yeast in terms of degree tolerance to higher concentrations of fructose, glucose, and sucrose. This is consistent with Teixeira *et al*. (2010) finding that glucose impairs vacuolar function by reducing the cell's ability to sustain biological acidification of the vacuolar lumen. The factors that enhance yeast resistance to high-glucose stress are genes involved in vacuolar function, cell wall biosynthesis (ANP1), and transcriptional control of nutrient digestion (GCN4 and GCR1). The yeast isolates that showed a reduction in OD_{600nm} with increasing concentration, especially with glucose at OD values ≥ 1.000 is regarded as osmotolerant isolates. Similar results were described in an earlier study (Le and Le, 2015). Normally, cells shield themselves from damage and sustain metabolism by regulating metabolic patterns and gene expression in stresses like Snf1 protein kinase is a regulator of yeast in response to freezing stresses and trehalose acts as a protecting agent to the cell membranes under an osmotic environment (Balakumar and Arasaratnam, 2012; Meng *et al*., 2020) and by acquiring multi-stress tolerance to inhibitors (Murata *et al.*, 2021). Yeast cells develop osmotolerance due to the induction of the HOG-MAP (mitogen-activated protein) kinase cascade, which leads to an increased manufacturing and specific activity of GPDH, which is responsible for producing osmolyte glycerol, which counteracts long-term osmotic stress and is required for growth under high osmotic environments (Nass and Rao, 1999). It is also known that yeast cells respond differently to different inhibitory substances (Ok and Hashinaga, 1997; Sipiczki, 2003). *Candida tropicalis*^{B10}, *Candida tropicalis*^{B7}, *Candida tropicalis*^{CY}, *Candida tropicalis*^{H4} and *Clavispora lusitaniae*^{H7} can be referred to as osmotolerant yeasts for tolerating 20% (g/v) glucose and 30 % (g/v) sucrose, is in agreement with the report that, stress-tolerant in non-Saccharomyces emerges as alternative industrial yeasts (Mukherjee *et al.*, 2017a) as yeast for vinification metabolise 220 - 240 (g/l) of sugar (Suárez-Lepe and Morata, 2012). It is well known that the ability to efficiently transport glycerol into the cells is an essential mechanism to combat osmotic stress in many yeast species The majority of these osmotolerant strains were isolated from sugar-rich environments such as honey, maple syrup, beet sugar thick juice, molasses, and floral nectar and some were isolated from fermented cacao beans. As most of these species evolved independently from one another For example, 2 *Z. rouxii* plasma membrane sugar transporters, ZrFfz1 and ZrFfz2, with different substrate preferences (ZrFfz1 for fructose and ZrFfz2 for glucose) have been identified and are thought to play a role in the osmotolerance of this yeast (Mukherjee *et al.*, 2017a).

Sulphite (metabisulphite) is an essential chemical in winery used for control of microbial contaminants in must and winery material surfaces. However, if the amount of metabisulphite used is not regulated could also inhibit fermenter microorganisms, thus, tolerance to metabisulphite became a criterion for selecting yeasts for wine production. The wild yeasts isolates as well as the commercial yeast showed growth in media containing various concentrations of metabisulphite with OD value ≥ 0.5. My observation agrees with the finding of Renouf *et al*. (2006) that *Brettanomyces bruxellensis* was the most adapted non-Saccharomyces yeast to the level of sulphating at harvest time and cold maceration, which could be enhanced by exhibiting a higher iron uptake (Berner and Arneborg, 2012) or linked to the genotypic made up (Curtin *et al*., 2012).

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One of the technological properties of the selection of traditional yeasts includes resistance to Sulphur dioxide (Suárez-Lepe and Morata, 2012). There was a decrease in OD value with an increase in metabisulphite concentration, which collaborates with the report of Ogata *et al*. (2013), that SSU1-overexpressing strains, excreting high sulphite content showed a decrease in sulphite synthesis during the mid-fermentation phase, when particularly in comparison with the wild-type strains.

In the winery, pH is a parameter used in the control of non-acidophilic contaminating microorganisms. The pH tolerance of the yeast isolates examined showed an increase in tolerance (OD value) with an increase in pH and verse versa. There was a stationary phase observed from pH 3.5 – 5.0, which indicates that the yeast isolates were less stressed and tolerated pH 3.5 – 5.0. The finding from my study is following the work of Lahav *et al*. (2002) who reported that *Pichia* sp. belongs to the collection of organisms that are tolerant to lower pH values. A decrease in the specific rate of growth of lactobacilli resulting from the decrease in pH from 5.5 to 4.0 was reported, while in contrast, the yeast cells' specific growth rate was not significant affected in the medium (Narendranath and Power, 2005); as well as the relationship between improved acid resistance and potassium levels in the growth medium (Mira *et al*., 2010). Variation in pH tolerance of yeast cells is dependent on calcium metabolism (Brandão *et al*., 2014).

Cluster analysis was done to ascertain the similarity in inhibitory effect and resistance of the solutes and the yeast isolates. The dendrogram illustrated that alcohol and metabisulphite have strong similarities; fructose and sucrose have a similar inhibitory effect, while glucose and pH have a similar inhibitory effect. Although, the fructose-sucrose and glucose-pH clusters have a similarity of about 51.33 %. There is no report on the clustering of fructose, sucrose, glucose, and pH inhibitory effects for now. The clustering for the yeast isolates classified into 2 major clusters: cluster 1, isolates from honey (*Candida tropicalis*$^{(H4)}$ and *Clavispora lusitaniae*$^{(H7)}$) and, cluster 2, isolates from banana (*Candida tropicalis*$^{(B7)}$ and *Candida tropicalis*$^{(B10)}$) and the *Candida tropicalis*$^{(CY)}$, while cluster 2, *Candida tropicalis*$^{(B7}$ and *Candida tropicalis*$^{(CY)}$ were grouped: which implies that wild yeast isolate *Candida tropicalis*$^{(B7)}$ possess similar oenological properties.

The results of this study agree with the report that the potential of non-Saccharomyces yeast strains in winemaking has been acknowledged (Suárez-Lepe and Morata, 2012). Thus, studying the variety of yeasts in various conditions might identify strains with desirable properties for commercial uses (Ramos *et al*., 2013). The current finding indicated the possible use of non-Saccharomyces species and *Saccharomyces cerevisiae* combined for winemaking (Englezos *et al*., 2015).

Conclusion

The Nigerian tropical rain forest holds non-Saccharomyces population full of vigour for winemaking; as the wild yeasts, *Candida tropicalis*$^{(H4)}$ and *Clavispora lusitaniae*$^{(H7)}$ from honey; *Candida tropicalis*$^{(B10)}$ and *Candida tropicalis*$^{(B7)}$ from banana, possess similarity in stress tolerance with *Candida tropicalis*$^{(CY)}$, a commercial wine yeast.

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